The influence of the DnaA protein on transcription of the $ftsZ$ and $dnaA$ genes in *Escherichia coli*

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To my parents
Abstract

The investigation of the mechanisms governing the control of chromosomal DNA synthesis and cell division is fundamental to the understanding of the regulation of the cell cycle in *Escherichia coli*. Research over many years has shown that two proteins are central to these processes: DnaA in initiation of chromosomal replication and FtsZ in cell division. DnaA and FtsZ are not only thought to be essential to the biochemistry of these events but appear also to be involved in their timing within the cell-cycle. For this reason expression of both the *dnaA* and *ftsZ* genes are regulated by a number of different mechanisms, presumably to ensure efficient growth and division of the organism under a wide range of environmental conditions.

In addition to its aforementioned role, DnaA also functions as a regulatory protein in the expression of a number of genes. Its effect is mediated by a particular recognition sequence present at its site of action, known as the DnaA-box. Such a sequence is present in the promoter region of the *dnaA* gene and has in the past been reported to be involved in autoregulation of this gene. In this work this putative role of the DnaA protein is reassessed and cast in doubt.

The promoter region of the *ftsZ* gene also contains a number of DnaA boxes and some evidence exists that DnaA may be involved in *ftsZ* regulation. In this work evidence is presented to the contrary and it is shown that the apparent role of DnaA in *ftsZ* regulation is probably due to regulation of the gene by a growth-rate sensitive mechanism.
Acknowledgements

Foremost I wish to express a deep gratitude to my parents for their continuous support and generosity throughout my years of education and their apparent faith that these would one day be over. I am very grateful to Millie for supervision over the years and for allowing me the opportunity to experience life in her laboratory. For initial financial support, which permitted me to begin the PhD, I am indebted to The British Council in Amsterdam and for support during the final years, which allowed me to finish it, to the Department of Social Security in Edinburgh.

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# Contents

Declaration iii  
Abstract iv  
Acknowledgements v  
Abbreviations xii  

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>General introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Transcription</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1</td>
<td>RNA polymerase</td>
<td>3</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Promoter recognition</td>
<td>3</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Positive regulation of transcription</td>
<td>4</td>
</tr>
<tr>
<td>1.2.4</td>
<td>Negative regulation of transcription</td>
<td>5</td>
</tr>
<tr>
<td>1.2.5</td>
<td>Termination of transcription</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>Identification of the dnaA gene and isolation of its product</td>
<td>7</td>
</tr>
<tr>
<td>1.4</td>
<td>Nucleotide and amino acid sequence</td>
<td>8</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Sequence of the dnaA gene</td>
<td>9</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Sequence upstream of the structural gene</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>Biochemical properties of the DnaA protein</td>
<td>11</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Binding of DNA</td>
<td>12</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Binding of nucleotides and interaction with phospholipids</td>
<td>13</td>
</tr>
<tr>
<td>1.5.3</td>
<td>The Bacillus subtilis DnaA protein</td>
<td>14</td>
</tr>
<tr>
<td>1.6</td>
<td>dnaA mutants and their properties</td>
<td>15</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Temperature-sensitive mutants</td>
<td>16</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Cold-sensitive mutants</td>
<td>17</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Nonsense mutations</td>
<td>18</td>
</tr>
<tr>
<td>1.7</td>
<td>DnaA in initiation of chromosomal replication</td>
<td>19</td>
</tr>
<tr>
<td>1.7.1</td>
<td>The origin of replication</td>
<td>20</td>
</tr>
<tr>
<td>1.7.2</td>
<td>Genetic evidence for the sequence of events</td>
<td>21</td>
</tr>
<tr>
<td>1.7.3</td>
<td>The initial complex</td>
<td>22</td>
</tr>
<tr>
<td>1.7.4</td>
<td>The open complex</td>
<td>23</td>
</tr>
<tr>
<td>1.7.5</td>
<td>DNA topology and transcriptional activation</td>
<td>24</td>
</tr>
<tr>
<td>1.7.6</td>
<td>The prepriming and priming complexes</td>
<td>25</td>
</tr>
<tr>
<td>1.8</td>
<td>Timing and control of the initiation of chromosomal replication</td>
<td>26</td>
</tr>
</tbody>
</table>
1.8.1 Positive control
1.8.2 Negative control
1.8.3 Negative control through membrane attachment
1.8.4 Models for initiation control by DnaA

1.9 Regulation of \textit{dnaA} gene expression
1.9.1 Dam methylation and sequestration
1.9.2 Response to growth-rate
1.9.3 Autoregulation
1.9.4 Induction by DNA damage

1.10 Extragenic suppression of \textit{dnaA} mutations
1.10.1 Suppression by mutations in \textit{rpoB}
1.10.2 Suppression by a mutation in \textit{topA}
1.10.3 Suppression by deletion of \textit{seqA}
1.10.4 Suppression by a mutation in \textit{trxA}
1.10.5 Suppression by overproduction of GroEL and GroES
1.10.6 Constitutive stable DNA replication
1.10.7 Inducible stable DNA replication
1.10.8 Integrative suppression

1.11 DnaA in regulation of transcription
1.11.1 Repression of transcription initiation
1.11.2 Activation of transcription initiation
1.11.3 Termination of transcription

1.12 DnaA in plasmid and phage replication and in transposition
1.12.1 Plasmid and phage replication
1.12.2 Transposition of Tn5

1.13 Cell division

1.14 The FtsZ protein

1.15 The project

\textbf{Chapter II} \hspace{1em} \textbf{Materials and Methods}

2.1 Bacterial strains, phage strains and plasmids
2.1.1 Growth media and buffers
2.1.2 Growth of bacteria
2.1.3 Minimal medium supplements
2.1.4 Selection of antibiotic resistance

2.2 DNA techniques
2.2.1 Large-scale plasmid preparation
2.2.2 Small-scale plasmid preparation
2.2.3 Preparation of chromosomal DNA
2.2.4 Preparation of bacteriophage M13 DNA
2.2.5 Preparation of bacteriophage λ DNA
2.2.6 DNA precipitation
2.2.7 Determination of DNA concentrations
2.2.8 Digestion of DNA with restriction endonucleases
2.2.9 Partial digestion of DNA
2.2.10 Ligation of DNA
2.2.11 'Filling in' of recessed 3' termini
2.2.12 Agarose gel electrophoresis
2.2.13 Isolation of DNA from agarose gel slices
2.2.14 Labelling DNA fragments by random-priming
2.2.15 Southern blotting procedures
2.2.16 Site-directed mutagenesis after Taylor et al. (1985)
2.2.17 Site-directed mutagenesis after Kunkel (1985)
2.2.18 DNA sequencing techniques
2.2.19 Amplification of DNA using the Polymerase-Chain-Reaction
2.2.20 DNase I footprinting
2.2.21 Gel retardation

2.3 Bacterial techniques
2.3.1 Preparation of competent cells and transformation with plasmid DNA
2.3.2 Transformation by electroporation
2.3.3 Frozen storage of bacterial strains
2.3.4 F-factor mating of E. coli
2.3.5 Selection for the loss of tetracycline resistance
2.3.6 Sizing and counting of bacterial cells

2.4 Phage techniques
2.4.1 Preparation of λ plate lysates
2.4.2 Preparation and selection of λ lysogens not conferring antibiotic resistance
2.4.3 Selecting kan^R λ lysogens
2.4.4 UV induction of λ lysogens
2.4.5 Induction of λ lysogens with mitomycin C
2.4.6 Purification of bacteriophage λ particles from liquid lysates
2.4.7 Preparation of phage P1 plate lysates
2.4.8 Phage P1-mediated transduction
Chapter III  Autogenous regulation of dnaA gene expression

3.1 Introduction

3.2 Isolation of integratively suppressed derivatives
of a dnaA46 strain

3.2.1 Isolation and characterisation of pKN500 integrants

3.2.2 Isolation of R1дрл-19 integrants

3.2.3 Isolation of F::Tn5 integrants

3.3 The activity of λRB1 in integratively
suppressed dnaA46 derivatives

3.3.1 TPK strains

3.3.2 TPR strains

3.3.3 TPF strains

3.4 Efficiency of integrative suppression

3.4.1 Growth on agar

3.4.2 Cell-size distributions

3.4.3 Discussion

3.5 The effect of integrative suppression of various dnaA
alleles on dnaA promoter activity

3.5.1 Construction of integratively suppressed dnaA46
derivatives of TP91

3.5.2 lacZ expression from λRB1 in integratively suppressed dnaA5
dnaA601, dnaA604 and dnaA204 strains

3.6 Sensitivity of the dnaA promoters to DnaA overproduction in an
integratively suppressed strain

3.7 Integrative suppression by the R1 minimal origin of replication

3.7.1 Attempt to clone the R1 minimal origin of replication using PCR

3.7.2 Construction of pGWIS

3.7.3 Integrative suppression of dnaA46 with pGWIS

3.8 Construction and characterisation of λRWS945

3.8.1 Construction of λRWS945

3.8.2 Characterisation of λRWS945

3.9 The influence of secondary mutations

3.9.1 Additional mutations accumulate in integratively suppressed strains

3.9.2 Curing of pKN500 from an integratively suppressed strain

3.9.3 dnaA promoter activity in NF279 and NF#B(pGW71)
3.10 The behaviour of the dnaA promoters on a plasmid in an integratively suppressed strain

3.11 The effect of extragenic suppression of dnaA on dnaA promoter activity
   3.11.1 Isolation of spontaneous pseudo-revertants of ED945
   3.11.2 Behaviour of λRWS945 in other suppressed strains

3.12 Involvement of the DnaA-box
   3.12.1 Mutation of the DnaA-box in the dnaA promoter region
   3.12.2 Characterisation of λRWS945M in a dnaA46 strain
   3.12.3 Activity of λRWS945M in stationary phase
   3.12.4 Overproduction of DnaA
   3.12.5 Titration of DnaA
   3.12.6 Integrative suppression
   3.12.7 Overproduction of Dam methylase
   3.12.8 Resequencing of the DnaA-box mutation in λRWS945M

3.13 Binding of DnaA to the mutant DnaA-box in vitro
   3.13.1 DNase I footprinting
   3.13.2 Gel retardation

3.14 Behaviour of λRWS945 and λRWS945M in a dnaA0 strain
   3.14.1 Construction of isogenic dnaA+ and dnaA0 strains
   3.14.2 Behaviour of λRWS945 and λRWS945M in EH3791 and its derivatives

3.15 Discussion
   3.15.1 Thermal inactivation of DnaA18 protein; DnaA as a transcriptional regulator
   3.15.2 Indirect effects of DnaA inactivation on dnaA promoter activity
   3.15.3 Mutation of the DnaA-box
   3.15.4 Is dnaA autoregulated?

Chaper IV The role of DnaA in transcription of ftsZ
4.1 Introduction
   4.1.1 Regulation of transcription of ftsZ
   4.1.2 DnaA as a possible transcriptional regulator of ftsZ

4.2 Mutation of the DnaA-box in ftsQ

4.3 Construction of new transcriptional fusions of ftsZ promoters and lacZ
4.4 Behaviour of λRWS100, λRWS200 and λRWS201 in a dnaA46 host 212
4.5 Behaviour of λRWS100 and λRWS200 in a dnaC325 host 216
4.6 Mutagenesis of the ftsA and ftsQ DnaA-boxes 218
4.6.1 Mutagenesis 218
4.6.2 Construction of pRWS20M and λRWS20M 219
4.7 Behaviour of λRWS20M in a dnaA46 host 220
4.8 Overproduction of DnaA 222
4.9 Introduction of oriC plasmids 225
4.10 Integrative suppression 227
4.11 Behaviour of the ftsZ promoters in a dnaA0 strain 228
4.12 Influence of the DnaA-boxes on transcription from ddlB 229
4.12.1 Construction of pTL/ZAD and pTL/MAD 229
4.12.2 lacZ expression from pTL/ZAD and pTL/MAD 231
4.13 Replacement of the chromosomal ftsQ and ftsA genes with those carrying mutant DnaA-boxes 233
4.13.1 First strategy 233
4.13.2 Gene replacement using pMAK705 235
4.13.3 Initial characterisation of the chromosomal DnaA-box mutations 238
4.14 Discussion 240
4.14.1 DnaA is not a regulator of ftsZ transcription 240
4.14.2 Importance of ftsZ transcription in the timing of cell division 244

Chapter V Growth-rate sensitive transcription of ftsZ 245
5.1 Introduction 246
5.2 Growth-rate sensitivity of the proximal ftsZ promoters 247
5.3 Behaviour of the ftsZ promoters during a nutritional shift-up 250
5.4 Behaviour of the ftsZ promoters during entry into stationary phase 253
5.5 Discussion 256

Bibliography 260
Appendix Published work 287
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
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<td>amp</td>
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</tr>
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<td>Adenosine-5'-triphosphate</td>
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<td>Kilobase pair(s)</td>
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<td>kD</td>
<td>Kilodalton</td>
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<tr>
<td>moi</td>
<td>Multiplicity of infection</td>
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<td>R</td>
<td>Resistant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>S</td>
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<td>Streptomycin</td>
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</tr>
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<td>Dodecylpoly(ethyleneglycolether)$_n$</td>
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</tbody>
</table>
Chapter I
Introduction
INTRODUCTION

1.1 General introduction

The purpose of life has long been the subject of debate. From a purely biological perspective one can argue that life is nothing but a highly adaptable system which ensures the immortality of genetic information stored on the DNA molecule. For its continued existence the genetic information is dependent upon the viability and reproductive capacity of the organism which carries it and in turn these factors are a consequence of the information present on the DNA of the organism. To ensure survival under changing conditions the DNA molecule can alter subtly from generation to generation giving rise to an ever more diverse range of hosts, some of which will be adapted to new conditions, a process known as evolution. As a consequence the content of the genetic information is forever changing, nevertheless one element must remain the same: the ability of the DNA to be replicated, which can therefore be considered to be the fundamental basis of life.

The process of DNA replication has been studied in a variety of organisms and most extensively in the gram-negative enterobacterium Escherichia coli. This bacterium grows by lateral elongation and divides into two daughter cells each carrying an identical copy of the chromosome. This process of growth and division must continue in an orderly fashion and is termed the cell-cycle. Each cycle consists of the replication of the chromosome and the partitioning of the newly replicated chromosomes into the daughter cells. These are complex processes and require the participation of many proteins; among these are the product of the dnaA gene, which is essential for the onset of chromosomal replication, and that of the ftsZ gene which is fundamental to cell division. Apart from their well established roles in the biochemistry of the processes in which they play a part, the DnaA and FtsZ proteins appear to be central to the timing of initiation of DNA replication and cell division within the cell-cycle. Although these functions of the two proteins are still poorly understood, it appears that their concentrations in the cell are important to maintain a regular cell-cycle resulting in efficient inheritance of chromosomal DNA. One level at which the intracellular abundance of a protein is regulated is that of the transcription of its gene into messenger RNA. The cell has developed a number of mechanisms whereby certain proteins can influence the level of transcription of a particular gene depending upon the requirement for its product under particular conditions. There is evidence that the DnaA protein may be acting as a transcriptional regulator of both the dnaA and ftsZ genes and further study of these putative roles of the DnaA protein is the subject of this work.
This chapter deals with three main topics. Firstly, a brief description is given of the process of transcription and of some of the factors which are involved therein. Secondly, a review is presented of the literature concerning the DnaA protein and its reported activities in the cell, notably that in initiation of chromosomal DNA replication. Finally an account is given of the function of the FtsZ protein in cell division.

1.2 Transcription

Gene expression starts with the synthesis of an RNA transcript of the gene. Transcription is initiated at a sequence, the promoter, which is recognised and bound by RNA polymerase holoenzyme to form a stable, closed initiation complex. This is then transformed into a transcriptionally active open complex through the melting of a short stretch of DNA within the sequence bound by the enzyme. Polymerisation of the first few nucleotides of the nascent RNA chain then commences. At this stage the polymerase/DNA complex is unstable and transcription is prone to premature abortion. Once the transcript is eight to nine nucleotides long the σ-subunit is ejected from the enzyme complex and the tendency for the polymerase to dissociate from the DNA template is lost. Transcription will now continue until the RNA polymerase encounters signals which lead to termination of transcription.

1.2.1 RNA polymerase.

The RNA polymerase holoenzyme consists of five subunits: α2ββ'σ. The σ-subunit is associated with the complex only during initiation of transcription and its role is in recognition of the promoter sequence. The majority of promoters in *E. coli* are recognised by the σ70-factor. However, several distinct classes of promoters have now been discovered which differ significantly in sequence from those recognised by σ70; these promoters require other σ-factors to direct RNA polymerase to them. Genes dependent on a particular minor species of σ, are often involved in a common process such as a response to a particular condition in which the organism finds itself. σ-factors can thus serve to mediate differential gene expression under conditions such as high temperature or other cellular stress (σ32 and σ24) and nitrogen starvation (σ54). Entry into the stationary growth phase requires the expression of a set of genes recognised by σ38. It has also been proposed that genes involved in flagella synthesis
are transcribed with the aid of yet another σ-factor. The function of various σ-factors has been reviewed by Helmann and Chamberlin (1988).

In addition to its role in promoter recognition, σ70 has been implicated in binding a particular class of transcriptional activators (class II) which bind at or close to the –35 region of certain promoters. A feature of these promoters is a poor match to the consensus of the –35 sequence normally recognised by the σ70 C-terminus. It appears therefore that σ70 can recognise both DNA and protein signals at the promoter (Kumar et al., 1993 and 1994), although absolute proof of this is still lacking.

The α-subunit is required for assembly of the core enzyme complex; it appears that it is the N-terminal part of the protein which is responsible for interactions with another α-subunit and the β-subunit (Hayward et al., 1991). In addition, the α-subunit interacts with DNA elements present upstream of the –35 sequence in some promoters (Ross et al., 1993; Blatter et al., 1994) and with a number of transcriptional activators such as catabolite gene activator protein, CAP (Zou et al., 1992), Fnr (which is an activator of genes involved in anaerobic respiratory pathways; Lombardo et al., 1991) and other activating factors which bind upstream of the promoter (class I activators; Ishihama, 1992).

The β and β'-subunits are the most evolutionarily conserved and both seem to be involved in formation of the catalytic centre of RNA polymerase; nucleotides can be cross-linked to amino-acids in the β-subunit and the 3' terminus of the nascent RNA has been found to cross-link to the β'-subunit (Mustaev et al., 1991; Borukhov et al., 1991).

1.2.2 Promoter recognition.

It is believed that a large part of the difference in the relative efficiencies of promoters, which can vary over four orders of magnitude, is due to differences in the DNA sequence of the promoter (McClure, 1985; Harley and Reynolds, 1987). Comparison of σ70 promoter sequences led to the identification of two conserved hexamers: the –35 sequence (TTGACA) and, separated by a 16 bp to 18 bp spacer region, the –10 sequence (TATAAT). In each region three bases are highly conserved: TTG— at –35 and TA—T at –10. Weakly conserved homologies flank these sequences. The efficiency by which a promoter directs the synthesis of a message is therefore largely dependent on its DNA sequence. This conclusion is supported by the observation that mutations which decrease the similarity of the promoter to that of the
consensus generally tend to decrease promoter activity while, conversely, those that increase similarity to the consensus sequence increase the activity of the promoter.

Attachment of RNA polymerase to the promoter is thought to occur in two stages (McClure, 1985). A reversible loose binding of the enzyme complex to the DNA is followed by open complex formation and tight binding. Both initial binding and open complex formation can be affected by promoter mutations (McClure, 1985).

**1.2.3 Positive regulation of transcription.**

An increase in the basal strength of a promoter may be achieved in various ways: interaction with a protein activator, alteration of DNA topology and methylation of the DNA are examples of this. *In vitro* studies have demonstrated that activators can increase promoter function over a wide range. For example, λcI protein activates the λPRM promoter approximately ten-fold (Hawley and McClure, 1983) and λcII activates λPRE in excess of a hundred-fold (Shih and Gussin, 1984). It was shown that cII, which binds sequences flanking the −35 region of the promoter, increases both the initial binding of RNA polymerase to the promoter and the rate of open complex formation (Shih and Gussin, 1984).

It was stated above that many transcriptional activators fall into one of two categories. Class I activators such as OmpR, Ada, IHF and OxyR bind upstream of the −35 promoter element and interact with the α-subunit. Class II activators such as cI, cII, MerR and PhoB generally bind at or downstream of the −35 sequence and do not show an interaction with the α-subunit but have an alternative mode of activation, perhaps mediated by contact with σ70 as has been demonstrated in the case of PhoB. CAP is unique in that it can be either a class I or a class II activator depending on the location of its binding site (Ishihama, 1992; Kumar *et al.*, 1994).

Another class of activator is formed by the enhancer binding proteins (EBP) which are present in a wide range of bacteria and which generally activate promoters recognised by the alternative σ54-factor (Merrick, 1993). As well as differing in the type of promoters which they activate, EBPs differ from class I and class II activators in the fact that their binding sites are located at distances of more than 100 bp from the transcription start (Kustu *et al.*, 1991). A well studied example of an EBP is the NtrC protein which catalyses open complex formation and is dependent upon ATP hydrolysis for its activity (Foster-Hartnett *et al.*, 1994 and references therein). Recently, sequence similarity has been found between NtrC and the DnaA protein which is the subject of this thesis (W. Messer, personal communication). DnaA has
been implicated both in ATP dependent DNA strand separation at the origin and in transcriptional activation. The function of the region of similarity of these proteins is not yet known but elucidation of this would shed light on the biochemistry of their actions.

DNA topology can influence promoter activity. An example is the activation of the bgl promoter by gyrase mutations (DiNardo et al., 1982). It is unclear whether this is due to an effect on RNA polymerase binding or due to an indirect effect on a possible repressor which would fail to bind DNA of lowered negative superhelicity.

DNA methylation can influence promoter activity in several different ways. Expression of the mom gene of phage Mu is decreased in a strain containing mutations (dam) that negatively affect DNA methylation (Plasterk et al., 1984). It is not known how methylation increases expression of the mom gene. The dnaA promoter also responds positively to Dam methylation (Kücherer et al., 1986; Braun and Wright, 1986). An opposite effect of methylation is seen at the PIN promoter of Tn10. In wild-type cells Tn10 transposition is coupled to chromosomal DNA replication. Passage of the replication fork over the target sites for Dam methylation (GATC) generates unmethylated sequences and activates the transposon by increasing transcription of the transposase gene from PIN (Kleckner et al., 1984).

1.2.4 Negative regulation of transcription.

Repressor proteins are thought to act through interference with RNA polymerase/promoter interactions. The ability of a molecule to act positively in the regulation of transcription does not prohibit it from also functioning as a repressor. An example is the cI protein which plays a fully bifunctional role in controlling gene expression.

The positions of the binding sites for LexA, a protein which represses transcription of a number of genes, illustrates that repression may occur by binding either at the actual promoter sequence or just upstream or downstream of it. LexA may interfere with transcription in a different way in each case and this may ensure different basal levels of expression of the various genes under uninduced circumstances (Hoopes and McClure, 1987 and references therein).
1.2.5 Termination of transcription.

The primary signal for termination of transcription lies within the DNA transcribed by the RNA polymerase complex. Terminators have been distinguished in *E. coli* according to whether or not the RNA polymerase requires additional factors to terminate transcription *in vitro*.

So-called simple terminators consist of a hairpin structure in the transcript, which is generated by base-pairing between inverted repeats of GC rich DNA, followed by a run of U residues. It is believed that the RNA polymerase pauses when it encounters the hairpin structure and that the U residues effect termination by forming a particularly unstable stretch of RNA-DNA hybrid duplex with the template. Its disruption causes reannealing of the template DNA with the non-coding DNA strand (collapse of the transcription bubble) and subsequent release of RNA polymerase. A detailed examination of the efficiency of a number of terminators *in vitro* has been carried out by Reynolds *et al.* (1992). This study suggests that not only destabilisation of the RNA-DNA hybrid, but also destabilisation of protein/protein and protein/nucleic-acid interactions are important for termination.

A different class of terminators depends upon a protein factor, Rho (ρ), to effect release of RNA polymerase. A requirement is that the RNA polymerase is stalled at a specific site; release of the polymerase then occurs through the action of ρ. It is thought that the ATP dependent RNA-DNA helicase activity of ρ brings about a destabilisation of the hybrid duplex at ρ-dependent termination sites (Geiselmann *et al*., 1993). It has recently been shown that another factor, NusG, is required for ρ-dependent termination. It appears that NusG interacts with RNA polymerase and may alter its interaction with DNA and RNA so as to facilitate a rapid release by ρ (Sullivan and Gottesman, 1992).

A number of other protein factors which affect termination in various ways have been identified. NusA, for instance, has been shown to increase the termination efficiency of several ρ-independent promoters (Schmidt and Chamberlin, 1987). It has however also been implicated in antitermination by λN protein at both ρ-dependent and ρ-independent terminators in phage λ (Das and Walska, 1984; Yager and von Hippel, 1987). NusA appears to bind RNA polymerase core enzyme and stays associated with it throughout elongation of the transcript. It has recently been proposed that it enhances pausing of the transcription complex. Depending on the site at which this occurs, pausing could either facilitate coupling of transcription and translation blocking termination or, alternatively, facilitate the access of ρ leading to termination (Zheng and Friedman, 1994). For further reading on the processes of termination and antitermination the reader is referred to a recent review by Das (1993).
1.3 Identification of the dnaA gene and isolation of its product

The dnaA gene of E. coli was identified through the isolation of conditionally lethal mutants deficient in replication of chromosomal DNA at restrictive temperature (Kohiyama et al., 1966). Characterisation of such mutants resulted in their division into two groups according to their phenotypes upon a shift from permissive to restrictive temperature, the so-called "quick-stop" mutants in which DNA replication ceases immediately and the slow-stop mutants which show some residual DNA synthesis (Hirota et al., 1968). Mutants belonging to the former group were later shown to be deficient in the elongation process of chromosomal replication whereas the slow-stop mutants were shown to be unable to initiate DNA synthesis at the restrictive temperature but could complete ongoing rounds of replication. The isolation of many initiation mutants has been reported (Kohiyama, 1968; Kuempel, 1969; Carl, 1970; Hirota et al., 1970; Abe and Tomizawa, 1971; Beyersmann et al., 1974; Wada and Yura, 1974) most of which were found to reside at three locations on the genetic map, designated dnaA, dnaB and dnaC (Wechsler and Gross, 1971; Sevastopoulos et al., 1977). The dnaA gene was isolated in specialised transducing phages, λ-tna or λ-dnaA, cloned into a plasmid (Miki et al., 1978 and 1979) and was shown to code for a diffusable product of about 50 kD which complements temperature-sensitive dnaA mutants (Hansen and von Meyenburg, 1979; Murakami et al., 1980; Schaus et al., 1981a).

dnaA was mapped to 83 minutes on the chromosomal map (Bachmann, 1990), 42 kb to the left of the chromosomal origin of replication, oriC (von Meyenburg and Hansen, 1980), and is located between rpmH and dnaN which code for the ribosomal protein L34 (Hansen et al., 1982b) and the β-subunit of DNA polymerase III respectively (Sakakibara and Mizukami, 1980). dnaA and dnaN on the one hand and rpmH on the other are divergently transcribed, each from several promoters (Hansen et al., 1982b; Figure 1.2). It has been suggested that dnaA and dnaN are the first two genes of an operon also comprising recF and possibly gyrB. Although it has been shown that dnaN is transcribed from the dnaA promoters and that transcription could proceed through all four genes (Sako and Sakakibara, 1980) disco-ordinate expression of the genes has been demonstrated (Quiñones and Messer, 1988). Several investigators have found promoters for dnaN within the dnaA coding region (Quiñones and Messer, 1988; Armengod et al., 1988) and similarly the dnaN coding region contains promoters for recF as well as sequences which negatively modulate recF transcription (Armengod and Lambès, 1986). As the dnaN stop-codon and the recF start-codon overlap, translational coupling between these genes may also play a
part in recF regulation; indeed it has been shown that prevention of dnaN translation reduces recF expression (Armengod et al., 1988). Also, a promoter for gyrB within the recF structural gene has been reported (Adachi et al., 1984) and recently it has been shown that there are strong termination signals within recF and that gyrB is predominantly transcribed from the promoter within recF (Macián et al., 1994). It is clear that even if these genes do form an operon, independent regulation of each gene is possible. It is noteworthy that the arrangement of these genes is highly conserved among eubacteria (Yoshikawa and Ogasawara, 1991) which may point to a functional significance of such an organisation.

The product of the dnaA gene was identified through the analysis of the proteins encoded by specialised transducing phages such as λ-tna and λ-dnaA (Hansen and von Meyenburg, 1979; Miki et al., 1979; Murakami et al., 1980) and through the isolation and study of various amber and insertional mutants in dnaA and dnaN (Kimura et al., 1979 and 1980; Yuasa and Sakakibara, 1980). The reported molecular weight of the dnaA gene product varied from 48 to 54 kD. On the basis of the complete sequence of the dnaA gene the molecular weight has been calculated to be 52.574 kD (Hansen et al., 1982a; Ohmori et al., 1984). Purification of the DnaA protein was achieved by two groups. Chakraborty et al. (1982) used a DNA binding assay with oriC plasmids as substrate, based on the assumption that the DnaA protein would bind to origin sequences. Fuller and Kornberg (1983) made use of an in vitro replication complementation assay based on the observation that in vitro replication of oriC plasmids is absolutely dependent upon the product of the dnaA gene (Fuller et al., 1981; Kaguni et al., 1982). Both purification procedures yielded a protein of around 52 kD. An improved method has since been reported which is based on coprecipitation of DnaA with phospholipids and which is less prone to contamination (Sekimizu et al., 1988b).
1.4 Nucleotide and amino acid sequence

1.4.1 Sequence of the dnaA gene.

The dnaA genes of E. coli and of many other bacteria have been sequenced. E. coli dnaA encodes a basic protein of 467 amino acids which has a calculated isoelectric point of 9.6, possibly indicative of a direct interaction between the protein and DNA, although there is no apparent sequence similarity between DnaA and other known DNA binding proteins active in initiation of DNA replication, such as λO protein and RepA (Hansen et al., 1982a). Homology amongst DnaA proteins from various bacteria, however, is striking; Figure 1.1 shows a comparison of the amino acid sequences of the DnaA proteins of twelve different bacteria some of which diverged over a billion years ago (Yoshikawa and Ogasawara, 1991) and it is clear that these proteins are homologous, varying from 30% to 87% in identity with E. coli DnaA, depending on the degree of evolutionary divergence. It is also apparent that certain regions of the protein are more highly conserved than others; on the basis of this the protein has been divided into four domains differing in degree of similarity between the various bacteria (Fujita et al., 1990).

The amino terminus (domain I) is well conserved among most DnaA proteins, however the highest degree of similarity is in domain III which contains an amino acid sequence known as the P-loop or Walker "Type A" Motif, typical of nucleotide binding proteins (Saraste et al., 1990). DnaA contains a good match to the consensus of this sequence (G—G—GKT) but more extensive similarity of this region has recently been found with ATPases of the NtrC superfamily which comprises proteins involved in transcriptional activation, and with the helicase superfamily III, which comprises mostly viral DNA and RNA helicases (Koonin, 1993; Messer and Weigel, in press). Another region of high similarity with NtrC proteins is found towards the end of domain III with the sequence NVRELEGAL (cited in Messer and Weigel, in press). Between this and the P-loop motif there is a stretch of around fifty amino acids with some similarity with the Gene 69 product of phage T4, a protein involved in DNA replication which has been found to be associated with membrane fractions (Mosig and Macdonald, 1986). Since this region of similarity corresponds to one of four possible hydrophobic α-helices present in domain III and IV of DnaA (Skarstad and Boye, 1994) it is possible that both DnaA and gp69 interact with similar cellular components. However, no evidence for transmembrane regions has been found. A sequence of very limited similarity with cAMP binding proteins has been reported to be near the end of domain III (residues 307–334). It is as yet unclear if this is
A comparison of the dnaA genes of twelve different bacteria. Capital letters indicate identity or strong homology of a residue amongst nine or more species. The region marked ATP denotes the putative ATP binding site. Division into domains I to IV is after Fujita et al. (1990). Shaded bars indicate common conserved motifs of DnaA proteins and transcriptional activators of the NtrC family. The horizontal brackets mark the positions of the putative amphipathic α-helices. Dots indicate gaps. The figure was adapted from Richter and Messer (in press) and from Skarstad and Boye (1994).
significant but it is noteworthy that five of the nine conserved amino acids are known to interact with, or be close to, cAMP in the CAP protein (Hughes et al., 1988). This sequence is not well conserved in DnaA proteins of other bacterial species.

A region close to the carboxy terminal part of the protein is conserved. This domain IV seems to be involved in the binding of DNA as it has recently been shown that the 160 carboxy terminal amino acids are sufficient for specific binding to DNA (A. Roth, personal communication).

Domains I and III are separated by domain II which shows little similarity in sequence amongst the various organisms. It is in this domain that the sequence divergence between organisms with genomes of high or low GC content is most apparent. The difference in length of various DnaA proteins is mainly due to additional amino acids in domain II and has been reported to be because of the acquisition of extra proline and alanine residues (both of which have corresponding codons with G or C in the first two positions) by organisms with a high genomic GC content such as Micrococcus luteus or Streptomyces coelicolor (Fujita et al., 1992; Calcutt and Schmidt, 1992). This however does not account for the long domain II of Mycoplasma capricolum which is derived from gram positive bacteria of low GC content (Fujita et al., 1992).

1.4.2 Sequence upstream of the structural gene.

The nucleotide sequence of DNA upstream of the E. coli dnaA gene shows two promoter sequences which were identified by S1 mapping, dnaA1p and dnaA2p, located 230 bp and 150 bp upstream of the coding region respectively (Hansen et al., 1982b; Figure 1.2). The proximal promoter, dnaA2p, has a sequence downstream of the Pribnow box which is similar to that of stringently controlled ribosomal protein genes (Travers, 1984) and regulation of dnaA by ppGpp has been suggested (Chiaramello and Zyskind, 1990).

The promoter region shows six recognition sites (GATC) for deoxyadenine-methyltransferase (Dam) within 140 bp; it has been shown that methylation is an important control element in dnaA transcription (Braun and Wright, 1986; Kücherer et al., 1986; Campbell and Kleckner, 1990). Another striking feature of this region is the presence of a particular nine basepair sequence between the two promoters. Five similar sequences, known as DnaA-boxes, are present in oriC and have been shown to bind DnaA protein (Fuller et al., 1984; Matsui et al., 1985). The presence of a DnaA-box at the promoter has been implicated in possible autoregulation of the dnaA gene (Braun et al., 1985; Wang and Kaguni, 1987). Another putative DnaA binding site is
present within the coding region of the gene but its role, if any, is as yet unclear (Wende et al., 1991).

Comparison of the *E. coli dnaA* nucleotide sequence with that of other organisms shows that in closely related species, *Salmonella typhimurium* and *Serratia marcescens*, the proximal promoter and the upstream DnaA-box are conserved but the distal promoter and the internal DnaA-box less so (Skovgaard and Hansen, 1987). In more distantly related organisms there is a great deal of variation in the number and arrangement of DnaA-boxes flanking the gene. In all gram-positive bacteria which have been studied so far, *Bacillus subtilis*, *Micrococcus luteus*, *Mycoplasma capricolum* and *Streptomyces coelicolor*, there appear to be two clusters of DnaA-boxes on either side of the *dnaA* gene (Moriya et al., 1985; Fujita et al., 1990; Fujita et al., 1992; Calcutt and Schmidt, 1992) whereas in gram-negative bacteria such as *Pseudomonas putida* there is at most one such cluster upstream of the gene (Fujita et al., 1989). There is evidence that at least in some cases these clusters are locations for initiation of chromosomal replication (Moriya et al., 1985; Fujita et al., 1989; Calcutt and Schmidt, 1992). There is insufficient information to say whether these regions have any regulatory role with respect to the *dnaA* gene.

**Figure 1.2** The *dnaA* operon and promoter region.

1.5 Biochemical properties of the DnaA protein

DnaA is a stable protein (Sakakibara and Yuasa, 1982) which is present in the cell at between 400 and 2000 monomers (Sakakibara and Yuasa, 1982; Sekimizu et al., 1988b; Hansen et al., 1991a).

1.5.1 Binding of DNA.

DnaA protein binds double stranded DNA carrying oriC; binding is enhanced by supercoiling of the DNA but is not dependent on it (Chakraborty et al., 1982; Fuller and Kornberg, 1983). Numerous studies, using electron-microscopy and both in vitro and in vivo DNA footprinting, have shown that twenty to forty monomers of DnaA, in conjunction with other proteins, form a complex with the origin DNA (Kaguni et al., 1982; Fuller et al., 1984; Funnell et al., 1987; Samitt et al., 1989; Holz et al., 1992). Filter binding assays with various DNA fragments showed that binding of DnaA is not limited to oriC alone but occurs specifically with stretches of DNA bearing nine basepair sequences identical to or closely resembling those present at oriC. These sequences, four of which are found in the origin (R1-R4), seem to form the target site for the DnaA protein and a consensus sequence was derived by Fuller et al. (1984): TTAT(C/A)CA(C/A)A and termed the DnaA-box of which positions 1,2,4,5,6,7 and 9 are essentially invariant but certain mismatches can be tolerated at positions 3 and 8. DNase I footprinting experiments show that the protein binds asymmetrically to its target site as protection patterns on the two DNA strands differ (Fuller et al., 1984).

It was clear that other as yet unidentified elements such as perhaps the environment of the DnaA-box are of influence as identical DnaA-boxes (TTTTCCACA) present in the mioC promoter, the dnaA structural gene and the uncA gene bind DnaA in the first two cases but not in the last. In vivo work presented by Hansen et al. (1987), examining titration of DnaA by plasmids carrying various combinations of DnaA-boxes, demonstrated that DnaA-boxes may act cooperatively and that the mioC DnaA-box binds far more efficiently than the origin. However, when isolated boxes are tested for their binding capacity in vitro the comparative binding capacities are reversed (C. Weigel and W. Messer, personal communication) adding weight to the idea that the nine basepair sequence is not the sole determinant of a recognition site. This is supported by the discovery by de Wind et al. (1987) that the mioC promoter from E. coli B/r is repressed by DnaA to a lesser extent than that from E. coli K-12 and that the two differ only at a site which is located more than twenty bases from the DnaA-box.
Further analysis of what constitutes an active DnaA-box was carried out by Schaefer and Messer (1991) using the property of the DnaA/DnaA-box complex to act as a transcriptional terminator (Schaefer and Messer, 1988 and 1989). From quantitative in vivo termination assays they derived a new consensus sequence: (T/C)(T/C)(A/C/T)T(A/C)C(A/G)(A/C/T)(A/C). Also, based on the assumption that the nucleotides within the nine basepair sequence contribute separately to the relative affinity of the DnaA-box, they derived a system which allows predictions to be made of the binding capacity of various nine basepair combinations. That these predictions are not definitive is demonstrated by the fact that a box not fitting the consensus, present in the rpoH promoter (TTATTCACA) was shown to bind DnaA (Wang and Kaguni, 1989). It has been suggested that its proximity to a perfect DnaA-box may influence its binding capacity.

Binding of DnaA in vitro has been shown in the following cases: the mioC, dnaA, uvrB, rpoH and nrd promoters (Fuller et al., 1984; Braun et al., 1985; van de Berg et al., 1985; Wang and Kaguni, 1989; Augustin et al., 1994), the dnaA structural gene, the IRL of Tn5 and the origins of M13, pSC101, ColE1 (Fuller et al., 1984), RK2 (Gaylo et al., 1987), R6K (Wu et al., 1992) and R1 (Masai and Arai, 1987). The kinetics of binding of the DnaA protein to oriC suggest it is a cooperative process (Chakraborty et al., 1982; Fuller and Kornberg, 1983).

DnaA bound to the dnaA promoter area covers a region of fifty basepairs between the two promoters centred around the DnaA-box and two other sites, one upstream of dnaA1p and the other downstream of dnaA2p, which both contain the sequence TTAT (Braun et al., 1985). Whether this sequence is an additional element in recognition of a target site for DnaA is not known but the results again imply that the environment of the DnaA-box could be important for recognition. Although such additional recognition elements may exist, unpublished results by Messer and co-workers indicate that DNA outside the immediate vicinity of the DnaA-box is not tightly bound by the protein; a restriction site within the region covered by DnaA protein is still accessible to the restriction enzyme which suggests that protein/protein interactions around the target site are more important than those between protein and DNA (C. Weigel, personal communication).

1.5.2 Binding of nucleotides and interaction with phospholipids.

DnaA has a high and almost equal affinity for ATP and ADP (Sekimizu et al., 1987) with respective $K_D$ values of 30 and 100 nM; Mg$^{2+}$ is presumably required for binding as chelation of the cation releases the nucleotide (Sekimizu and Kornberg,
DnaA complexed with either nucleotide is present in solution in monomeric or dimeric form whereas the nucleotide free form aggregates. DnaA-ATP and DnaA-ADP both bind oriC DNA with similar kinetics and form complexes which are indistinguishable by footprinting, though clearly distinct from that formed with nucleotide free DnaA (Crooke et al., 1993). Mild tryptic digests of the various complexes show that binding of oriC by the nucleotide forms of DnaA leaves its carboxy terminus accessible whereas this is not the case with nucleotide free DnaA. More vigorous digestion is necessary to show a difference between DnaA-ATP and DnaA-ADP (Yung and Kornberg, 1989; Yung et al., 1990). Thus, nucleotide binding causes a conformational change in the protein which is similar, though not identical, for both ATP and ADP. A clear difference between the nucleotide bound forms of DnaA emerges in their ability to promote replication in an in vitro assay; DnaA-ATP is required for an active initiation complex, DnaA-ADP is inactive although it can augment the activity of limiting amounts of the ATP form (Yung et al., 1990). Bound ATP is slowly hydrolysed to ADP in a DNA dependent manner. ADP remains bound to the protein and renders it inactive for replication (Sekimizu et al., 1987).

Sekimizu et al. (1988b) showed that about twenty percent of DnaA present in the cell is recovered in particulate membrane fractions and that DnaA forms a complex with phospholipids. An important property of acidic phospholipids such as cardiolipin and to a lesser extent phosphatidylglycerol is their ability to effect a rapid release of bound nucleotides from DnaA. In the presence of ATP cardiolipin can in this way catalyse the rejuvenation of an inactive initiation complex by providing a means of regenerating DnaA-ATP from inert DnaA-ADP (Sekimizu and Kornberg, 1988); this reactivation occurs only if the protein is complexed with oriC DNA. In the absence of oriC DNA the DnaA protein is rendered incapable of binding DNA after release of the nucleotide. This has been taken to indicate that there is competition between DNA and cardiolipin for the same site on the protein; indeed the cardiolipin backbone has a similar structure to the phosphodiester backbone of DNA and immunological cross-reactivity has been observed (Crooke et al., 1992). On the other hand it has been shown that it is the carboxy terminal part of the protein that binds DNA, whereas phospholipids interact with a 30 kD amino terminal part of the protein created by tryptic digestion (Yung and Kornberg, 1989). For this reason it seems unlikely that DNA and cardiolipin would compete for the same site on the protein; rather, it could be that a conformational change caused by cardiolipin action prevents subsequent binding to oriC, perhaps by causing aggregation of the nucleotide free form of DnaA. The mechanism of cardiolipin action is unknown. It does not appear to act through chelation of Mg\(^{2+}\), which normally stabilises the DnaA-ATP/ADP complexes, as high
concentrations of this cation do not inhibit cardiolipin catalysed nucleotide release (Sekimizu et al., 1988a).

In vivo involvement of cardiolipin in interaction with DnaA or control of initiation of replication has not been directly demonstrated. However, it has been shown that cells can survive well with very little cardiolipin but not at all with none (Ohta et al., 1981). This implies that its structural role is not as important as some other essential functions it may perform; indeed a requirement for phospholipid synthesis for initiation of replication has been suggested in the past (Pierucci and Rickert, 1985). An inhibitory effect of phenethyl alcohol (PEA), which causes strong inhibition of phospholipid synthesis, on initiation of replication in vivo has been observed (Wada and Yura, 1974). Very recently further evidence has emerged which strongly supports a crucial role of phospholipids in initiation of replication at oriC (Xia and Dowan, 1995). Inactivation of the pgsA gene (encoding phosphatidylglycerophosphate synthase) limits the synthesis of the major acidic phospholipids and leads to arrest of cell growth. A mutation in the rnh gene (which encodes RNase H) that bypasses the need for the DnaA protein through induction of constitutive stable DNA replication (Section 1.10.6) also suppresses the arrested growth associated with the pgsA mutation. The maintenance of oriC plasmids, which remain dependent upon DnaA, was still compromised indicating a role of phospholipids specifically in DnaA-mediated initiation of replication at oriC.

A requirement for phospholipid catalysed nucleotide exchange is that the phospholipids are present in a fluid lipid bilayer. Phospholipids derived from membranes lacking oleic acid, an unsaturated fatty acid, are not active in vitro. Unsaturated fatty acids contribute to the fluidity of the membrane by destabilising the regular stacking of the saturated fatty acids (Yung and Kornberg, 1988; Castuma et al., 1993). These in vitro results are supported by the in vivo observation by Fralick and Lark (1973) that 3-decynoyl-N-acetyl cysteamine, a drug which inhibits the synthesis of unsaturated fatty acids in E. coli, prevents reinitiation of replication in temperature-sensitive dnaA and dnaC mutants after a shift back from the restrictive temperature. Addition of oleic acid relieves this block.

Aggregated DnaA protein complexed with phospholipids is inactive in a purified in vitro replication system but active in a crude system; therefore the latter must contain a factor which can activate the aggregated protein. Two candidates have been found: phospholipase A2 (or its counterpart in E. coli, phospholipase A1) and DnaK (in the presence of ATP) are both able to disaggregate the complexed DnaA, each with a different mode of action. Phospholipase is likely to act by degrading the phospholipids, whereas DnaK seems to interact with the DnaA protein as it, in
contrast to phospholipase, is not inhibited by increased amounts of phospholipids. Neither enzyme enhances the activity of the monomeric form of DnaA so their role in reactivation is limited to liberating DnaA from the aggregate (Hwang et al., 1990).

A separate pathway for the regeneration of DnaA-ATP has been reported by Hughes et al. (1988). cAMP rapidly eliminates bound ADP and in the presence of ATP the protein can be restored to its active form; cAMP does however not release ATP. cAMP binds to DnaA with a $K_D$ of 1 $\mu$M, which is in the concentration range of cAMP in vivo, and stimulates the binding of DnaA to $oriC$ and the $mioC$ promoter. The binding site for cAMP is probably distinct from that of ATP and ADP as ATP binding is not inhibited by cAMP, cAMP binding is not inhibited by ADP and the ATP inhibition shows non-competitive kinetics. Presumably ATP induces a conformational change that renders DnaA refractory to cAMP.

There is some evidence for a second ATP binding site on the DnaA protein with a lower affinity for the nucleotide. High levels of ATP (5 mM) are needed to form the open complex at $oriC$ (Sekimizu et al., 1988a). When DnaA-ATP is exposed to 4 mM ADP the protein is prevented from responding to higher levels of ATP by subsequently forming the open complex. Presumably irreversible binding of ADP to this low affinity site is responsible for the inactivation (Yung et al., 1990).

1.5.3 The Bacillus subtilis DnaA protein.

The Bacillus subtilis DnaA homologue has been purified and characterised (Fukuoka et al., 1990). It binds ATP with high affinity (20 $\mu$M) but does not bind cAMP. Binding of DnaA to DNA occurs most readily to the sequence TTATCCACA but also to sequences differing from it by one base or even by two bases but only if located adjacent to a perfect binding site.

B. subtilis DnaA protein is toxic when introduced in high amounts into E. coli as it interferes with the activity of the host protein, probably by forming inactive mixed oligomers (Andrup et al., 1988).
1.6 *dnaA* mutants and their properties

1.6.1 Temperature-sensitive mutants.

The physiology of strains carrying temperature-sensitive mutations in the *dnaA* gene has been well studied. Hirota *et al.* (1968) isolated two types of slow-stop mutants both of which showed residual DNA synthesis after a shift to restrictive temperature: one class which showed dependence of the amount of residual synthesis on external conditions such as temperature and growth medium and another class in which this synthesis was independent of these conditions and where the increase of the amount of DNA was found always to be about 40% of that initially present. This increase is what is theoretically expected if all ongoing rounds of replication are able to be completed but no new rounds can be initiated (Maaløe and Hanawalt, 1961) and it is to this latter class that all temperature-sensitive *dnaA* alleles have been found to belong.

Mutated sites within *dnaA* were mapped using marker rescue analysis (Hansen *et al.*, 1984) and correlations were found between the locations of the mutations and phenotypic characteristics such as reversibility and suppression, providing further evidence for functional domains within the protein. A more detailed sequence analysis of the mutants revealed that several pairs of mutants initially thought to be distinct share the same sequence and that a number contain more than one point mutation (Table 1.1). For instance *dnaA508* allele is mutated in two positions in the amino terminal part of the protein and each of the mutants found to be altered in the putative ATP binding site also has a second mutation elsewhere, suggesting that the initial mutation is too severe to permit viability and must be partly suppressed by secondary mutation (Hansen *et al.*, 1992). The mutants vary in the degree of their temperature-sensitivity and a major distinction occurs between those that are reversible (i.e. they can resume DNA synthesis upon a shift back from the restrictive to the permissive temperature without additional protein synthesis) and those that are not. The reversible mutants are all altered in the ATP binding site with the exception of *dnaA167*. All the mutant proteins retain some activity at the restrictive temperature as their phenotypes can be partly suppressed by overproduction of mutant protein. A recent study showed that two mutants (*dnaA508* and *dnaA204*) which have been classified as irreversible do possess significant activity upon a return from the restrictive to the permissive temperature; initiation of replication takes place after only a very short period of protein synthesis at low temperature suggesting that the DnaA204 and DnaA508 proteins have initiation capacity after a return to the permissive temperature which is just below the threshold required for initiation to occur (Hansen, 1995; Hansen and
Table 1.1 Characteristics of dnaA mutants.

**dnaA<sup>ts</sup> alleles**

<table>
<thead>
<tr>
<th>dnaA allele</th>
<th>508</th>
<th>167</th>
<th>46</th>
<th>601</th>
<th>602</th>
<th>604</th>
<th>606</th>
<th>5</th>
<th>205</th>
<th>203</th>
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<tbody>
<tr>
<td>Position of amino acid change&lt;sup&gt;1&lt;/sup&gt;</td>
<td>28</td>
<td>157</td>
<td>184</td>
<td>184</td>
<td>184</td>
<td>184</td>
<td>383</td>
<td>389</td>
<td>411</td>
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<tr>
<td></td>
<td>80</td>
<td>252</td>
<td>296</td>
<td>347</td>
<td>427</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation in ATP binding site</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reversibility&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cold-sensitivity of merodiploid&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>nt</td>
<td></td>
<td></td>
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<tr>
<td>Asynchrony index&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.3</td>
<td>0.4</td>
<td>1.3</td>
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<td>1.3</td>
<td>1.2</td>
<td>0.7</td>
<td>0.2</td>
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</tr>
<tr>
<td>Suppression by GroEL&lt;sup&gt;4&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>nt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seqA&lt;sup&gt;12&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Suppression by rpoB&lt;sup&gt;5&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nt</td>
<td>-</td>
<td>nt</td>
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<tr>
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<tr>
<td>rpoB&lt;sup&gt;904&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>nt</td>
<td>-</td>
<td>nt</td>
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</table>

**Intragenic suppressors of dnaA<sup>ts</sup> alleles**

<table>
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<th>amino acid position of suppressor</th>
</tr>
</thead>
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<td>156</td>
</tr>
<tr>
<td></td>
<td>252</td>
<td>271</td>
</tr>
<tr>
<td>dnaA&lt;sup&gt;508cos87&lt;/sup&gt;</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

**Other dnaA alleles**

<table>
<thead>
<tr>
<th>dnaA allele</th>
<th>type</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaA&lt;sub&gt;215&lt;/sub&gt;&lt;sup&gt;8&lt;/sup&gt;</td>
<td>opal (trp&lt;sub&gt;117&lt;/sub&gt;-UGA)</td>
</tr>
<tr>
<td>dnaA&lt;sub&gt;91&lt;/sub&gt;,&lt;sub&gt;311&lt;/sub&gt;,&lt;sub&gt;366&lt;/sub&gt;&lt;sup&gt;9&lt;/sup&gt;</td>
<td>amber, not sequenced</td>
</tr>
<tr>
<td>dnaA&lt;sub&gt;17&lt;/sub&gt;,&lt;sub&gt;452&lt;/sub&gt;&lt;sup&gt;10&lt;/sup&gt;</td>
<td>amber, not sequenced</td>
</tr>
<tr>
<td>dnaA&lt;sub&gt;801&lt;/sub&gt;,&lt;sub&gt;803&lt;/sub&gt;,&lt;sub&gt;806&lt;/sub&gt;&lt;sup&gt;11&lt;/sup&gt;</td>
<td>amber, not sequenced</td>
</tr>
</tbody>
</table>

INTRODUCTION

Atlung, 1995a). The dnaA205 mutation on the other hand appears to be truly irreversible (Hansen, 1995).

The dnaA mutants share another common characteristic in that they show asynchrony of initiation of replication even at the permissive temperature. In wild-type *E. coli* replication is triggered simultaneously at all origins present in the cell at a certain stage in the cell-cycle (Skarstad et al., 1985 and 1986). In the dnaAts mutants this co-ordination is lost, a phenomenon termed asynchrony, the degree of which is allele specific. Mutants altered in the ATP binding domain show far greater asynchrony than those with mutations elsewhere and this asynchrony associated with the ATP binding domain is independent of temperature; in other mutants asynchrony becomes more pronounced with increasing temperature. Taking the dnaA46 allele as an example it seems that maintenance of synchrony is an intrinsic property of the protein; asynchrony is not due to a lack of active protein as it occurs at all temperatures in this and similar mutants and is not relieved by overproduction of the mutant protein (Skarstad et al., 1988).

The alleles vary in their patterns of suppression by mutations in rpoB (Table 1.1). Although all dnaAts alleles tested can be suppressed by an rpoB mutation, they cannot all be suppressed by the same one (Atlung, 1984). This will be discussed further in Section 1.10.1.

It has been shown that all the mutant alleles are recessive to the wild-type (Gottfried and Wechsler, 1977; Wechsler, 1980; Hansen et al., 1984). This is at variance with earlier reports stating the reverse (Beyersmann et al., 1974; Zahn et al., 1977) or that dominance is allele and even medium dependent (Murakami et al., 1980). This contradiction could be due to the fact that many merodiploids are cold-sensitive. In early experiments, when this fact was still unnoticed, this would have interfered with the construction of stable merodiploids at low temperature and would account for erroneous conclusions being drawn. This notion is supported by the fact that Wechsler (1980) showed that certain heterozygous diploids are cold-sensitive. In early experiments, when this fact was still unnoticed, this would have interfered with the construction of stable merodiploids at low temperature and would accumulate of additional mutations at low temperature. Cold-sensitivity is only seen in heterozygotes carrying the ATP binding site mutations; these mutants also show cold-sensitivity if the mutant protein is overproduced in the absence of wild-type DnaA (Hansen et al., 1992). Cold-sensitivity is probably due to overinitiation of replication at low temperature (von Meyenburg and Hansen, 1987).

A positive role of DnaA in replication has been inferred from studies of the behaviour of dnaAts mutants. If reversible mutants such as dnaA46, dnaA5 or dnaA167 are incubated at the restrictive temperature and subsequently returned to the permissive temperature a burst of initiation is observed. The amount of reinitiation was
INTRODUCTION

found to be proportional to the length of the incubation at high temperature (Abe and Tomizawa, 1971; Evans and Eberle, 1975; Tippe-Schindler et al., 1979; Eberle and Forrest, 1982). A similar accumulated capacity for initiation can be expressed by treating reversible dnaA\textsuperscript{ts} mutants grown at temperatures between permissive and restrictive with chloramphenicol or through a nutritional shift up (LaDuca and Helmstetter, 1983); in both cases stable RNA synthesis is stimulated (Shen and Bremer, 1977; Shepherd et al., 1980). In conjunction with other data this indicates that DnaA acts at initiation of replication at a stage requiring RNA synthesis. This will be discussed further in Sections 1.7.4 and 1.7.5.

In wild-type E. coli initiation occurs at a virtually constant ratio of origins per cell mass, known as the initiation mass (Donachie, 1968); further evidence for a role for DnaA in regulation of initiation comes from the fact that the initiation mass of dnaA\textsuperscript{ts} mutants is higher than that of a wild-type strain at permissive temperature and increases with temperature indicating that the mutant product is less active than the wild-type under all conditions (Frey et al., 1981; LaDuca and Helmstetter, 1983).

The products of the dnaA46 and dnaA5 genes have been purified and characterised in vitro (Hwang and Kaguni, 1988a; Hupp and Kaguni, 1993a). DnaA46 protein does not bind ATP, binds oriC with much less affinity than wild-type DnaA and does not bind other DnaA-boxes such as are present in the mioC promoter and the pBR322 origin. In vitro as in vivo (Tippe-Schindler et al., 1979) it is reversibly thermolabile; its reduced ability to bind oriC is, however, not diminished at high temperature though its replication activity in a crude enzyme system is diminished. DnaA46 at permissive temperature in such a system shows a pronounced lag compared with wild-type protein before incorporation of deoxyribonucleotides and unlike wild-type DnaA is totally inactive in replication systems using purified enzymes (Hwang and Kaguni, 1988b). This difference is due to the fact that DnaA46 is activated by factors present in the crude system, one of which was identified as DnaK, the other probably being GrpE (Hupp and Kaguni, 1993c). It seems that the thermolability of DnaA46 is, at least in vitro, in the interaction with these activating factors rather than in its subsequent role in initiation of replication. It also seems likely that the lag before replication with the DnaA46 protein reflects time needed for the mutant protein to be activated (Hwang and Kaguni, 1988a, 1988b and 1991).

DnaA5 is similar to DnaA46 in its failure to bind ATP in vitro, in the lag preceding replication in vivo and in a crude system, as well as in its inability to promote replication in a purified enzyme system. It differs in its ability to bind DnaA-boxes; it binds with high affinity to the dnaA and rpoH promoter regions and forms complexes indistinguishable from those with the wild-type protein. It binds oriC well
but forms an altered nucleoprotein complex especially at the AT-rich region to the left of the origin (Hupp and Kaguni, 1993a). DnaA5 forms inactive complexes with wild-type DnaA and it has been suggested that the \textit{in vivo} cold-sensitivity of merodiploids may be due to this fact; it is odd in this light that the \textit{dnaA46} product, which also leads to cold-sensitivity in heterozygous diploids \textit{in vivo}, does not seem to interfere with replication when mixed with wild-type DnaA \textit{in vitro} (Hwang and Kaguni, 1988a). An indication that it may do so \textit{in vivo} comes from work by Boye \textit{et al.} (1988) who demonstrated that a wildtype strain carrying a plasmid with the \textit{dnaA46} allele still initiates replication asynchronously.

DnaA5 is only active in purified enzyme systems if DnaK and GrpE are present (Hupp and Kaguni, 1993b and 1993c). An absolute requirement for RNA polymerase has also been shown. It seems that DnaA5, in addition to its need for "activating factors" may have a greater requirement for transcriptional activation of the origin than does the wild-type protein because of a reduced capacity in the strand opening reaction. This may be in keeping with the observation that \textit{dnaAts} mutants are exceptionally sensitive \textit{in vivo} to rifampicin (Orr \textit{et al.}, 1978).

What exactly is the action of DnaK and GrpE in promoting DnaA5 activity is uncertain. It seems different from the action of DnaK in disaggregating DnaA/phospholipid complexes in that this is independent of GrpE. It is also distinct from their role in \textit{\lambda} replication since here GrpE is again not absolutely required, though DnaJ is, which in contrast acts negatively on activation of DnaA mutant proteins. In \textit{\lambda} replication DnaK acts after the formation of an initiation complex by releasing DnaB from \textit{\lambda}P protein (Zylicz \textit{et al.}, 1989) whereas its action is before formation of a complex in the \textit{in vitro} ori\textit{C} systems (Hupp and Kaguni, 1993c). Whether wild-type DnaA interacts with DnaK and GrpE is not clear. \textit{dnaK} mutants are deficient in initiation of replication (Sakakibara, 1988; Ohki and Smith, 1989) and DnaA and DnaK have been shown to interact \textit{in vitro} (Malki \textit{et al.}, 1991). However, there is no effect of DnaK on DnaA promoted replication in extracts made from \textit{dnaK} mutants (Malki \textit{et al.}, 1991) so it is possible that DnaK only acts on particular forms of DnaA such as inactive aggregates or misfolded protein or that it acts only under extreme conditions such as high temperature; a \textit{dnaK} null mutant is viable at 30°C (Bukau and Walker, 1989).

Two reports show that DnaA46 is degraded at high temperature (Sakakibara and Yuasa, 1982; Katayama and Nagata, 1991). The same has been reported concerning DnaA167 (Sakakibara and Yuasa, 1982). This is in contrast to the wild-type protein which appears stable at all temperatures (Sakakibara and Yuasa, 1982). The fact that there is a build-up of DnaA\textit{ts} protein at high temperature, which can
trigger initiation upon a return to the permissive temperature, must indicate that this degradation is less rapid than the synthesis of new protein.

1.6.2 Cold-sensitive mutants.

The isolation of cold-sensitive mutants in dnaA has been reported (Wehr et al., 1975). These show different kinetics from the temperature-sensitive mutants in that the residual DNA synthesis at low (restrictive) temperature is approximately 100% over fourteen hours. This implies that initiation has not been completely blocked but has been drastically reduced. That it is initiation and not chain elongation which is affected is demonstrated by the fact that these mutants can be suppressed by introduction of an alternative, DnaA independent, origin into the chromosome at which replication will then be initiated.

A different class of cold-sensitive mutant consists of the intragenic suppressors of dnaA46 and dnaA508, named dnaA46cos and dnaA508cos (Kellenberger-Gujer et al., 1978; Eberle et al., 1989). Their phenotypes are similar; both grow as well as the wild-type parent at high temperature but overinitiate replication at low temperature. Sequencing of the mutants revealed that dnaA46cos contains two mutations in addition to the two present in dnaA46 (Braun et al., 1987); dnaA508cos contains a single additional mutation changing its start-codon from GTG to ATG (Eberle et al., 1989). Overinitiation in dnaA508cos could simply be due to higher levels of mutant protein as an ATG start-codon has been shown to be more efficient in translation than GTG, though less so at low temperature than at 37°C (Shinedling et al., 1987). Also, levels of dnaA508cos transcripts were elevated compared with the wild-type implying that autoregulation may also be affected. Presence of DnaA508cos cannot complement dnaA5 showing that the mutation does not restore DnaA508cos to a conformation with equal activity to that of the wild-type (Eberle et al., 1989).

It was proposed that the dnaA46cos mutation may also lead to overproduction of the mutant protein resulting in overinitiation at low temperature (Braun et al., 1987). Recently, however, this has been shown not to be the case. The DnaA46cos protein has been purified and studied in vitro (Katayama, 1994). It binds neither ATP nor ADP and shows a reduced in vitro replication activity at high temperature. DnaA46cos remains competent for replication at low temperature for a longer period of time than the wild-type protein in a crude extract. In addition, it has been found that wild-type DnaA is gradually inactivated by an unidentified soluble factor to which DnaA46cos is immune (Katayama and Crooke, 1994). It therefore seems that the overinitiation caused by DnaA46cos is due to its being inert to a negative regulator of
DnaA activity which may be linked to the conversion of DnaA·ATP to DnaA·ADP. This could explain why de novo protein synthesis, which is normally required for replication, is not required for initiation to occur upon a shift of a dnaA46cos mutant to the restrictive temperature.

In vivo suppression of the dnaA46cos mutation by λdv, a plasmid using the λ replication system, indicates an interaction between DnaA and DnaB (Kellenberger-Gujer and Podhajska, 1978). λdv produces λP protein which is known to interact with DnaB (Dodson et al., 1985) and sequesters it; this is lethal in a dnaA46 strain at permissive temperature, though not in a wild-type, and relieves cold-sensitivity in a dnaA46cos strain. Also, high levels of a DnaB analogue from P1 phage (Ban), which produces heteromultimers of reduced activity, suppresses dnaA46cos (Frey et al., 1984). Hence, an alternative explanation for the overinitiation by dnaA46cos is an increased capacity to deliver DnaB to the replication complex.

1.6.3 Nonsense mutations.

Several nonsense mutations in dnaA have been isolated (Table 1.1). Schaus et al. (1981a) isolated three amber mutants (dnaA91, dnaA311 and dnaA366) which lead to a rapid cessation of replication when shifted to high temperature in strains carrying a temperature-sensitive suppressor. One of the mutations, dnaA311, produces a partially active product as it can be suppressed by a mutation in rpoB (Schaus et al., 1981b).

Amber mutants dnaA801, dnaA803 and dnaA806 isolated by Kimura et al., (1979) are probably located within dnaN. Further amber mutations isolated by them (Kimura et al., 1980), dnaA17 and dnaA452, have not been further characterised. One opal mutation, dnaA215, has been sequenced (Wende et al., 1991).
1.7 DnaA in initiation of chromosomal replication

Over the years many roles have been attributed to the DnaA protein but there is no doubt that its primary function is in the biochemistry of initiation of chromosomal replication; it fulfils this capacity in all prokaryotes so far studied. In *E. coli* initiation takes place at a unique site, oriC (Oka *et al.*, 1980); replication proceeds bidirectionally around the chromosome (Masters and Broda, 1971) and is terminated at sequences named terC, which are located more or less diametrically opposite oriC (Pelletier *et al.*, 1988). The origin of replication, the events which take place there and the involvement of DnaA therein are the subject of this section.

1.7.1 The origin of replication.

oriC was found to be located at 84 minutes on the genetic map (Miki *et al.*, 1978; Bachmann, 1990) and was sequenced by several groups (Meijer *et al.*, 1979; Sugimoto *et al.*, 1979; Bühl and Messer, 1983). The minimal part of this region carrying the cis acting elements necessary for initiation to occur successfully was delimited by Oka *et al.* (1980) to a 245 bp segment, though it has since been shown that a small additional AT-rich sequence located just outside this segment is also essential for efficient initiation (Asai *et al.*, 1990).

Comparison of the sequences of the replication origins from various bacteria shows that those of the enterobacteriaceae are very well conserved and that even the origin of a relatively distantly related bacterium *Vibrio harveyi* functions in *E. coli* (Harding *et al.*, 1982; Zyskind *et al.*, 1983). This and early mutational analysis revealed that the four DnaA-boxes, eight GATC sites and three AT-rich sequences in the left part of the origin are particularly well conserved and that deletions or insertions are generally less well tolerated than single base changes, even if these are located in conserved elements (Oka *et al.*, 1984).

Several promoters have been identified within the minimal origin: ori-pL in the right half of the region with transcription leftward and ori-pR at the right border transcribing rightward were discovered in vitro (Lother and Messer, 1981) and later in vivo and their transcripts were shown to terminate after about 150 bases (Schauzu *et al.*, 1987). An additional rightward promoter has since been found (Junker *et al.*, 1986; Asai *et al.*, 1992) in the left half of the minimal origin (ori-pR1). Other features of possible importance are binding sites for integration host factor (IHF) (Polaczek, 1990) and for factor for inversion stimulation (Fis) (Filutowicz *et al.*, 1992), both DNA bending proteins. A binding site in the AT-rich region exists for IciA (Hwang
and Kornberg, 1990; Thöny et al., 1991; Hwang et al., 1992), a putative negative regulator of initiation, and a site for right origin binding protein (Rob), a 33 kD protein of unknown function, was recently identified adjacent to DnaA-box R4 (Skarstad et al., 1993). A complex of oriC and gyrase has been visualised by electron microscopy and gyrase seems to bind between DnaA-boxes R3 and R4 (Lother et al., 1983). Finally, many reports show that oriC has affinity for certain membrane fractions (Wolf-Watz and Masters, 1979; Jacq and Kohiyama, 1980; Hendrickson et al., 1982) and that there are specific sites within and around the minimal origin which are bound by membrane proteins (Jacq et al., 1983; Kusano et al., 1984). Figure 1.3 shows the origin and some of its features.

Figure 1.3 The origin of replication and its environment.

The organisation of the oriC region. Above: Genes in the vicinity of oriC are shown and their direction of transcription is indicated. Below: Structural elements, protein binding sites and promoters within oriC are shown. The AT rich cluster and the AT rich 13-mers are shown to the left of oriC as boxes. DnaA-boxes are shown as pentagons indicating their orientation and are labelled R1—R4 and M. IHF and Fis binding sites are indicated by white boxes and promoters are represented by black arrow-heads indicating the direction of transcription. See text for details. Note that the top and bottom parts of the figure are drawn to different scales. Adapted from Woelker and Messer (1993).

The sequence of events at oriC leading up to the formation of two replication forks and the products involved in this process have been studied in vivo by looking at the phenotypes of various initiation mutants but far more extensively in vitro owing to the possibility of carrying out minichromosome replication in either crude cell extracts.
INTRODUCTION

(Fuller et al., 1981) or in a system reconstituted from purified enzymes (Kaguni and Kornberg, 1984; Funnell et al., 1986). As few as nine enzymes are sufficient for replication in vitro of a supercoiled oriC template: DnaA, HU or IHF, DnaB helicase, DnaC, DnaG primase, single strand binding protein (SSB), DNA polymerase III holoenzyme, gyrase and ligase (Skarstad et al., 1990). On the basis of studies with this and similar systems initiation of replication has been divided into several successive stages (Funnell et al., 1986; Sekimizu et al., 1988a) which will be discussed here.

1.7.2 Genetic evidence for the sequence of events.

Initiation of replication requires de novo protein synthesis. If this is prevented either by the addition of chloramphenicol or by amino acid starvation new rounds of replication are not initiated but elongation continues (Maaløe and Hanawalt, 1961; Bird and Lark, 1968). Similarly, untranslated de novo transcription by RNA polymerase is required: initiation is prevented by treatment with rifampicin or streptolydigin at a stage which is no longer sensitive to chloramphenicol, in other words after all proteins necessary for initiation have been synthesised (Lark, 1972; Messer, 1972).

More insight into the events emerged from the study of temperature-sensitive initiation mutants in dnaA and dnaC. Kung and Glaser (1978) constructed a dnaCcos, dnaAs double mutant with a restrictive temperature for DnaC at 20°C and for DnaA at 41°C. The fact that a shift from 20–41°C resulted in no synthesis of DNA in contrast to the reverse shift which led to a 35% increase in DNA synthesis, shows that the action of DnaA must precede that of DnaC. This was corroborated through the investigation by Zyskind et al. (1977) of the effects of various antibiotics on reinitiation of replication after the return to the permissive temperature of reversible temperature-sensitive initiation mutants, following incubation at high temperature. Reinitiation in neither dnaAs nor dnaCts strains is prevented by chloramphenicol when added just before the shift back to low temperature. Rifampicin does inhibit reinitiation in a dnaAs strain but not in a dnaCts strain, indicating that DnaA acts before or during a stage requiring transcription and DnaC acts later. Experiments with nalidixic acid (a gyrase inhibitor) showed that DnaA acts before and DnaC during a stage requiring the action of gyrase. The molecular basis of the action of these and other proteins involved in the initiation process has to some extent been solved by in vitro studies.
1.7.3 The initial complex.

DnaA protein binds to the four DnaA-boxes identified by Fuller et al. (1984) in oriC (R1-R4) and a fifth box, M (Matsui et al., 1985). The interaction with R3 and M is weaker than that with the other three DnaA-boxes (Matsui et al., 1985). The complex can be visualised by electron-microscopy and its size is dependent on the amount of DnaA added but a particular structure containing about twenty DnaA monomers per oriC template has been shown to be capable of promoting replication (Fuller et al., 1984; Crooke et al., 1993). Initial complexes formed with DnaA-ATP or DnaA-ADP are indistinguishable by footprinting (Crooke et al., 1993) as are complexes formed with supercoiled or relaxed template DNA (Woelker and Messer, 1993). However, DnaA-ATP and negative superhelicity of the DNA are absolutely essential for the complex to advance through its subsequent stages. In vivo footprinting of non-synchronised cultures showed that the DnaA-boxes R1, R2 and R4 are protected throughout the cell-cycle; protection of R3 and M was not detected but this does not preclude a transient interaction of the protein at these sites at a particular time during the cycle (Samitt et al., 1989). It is also possible that these sites are not bound in vivo and that the results of Fuller et al. (1984) were due to non-physiological concentrations of DnaA being used.

Mutational analysis revealed, as would be expected, that the DnaA-boxes are essential for the formation of the initial complex; a single DnaA-box is insufficient (Crooke et al., 1993). Surprisingly, mutations changing the binding capacity of individual DnaA-boxes, leaving the rest unchanged showed no phenotype in vivo; mutations decreasing the affinity for individual boxes as well as those increasing the affinity for R1 or R4 were tested (Holz et al., 1992). Even a double mutant with reduced binding at R1 and R4 still allowed in vivo replication of an oriC plasmid albeit at a lower rate. Footprinting analysis showed that mutation of one or two boxes did not affect binding at others, suggesting a model where oriC is arranged around a core of DnaA monomers and where the monomers bind to the individual DnaA-boxes independently of one another. The cooperativity seen in the kinetics of initial complex formation is therefore probably due to protein interactions rather than protein/DNA interactions (Holz et al., 1992). Certain mutations have been found which allow replication to occur in vitro but not in vivo: these are in the DnaA-box M and in the Fis binding site and indicate that initiation in vivo has additional requirements not elicited by the system reconstituted from purified components (Crooke et al., 1993).

Mutations altering the spacing between the DnaA-boxes show that increasing or decreasing the distance by one helical turn between DnaA-boxes R3 and R4 or between R2 and R3 results in a functional origin. Alterations by less than one helical
turn or by two helical turns abolish origin function showing that correct helical phasing is essential but not sufficient for activity (Messer et al., 1992; Crooke et al., 1993). Insertions and deletions are not tolerated between R1 and R2 so here the precise distance between the boxes is of importance (Messer et al., 1992). The AT-rich region is not required for initial complex formation (Crooke et al., 1993).

It seems likely that other proteins besides DnaA such as HU, IHF and Fis form part of the initial complex; a stimulatory effect of the histone-like protein HU was found in an *in vitro* replication system and could be involved in the formation or maintenance of the structure of the complex (Dixon and Kornberg, 1984); at high levels it appears to stabilise the structure (Baker and Kornberg, 1988). An indirect effect of HU on the efficiency of initiation of replication has been proposed by Bonnefoy and Rouvière-Yaniv (1992); they showed that HU modulates the binding of IHF to oriC. Low levels of HU stimulate binding whereas high levels inhibit it. IHF in turn could be involved in creating a bend in the DNA in such a way as to facilitate an interaction between DnaA protein and the AT-rich region in the formation of an open complex at oriC (Polaczek, 1990).

A role in replication *in vivo* for these proteins can be inferred from the fact that minichromosomes are not well maintained in mutants lacking HU or Fis and that cells carrying mutations in the *fis* or *him* genes (coding for Fis and an IHF subunit respectively) initiate replication asynchronously (Boye et al., 1993). On the other hand mutants with a defect in either the genes coding for HU or in *fis* or *him* are viable and even a combination of mutations is tolerated (Ogawa et al., 1989; Kano and Imamoto, 1990; Filutowicz et al., 1992). It is possible that the requirement for these proteins is not absolute and depends on conditions such as temperature or superhelicity of the chromosome; a *fis* null mutation increases the temperature-sensitivity of *dnaA*46 or *gyrB*ts mutants (Filutowicz et al., 1992).

A model for the initial complex has recently been proposed by Woelker and Messer (1993). It is based on extensive mutagenesis experiments and footprinting experiments using KMnO4 and T4 endonuclease VII to identify distortions and bulges in the complexed origin DNA and suggests that the DNA is wrapped around a core of DnaA protein. Distortions were found particularly in the left part of the origin, in the AT-rich region and in the right part of the origin, close to the start sites for DNA synthesis (Figure 1.4).
Figure 1.4 Model for the initiation complex at oriC.

Large black arrows represent DnaA-boxes, small black arrows represent KMnO$_4$ sensitive sites (distortions in the DNA). The IHF and Fis binding sites are shown by darker regions on the DNA where it requires bending. The AT rich regions are indicated by lighter shading. DNA synthesis start sites are represented by white arrow-heads. In this model 10 DnaA monomers, arranged as five dimers are bound to the DNA forming a structure in which all DnaA-boxes are oriented in the same direction. From Woelker and Messer (1993).

1.7.4 The open complex.

A key step in initiation is that of strand separation in the origin to allow assembly of the components required to prime and continue DNA synthesis. Bramhill and Kornberg (1988b) discovered that the function of the three conserved AT-rich 13-mers at the left of the origin is at this stage. Such AT-rich regions, although variable in number, length and sequence, are a general feature of origins which function in prokaryotes irrespective of whether or not they are DnaA dependent (Bramhill and Kornberg, 1988a) indicating that whatever the nature of the initial complex, strand separation is always achieved by a general mechanism (Figure 1.5).
**Figure 1.5** A comparison of AT-rich repeats in prokaryotic origins.

<table>
<thead>
<tr>
<th>Origin</th>
<th>AT-rich</th>
<th>Tightly bound</th>
<th>AT-rich repeat consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli oriC</td>
<td>13 13 13</td>
<td>DNA protein</td>
<td>GATCTnTTnTTTT</td>
</tr>
<tr>
<td>B. subtilis oriC</td>
<td>16 16 16</td>
<td>DNA protein</td>
<td>GATACCTAnTTTTTC</td>
</tr>
<tr>
<td>pSC101</td>
<td>13 13</td>
<td>RepA</td>
<td>GATCTnTTnTTTT</td>
</tr>
<tr>
<td>P1</td>
<td>7 7 7 7 7</td>
<td>P1 RepA</td>
<td>AGATCC(A,T)</td>
</tr>
<tr>
<td>F</td>
<td>8 8 8 8</td>
<td>E protein</td>
<td>TTTTTA(T,G)A</td>
</tr>
<tr>
<td>R1</td>
<td>9 9 9</td>
<td>R1 RepA</td>
<td>TTTAAnGA</td>
</tr>
<tr>
<td>R6K oriY</td>
<td>10 10 10</td>
<td>π protein</td>
<td>TATTnATTT</td>
</tr>
<tr>
<td>λ</td>
<td>11 11 11</td>
<td>O protein</td>
<td>TnTCTTTTGT</td>
</tr>
<tr>
<td>φ82</td>
<td>11 11</td>
<td>O protein</td>
<td>TTTGTCTTTTGT</td>
</tr>
<tr>
<td>φ80</td>
<td>6 6 6</td>
<td>O protein</td>
<td>TCTTGT</td>
</tr>
<tr>
<td>RK2</td>
<td>9 9 9</td>
<td>TrfA protein</td>
<td>GGT(T,A)AAAA</td>
</tr>
<tr>
<td>P4</td>
<td>9 9 9</td>
<td>α protein</td>
<td>CACTTAAG</td>
</tr>
</tbody>
</table>

The numbers indicate the lengths in nucleotides and approximate locations of the AT-rich repeats in each origin. Regions tightly bound by an origin specific protein are hatched. Boxes marked 'A' indicate DNA binding sites, based on either experimental evidence of binding or on close sequence similarity to known binding sites. In the consensus sequences for the AT-rich repeats the nonspecific positions are marked 'n'. Adapted from Bammell and Kornberg (1988a).
The location of initial duplex opening coincides with that of the AT-rich sequences and deletion of either the left or middle 13-mers results in a non-functional origin. Duplex opening still occurs to some extent in such mutants but is abolished if the right 13-mer is deleted. This and consideration of the energetics involved in the strand separation reaction led to a model where the 13-mers are opened sequentially starting from the right (Bramhill and Kornberg, 1988b). An indication for this *in vivo* is that if initiation is arrested in a dnaC<sup>ts</sup> mutant, only the right 13-mer is unwound (Gille and Messer, 1991). An additional 12 bp AT-rich sequence to the left of the 13-mers has been identified to be essential for the strand opening reaction *in vivo*. It has no sequence requirement other than a high AT content (Asai *et al.*, 1990).

It has been shown that the AT-rich region unwinds *in vitro* to some extent without the action of the replication proteins if the DNA is sufficiently supercoiled and in the absence of Mg<sup>2+</sup> (Kowalski and Eddy, 1989; Gille and Messer, 1991; Messer *et al.*, 1991). With Mg<sup>2+</sup> melting was dependent upon the addition of DnaA protein (Gille and Messer, 1991) and despite the fact that footprinting experiments do not show an interaction between DnaA and the AT-rich region (Hwang and Kornberg, 1992b) several studies point to an active role for DnaA in formation of the open complex, thought to be an interaction with the right and middle 13-mers. Firstly, the integrity of the sequence of the right and middle 13-mers is important unlike that of the left 13-mer which can be replaced with heterologous DNA of high AT content (Kowalski and Eddy, 1989; Asai *et al.*, 1990; Hwang and Kornberg, 1992b). The sequence of the right 13-mer is also better conserved than that of the other two (Zyskind *et al.*, 1983). Secondly, although a change in the spacing between the middle and left 13-mers is tolerated (Bramhill and Kornberg, 1988b) any alteration, even by only one basepair, in the distance between the 13-mers and the DnaA-boxes drastically reduces *in vivo* and *in vitro* replication activity. The absolute requirement for correct spacing implies a direct interaction of a protein with both elements, the prime candidate for which is DnaA (Hsu *et al.*, 1994). Thirdly, sensitivity of one strand of the 13-mers to P1 endonuclease (which cleaves single stranded DNA) is much greater than that of the other, indicating that one strand interacts tightly with a factor, possibly DnaA (Hwang and Kornberg, 1992a). Fourthly, Yung and Kornberg (1989) showed that DnaA interacts specifically with the 13-mers in filter binding assays and that DnaA can be displaced from the open complex by oligomers carrying the AT-rich 13-mers. A difference between the ATP and ADP bound forms emerges here: DnaA-ADP is more readily displaced than DnaA-ATP, indicating that an open complex formed with the latter is more stable.
Open complex formation requires a relatively high concentration of ATP (5 mM), which can be substituted by CTP or dATP but not by the non-hydrolysable analogue ATP\textsubscript{y}S (Bramhill and Kornberg, 1988b). Two separate requirements seem to exist for ATP: initially an allosteric effect in binding to a high affinity site in DnaA for which ATP can be substituted by ATP\textsubscript{y}S (Sekimizu et al., 1987) and subsequently a role at a putative low affinity site which requires hydrolysis, presumably to produce energy for the strand opening or helicase entry, again implying an active role for DnaA beyond the formation of the initial complex. It seems that the DnaA monomers within the initial complex are assigned different roles depending upon their exact location: some will interact only with a DnaA-box and other monomers, others will interact also with the AT-rich 13-mers and still others may interact with the DnaB/DnaC complex at a later stage. This model reconciles the need for hydrolysable nucleotides with the observed low level of hydrolysis between formation of the initial complex and the transition through the strand opening reaction to a prepriming complex (Sekimizu et al., 1987). Perhaps hydrolysis occurs only in a subset of molecules. Also this model proposes that the DnaA protein exhibits a dual sequence specificity; it would not be unique in this respect, as a similar property is found with the λ Int protein (Richet et al., 1986).

Yung and Kornberg (1989) found that in vitro open complex formation could only be achieved at a relatively high temperature but that this requirement was alleviated to an extent by the addition of low levels of HU protein, though not by high levels. IHF can substitute for HU and does not become inhibitory at higher concentrations. It seems likely that both proteins act via the introduction of bends in the DNA and it is known that this can favour strand separation (Ramstein and Lavery, 1988). HU which binds non-specifically to DNA could, when present at high concentrations, become inhibitory to strand separation by constraining the superhelicity of the template.

Each of the 13-mers contains a GATC sequence which is the target for Dam methyltransferase. Methylation induces bending in the origin (Kimura et al., 1989) and is known to facilitate unwinding of DNA (Yamaki et al., 1988), yet the GATC sites in the middle and left 13-mers appear not to be essential for their function. The one present in the right 13-mer cannot be changed but this could be due to its possible role as a recognition sequence for DnaA (Asai et al., 1990).
INTRODUCTION

1.7.5 DNA topology and transcriptional activation.

The study of minichromosomes has shown that their replication is particularly sensitive to mutations which affect chromosomal supercoiling. Their superhelical density is less than that of most other plasmids and is comparable to that of the chromosome. Minichromosomes are unstable in mutants such as gyrB with decreased superhelicity (Leonard et al., 1985) and in vitro negative superhelicity is essential to effect open complex formation (Sekimizu et al., 1988a; Woelker and Messer, 1993). If superhelicity is low the open complex can be formed if an RNA-DNA hybrid (R-loop) is present at, or close to, oriC (within 500 bp) (Baker and Kornberg, 1988); it is proposed that in this case unwinding of DNA is initiated outside oriC and is propagated into the origin as intervening GC rich stretches block the activating action of the R-loop (Skarstad et al., 1990). Open complex formation is, however, still dependent on the presence of the AT-rich region (Baker and Kornberg, 1988). Apart from allowing initiation of replication to proceed with relaxed DNA, the R-loop assists open complex formation under a number of conditions where this would otherwise be impaired: at low temperature and when free negative superhelicity is constrained by excess HU protein (Skarstad et al., 1990) and therefore its action is described as "transcriptional activation" of the origin.

Whether this represents the transcriptional step in initiation which is affected by rifampicin in vivo (Lark, 1972) is uncertain as another form of transcriptional activation by RNA polymerase can be envisaged. A transcribing RNA polymerase generates positive supercoils in front of it and negative supercoils behind it (Liu and Wang, 1987) and therefore transcription away from the AT-rich region would be an activating factor of possible regulatory significance in open complex formation. Examination of the promoters in and around oriC show that two of these promoting transcription towards the 13-mers (mioCp and ori-pL) are repressed by DnaA (Asai et al., 1992; Schauzu et al., 1987; Lother et al., 1985; Nozaki et al., 1988) and one promoter for transcription away from them is activated by DnaA (Asai et al., 1992). This is an indication that DnaA may be involved in controlling the efficiency of initiation by differentially regulating positively and negatively acting elements in open complex formation. In keeping with the principle of activation of the origin by RNA polymerase, Theisen et al. (1993) and Ogawa and Okazaki (1994) showed cyclic variations of the levels of transcription in synchronous cultures from the mioC promoter and from the gidA promoter which is located to the left of the origin and promotes transcription away from it; gidAp transcription is most active immediately before the time of initiation of replication and is inhibited for a time thereafter, whereas mioCp transcription is shut off just prior to initiation.
On this basis it is tempting to assign to mioC transcription a regulatory role in the timing of initiation of chromosomal replication but this is not borne out by the fact that strains carrying various deletions of the mioC promoter show no deviation in cell-cycle parameters from those of the wild-type. Growth-rate, cell size, number of origins per cell and timing of initiation were normal under a variety of conditions (Løbner-Olesen and Boye, 1992).

This is in sharp contrast to its influence on the stability of minichromosomes. Inactivation of the minichromosomal mioC promoter, but not deletion of the coding region reduces minichromosome copy-number (Stuitje and Meijer, 1983). Removal of the DnaA-box in the promoter region or replacement of the natural promoter with one that is not regulated by DnaA also reduces copy-number (Stuitje et al., 1986; Løbner-Olesen et al., 1987) indicating the necessity for correct control of mioC transcription. In cells lacking HU protein, where minichromosome replication is impaired, the presence of mioC has been found to be particularly important for minichromosome maintenance (Ogawa et al., 1989).

A role in priming initiation by mioCp transcripts was inferred by Rokeach et al. (1987) from the discovery that some of these transcripts terminate in the origin at sites where RNA-DNA transitions were previously identified by Kohara et al. (1985); this role now seems unlikely as transcription from mioCp was shown to cease before the action of DnaC and therefore before the priming stage in the initiation process (Theisen et al., 1993). Despite its importance in minichromosome maintenance and the knowledge that mioCp is subject to complex control through Dam methylation (Schauzu et al., 1987) and the stringent response (Rokeach and Zyskind, 1986) its function in chromosomal replication remains obscure as does the exact nature of the rifampicin sensitive event in initiation.

The gidA promoter has not been as well studied as mioCp. A positive effect of its presence on the replication of minichromosomes has been reported as transcription promoted by it can relieve the slight negative effect on replication of certain DnaA-box mutations in the origin (Asai et al., 1990). gidAp, like mioCp, is subject to stringent control and a link between growth-rate and control of initiation of replication through the action of these two promoters has been suggested (Ogawa and Okazaki, 1994) although supporting evidence is lacking; the effect of a gidAp deletion on chromosomal replication would shed light on this matter but such a mutant has not yet been constructed.

If the importance of superhelicity for DNA unwinding is borne in mind and if the concept of transcriptional activation is correct, then genetic evidence supports a direct role of DnaA in open complex formation. This stems from the fact that dnaA18
mutants are particularly sensitive to both gyrase inhibitors such as coumermycin, nalidixic acid and novobiocin (Filutowicz, 1980) and to the transcriptional inhibitor rifampicin (Orr et al., 1978), both of which would be detrimental to strand separation through either a change in DNA topology or prevention of transcriptional activation. Furthermore, dnaAts and gyrBts mutants share a common suppressor in an rpoB mutation which increases negative supercoiling (Filutowicz and Jonczyk, 1981) and finally, some dnaA1ts mutations are suppressed by a topA mutation (an allele of the gene coding for topoisomerase I) which leads to a similar effect (Louarn et al., 1984). In combination with the observation that the DnaA5 protein requires the action of RNA polymerase to function in an in vitro replication system (Hupp and Kaguni, 1993b), it seems that transcriptional activation operates during an event which requires an active rather than a passive contribution on the part of the DnaA protein.

1.7.6 The prepriming and priming complexes.

Once initial strand separation at the origin has been achieved the DnaB protein is recruited to further unwind the DNA. DnaB is a helicase with affinity for single-stranded DNA and binding is improved through an allosteric effect of ATP complexed with the protein (Arai and Kornberg, 1981a). DnaB proceeds in the 5’→3’ direction along the strand of DNA and its action requires a hydrolysable ribonucleoside triphosphate (LeBowitz and McMacken, 1986). In vitro DnaB is required to direct the replication machinery to oriC as without its presence DNA polymerase III and primase do not show a preference for establishing themselves at that particular site (Baker et al., 1987). A complex containing only DnaA, DnaB and HU can be isolated from an in vitro replication reaction and is known as the prepriming complex. Electronmicroscopy reveals that it is larger than the open complex as an additional 50 bp from the left of oriC are involved in the structure (Funnell et al., 1987).

The functioning of DnaB depends entirely on the presence of another protein, DnaC, which appears to have no other function than to deliver DnaB to the initiation complex (Wahle et al., 1989b). DnaB and DnaC form a tight complex in the presence of ATP. A DnaB hexamer binds six DnaC monomers which in turn each bind one molecule of ATP (Kobori and Kornberg, 1982); nucleotide binding stabilises the complex and can be substituted for this purpose by ATPyS although this interferes with subsequent events (Wahle et al., 1989a). This is because DnaC must be released in order for DnaB to become active as a helicase; release requires ATP hydrolysis (Wahle et al., 1989b). Genetic evidence for an interaction between DnaB and DnaC exists; mutations in either dnaB or dnaC result in both initiation and elongation
phenotypes (Zyskind and Smith, 1977) suggesting that both proteins are involved in the same function in replication and Sclafani and Wechsler (1981) showed that the dnaB252 initiation mutant can be suppressed by oversupply of DnaC.

In addition to the genetics described earlier which imply an interaction between DnaA and DnaB through the suppression of the dnaA46cos mutation by λdv, recent work implicates the DnaA protein in formation of the prepriming complex. An interaction between DnaA and DnaB, either alone or complexed with DnaC, has been demonstrated in vitro using affinity chromatography (Marszalek and Kaguni, 1994). It has been suggested that this interaction may serve to help relocate DnaB from its presumed site of entry at the 13-mers in the left of the origin to where the start sites for DNA synthesis have been found at the right of oriC (Seufert and Messer, 1987; Messer and Weigel, in press). Unwinding of the template then occurs through strand separation by the helicase and the swiveling action of gyrase to remove positive supercoils that would otherwise accumulate (Baker et al., 1986).

The next step appears to involve a direct interaction of DnaB and DnaG primase (Masukata and Ogawa, 1980; Arai and Kornberg, 1981b) to form the priming complex. Despite speculation in the past that transcription by both RNA polymerase and by primase gives rise to primers (Ogawa et al., 1985; van de Ende et al., 1985) it is now generally accepted that in vivo the primers for replication are generated by primase alone and that the role of RNA polymerase is in open complex formation.

The events which follow formation of a prepriming complex are no longer dependent upon the action of DnaA. In vitro the protein can at this stage be recycled to promote initiation on a fresh template (Yung et al., 1990). In vivo there is some genetic evidence that DnaA may have some part to play in the elongation process as particular dnaAcos alleles can suppress a temperature-sensitive mutation in dnaX (formerly dnaZ), which encodes both the τ and γ subunits of DNA polymerase III holoenzyme (Walker et al., 1982; Blinkowa and Walker, 1983). Curiously, the suppressing dnaAcos alleles must be present on the chromosome and not on a plasmid for suppression to occur (Ginés-Candelaria et al., 1995). The basis of this suppression is obscure as DnaA has never otherwise been implicated in the elongation process and is certainly not an essential element of this.
1.8 Timing and control of the initiation of chromosomal replication

During a single cycle of growth and division the genetic material present in the cell must be duplicated once and once only. The frequency of chromosome replication is determined at the level of initiation of DNA synthesis and the understanding of the mechanism involved in this has been the aim of much research into the *E. coli* cell-cycle.

Observations made in the nineteen fifties and sixties laid the foundation upon which future models of cell-cycle control would be based. Early work by Meselson and Stahl (1958) showed that replication is a very regular process; each part of the chromosome is replicated at intervals of exactly one generation time. Another important but puzzling observation at the time was the fact that the DNA content of cells varies with growth-rate (Schaechter *et al.*, 1958). Furthermore, work by Cooper and Helmstetter revealed that initiation of replication occurs at a particular point in the cell-cycle which is different at different growth-rates (Cooper and Helmstetter, 1968; Helmstetter and Cooper, 1968). Also they defined the parameters which are still used to describe the timing of key events in the bacterial cell-cycle. The most important of these are the time required to replicate the chromosome (C) and the time interval between termination of replication and cell division (D), both of which are virtually constant at generation times of less than sixty minutes; C is equal to approximately forty minutes and D is around twenty minutes. At slower growth-rates the C period gradually increases and can become as long as 200 minutes at a generation time of ten hours (Kubitschek and Newman, 1978). Variations in the D period also occur at slow growth-rates but these are smaller and appear to be strain dependent (Kubitschek, 1974). Under conditions used in most laboratory experiments C and D will be more or less constant and together will amount to roughly sixty minutes. However, *E. coli* can grow with a generation time as short as twenty minutes. To achieve this the organism has developed the capacity to initiate a round of replication on a chromosome before the previous round has reached termination; as many as six replication forks can therefore be active on a single chromosome.

Donachie (1968) defined a new parameter by combining estimates of the average initiation age in *E coli B/r* with measurements of the average cell mass of *Salmonella typhimurium* and calculating from this the average cell mass per origin at the time of initiation, the so-called initiation mass. This was found to be independent of growth-rate. Direct measurements showed this to be true at growth-rates with a generation time of less than sixty minutes but showed also that at slower growth-rates the initiation mass increases with increasing growth-rate (Churchward *et al.*, 1981).
Independent support for a constancy of initiation mass from cell to cell comes from Koppes et al. (1978) who found that the volumes of cells at the time of initiation vary little.

The idea of a constant initiation mass led to two basic concepts of control of the timing of initiation: the "inhibitor dilution model" (Pritchard et al., 1969) and the "initiator accumulation model" (Sompayrac and Maaløe, 1973). The former requires a stable initiation repressor to be synthesised in a burst immediately following initiation of replication or alternatively, an unstable repressor to be expressed constitutively from a gene close to the origin of replication, in which case the gene dosage would double soon after initiation. Cell growth would then dilute the repressor until its concentration drops below a threshold at which initiation is no longer repressed, one generation time later. The initiator accumulation model proposes that a positive factor must increase to a certain critical level, either in amount or in concentration, in order to trigger replication and that this factor must then be inactivated. In order for there to be a coupling between the rate of synthesis of the initiator and increase in cell mass the initiator is proposed to be subject to autoregulation. These models and variations on them are discussed at length in a critical review by Bremer and Churchward (1991).

Objections are raised to both models and modifications and alternatives are presented; the authors conclude that a model based on positive regulation would best account for the relative constancy of initiation mass. Depending on the nature of the initiator molecule two types of control for its synthesis can be envisaged. Assuming that the initiator molecule executes its function of prompting initiation by binding to particular target sites and that this binding is rapid and tight, the absolute amount of initiator would determine the rate of filling of the target sites. On the other hand, if binding is slow or reversible it would be the concentration of the initiator which would determine the rate of binding to the target. In order to account for growth-rate invariance of initiation mass, the differential rate of synthesis of a tightly binding initiator must decrease with increasing growth-rate and the synthesis of a reversibly binding initiator per total protein should be constant with growth-rate.

A control mechanism for initiation must also take into account the more recent observation that all origins which are present in a normal rapidly growing cell initiate replication simultaneously, as determined by flow cytometric studies (Skarstad et al., 1985 and 1986). Strains in which this is altered include dam mutants and most dnaA18 mutants which exhibit variable interinitiation times (Tippe-Schindler et al., 1979; Bakker and Smith, 1989; Løbner-Olesen et al., 1994). In dnaA mutants the capacity to promote initiation and the capacity to achieve synchrony of initiation at multiple origins can be regarded as separate functions; suppression of temperature-sensitivity, either by
overproduction of the mutant protein or by introduction of a suppressing allele of \( rpoB \), and even complementation with a wild-type copy of the \( dnaA \) gene do not relieve asynchrony (Boye et al., 1988).

Experimental evidence strongly suggests that timing of initiation is indeed at least in part governed by the rate of synthesis of a positively acting factor, the DnaA protein, but that this protein acts in conjunction with negatively acting elements, the nature of which are only now coming to light.

1.8.1 Positive control.

A positive role for DnaA was first inferred from the accumulation of initiation potential in \( dnaA^{ts} \) mutants mentioned in Section 1.6.1. This was studied in detail by Eberle and Forrest (1982). If strains are kept at non-permissive temperature for a period in excess of what would be one generation time at the permissive temperature, they acquire a capacity to initiate more than one round of replication upon a subsequent return to the permissive temperature. This is thought to be due to a build up of reversibly denaturable DnaA\(^{ts} \) protein which cannot be used at the high temperature but which is initiation competent at the low temperature. In order to fully express the accumulated initiation capacity chloramphenicol must be added upon the return to the permissive temperature. It was originally supposed that this was to prevent synthesis of a negative regulator, however, it is also possible that chloramphenicol assists in open complex formation due to its stimulatory effect on RNA synthesis (LaDuca and Helmstetter, 1983).

The initiation mass is very sensitive to changes in the levels and activity of DnaA. Temperature-sensitive mutants such as \( dnaA46 \) and \( dnaA167 \) exhibit balanced growth over a range of temperatures between permissive and restrictive but there is a significant decrease in DNA content and origin concentration as the protein becomes progressively less active with increasing temperature (Hansen and Rasmussen, 1977; Fralick, 1978; Fralick, 1991). The introduction of multicopy oriC plasmids, presumed to titrate DnaA, does not normally affect chromosomal initiation in a wild-type strain, but becomes lethal in a \( dnaA^{ts} \) mutant (Stuitje and Meijer, 1983) suggesting that these mutant proteins are needed in higher concentrations than is the wild-type protein. A different kind of mutant with increased initiation mass has been reported which is neither sensitive to high or low temperature and which maps at or close to \( dnaA \) (Choung et al., 1981).

A decreased initiation mass is seen in strains carrying the \( dnaA46^{cos} \) mutation described earlier. In this case overinitiation occurs at low temperature (30°C) resulting
in cold-sensitivity (Kellenberger-Gujer et al., 1978). A similar phenotype is observed in strains carrying multiple copies of particular $dnaA^{ts}$ genes and in strains which are $dnaA^{+}/dnaA^{ts}$ heterozygous diploid. They have increased DNA content due to overinitiation at low temperature (Hansen et al., 1984; von Meyenburg and Hansen, 1987).

Thus, different mutations in DnaA have opposing effects on the initiation mass, indicating that DnaA is the limiting factor in initiation, at least in these mutant strains. When wild-type DnaA protein was initially overproduced no increase in the DNA content of the cell was observed, leading to the conclusion that the wild-type protein is in fact not limiting for initiation (Churchward et al., 1983). Further investigation showed this conclusion to be incorrect as overproduction of DnaA does lead to a greater concentration of oriC in the cell, but not to an overall increase in DNA because the extra forks initiated stall during elongation (Atlung et al., 1987; Pierucci et al., 1987); these effects are to some extent medium dependent (Xu and Bremer, 1988; Skarstad et al., 1989).

Løbner-Olesen et al. (1989) constructed a strain in which the $dnaA$ gene was expressed at a constant rate from the lac promoter induced by variable amounts of IPTG and showed that the initiation mass is increased at DnaA levels lower than wild-type but is reduced at high levels of $dnaA$ expression. At the $dnaA$ expression level resulting in cell physiology almost indistinguishable from that of the wild-type, initiation mass is very sensitive to small changes in the rate of DnaA protein synthesis, showing that under normal circumstances DnaA acts at the rate limiting step for initiation. Prolonged growth of cells having high levels of DnaA overproduction results in new steady states with a higher origin content compared with controls without induction of $dnaA$. The authors found that the maximum possible reduction in initiation mass was only twenty percent, which they attributed due to the fact that the availability of other components in the initiation process soon puts constraints on the amount of initiation that excess DnaA can promote. A later study using different experimental conditions showed that the reduction in initiation mass can in fact be as great as fifty percent (Atlung and Hansen, 1993).

As DnaA is involved in several successive stages of the initiation process it would seem likely that mutations altering other initiation factors which interact with DnaA, such as RNA polymerase and DnaB, would also influence the initiation mass. No DnaB mutant of this type has been reported; however, there are mutations in $rpoB$ and $rpoC$ which lead to an increased origin and DNA concentration (Tanaka et al., 1983). Initiation mass is reduced in a mutant carrying both an $rpoB$ allele known to suppress $dnaA^{ts}$ and a particular $rpoC^{ts}$ mutant (Rasmussen et al., 1983). Whether
this is due to an increased capacity in the strand opening reaction or to other effects is not clear. The rpoC<sup>ts</sup> mutation alone is sufficient to decrease the initiation mass and notably also increases levels of DnaA protein five-fold; therefore this could well be the mechanism by which other such mutations affect initiation (Petersen and Hansen, 1991). Increased chromosomal copy-number also results from increasing the temperature of a culture under conditions of lowered RNA polymerase activity. Low levels of rifampicin, mutation of rpoC, introduction of high copy-numbers of the rpoB and rpoC genes and high rpoH expression have this effect (Guzmán and Jiménez-Sánchez, 1986; Guzmán <i>et al.</i>, 1988; Jiménez-Sánchez <i>et al.</i>, 1994). Although this has been interpreted to be due to facilitated strand opening at high temperature in conjunction with a negative effect of reduced transcription on a putative inhibitor of initiation, an alternative explanation is that DnaA levels are altered under each of these circumstances and that this leads to reduced initiation mass. Evidence for this idea is derived from the discovery that heat shock in the presence of low levels of rifampicin increases expression from the dnaA promoters (cited in Messer and Weigel, in press). Thus, all known mutations which increase cellular DNA content (with the possible exception of an rpoB mutant) can, at least in part, be explained through an increase in levels of DnaA, though additional effects on strand opening and possible negatively acting elements are by no means excluded.

1.8.2 Negative control.

An argument for a form of post initiation negative control is implicit in the above. Overproduction of wild-type DnaA leads to only a small increase in overall DNA content while that in the rpoC mutant is two-fold higher and while overproduction of a mutant form of the protein such as DnaA46, or the presence of only a single copy of dnaA46<sup>cos</sup> causes lethality at low temperature due to runaway replication. Overproduction of wild-type protein has a greater effect on overall DNA content in a dnaAt<sup>ts</sup> strain than in a dnaA<sup>+</sup> strain (Churchward <i>et al.</i>, 1983). There appears to be a level of control which stalls or aborts replication forks if overinitiation is caused by the wild-type protein but which fails to work on replication initiated by, or even in the presence of, the mutant proteins. Overproduction of wild-type DnaA leads to a larger increase in DNA content if chloramphenicol or low levels of rifampicin are present, leading to the idea that these antibiotics may disengage the mechanism which inhibits replication fork movement after DnaA overproduction (Pierucci <i>et al.</i>, 1987); the rpoC mutant which increases the chromosomal DNA content of the cell (described in the previous section) may have a similar effect as it
also decreases transcriptional activity. If this were true it would seem that a mutation in dnaA would be able to circumvent this control mechanism only if DnaA itself were an activator or a part of this system. It has been observed that stalling of replication fork movement becomes greater with increasing DnaA concentration (Atlung and Hansen, 1993). Postinitiation control through stalling of a prematurely initiated round of replication has also been reported to occur in B. subtilis (Henckes et al., 1989).

Another form of negative control has already been mentioned in Section 1.6.2. Katayama (1994) has discovered a soluble factor which gradually inactivates DnaA but not DnaA46cos, thus explaining the occurrence of overinitiation in strains expressing this mutant protein. The same explanation could account for the cold-sensitivity seen in strains overexpressing DnaA46 or in dnaA46/dnaA+ heterozygotes. Owing to the lower activity of DnaA46 in initiation the inability for it to be inactivated will only be detrimental at high levels of the protein.

Limited evidence for a further negative regulator emerged from experiments to discover new proteins which bind the origin of replication. A 33 kD protein, IciA, was recovered which specifically binds as a dimer to the AT-rich 13-mers preventing strand opening by DnaA in vitro. Mutations in oriC on a minichromosome which abolish IciA binding result in a higher copy-number. However, deletion of iciA has no detectable phenotype in chromosomal initiation and overproduction of its product leads only to a slight delay in growth of cells diluted into fresh medium, a phenotype yet to be connected with initiation (Hwang and Kornberg, 1990; Thöny et al., 1991; Hwang et al., 1992).

1.8.3 Negative control through membrane attachment.

The best studied and clearest element of negative modulation of initiation comes through the study of the effects of methylation on initiation. If dnaAts mutants are grown at semi-permissive temperature for some time and then shifted back to the permissive temperature initiation capacity is increased, that is to say extra initiations take place (Hansen and Rasmussen, 1977). Initiations under these circumstances are triggered in waves where the first and subsequent rounds of initiation occur at intervals, which suggests that it takes some time for the cell to prepare itself for a new burst of initiation even if all the necessary proteins are present (LaDuca and Helmsatter, 1983; Hansen, 1995). Messer et al. (1985) observed that the spacing between bursts of initiation is reduced upon overexpression of the dam gene. They and Smith et al. (1985) also noted that dam- strains were transformed inefficiently by oriC plasmids, an observation that implied a positive role for methylation in initiation.
However, it was subsequently discovered that $dam^{-}$ strains could be easily transformed with unmethylated, although not with hemimethylated or fully methylated minichromosomes (Russel and Zinder, 1987). The difference in replication of unmethylated and hemimethylated DNA is only apparent in vivo, not in crude enzyme extracts (Landoulsi et al., 1989) suggesting the cell must contain an insoluble factor which prevents replication of hemimethylated DNA. A clue to the nature of this inhibitor came from the observation that complexes of oriC and outer membrane fractions can be isolated (Kusano et al., 1984; Yoshimoto et al., 1986), that these membrane fractions specifically bind hemimethylated DNA (Ogden et al., 1988) and that they can inhibit DNA replication in vitro on hemimethylated oriC templates (Landoulsi et al., 1990).

All DNA is transiently in a state of hemimethylation immediately after passage of the replication fork, before Dam methyltransferase has had the opportunity to methylate its target sites on the newly synthesised strand. Campbell and Kleckner (1990) made the key discovery that this state of hemimethylation persists for a longer than normal period of time at two specific locations on the chromosome. Remethylation at most sites is complete within five minutes but oriC and the dnaA promoter (though not sites in-between) fail to be remethylated for up to thirty percent of the cell-cycle; this period can be reduced by overproduction of Dam protein. A model was proposed in which oriC, immediately upon being replicated, is rendered refractory to further initiations through sequestration by the membrane and can only serve as a template for initiation once released, presumably through the action of Dam. In this way the levels of initiation proteins such as DnaA need not be exactly titrated but can rise beyond the threshold which triggers replication without giving rise to immediate reinitiation. Sequestration of the dnaA promoter would ensure that no further DnaA protein is produced until replication competent levels present in the cytoplasm have been sufficiently reduced, either by dilution or through inactivation.

Boye and Lønberg-Olesen (1990) studied DNA replication in vivo under conditions where the levels of Dam methyltransferase could be carefully controlled and found that initiation is normal only within a narrow window of dam expression. Both lower and higher levels of the protein result in more origins per cell and in asynchronous initiation, consequences which would be expected on the basis of Campbell and Kleckner’s model. Too little Dam would result in unmethylated origins which would escape sequestration; too much would reduce the period of sequestration and lead to premature reinitiation. Dam positively affects the expression of dnaA (Braun and Wright, 1986); however, such indirect effects cannot account for the
asynchronous phenotype observed as overexpression of dnaA does not affect the synchrony of initiation (Løbner-Olesen et al., 1989).

Factors involved in the sequestration process are beginning to be identified: Recently a protein, HobH, which is present in the outer membrane has been isolated. It specifically binds the methylated strand of hemimethylated origin DNA, preferentially in the left half of oriC where the majority of GATC sites are located. The unmethylated strand presumably remains partially accessible to Dam in order to eventually effect release of the DNA. A hobH mutant replicates asynchronously but not to the same extent as a dam mutant strain, indicating that other factors are also involved (Herrick et al., 1994). It has indeed been reported that origin binding capacity is not localised in a single membrane fraction. Both outer membrane fractions and fractions which differ in density and protein profile from inner and outer membrane have been shown to bind hemimethylated oriC DNA (Chakraborti et al., 1992). It may be that several distinct oriC/membrane complexes are formed.

In addition, a mutation, seqA, which allows transformation of dam mutant cells with fully methylated minichromosomes has been identified (Lu et al., 1994). In accordance with a presumed role in the sequestration process a seqA deletion results in a substantial (but not complete) reduction in the delay in remethylation of the origin following replication in a dam+ strain and multiple origins are initiated asynchronously. SeqA protein specifically binds hemimethylated origin DNA in vitro. In vivo it appears to modify the activity of the DnaA protein; seqA null mutants suppress the temperature-sensitivity of dnaA\textsuperscript{ts} mutants altered in the ATP binding site and overproduction of SeqA suppresses the overinitiation of a dnaA\textsuperscript{46cos} mutant, but exacerbates the temperature-sensitivity of dnaA\textsuperscript{ts} mutants. The genetically inferred interactions with the DnaA protein may be indirect but the possibility exists that DnaA itself is an effector in the sequestration process as suggested by the asynchrony phenotype of dnaA\textsuperscript{ts} mutants. Perhaps this is a clue to the different effects of overproduction of wild-type DnaA and excess initiation by DnaA\textsuperscript{46cos} on the cell; wild-type DnaA even in excess may still allow sequestration to occur (initiation is synchronous) whereas DnaA\textsuperscript{46cos} may escape this system.

1.8.4 Models for initiation control by DnaA.

It will be clear that many factors are potentially involved in the timing of initiation. However, computer models have shown that a simulation of experimentally obtained results concerning the cell-cycle can be achieved based on the simple assumption that it is the level of DnaA protein which sets the pace for rounds of
replication. Mahaffy and Zyskind (1989) based their model on the knowledge that DnaA is present in several forms in the cell, only DnaA-ATP being active in initiation, and on the premise that its synthesis is controlled by autoregulation and through the stringent response. A further feature of the model is the assumption that DnaA is released from the origin after initiation, immediately becoming available to bind to other origins which may be present, resulting in synchronous initiation. For this model to work growth-rate sensitive synthesis of the DnaA protein must be included in the parameters (Bremer and Churchward, 1991).

An alternative was proposed by Hansen et al. (1991b) with the assumption that free DnaA protein is bound, with high affinity, to DnaA-boxes present on the chromosome and that the binding sites in the origin have a lower affinity for the protein. An initial complex will only be formed if the number of DnaA molecules in the cell is in excess of the total number of binding sites (assuming one monomer per site). DnaA is released from the origin after initiation, as in the Zyskind model, and will be titrated by the new DnaA-boxes created during replication of the oriC proximal part of the chromosome. This model does not distinguish between active and inactive pools of DnaA protein and specifies no requirement for inactivation of the protein after initiation has taken place.

Though the two models differ in various respects they can both, through particular choices of parameters, reproduce, with the aid of a computer, a cell-cycle with constant initiation mass and regular divisions. In order to evaluate fully the role of DnaA in timing of initiation in a real organism it is essential that certain points be clarified: firstly, whether DnaA synthesis is in fact growth-rate sensitive, secondly, the relative abundances of DnaA-ATP and DnaA-ADP in the cell, and thirdly, what it is that determines their relative abundances. Further study of the membrane complexes seems crucial. The role of the membrane after initiation has occurred is beginning to be understood. However, there has been a report that periodically formed oriCl/membrane complexes exist before initiation and are a prerequisite for this event, implying that membrane attachment other than that through recognition of hemimethylated DNA occurs (Gayama et al., 1990). The fact that phospholipids interact with DnaA, at least in vitro (see Section 1.5.2), invites speculation that DnaA is an intermediary in initial membrane binding of the origin and that the protein may be activated at this stage. After initiation of replication has taken place, the origin is passed on to the sequestration machinery and is then released through an unknown mechanism; a detailed model for these events has been proposed by Norris (1993). At some point during this process excess initiation competent DnaA may be inactivated. It does not
INTRODUCTION

1.9 Regulation of dnaA gene expression

The structure and features of the dnaA promoter region were presented in Section 1.4. dnaA is transcribed from two promoters, the proximal one, dnaA2p, being responsible for 70-80% of steady state transcription during exponential growth (Kücherer et al., 1986; Polaczek and Wright, 1990).

1.9.1 Dam methylation and sequestration.

Five GATC sites are present within and just downstream of dnaA2p and one such site is present between the −10 and −35 sequences of dnaA1p, the distal promoter. The promoters respond differently to their state of methylation. In vivo a transcriptional fusion between the dnaA promoters and a lacZ reporter gene shows a two-fold reduction in promoter activity in a dam− strain compared with a dam+ (Braun and Wright, 1986). Quantitative S1 mapping showed that it is dnaA2p which is affected, although it is not clear whether this effect is a direct consequence of methylation on promoter activity or whether it is an indirect effect via some other regulatory system (Braun and Wright, 1986; Kücherer et al., 1986). Run off transcription experiments showed that the difference in activity of dnaA2p between its methylated and unmethylated states is still observed in vitro but that this difference is considerably less than in vivo (Braun and Wright, 1986) allowing for the possibility that methylation has both direct and indirect effects on dnaA expression.

Campbell and Kleckner (1990) studied the remethylation of one of the GATC sites within dnaA2p and found remethylation kinetics similar to those in the origin of replication (see Section 1.8.3). Evidence strongly suggests that the dnaA promoter region, when hemimethylated, is sequestered by the membrane as is hemimethylated oriC. During the period of sequestration, which is roughly one third of a generation time, transcription from both dnaA promoters is reduced to less than ten percent of steady state levels. It seems likely that the promoter region is inaccessible to RNA polymerase when attached to the membrane although a direct effect of hemimethylation on promoter activity has not formally been ruled out. The latter seems unlikely as dnaA1p activity is not normally affected by its state of methylation (Braun and Wright,
INTRODUCTION

1986). If high copy-number oriC plasmids are introduced into the cell, the level of hemimethylated DNA at both the origin and the dnaA promoter is reduced, suggesting that they are both subject to sequestration by the same system which is being titrated by excess copies of oriC (Campbell and Kleckner, 1990).

1.9.2 Response to growth-rate.

A model of replication control based on a growth-rate independent initiation mass with DnaA as the positively acting initiator requires the concentration of DnaA to be invariant with growth-rate. However, Chiaramello and Zyskind (1990) found that transcription from both promoters increases with increasing growth-rate. Sensitivity to growth-rate is far more pronounced at dnaA2p than at dnaA1p; during stationary phase transcription from dnaA2p is undetectable (Polaczek and Wright, 1990).

The promoters also showed sensitivity to levels of ppGpp, an alarmone which is induced as a result of amino acid starvation giving rise to the stringent response; this affects primarily promoters for stable RNA synthesis, the structures of which resemble dnaA2p. It has recently been reported that ppGpp seems to affect RNA chain elongation from dnaA2p rather than initiation of transcription (Gil and Zyskind, 1994). Levels of ppGpp are inversely proportional to growth-rate (Baracchini and Bremer, 1988), but it seems that the growth-rate sensitivity of dnaA transcription is not governed solely by this nucleotide as a ΔrelA ΔspoT double mutant which is completely deficient in its synthesis still shows some growth-rate sensitivity of dnaA2p transcription (Gil and Zyskind, 1994). Completely opposing results were obtained by Hansen et al. (1991a) who reported that a transcriptional fusion between the dnaA promoters and lacZ showed no growth-rate sensitivity.

Whether the actual intracellular concentration of DnaA varies with growth-rate is also controversial. Chiaramello and Zyskind (1989) reported a linear increase in the ratio of DnaA to total protein with increasing growth-rate. Polaczek and Wright (1990) found that there was a slight increase in DnaA protein concentration at faster growth-rates whereas, in contrast, Hansen et al. (1991a) reported that the DnaA concentration was invariant with growth-rate in E. coli K-12 and varied slightly with growth-rate in E. coli B/r: in neither strain, however, did the concentration of DnaA per origin change with growth-rate. A reason for the conflicting results from identical experiments performed in the various laboratories remains to be found.
1.9.3 Autoregulation.

Based on their experiments with temperature-sensitive dnaA mutants, Hansen and Rasmussen (1977) proposed that the dnaA gene is subject to autoregulation. A dnaA<sup>ts</sup> mutant, when incubated at high temperature for a particular length of time accumulates more initiation capacity than is used up by a dnaA<sup>+</sup> strain during the same period, leading to the idea that more of the DnaA<sup>ts</sup> product is produced at high temperature due to a failure to regulate its own synthesis. Compelling evidence exists that the dnaA gene is in fact autoregulated. dnaA promoter fusions with various reporter genes show repression of transcription upon overproduction of DnaA protein and show derepression of transcription upon temperature inactivation of DnaA<sup>ts</sup> mutant proteins (Braun et al., 1985; Atlung et al., 1985; Masters et al., 1989). Direct study of dnaA transcripts by in vivo S1 mapping gave similar results (Kücherer et al., 1986) as did in vitro transcription run off studies using purified DnaA protein (Wang and Kaguni, 1987).

Despite this body of evidence, indications exist that autoregulation may not simply be due to binding of the DnaA protein to the DnaA-box present between the two promoters, resulting in repression, as had previously been assumed (Polaczek and Wright, 1990). Also it has been shown that temperature inactivation of a mutant DnaA protein does not always lead to a derepression of dnaA transcription: in an integratively suppressed dnaA<sup>46</sup> strain derepression of a dnaA<sup>p–lacZ</sup> fusion does not occur at high temperature (Masters et al., 1989). The question of autoregulation of dnaA will be addressed in greater detail in Chapter III.

1.9.4 Induction by DNA damage.

DNA damage which inhibits replication induces transcription of dnaA and leads to a three-fold increase in concentration of the DnaA protein (Quiñones et al., 1991a; Quiñones et al., 1991b). This induction appears to be linked to the SOS response (Walker, 1984) as recA and lexA mutations abolish it; however, there is no LexA-box present in the DnaA promoter region implying that an SOS inducible product, rather than cleavage of LexA directly, may effect induction of dnaA (Quiñones et al., 1991b).

It appears therefore that, in addition to its normal role in initiation of replication, DnaA is also required for replication to reinitiate after cellular stress and that the normal control of its synthesis must be overruled for this to occur. The exact nature of the process in which it participates is, however, not clear. It is in a sense puzzling why the cell should produce a large amount of DnaA during the SOS
response as this same condition induces a form of chromosomal replication which is independent of the DnaA protein (Stable DNA Replication, see below). Nevertheless there are processes in which DnaA could participate. Verma et al. (1989) showed that initiation of replication is transiently inhibited after UV damage and that this inhibition is followed by a large stimulation of initiation of replication. It was proposed that induction of dnaA may be responsible for this increased reinitiation (Quiñones et al., 1991b). Reinitiation is, however, apparently not dependent upon recA (Verma et al., 1989) whereas induction of dnaA is, therefore it is more likely that DnaA participates in another, recA dependent, form of recovery from DNA damage such as induced replisome reactivation (IRR) (Witkin et al., 1987).

1.10 Extragenic suppression of dnaA mutations

Temperature-sensitive mutations in dnaA can be suppressed by additional mutations at a variety of loci. Wechsler and Zdzienicka (1975) and Atlung (1981) identified a number of these so-called das mutations, most of which have not yet been precisely defined. In addition, four separate attempts to clone the dnaA gene have yielded cloned suppressors (Projan and Wechsler, 1981; Takeda and Hirota, 1982; Jenkins et al., 1986; Fayet et al., 1986). Given the complexity of the process or processes in which DnaA is involved this array of different suppressors is not surprising.

There are at least four general mechanisms by which genetic suppression can occur. Firstly, interactive suppression, which can be very informative with respect to determining functional domains of the mutant proteins in question and for identifying other gene products which are capable of interaction with these proteins. Secondly, bypass suppression which obviates the requirement for a particular gene product by activating pathways alternative to the one in which the product is required. Thirdly, informational suppressors which are frameshift and missense suppressing tRNAs. It is characteristic of these suppressors that, in contrast to interactive suppressors, they exhibit allele specificity independent of the location of the mutation within the gene as suppression depends on the type of mutation rather than its functional consequences. Such suppressors will act on a wide range of otherwise unconnected genes and are therefore not very informative with regard to protein/protein interactions. Finally, increased expression of the mutant gene under conditions which would otherwise be non-permissive can in some cases lead to increased viability, presumably through an
increased amount of the mutant protein compensating for a reduced specific activity. This form of suppression of the dnaAts mutations has already been mentioned; its mode of operation at the molecular level is not understood.

1.10.1 Suppression by mutations in rpoB.

Bagdasarian et al. (1977) showed that a number of dnaAts mutations could be suppressed by mutations in rpoB, the gene encoding the β-subunit of RNA polymerase, which confer resistance to rifampicin. Atlung (1984) extended this study and discovered that all known dnaAts mutants can be suppressed in this way and that allele specificity is observed; a specific rpoB mutation will suppress one or more particular dnaAts mutations but not others (Atlung, 1984; Hansen et al., 1984; Hansen et al., 1992). Even an amber mutation in dnaA can be suppressed by an rpoB mutation (Schaus et al., 1981b); this is, however, not an indication of bypass suppression as other dnaA amber mutations cannot be suppressed in this way, implying that at least a partially active DnaA protein is required. The suppressed amber mutation is indeed located at the distal end of the gene, leaving most of the protein intact (Schaus et al., 1981b).

It could be argued that mutations in rpoB effect suppression by allowing increased transcription of the dnaA gene resulting in overproduction of the mutant product which is known to increase the permissive temperature. A particular allele, dnaA601, has, however, been shown to be poorly suppressed by overexpression but can be suppressed by a mutation in rpoB (Hansen et al., 1992). This argues against the possibility that rpoB acts simply through increased transcription of the mutant gene. In addition, if this were the case, then the observed allele specificity would not be expected. It would seem far more likely that a form of interactive suppression is occurring at the stage of transcriptional activation of the origin.

1.10.2 Suppression by a mutation in topA.

The dnaA46 mutation can be suppressed by deletion of the topA gene, which encodes topoisomerase I (Louarn et al., 1984), resulting in increased negative supercoiling of the chromosome (Pruss et al., 1982). As superhelicity is a factor in controlling gene expression (Smith, 1981) it is possible, although this has never been demonstrated, that the topA deletion leads to increased DnaA production. Alternatively, negative superhelicity could compensate for decreased activity of the DnaA46 protein in open complex formation.
1.10.3 Suppression by deletion of seqA.

It was mentioned in Section 1.8.3 that deletion of the seqA gene leads to suppression of temperature-sensitivity in strains carrying certain dnaA<sup>ts</sup> alleles (Table 1.1). This implies a form of interactive suppression. In addition, overproduction of SeqA protein in the cell suppresses the cold-sensitive phenotype of the dnaA<sup>46cos</sup> mutation (Lu <i>et al.</i>, 1994; von Freiesleben <i>et al.</i>, 1994). These effects indicate that SeqA is involved in controlling the activity of DnaA in initiation of replication. How SeqA functions is still unclear; it could be involved in the sequestration of oriC by the membrane as originally assumed or alternatively it could be involved in ensuring that only DnaA-ATP is initiation competent under normal circumstances. If the latter possibility were correct, a seqA deletion would result in overreplication and would also permit DnaA mutant proteins, incapable of binding ATP, to become initiation competent. A seqA mutant does indeed show an increased frequency of initiation and the dnaA mutants which it suppresses all are altered in the putative ATP-binding site (von Freiesleben <i>et al.</i>, 1994).

1.10.4 Suppression by a mutation in trxA.

The dnaA suppressor originally named dasC (Atlung, 1981) appears to be, at least in part, allelic with trxA, the gene encoding thioredoxin (Hupp and Kaguni, 1988), a cofactor for the reduction of ribonucleoside triphosphates by ribonucleotide reductase. The mechanism of this suppression is still far from clear; it appears to require an additional unlinked mutation as transfer of the trxA mutation to another dnaA<sup>46</sup> strain does not confer temperature resistance (Hupp and Kaguni, 1988).

1.10.5 Suppression by overproduction of GroEL and GroES.

Several separate reports exist of cloned suppressors of dnaA<sup>ts</sup> mutations (Projan and Wechsler, 1981; Takeda and Hirota, 1982; Jenkins <i>et al.</i>, 1986; Fayet <i>et al.</i>, 1986). Three of these have been characterised and have been found to be identical. Suppression results from overexpression of GroEL and GroES, proteins which act in concert in the folding of a number of different polypeptides and are known as molecular chaperones (Langer <i>et al.</i>, 1992). Temperature-sensitive groE mutants are deficient in chromosomal replication and in RNA synthesis at the non-permissive temperature (Wada and Itikawa, 1984). Whether these proteins normally play a part in initiation of replication is unclear.
Suppression of \textit{dnaA}^ts mutations by \textit{groE} requires overproduction of both GroEL and GroES (Fayet et al., 1986) and shows strong allele specificity; only the reversible \textit{dnaA} mutations are suppressed. The mutants which are altered in the ATP binding site also exhibit cold-sensitivity upon overproduction of the GroE proteins (Fayet et al., 1986; Jenkins et al., 1986). The phenotypic parallels between the intragenic suppression of \textit{dnaA}46 by \textit{dnaA}46\textit{cos} and the extragenic suppression by \textit{groE} is striking. A report by Katayama and Nagata (1991) showed that the cold-sensitivity seen when the GroE proteins are overproduced in a \textit{dnaA}46 strain is caused by overinitiation of chromosomal replication which is independent of protein synthesis, indicating that the GroE proteins increase the initiation capacity of the DnaA46 protein. This is not due to a change in the rate of synthesis of the protein as its intracellular levels in the suppressed and non-suppressed strains are similar.

A shift down in temperature from 42°C–30°C of GroE suppressed \textit{dnaA}46 cultures leads to greater levels of DNA accumulation in the presence of chloramphenicol than in its absence, reminiscent of the effect of this antibiotic on stalled replication forks due to overproduction of wild-type DnaA (Pierucci et al., 1987). Overinitiation was seen to occur repeatedly over a four hour period without any additional protein synthesis, whereas in the case of \textit{dnaA}46\textit{cos} the burst of overinitiation only lasts for one hour after a temperature shift down. The difference may indicate that in the \textit{dnaA}46 strain suppressed by \textit{groE}, the mutant protein is maintained in an initiation competent state for longer than in the \textit{dnaA}46\textit{cos} strain.

It was also shown that overproduction of the GroE proteins in a \textit{dnaA}46 \textit{cyA}283 strain, which is deficient in the synthesis of cAMP, allows temperature resistant growth but does not lead to cold-sensitivity in the absence of cAMP. When cAMP is added the cold-sensitive phenotype returns. cAMP has been shown to aid the release of ADP from DnaA to restore the ATP bound form of the protein (Hughes et al., 1988). As DnaA46 does not bind ATP (Hwang and Kaguni, 1988a) it is not obvious what the effect of cAMP on overreplication is. It was mentioned earlier that there appears to be a system which inactivates DnaA protein and to which the DnaA46\textit{cos} protein is insensitive (Katayama, 1994). Perhaps the presence of cAMP in the \textit{groE} suppressed strain permits initiation to escape this same system as a consequence of an effect on protein conformation. It is also possible that the action of GroEL and GroES is to restore ATP binding activity to the protein, in which case the absence of cAMP may allow the accumulation of enough replication incompetent DnaA-ADP to avert overinitiation at low temperature. The exact nature of this example of interactive suppression remains to be clarified. It would be interesting to determine whether the cold-sensitivity of a \textit{dnaA}46\textit{cos} strain is also relieved by a \textit{cyA} mutation.
1.10.6 Constitutive stable DNA replication.

It has been shown that secondary mutations (originally dasF) in the rnh gene, which codes for RNase H, can suppress all temperature-sensitive mutations in dnaA as well as dnaA null mutations (Horiuchi et al., 1984; Lindahl and Lindahl, 1984). These suppressed strains also lose their requirement for oriC (Kogoma and von Meyenburg, 1983), showing that the suppression is bypass in nature. An alternative form of chromosomal replication is initiated at at least four secondary origins, oriKs, two of which have been mapped to terC, the others to 45 minutes and 95 minutes on the genetic map (de Massy et al., 1984). This is known as constitutive stable DNA replication (cSDR) as it does not require protein synthesis (Kogoma, 1978).

The function of RNase H is to degrade the RNA moiety of RNA-DNA hybrid duplexes and it seems likely that cSDR is normally suppressed by the action of RNase H. In its absence these RNA-DNA hybrids may act by generating R-loops at the alternative origins to allow initiation of replication (von Meyenburg et al., 1987). In vitro the action of RNase H has also been shown to confer specificity of initiation to oriC (Ogawa et al., 1984).

cSDR requires the recA gene product (Kogoma et al., 1985). In a recA background initiation at oriKs can take place only if the LexA regulon, normally involved in the SOS response, is derepressed; initiation under these circumstances also requires DNA polymerase I (Cao and Kogoma, 1993). The exact nature of this and the recA dependent initiation processes of cSDR are not completely understood. It has been shown that their mode of replication is asynchronous, stressing the importance of DnaA and oriC in the timing of initiation (von Meyenburg et al., 1987).

1.10.7 Inducible stable DNA replication.

Another dnaA independent form of DNA replication which does not require protein synthesis is induced as a consequence of the SOS response to DNA damage and is known as inducible stable DNA replication (iSDR). Like cSDR this form of replication is dependent upon the recA gene product, both upon its activity in SOS induction and upon its recombinase activity to generate D-loops which are required for initiation (Asai et al., 1993). The sequences at which iSDR is initiated are known as oriMs, two of which are located within oriC and one at terC (Asai et al., 1994; Magee et al., 1992). In contrast to cSDR, iSDR initiation does not require transcription (Magee et al., 1992) and is not inhibited by RNase H.

It has recently been reported that both cSDR and iSDR require the PriA protein which was originally discovered from studies on φX174 replication and has since also
been implicated in ColE1 replication (see Section 1.12.1) (Masai et al., 1994). It appears that the cell has two alternative mechanisms for primosome assembly: DnaB and DnaG can be delivered either to a DnaA/DNA complex or to a PriA/PriB/DNA complex. It seems likely that the latter mechanism operates at oriKs and oriMs.

1.10.8 Integrative suppression.

Nishimura et al. (1971) reported a form of bypass suppression of dnaAts strains carrying F plasmids. The strains become temperature resistant upon integration of the F factor into the chromosome. It was subsequently shown that integration of R1 and ColV2 can also effect such suppression which was termed integrative suppression (Nishimura et al., 1973). It was shown that at high temperature replication is initiated from the origin of the integrated plasmid but that at permissive temperature it is largely oriC which is the site of initiation (Chandler et al., 1977).

Integrative suppression can occur from a variety of sites on the chromosome but the distribution appears non-random (Nishimura et al., 1971). Integration of the plasmid near terC results in loss of viability on rich medium at high temperature, presumably due to underinitiation of replication relative to mass increase. Constraints are also imposed on the orientation of an integrated R1 replicon which, at least in some cases, influences the ability of the replication fork to traverse terC (Louarn et al., 1982).

A wide variety of replicons have the ability to integratively suppress dnaAts mutations. Although originally only low copy number plasmids and a P2 prophage (Lindahl et al., 1971) promoted integrative suppression, it has since been reported for high copy number plasmids such as ColE1 (Yamaguchi and Tomizawa, 1980) and R1162 (Brasch and Meyer, 1988); in these cases the cells are viable only if they carry sufficient cytoplasmic copies of the plasmid to avoid runaway replication at the integrated plasmid origin.

Although the F factor can integratively suppress dnaAts strains it has been shown that F replication itself has an absolute requirement for the DnaA protein (Kline et al., 1986; Kogoma and Kline, 1987) and that F cannot integratively suppress a dnaA null strain (Hansen and Yarmolinsky, 1986). It is probable that the requirement for DnaA in F replication is such that a product which is only partially active for chromosomal replication at oriC suffices to promote replication at the F origin. Although an absolute requirement for DnaA in R1 and R100 replication has also been reported (Masai and Arai, 1987; Nagata et al., 1988) dnaA null strains do exist which initiate replication by virtue of an integrated R1 derivative (Hansen and Yarmolinsky,
1986; Bernander et al., 1991). The apparent contradiction between these reports could stem from the rich medium sensitivity exhibited by strains integratively suppressed by R1 derivatives (Hansen and Yarmolinsky, 1986). Some aspects of integrative suppression will be discussed further in Chapter III.

1.11 DnaA in regulation of transcription

Apart from a role in the regulation of its own synthesis, DnaA has been shown to be involved in the control of the expression of a variety of genes at the transcriptional level. Three ways in which DnaA exerts this activity have been reported: repression of promoters, activation of promoters and termination of transcription.

1.11.1 Repression of transcription initiation.

DnaA has been shown to act as a repressor of transcription at the dnaA promoter (Braun et al., 1985; Atlung et al., 1985; Kücherer et al., 1986; Wang and Kaguni, 1987). Similarly, evidence exists that the mioC promoter is subject to repression through DnaA binding to a target site in its promoter region (Stuitje et al., 1986; Lother et al., 1985; Løbner-Olesen et al., 1987).

Two of the four promoters which are responsible for the transcription of rpoH, the gene coding for the $\sigma^{32}$ factor involved in the recognition of heat shock responsive genes, are repressed by DnaA (Wang and Kaguni, 1989). In common with dnaA and mioC, rpoH has DnaA-boxes in its promoter region, one which fits the consensus sequence determined by Schaefer and Messer (1991) and another which differs from this at one position, but which appears also to be bound by the protein.

Limited evidence exists also that DnaA may repress transcription from drpA, a gene involved in DNA and RNA synthesis (Zhou and Syvanen, 1990). Finally, it has been reported that the origin promoter ori-pL is negatively regulated by DnaA in vivo (Asai et al., 1992) and in vitro (Nozaki et al., 1988). DnaA-boxes R2 and R4 are both thought to be involved in this.

There is no apparent correlation between the position and orientation of the DnaA-boxes with respect to the promoter sequences and their effect on transcription.
This indicates that repression of transcription may not occur by an identical mechanism in each case.

The *uvrB* gene, which is involved in excision repair of DNA damage, contains a pair of invertedly repeated DnaA-boxes in the most distal of its three possible promoters. It has not been demonstrated that transcription from this particular promoter is in fact involved in expression of *uvrB* and therefore it is unclear what the role of DnaA is in this case (van de Berg *et al.*, 1985). The *dam* gene has also been reported to be negatively regulated by DnaA (Jonczyk *et al.*, 1989). Further characterisation of *dam* expression revealed, however, that its major promoters show little or no derepression upon inactivation of DnaA<sup>ts</sup> protein (Løbner-Olesen *et al.*, 1992).

1.11.2 Activation of transcription initiation.

Although DnaA has generally been reported to repress transcription of its gene, there is evidence that under physiological conditions this is only the case at *dnaA1p*, the minor promoter, and that *dnaA2p* is in fact activated by the DnaA protein (Polaczek and Wright, 1990). Two other examples exist of positive regulation by DnaA. In the case of the *nrd* operon which encodes the two subunits of ribonucleoside diphosphate reductase, two DnaA-boxes are present upstream of the promoter. Mutation of these sites, resulting in loss of DnaA binding capacity, decreases *nrd* expression two-fold (Augustin *et al.*, 1994). The other example is positive regulation of the origin promoter *ori-PR* by DnaA, reported by Asai *et al.* (1992). Although the position of the DnaA-boxes in the activated promoters is similar in the case of *dnaA2p* and *nrdp*, their orientation with respect to the direction of transcription is different.

Recently, inactivation of DnaA46 protein has been shown to influence negatively the expression of the *fliC* gene, involved in the production of flagellin (Mizushima *et al.*, 1994). It is as yet unclear whether this effect is direct or indirect.

1.11.3 Termination of transcription.

The *asnC* gene is located upstream of *mioC* and is transcribed towards *oriC*. Although this gene contains a transcriptional terminator, some transcripts extend into *oriC in vivo* (Gielow *et al.*, 1988). Schaefer and Messer (1988) found that termination of *asnC* transcription is increased at elevated intracellular levels of DnaA protein and that this DnaA dependent termination occurs not at the *asnC* terminator but downstream, at the DnaA-box in the *mioC* promoter.
The ability of various DnaA-boxes to effect termination was tested and it was at one time reported that transcription termination depends on the orientation of the DnaA-box (Schaefer and Messer, 1989). It now seems that this was due to an interaction with an additional DnaA-box present in the reporter gene and it is now believed that for termination to occur two DnaA-boxes must be present in the same orientation with respect to each other, but in either orientation with respect to the direction of transcription (W. Messer, personal communication). It is not at all clear what the molecular mechanism of DnaA mediated transcriptional termination might be, nor is it clear whether there are parallels with the action of other known transcriptional termination factors described in Section 1.2.5.

The guaBA operon appears to be controlled by DnaA modulating the level of transcription of the guaB gene. One putative DnaA-box is present in the promoter and another in the guaB gene, both of which are required in order for DnaA to exercise an effect on gua transcription (Tesfa-Selase and Drabble, 1992). The internal DnaA-box has been shown to bind DnaA (Tesfa-Selase, personal communication) and to act as a terminator (Schaefer et al., 1992); whether this effect was again influenced by an additional DnaA-box in the reporter gene has not been addressed.

The dnaA gene contains a putative DnaA-box within the coding region. This too was thought to be involved in transcription termination (Messer et al., 1988) but was later shown by mutational analysis not to be (Wende et al., 1991).

Some evidence for an effect of DnaA on attenuation of the tryptophan synthetase gene was reported by Atlung and Hansen (1983). dnaAts mutants showed decreased expression of the trp operon at high temperature as long as the trp attenuator was intact. It is unclear what the nature of this effect is as the trp attenuator contains no DnaA-box.

Finally, a role for DnaA as a terminator was suggested in the transcription of ftsZ, a major cell division gene. Three DnaA-boxes are present in the upstream ftsQ and ftsA genes which are to some extent cotranscribed with ftsZ (Masters et al., 1989). An investigation into the role of DnaA in ftsZ transcription will be presented in Chapter IV.
1.12 DnaA in plasmid and phage replication and in transposition

1.12.1 Plasmid and phage replication.

DnaA has been found to be required for normal chromosomal replication in all bacteria so far studied. For the replication of extrachromosomal elements such as plasmids and bacteriophages the requirement for the DnaA protein varies. Phages λ and P2, for instance, show no dependence at all upon DnaA whereas phage P1 cannot replicate in a dnaA null strain (Hansen and Yarmolinsky, 1986) and requires DnaA for its replication in vitro (Wickner and Chattoraj, 1987) apparently to cooperate with the P1 RepA protein in the strand opening reaction (Mukhopadhyay et al., 1993). The origin of the plasmid pSC101 and the oriS of the F-factor show similarity to the P1 origin in the position of the DnaA-boxes in the arrangement of the origin region (Figure 1.5). Although all three elements encode an initiator protein and F has an alternative origin (oriV) (Kline, 1985) with no DnaA-boxes, the dependence of these replicons on DnaA is absolute (Hansen and Yarmolinsky, 1986). Its role may be similar to that in P1 replication although this remains to be determined.

The origins of the broad host range plasmids R6K and RK2 contain DnaA binding sites, which are required for replication, and these plasmids fail to replicate in a dnaA null strain (Wu et al., 1992; Gaylo et al., 1987). The exact role of DnaA here has not been well studied.

In some cases the requirement for DnaA has turned out to be indirect. M13 cannot successfully infect a dnaA<sup>ts</sup> strain at high temperature, yet is able to do so if this strain is integratively suppressed (Mitra and Stallions, 1976), indicating that it is chromosomal replication of the host and not dnaA function per se which is important for M13 replication. Similarly, Williams and Egan (1994) recently showed that phage 186, which cannot productively infect dnaA<sup>ts</sup> strains at high temperature, can infect a suppressed dnaA null strain. The fact that other host mutations which affect chromosomal replication also block 186 replication implies that a host gene, the expression of which is dependent upon DNA synthesis, could be required for 186 replication.

The ColEl plasmid, pBR322, can replicate in the absence of DnaA, as the entry of DnaB and DnaG is mediated by a preprimosome assembled from PriA, PriB, PriC and DnaT (Minden and Marians, 1985), proteins discovered through work on ϕX174 replication. In the absence of the primosomal proteins pBR322 replication becomes DnaA dependent (Seufert et al., 1988). Although not normally DnaA dependent, the rate of ColEl replication is reduced in a dnaA<sup>ts</sup> host at the restrictive temperature. Chiang et al. (1991) showed this to be due to an indirect effect. Cessation
of host DNA synthesis leads to an increase in the pool of free RNA polymerase. This in turn gives rise to an imbalance of transcription from the promoters for RNA I and RNA II, transcripts which are involved in plasmid replication and in the control of plasmid copy number.

1.12.2 Transposition of Tn5.

A novel function of DnaA was reported by Yin and Reznikoff (1987). dnaA null strains and dnaA amber mutants show a ten-fold reduction in Tn5 tranposition frequency compared with a wild-type strain. DnaA binds to DnaA-boxes at each outer end of Tn5 which presumably mediate its function. No other transposons show a requirement for DnaA or contain DnaA-boxes in their sequences. It is not clear what the exact nature of the DnaA activity in Tn5 transposition is; transcription, either initiating in Tn5 or entering into it does not appear to be affected.
1.13 Cell division

The means by which *E. coli* reproduces is through the division of each cell into two genetically identical daughter cells. In order to achieve this the cell must successfully duplicate its genetic material which must then be partitioned along with the rest of the cell content into two compartments which must finally be separated. Separation occurs by ingrowth of all three layers of the cell envelope; this consists of a cytoplasmic (inner) membrane, a peptidoglycan saccus and an outer membrane. In normal cells this process is extremely efficient and very rarely results in the production of anucleate cells (Hiraga *et al.*, 1989). Division of the cell could be considered due to ingrowth of the peptidoglycan saccus to push the cytoplasmic membrane in and pull the outer membrane behind it (Cook *et al.*, 1989). However, it has been shown that cells lacking a cell wall, so-called L-forms, are also able to divide (Onoda *et al.*, 1987) indicating that the peptidoglycan saccus is not an essential part of the division process. Owing to recent insights into the function of the FtsZ protein it is now believed that it is contraction of the cytoplasmic membrane which drives constriction of the cell.

As with the initial study of DNA replication the study of cell division has largely been based upon the isolation of conditionally lethal mutants. Mutants which are deficient in the cell division process at restrictive temperature continue to grow and form long filaments and became known as "filamentous temperature-sensitive" (*fts*) mutants. Identification of such mutants revealed the existence of several proteins that appear to act sequentially in the division process.

The genes affected in six *fts* mutants are located in the *mra* operon at 2.5 minutes on the genetic map. This operon contains sixteen open reading frames from which fourteen protein products have been identified, all of which are either involved in peptidoglycan synthesis or in cell division (Donachie, 1993). The latter include FtsA, FtsI, FtsQ, FtsW, FtsZ and EnvA. Chapter IV deals with aspects of transcriptional control of *ftsZ*, one of the most distal genes in the *mra* operon.
1.14 The FtsZ protein

Cells in which the activity of FtsZ is inhibited continue to grow and form filaments. These filaments are smooth indicating that FtsZ acts early in the division process, before the appearance of indentations in the filament due to localised septum formation. Plasmolysis of ftsZ mutant filaments causes the appearance of irregularly spaced "plasmolysis bays", in contrast to the regularly spaced bays seen in other fts mutants. Based on the assumption that these regularly spaced bays indicate potential division sites, this was taken to indicate that FtsZ is required for cell division to initiate at all (Cook and Rothfield, 1991).

FtsZ is the most abundant cell division protein, estimated to be present in 5000 to 20000 copies per cell (Bi and Lutkenhaus, 1991). Recently it has been shown that in cells which are about to divide FtsZ aggregates on the inner surface of the cytoplasmic membrane at the site of division to form a circumferential ring. The protein remains localised at the leading edge of constriction during the septation process (Bi and Lutkenhaus, 1992; Lutkenhaus, 1993). It has been speculated, though not proven, that the FtsZ ring is contractile and pulls the inner membrane with it leading to constriction.

FtsZ shares a number of regions of homology with eukaryotic tubulins (Lutkenhaus, 1993); one of these regions represents a highly conserved GTP/GDP binding site. FtsZ has been shown to be a weak GTPase. This function requires activation of the protein which can be brought about by K+ or by high concentrations of the protein itself, perhaps after attaining some critical concentration (Mukherjee et al., 1993; de Boer et al., 1992). In vitro, FtsZ can assemble into polymeric structures in a GTP dependent manner (Mukherjee et al., 1994); in vivo such polymerisation may be the first step in the division process.

An interaction between the FtsZ ring and the peptidoglycan sacculus has been inferred since alterations in the geometry of the FtsZ ring affect polar morphology (Bi and Lutkenhaus, 1992). It is likely that this interaction involves the cytoplasmic domains of membrane associated cell division proteins such as FtsQ, PBP3 and FtsW (Lutkenhaus, 1993; Khattar et al., 1994). Evidence of a physical interaction of FtsZ with a cytoplasmic division protein of as yet unknown function, FtsA, has recently emerged (Hale et al., 1994). Further studies will be necessary to define which proteins form the postulated "cell division complex" and to elucidate its exact biochemical function.

During normal cell growth the FtsZ protein can potentially localise at one of three division sites. The first is the cell centre, which represents the future division site
and the other two are the cell poles which represent old division sites. FtsZ is normally prevented from assembling at the cell poles by the presence of a protein complex consisting of MinC, MinD and MinE. It seems that it is MinC which interacts with FtsZ (Bi and Lutkenhaus, 1990) blocking division at all sites and that MinE antagonises this effect specifically at the centre of the cell allowing normal division to occur (de Boer et al., 1989).

FtsZ is the target for another cell division inhibitor, SfiA, which is induced as a result of the SOS response to DNA damage. This interaction leads to transient filamentation of cells, which is reversed upon degradation of SfiA by the Lon protease (Lutkenhaus, 1983; Mizusawa and Gottesman, 1983). The interaction of MinC and SfiA with FtsZ may be similar in nature as division inhibition by either protein can be overcome by a common suppressor in FtsZ (Lutkenhaus, 1990).

In parallel with the function of DnaA in replication, FtsZ appears to be the limiting factor in the onset of division as its overproduction leads to an increased number of septation events per increase in cell mass (Ward and Lutkenhaus, 1985). In accordance with the view of FtsZ as an activator of the division process, high concentrations of FtsZ can prevent filamentation induced by SfiA and MinC (Lutkenhaus et al., 1986; Bi and Lutkenhaus, 1990; de Boer et al., 1990). Although moderate overproduction of FtsZ in a wild-type cell stimulates division, shown by the production of minicells, a drastic overproduction (greater than ten-fold) of FtsZ results in complete inhibition of division (Ward and Lutkenhaus, 1985). The reason for this is not clear but could be due to an upset of the stoichiometry of the "division complex".

The regulation of ftsZ expression is complex and appears to occur at the transcriptional and translational levels as well as at a functional level described above. Transcriptional control of ftsZ will be addressed in Chapters IV and V.
1.15 The project

The work described in this thesis is based mainly upon observations published by Masters et al. (1989). They found that expression from cloned ftsZ promoters responds to thermal inactivation of DnaA15 protein and proposed that DnaA may be a transcriptional regulator of ftsZ. The investigation into this possible role of the DnaA protein was continued and the results are presented in Chapter IV. During the course of the original work into DnaA mediated regulation of ftsZ, Masters and co-workers compared expression of the dnaA gene, which is documented to be under control of the DnaA protein, to expression of ftsZ and found that in integratively suppressed strains the promoters of both genes are insensitive to DnaA15 inactivation. This was a surprising result and does not fit the model of dnaA autoregulation. Control of dnaA expression was therefore also the subject of further research which is described in Chapter III. Finally, promoter fusion constructs made during these studies were found useful for reinvestigating growth-rate sensitivity of ftsZ transcription, previously described by Dewar et al. (1989). Her results were confirmed and this is presented in Chapter V.
Chapter II
Materials and Methods
2.1 Bacterial strains, phage strains and plasmids

2.1.1 Growth media and buffers.

Bacterial strains used in this study are listed in Table 2.1.1. Bacteria were either maintained on L-broth plates stored at room temperature, or for longer term storage, in frozen storage buffer at −70°C.

Bacteriophages used in this study are listed in Table 2.1.2. Phage lysates of P1 and λ were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth. M13 phage lysates were stored without chloroform as M13 is chloroform sensitive. Plasmids used and constructed in the course of this study are listed in Table 2.1.3.

Growth media and commonly used buffers are listed in Tables 2.1.4 and 2.1.5. L-broth and L-agar were used routinely for all bacterial manipulations, except where stated. For work with phage λ the media were supplemented with 10mM MgSO₄ and 0.2% maltose to maximise expression of the λ receptor protein; for phage P1, 2.5 mM CaCl₂ was added. VB minimal agar, supplemented with appropriate carbon sources, vitamins and amino acids, was used for selection of nutritional markers.

2.1.2 Growth of bacteria.

Bacteria were routinely grown as liquid cultures at 37°C (or 30°C for temperature-sensitive strains). Usually fresh overnight cultures that had been inoculated from a single colony were diluted back the following day and grown as required.
### Table 2.1.1 Bacterial strains.

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<th>Genotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>BW313</td>
<td><em>dut, ung, thi-1, relA, spoT1, FlysA</em></td>
<td>Laboratory stock</td>
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<tr>
<td>DSB1</td>
<td><em>rodAsui₄₃, INV: rrnD-rrnE, sup⁰, recD::miniTn10, leu+, leu::Tn9</em> [due to chromosomal duplication], tetR, kanR, chlR*</td>
<td>D. S. Boyle</td>
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<tr>
<td>ED100</td>
<td>as ED419 except carries λRWS100 as prophage</td>
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</tr>
<tr>
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<td>as ED419 except carries λRWS200 as prophage</td>
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<td>as ED419 except carries λRWS201 as prophage</td>
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<td>EH3791</td>
<td>$asnB32$, $relA1$, $spoT1$, $thi-1$, $fuc-1$, $lysA$, $ilv-192$, $dnaA::Tnl\ 0$, $mad-1$, $zia::pKN500$, $kanR$, $tetR$</td>
<td>Hansen and Yarmolinsky (1986)</td>
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<td>MF746</td>
<td><em>thr-1, dnaK756, thi-1, lacY1, tonA21, supE44, λ−</em></td>
<td>B. Bachmann</td>
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<td>MM18</td>
<td>*F−, argG6, hisG1, asnA31, asnB32, leuB6, metB1, pyrE, galE, lac, xyl7,</td>
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<td></td>
<td><em>supE44, uhp, bgl+ fhuA2, gyrA, naiR, rpsL, tsx, λ−</em></td>
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<tr>
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<td>as MM18 except <em>asn+</em></td>
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<td>MM182</td>
<td>as MM18 except <em>asnA+, bgl−, dnaA5</em></td>
<td>March (1988)</td>
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<td>MM183</td>
<td>as MM18 except <em>asnA+, bgl−, dnaA204</em></td>
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<tr>
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<td>as MM18 except <em>asnA+, bgl−, dnaA508</em></td>
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<td>MM185</td>
<td>as MM18 except <em>asnA+, bgl−, dnaA46</em></td>
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<td>as MM18 except <em>asnA+, bgl−, dnaA203</em></td>
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<td>as MM18 except <em>asnA+, bgl−, dnaA601</em></td>
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<td>as MM18 except <em>asnA+, bgl−, dnaA604</em></td>
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<tr>
<td>MM190</td>
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<td>as MMB181 except spontaneous <em>bgl+</em></td>
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<tr>
<td>MMB182</td>
<td>as MMB182 except spontaneous <em>bgl+</em></td>
<td>This work</td>
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<td>as MMB183 except spontaneous <em>bgl+</em></td>
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<td>as MMB185 except spontaneous <em>bgl+</em></td>
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<td>as MMB186 except spontaneous <em>bgl+</em></td>
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<td>MMB187</td>
<td>as MMB187 except spontaneous <em>bgl+</em></td>
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<td>MMB189</td>
<td>as MMB189 except spontaneous <em>bgl+</em></td>
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<td>MMB190</td>
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<td>MMBT184</td>
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<td>MMT186</td>
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<td>NF279</td>
<td>his, argG, leu, metB, ilv, pyrB, rbs, mtl, xyl, gal, lac, malA, nalR, strR, spcR</td>
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<td>NF#31 to NF#39</td>
<td>as NF279 except zii::pKN500 by P1 transduction from TPK88</td>
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<td>NF#A to NF#I</td>
<td>as NF#31 except metB+ by P1 transduction from TP8503</td>
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<td>NM621</td>
<td>leu, proB1, hsdR, mcrA−, mcrB+, recD supE, tsx, tonA</td>
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<td>SHA5</td>
<td>rodAusi, INV: rrdD-rrnE, sup0, leu+, leu::TN10 [due to chromosomal duplication], tetR, kanR</td>
<td>Addinall (1994)</td>
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<td>TC182</td>
<td>dnaA46, thi, ilv-42, argH, metB, pyrE, xyl4, trp, his, lacY, tna, φ805, λR, strR, T6R</td>
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<td>TG1</td>
<td>F[traD36, proAB+, lacIQ, lacZΔM15], thi-1, supE, hsdΔ5, Δ[lac-proAB]</td>
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<td>TOE13</td>
<td>thyA, argE, leu, his, pro, thr, thi, ftsA13ts</td>
<td>K. J. Begg</td>
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<td>TP30</td>
<td>as ED419 except carries λJFL100 as prophage</td>
<td>T. Paterson</td>
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<td>TP48</td>
<td>as TP8503 except carries λJFL100 as prophage</td>
<td>T. Paterson</td>
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<tr>
<td>TP88</td>
<td>as ED419 except carries λRB1 as prophage</td>
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<td>TP91</td>
<td>as TP8503 except carries λRB1 as prophage</td>
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<td>TP93 as TP88 except <em>dnaA+</em> by P1 transduction from TP91</td>
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<td>TP100 as TP8503 except carries λRWS100 as prophage</td>
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<td>TP182 as TP92 except <em>dnaA5</em> by P1 transduction from MMB182</td>
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<td>TP201 as TP8503 except carries λRWS201 as prophage</td>
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<td>TP201#2 as TP201 except re-infected with λRWS201</td>
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<td>TP20M as TP8503 except carries λRWS20M as prophage</td>
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<td>TP20M#2 as TP20M except re-infected with λRWS20M</td>
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<td>TP945 as TP8503 except carries λRWS945 as prophage</td>
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<td>TP8503 as ED419 except <em>dnaA+ Tn7</em></td>
<td>T. Paterson</td>
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<td>as TP88 except integratively suppressed by F::TN5</td>
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<td>as TP185 except (zii::pKN500)</td>
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<td>as TP186 except (zii::pKN500)</td>
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<td>as TP187 except (zii::pKN500)</td>
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<td>as TP190 except (zii::pKN500)</td>
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<td>TP/MAD</td>
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<td>(kan)</td>
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<td>TPR1 to</td>
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<td>TPR3</td>
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<td>as TP200 except (rpoS::Tn10) introduced by P1 transduction</td>
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<tr>
<td>TP/ZAD</td>
<td>as TP8503 but <em>leu</em>&lt;sup&gt;+&lt;/sup&gt;, <em>ftsQ</em> and <em>ftsA</em> replaced by equivalent fragment from pMAK/ZAD</td>
<td>This work</td>
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<td>TP/ZAD (kan)</td>
<td>as TP/ZAD except carries the <em>aphA</em> gene at 94 min., <em>kan</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>W3110</td>
<td>F&lt;sup&gt;-&lt;/sup&gt;, INV:<em>rrnD-rrnE</em>, <em>sup</em>&lt;sup&gt;0&lt;/sup&gt;, <em>λ</em>&lt;sup&gt;S&lt;/sup&gt;, <em>λ</em>&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Laboratory stock</td>
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<td>W3110/MAD</td>
<td>as W3110 except contains triple DnaA-box mutation in <em>ftsQ</em> and <em>ftsA</em></td>
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<tr>
<td>W3110/ZAD</td>
<td>as W3110, <em>ftsQ</em> and <em>ftsA</em> replaced by equivalent fragment from pMAK/ZAD</td>
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<td>W311A</td>
<td>as W3110 except <em>ftsAl2</em>&lt;sup&gt;ts&lt;/sup&gt;</td>
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<td>W311K</td>
<td>as W3110 except carries a <em>kan</em>&lt;sup&gt;R&lt;/sup&gt; cassette at 94 min.</td>
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Table 2.1.2 Bacteriophages.

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<td>λJFL100</td>
<td>carries the 1.8 kb <em>EcoRI–HindIII</em> fragment with <em>ftsQ</em> and <em>ftsA'</em></td>
<td>J. F. Lutkenhaus</td>
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<tr>
<td></td>
<td>transcriptionally fused to <em>lacZ</em>, <em>imm21</em>, <em>ninR5</em></td>
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<tr>
<td>λRB1</td>
<td>carries a translational fusion of the 945 bp <em>EcoRI</em> fragment with the <em>dnaA</em> promoter region and N-terminal part of <em>dnaA</em> with <em>lacZ</em>, <em>immλ</em></td>
<td>Braun <em>et al.</em> (1985)</td>
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<td>λRS45</td>
<td>transcriptional promoter fusion vector with <em>lacZ</em> as reporter gene, <em>imm21</em>, <em>ninR5</em></td>
<td>Simons <em>et al.</em> (1987)</td>
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<td>λRWS100</td>
<td>recombinant of λRS45 and pRWS100, <em>kanR</em></td>
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<td>λRWS200</td>
<td>recombinant of λRS45 and pRWS200, <em>kanR</em></td>
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<td>λRWS201</td>
<td>recombinant of λRS45 and pRWS201, <em>kanR</em></td>
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<td>λRWS20M</td>
<td>recombinant of λRS45 and pRWS20M, <em>kanR</em></td>
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<td>λRWS945M</td>
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<td>M13#1</td>
<td>M13mp18 with the 1.8 kb <em>EcoRI–HindIII</em> fragment carrying <em>ftsQ</em> and <em>ftsA'</em></td>
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<td>M131.8/m3/6</td>
<td>as M13#1 except carries the triple DnaA-box mutation</td>
<td>This work</td>
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<td>M13M1</td>
<td>as M13#1 except carries a mutated DnaA-box in <em>ftsQ</em></td>
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<td>M13 based cloning vector</td>
<td>Messing (1979)</td>
</tr>
<tr>
<td>M13mp19</td>
<td>M13 based cloning vector</td>
<td>Messing (1979)</td>
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<td>M13pdnaA1</td>
<td>M13mp19 with the 945 bp EcoRI fragment carrying the dnaA promoter region</td>
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<td>M13pdnaA8</td>
<td>as M13pdnaA1 except carries a mutated DnaA-box in the dnaA promoter</td>
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<td>P1</td>
<td>wild-type transducing phage</td>
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### Table 2.1.3 Plasmids.

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<td>F::Tn5</td>
<td>from <em>in vivo</em> recombination of F and Tn5, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hansen <em>et al.</em> (1984)</td>
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<td>pBR322</td>
<td>pMB1 replicon, amp&lt;sup&gt;R&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bolivar (1978)</td>
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<tr>
<td>pBR328</td>
<td>pMB1 replicon, amp&lt;sup&gt;R&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;, chi&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Soberon <em>et al.</em> (1980)</td>
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<tr>
<td>pdam118</td>
<td>pBR322, contains 1.14 kb insert with the <em>dam</em> gene, amp&lt;sup&gt;R&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Brooks <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>pGW71</td>
<td>R1 minimal replicon, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bernander <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>pGWΩ</td>
<td>Ω fragment inserted into <em>PstI</em> site in pGW71, amp&lt;sup&gt;R&lt;/sup&gt;, str&lt;sup&gt;R&lt;/sup&gt;, spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pGW'IS</td>
<td>R1 minimal replicon, contains IS1 sequence, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pHCΩ1</td>
<td><em>ColD</em> replicon, contains Ω fragment flanked by <em>PstI</em> sites, kan&lt;sup&gt;R&lt;/sup&gt;, str&lt;sup&gt;R&lt;/sup&gt;, spc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>McLennan (1993)</td>
</tr>
<tr>
<td>pH4</td>
<td>pBR325 derivative containing <em>oriC</em> and <em>mioC</em> flanked by sequences of unknown extent, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>J. H. Pringle</td>
</tr>
<tr>
<td>pJM87</td>
<td>pBR325, contains a 2.7 kb insert with the <em>dnaA</em> gene, amp&lt;sup&gt;R&lt;/sup&gt;, chi&lt;sup&gt;R&lt;/sup&gt;</td>
<td>March (1988)</td>
</tr>
<tr>
<td>pKN500</td>
<td>derivative of R1<em>drd</em>-19, contains IS1 sequence, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Molin <em>et al.</em> (1979)</td>
</tr>
<tr>
<td>pLSK5</td>
<td>pJF118EH carrying the <em>dnaA</em> gene under control of the <em>tac</em> promoter, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kücherer (1985)</td>
</tr>
<tr>
<td>pMAK705</td>
<td>pSC101, temperature-sensitive for replication, chi&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hamilton <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>pMAK/MAD</td>
<td>pMAK705 with the 4.7 kb <em>BamHI–ClaI</em> fragment carrying the <em>ddlB–envA'</em> genes with the triple DnaA-box mutation, chi&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pMAK/ZAD</td>
<td>pMAK705 with the 4.7 kb <em>BamHI–ClaI</em> fragment carrying the <em>ddlB–envA'</em> genes, chi&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source/reference</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>pND5</td>
<td>pBR325 derivative carrying the 8.1 kb EcoRI fragment with the groE operon</td>
<td>Jenkins <em>et al.</em> (1986)</td>
</tr>
<tr>
<td></td>
<td>amp&lt;sup&gt;R&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pOC142-R</td>
<td>pBR322 derivative with a 4.0 kb XmnI fragment containing ori&lt;sub&gt;C&lt;/sub&gt;, mio&lt;sub&gt;C&lt;/sub&gt; and gidA, amp&lt;sup&gt;R&lt;/sup&gt;, tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>W. Messer</td>
</tr>
<tr>
<td>pOC161</td>
<td>pBR322 derivative with a 1.5 kb SmaI–HincII fragment containing ori&lt;sub&gt;C&lt;/sub&gt; and mio&lt;sub&gt;C&lt;/sub&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>W. Messer</td>
</tr>
<tr>
<td>pRS551</td>
<td>promoter fusion vector containing lacZ as reporter gene, pMB1 replicon, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simons <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>pRWS100</td>
<td>pRS551 with the 2.3 kb EcoRI insert carrying ftsQ&lt;sup&gt;+&lt;/sup&gt;, ftsA&lt;sup&gt;+&lt;/sup&gt; and ftsZ&lt;sup&gt;+&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRWS200</td>
<td>pRS551 with the 1.8 kb EcoRI–HindIII insert carrying ftsQ&lt;sup&gt;+&lt;/sup&gt; and ftsA′&lt;sup&gt;+&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRWS201</td>
<td>as pRWS200, contains HindIII–BamHI linker, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRWS0M</td>
<td>as pRWS201 except carries triple DnaA-box mutation, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRWS945</td>
<td>pRS551 with the 945 bp EcoRI insert carrying the dnaA and rpmH promoter region, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pRWS945M</td>
<td>as pRWS945 except carries a mutated DnaA-box in the dnaA promoter, amp&lt;sup&gt;R&lt;/sup&gt;, kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pSU200</td>
<td>pMB1 replicon, contains the 1.8 kb EcoRI–HindIII fragment with ftsQ&lt;sup&gt;+&lt;/sup&gt; and ftsA′&lt;sup&gt;+&lt;/sup&gt; fused to lacZ, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>S. J. Dewar</td>
</tr>
<tr>
<td>pSZ24</td>
<td>pTZ18R, f&lt;sub&gt;1&lt;/sub&gt; and pBR325 origins, contains the 2.3 kb fragment with ftsQ&lt;sup&gt;+&lt;/sup&gt;, ftsA&lt;sup&gt;+&lt;/sup&gt; and ftsZ&lt;sup&gt;+&lt;/sup&gt;, amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Dewar (1988)</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Source/reference</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>pT7-3</td>
<td>CoLE1 replicon designed for expression from the phage T7 Φ10 promoter, ampR</td>
<td>S. Tabor</td>
</tr>
<tr>
<td>pT7-4ddl</td>
<td>pT7-4 with the 1.1 kb BamHI fragment containing the ddlB gene, ampR</td>
<td>M. M. Khattar</td>
</tr>
<tr>
<td>pT7/MAD</td>
<td>as pT7/ZAD except carries the triple DnaA-box mutation, ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pT7/tet</td>
<td>as pT7/ZAQ except carries the tet gene in the BglII site in ftsA, ampR, tetR</td>
<td>This work</td>
</tr>
<tr>
<td>pT7/ZAM</td>
<td>as pT7/ZAQ except carries the triple DnaA-box mutation, ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pT7/ZAQ</td>
<td>pT7-3 with the 4.3 kb PstI–ClaI fragment containing ddlB', ftsQ, ftsA, ftsZ and envA', ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pT7/ZAD</td>
<td>pT7-3 with the 4.7 kb BamHI–ClaI fragment containing ddlB, ftsQ, ftsA, ftsZ and envA', ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pTL61T</td>
<td>promoter fusion vector carrying lacZ as reporter gene, pMB1 replicon, ampR</td>
<td>Linn and St. Pierre</td>
</tr>
<tr>
<td>pTLM1</td>
<td>as pTLQA except carries a mutated DnaA-box in ftsQ, ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pTLQA</td>
<td>pTL61T with the 1.8 kb EcoRI–HindIII fragment carrying ftsQ and ftsA', ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pTL/MAD</td>
<td>as pTL/ZAD except carries a double DnaA-box mutation, ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pTL/ZAD</td>
<td>pTL61T with the 2.1 kb BamHI–BglII fragment carrying ddlB, ftsQ and ftsA', fused to lacZ, ampR</td>
<td>This work</td>
</tr>
<tr>
<td>pZAQ</td>
<td>pBR322 derivative carrying the ftsQ, ftsA and ftsZ genes on a 4.3 kb PstI–ClaI fragment, tetR</td>
<td>Ward and Lutkenhaus (1985)</td>
</tr>
<tr>
<td>R1дрd-19</td>
<td>R1 derivative, derepressed for transfer, ampR, chlR, kanR, spcR, strR</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>
## Table 2.1.4 Growth media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Components</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-broth</strong></td>
<td>Difco bacto tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>Difco bacto yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>pH to 7.2 with NaOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distilled water to 1 litre</td>
<td></td>
</tr>
<tr>
<td><strong>L- agar</strong></td>
<td>L-broth + 15 g Difco agar per litre</td>
<td></td>
</tr>
<tr>
<td><strong>LB top agar</strong></td>
<td>L-broth + 6.5 g Difco agar per litre</td>
<td></td>
</tr>
<tr>
<td><strong>Nutrient broth (NB)</strong></td>
<td>Oxoid No.2 nutrient broth</td>
<td>25 g</td>
</tr>
<tr>
<td></td>
<td>Distilled water to 1 litre</td>
<td></td>
</tr>
<tr>
<td></td>
<td>It should be noted that NB has insufficient</td>
<td></td>
</tr>
<tr>
<td></td>
<td>thymine, this was therefore routinely added</td>
<td></td>
</tr>
<tr>
<td></td>
<td>at a concentration of 40 μg ml⁻¹</td>
<td></td>
</tr>
<tr>
<td><strong>NB agar</strong></td>
<td>Nutrient Broth + 12.5 g Davis NZ agar</td>
<td></td>
</tr>
<tr>
<td><strong>20x VB salts</strong></td>
<td>20x VB salts</td>
<td>25 ml</td>
</tr>
<tr>
<td></td>
<td>20% carbon source</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>Supplements as required</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distilled water to 1 litre</td>
<td></td>
</tr>
<tr>
<td><strong>VB minimal agar</strong></td>
<td>As VB minimal medium + 15 g Difco agar per litre</td>
<td></td>
</tr>
<tr>
<td><strong>20x VB salts</strong></td>
<td>MgSO₄·7H₂O</td>
<td>4 g</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
<td>40 g</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>400 g</td>
</tr>
<tr>
<td></td>
<td>NaNH₄·HPO₄·4H₂O</td>
<td>70 g</td>
</tr>
<tr>
<td></td>
<td>Distilled water to 1 litre</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1.5 Commonly used buffers.

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Ingredients and Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage buffer</td>
<td>Na$_2$HPO$_4$ 7 g, KH$_2$PO$_4$ 3 g, NaCl 5 g, MgSO$_4$ (0.1 M) 10 ml, CaCl$_2$ (0.1 M) 10 ml, 1% gelatin solution 1 ml, Distilled water to 1 litre</td>
</tr>
<tr>
<td>Bacterial buffer</td>
<td>KH$_2$PO$_4$ 3 g, Na$_2$HPO$_4$ 7 g, NaCl 4 g, MgSO$_4$.7H$_2$O 2 g, Distilled water to 1 litre</td>
</tr>
<tr>
<td>TE buffer</td>
<td>Working solution: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>Working solution: 40 mM Tris–acetate, 2 mM EDTA, 50x Conc. stock solution: Tris base 242 g, Glacial acetic acid 57.1 ml, 0.5 M EDTA (pH 8.0) 100 ml, Distilled water to 1 litre</td>
</tr>
<tr>
<td>TBE buffer</td>
<td>Working solution: 89 mM Tris–borate, 89mM boric acid, 5x Conc. stock solution: Tris base 54 g, Boric acid 27.5 g, 0.05 M EDTA (pH 8.0) 20 ml, Distilled water to 1 litre</td>
</tr>
</tbody>
</table>
2.1.3 Minimal medium supplements.

Amino acid supplements were stored in stock solutions of pure amino acids at a concentration of between 2–10 mg/ml depending upon the solubility of the particular amino acid. Poorly soluble amino acids, such as tyrosine, were dissolved in 0.01M NaOH. The final concentration of the amino acids in the media was usually in the order of 20–100 μg/ml. If a rich minimal medium was required, vitamin-free casamino acids (CAA) were used. The stock concentration of CAA was 100 mg/ml and the final concentration in the medium was typically 1–5 mg/ml. It should be noted that casamino acids lack tryptophan and this should therefore be added to CAA medium if the bacterial strain to be used is auxotrophic for this amino acid.

Purines and pyrimidines were added to minimal media when required. Thymine and uracil were stored at a concentration of 2 mg/ml in water, and their final concentration in minimal medium was usually 20–40 μg/ml.

The only vitamin supplement used in this work was thiamine hydrochloride (vitamin B1). This was stored as a 1 mg/ml solution in water and its final concentration in minimal medium was 2 μg/ml.

2.1.4 Selection of antibiotic resistance.

The routine concentrations for the antibiotics used in this work are shown in Table 2.1.6. All antibiotics were used in both complex and minimal media with the exception of trimethoprim, which was only used in minimal medium as its function requires the absence of thymine.

Table 2.1.6 Antibiotic solutions.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Solvent</th>
<th>Conc. of stock solution (mg/ml)</th>
<th>Final conc. in media (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Amp</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Chl</td>
<td>Ethanol</td>
<td>20</td>
</tr>
<tr>
<td>Kanamycin sulphate</td>
<td>Kan</td>
<td>H2O</td>
<td>25</td>
</tr>
<tr>
<td>Naladixic acid</td>
<td>Nal</td>
<td>0.1 M NaOH</td>
<td>20</td>
</tr>
<tr>
<td>Spectinomycin dihydrochloride</td>
<td>Spc</td>
<td>H2O</td>
<td>50</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>Str</td>
<td>H2O</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline hydrochloride</td>
<td>Tet</td>
<td>50% ethanol</td>
<td>10</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Tmp</td>
<td>Methanol</td>
<td>5</td>
</tr>
</tbody>
</table>
2.2 DNA techniques

2.2.1 Large-scale plasmid preparation.

A single colony of the plasmid-carrying bacterial strain was inoculated into 5 ml of L-broth with the appropriate selection and incubated overnight at 37°C with vigorous shaking. 1 ml of this culture was then used to inoculate 500 ml of L-broth, with similar selection, in a 2 litre flask, which was then incubated at 37°C overnight, again with vigorous agitation. The culture was chilled on ice, transferred to two 250 ml centrifuge bottles and centrifuged in a Sorvall Superspeed centrifuge at 5000 rpm for 10 min at 4°C. The bacterial pellets were then each washed in 100 ml of TE buffer, pooled to give a total volume of 200 ml, and recentrifuged as above. The resultant cell pellet was resuspended in 5 ml of a solution containing 50mM Tris–HCl (pH 8.0), 25% sucrose and transferred to a 50 ml centrifuge tube. 1 ml lysozyme (20 mg/ml) was added, the solution mixed thoroughly, and incubated on ice for 10 min. 1 ml of 0.5M EDTA (pH 8.0) and 0.8 ml of RNase A solution (10 mg/ml) were added and incubation continued for a further 10 min on ice. Finally 5 ml of a lysis solution containing 100 mM Tris–HCl (pH 8.0), 125 mM EDTA, and 0.2% (w/v) Triton X-100 was added, the solution mixed thoroughly and incubated on ice for another 10 min. The resulting suspension was then centrifuged using a Sorvall SS-34 rotor at 15,000 rpm for 20 min at 4°C. The plasmid-containing supernatant could now be subjected to isopycnic gradient ultracentrifugation to separate plasmid and chromosomal DNA.

CsCl (17.1 g) was dissolved in the supernatant in a 25 ml measuring cylinder. Ethidium bromide (0.342 ml) solution (10 mg/ml) was added and the total volume made up to 23 ml with TE. This gave a CsCl density of 1.55 g/ml and an ethidium bromide concentration of 200 μg/ml. The solution was then transferred to two 11.5 ml Sorvall Ti-50 crimp-seal centrifuge tubes, balanced to within 0.05 g and then centrifuged in a Sorvall 50-B or 55-B ultracentrifuge at 38,000 rpm for 60 hours at 20°C in a Ti-50 rotor. At the end of the run the tubes were carefully removed from the rotor and the DNA bands could be visualized using a UV lamp. The lower (denser) plasmid bands were removed from the tubes using a syringe fitted with a wide-bore needle. The two samples were then pooled and the ethidium bromide extracted at least five times with isobutanol (isobutanol over CsCl-saturated TE). The sample was then dialysed against several changes of TE (1:2500) at 4°C over a period of 48 hours to remove the CsCl. The plasmid DNA could then be recovered from solution by precipitation.
2.2.2 Small-scale plasmid preparation.

Routine preparations of plasmid DNA were performed using a modification of the alkaline lysis method of Birnboim and Doly (1979). 5 ml of L-broth (plus suitable antibiotic selection) was inoculated with a single colony of the plasmid-bearing strain, and incubated overnight with continuous shaking at the appropriate temperature (typically 37°C). The culture was then centrifuged at 5000 x g for 10 min in a bench-top centrifuge. The supernatant was discarded, and the bacterial pellet resuspended in 0.1 ml of buffer containing 1% glucose, 10mM EDTA, and 25mM Tris–HCl (pH 8.0). To this cell suspension 0.2 ml of 0.2M NaOH/1%SDS was added, mixed by gentle inversion of the tube and incubated on ice for 5 min. 100 µl of 3 M Na acetate (pH 5.0) was then added, the solution mixed vigorously by extensive vortexing, and left on ice for a further 5 min. The mixture was then centrifuged in a micro-centrifuge for 10 min in order to pellet the precipitated chromosomal DNA and insoluble cellular debris. The resulting supernatant (~0.5 ml) was transferred to a fresh Eppendorf tube and 0.5 ml of phenol/chloroform (phenol saturated with TE (pH 8.0) plus an equal volume of chloroform) was added, mixed by vortexing and centrifuged in a micro-centrifuge for 2 min. The upper aqueous phase was transferred to a fresh tube and the plasmid DNA could then be recovered from solution by ethanol or isopropanol precipitation. In this case no extra salt needed to be added in order for precipitation to occur. Typically the final pellet of nucleic acid was resuspended in TE buffer containing RNase A (20 µg/ml). It was usually observed that 5 ml of overnight culture yielded approximately 3–5 µg of plasmid DNA.

2.2.3 Preparation of chromosomal DNA.

(i) Large-scale method. A single colony of the appropriate strain of *E. coli* was used to inoculate 5 ml of L-broth, which was incubated overnight at 37°C (or 30°C for temperature-sensitive strains) with vigorous shaking. 1 ml of this culture was used to inoculate 100 ml of L-broth, which was again incubated at a suitable temperature overnight with constant agitation. The culture was then chilled on ice and transferred to a 250 ml centrifuge bottle and centrifuged at 5000 rpm for 15 min at 4°C in a Sorvall Superspeed centrifuge. The supernatant was removed and the bacterial pellet was resuspended in 20 ml of STE, which is TE buffer with 10 mM sodium chloride. 1 ml of 10% SDS solution and 1 ml of proteinase K solution (4 mg/ml) were added, mixed gently and incubated at 50°C for 6 h without shaking. To this solution an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the suspension mixed gently by inversion and allowed to stand at room temperature for 10
MATERIALS AND METHODS

This was centrifuged in a benchtop centrifuge at 5000 x g for 15 min to separate the aqueous and phenolic phases. The upper aqueous phase was then carefully removed avoiding the protein interface. The nucleic acids in this phase were precipitated by adjusting the sample to 0.2 M sodium acetate (pH 5.5) and gently layering 2 volumes of ice-cold ethanol on top. The DNA was collected at the aqueous-ethanol interface by spooling it out with a glass rod. The spooled DNA was washed in 70% ethanol, dried briefly in air and dissolved overnight in 10 ml of TE at room temperature. To this solution 0.1 ml of RNase A (10 mg/ml) was added and the mixture incubated at 37°C for 1 h. 500 μl of a 10% SDS solution and 250 μl of proteinase K solution (4 mg/ml) were now added and the mixture incubated at 50°C for 1 hour. The sample was extracted with phenol:chloroform:isoamyl alcohol and the DNA precipitated by spooling as above. After washing in 70% ethanol the DNA was air dried and dissolved in 1 ml of TE. This solubilization took between 1 and 3 days. The yield of DNA was determined by UV spectrophotometry as described previously; from 100 ml of culture about 500 μg of chromosomal DNA was typically obtained.

(ii) Small-scale method. The method of Redfield and Campbell (1987) was found to be a reliable protocol for obtaining chromosomal DNA quickly. 1.5 ml of a fresh overnight culture of the appropriate bacterial strain was put into an Eppendorf tube and centrifuged for 2 min in a micro-centrifuge. The supernatant was discarded and the pellet gently resuspended in a solution containing 40 mM Tris-HCl (pH 8.0), 20 mM EDTA and 10 mg/ml lysozyme. This was incubated at 37°C for 30 min. To this 100 μl of a solution containing 5% SDS and RNase A (100 μg/ml) was added, mixed by gentle inversion and incubated at 37°C for 5 min. The resulting lysate was extracted twice with Tris-saturated phenol and once with chloroform taking care to mix the solutions very gently to avoid damaging the DNA. The upper (aqueous) phase was removed using a Gilson P1000 with a wide (cut off) pipette tip to avoid shearing the DNA. The DNA was then precipitated by adding 15 μl of 5 M NaCl and 1 ml of ethanol, gently inverting and leaving for at least 30 min. Routinely the pellet was resuspended in 50 μl of distilled water and then half of this was used for a restriction digest.

2.2.4 Preparation of bacteriophage M13 DNA.

In all preparations of M13 DNA the E. coli strain TG1 was used. A 5 ml overnight culture of TG1 was prepared in the usual way. This culture was used to set up a culture infected with bacteriophage M13. 50 μl of the overnight culture were used to inoculate 2 ml of L-broth. To this either 100 μl of an M13 phage suspension (about
1/10 of a single plaque) or an entire M13 plaque from an agar plate was added. This culture was incubated at 37°C with vigorous shaking for about 5 hours. 1.5 ml of this culture was transferred to an Eppendorf tube and centrifuged in a micro-centrifuge for 5 min. The resulting bacterial pellet could be used to prepare the double-stranded replicative form of M13 DNA, and the supernatant used to prepare single-stranded M13 DNA (or as a fresh bacteriophage suspension).

(i) Preparation of double-stranded M13 DNA. The bacterial pellet was washed once in bacterial buffer and the double-stranded DNA isolated in essentially the same way as was described for the small-scale isolation of plasmid DNA.

(ii) Preparation of single-stranded M13 DNA. 1.2–1.3 ml of the bacteriophage suspension was transferred to an Eppendorf tube and 200 µl of a solution containing 20% polyethylene glycol (PEG 8000) in 2.5 M sodium chloride was added, the solution mixed thoroughly and allowed to stand at room temperature for 15 min. The precipitated bacteriophage particles were recovered by centrifuging in a micro-centrifuge for 5 min and the supernatant was removed carefully leaving the pellet as dry as possible. The bacteriophage pellet was resuspended in 100 µl TE with vigorous vortexing. 50 µl of phenol (equilibrated with Tris–HCl [pH 8.0]) was added and the suspension mixed thoroughly by vortexing for 1 min. This was then centrifuged for 2 min and the upper aqueous layer carefully removed and placed in a fresh tube. The volume of the sample was adjusted to 0.5 ml and the single-stranded DNA recovered by phenol–chloroform extraction and ethanol precipitation as has been described earlier. An optional step was to chloroform extract the sample after the phenol–chloroform extraction. Using this method the yield of single-stranded DNA was usually approximately 5–10 µg DNA per millilitre of infected culture. The DNA was of sufficient quality for both sequencing and site-directed mutagenesis reactions.

2.2.5 Preparation of bacteriophage λDNA.

(i) Large-scale method. The λ particles were purified from liquid lysate phage stocks as described in Section 2.4.6. The phage band from the second step of CsCl step gradient centrifugation was dialysed overnight against 2 l of 1x TE buffer at 4°C. Pronase was added to the phage band (in the dialysis tubing) to a final concentration of 1 mg/ml. This was then dialysed for 2 hours against 1 l of pronase dialysis buffer (20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.002% Triton X-100) at 37°C. The phage DNA solution was transferred to Eppendorf tubes and the inside of the dialysis tubing was rinsed with 500 µl of 1x TE buffer to minimise loss of phage DNA. The pooled DNA solutions were extracted with an equal volume of phenol to
disrupt remaining phage heads, and then extracted with equal volumes of phenol/chloroform and chloroform to purify the λ DNA from denatured protein. The DNA was precipitated with ethanol, rinsed with 70% ethanol and resuspended in a total of 180 μl 1x TE buffer. The DNA was then precipitated with isopropanol and rinsed with 70% ethanol. The λ DNA pellet was resuspended in 100 μl 1x TE buffer and stored at −20°C. The DNA concentration was estimated using its absorbance at 260 nm.

(ii) Small-scale method. The λ particles were resuspended in 200 μl of phage buffer. The λ suspension was extracted with 200 μl of phenol to disrupt the phage heads, and then extracted with 200 μl of phenol/chloroform and chloroform to purify the λ DNA from denatured capsid proteins. The DNA was precipitated with ethanol, rinsed with 70% ethanol and resuspended in 36 μl 1x TE buffer. The DNA was then precipitated with isopropanol and rinsed with 70% ethanol. The λ DNA pellet was resuspended in 20 μl 1x TE buffer and stored at −20°C.

2.2.6 DNA precipitation.

DNA was precipitated from aqueous solution by: (i) adding 1/10 volume of 3M sodium acetate (pH5) and 3 volumes of absolute ethanol, mixing thoroughly and leaving on ice for a minimum of 10 min. This was then centrifuged in a microcentrifuge at 15000 rpm for at least 15 min. The supernatant was discarded, and the pellet washed in 70% ethanol by vortexing. This was recentrifuged as above for 10 min, the supernatant again discarded, and the pellet was dried under vacuum. The dried DNA pellet could then be resuspended in a suitable volume of TE buffer (with added RNase (20 μg/ml) if required). (ii) Instead of 3 volumes of absolute ethanol, 1 volume of isopropanol could be used. This had the advantage of keeping the total volume smaller and was therefore the preferred method. After isopropanol precipitation and centrifugation, the pellet was washed with 70% ethanol as above.

2.2.7 Determination of DNA concentrations.

DNA concentrations were determined by measuring the absorption of diluted solutions at 260 nm. For double-stranded DNA, an OD_{260} value of 1.0 represents a DNA concentration of 50 μg/ml, and for single-stranded DNA a similar value represents a DNA concentration of 40 μg/ml. DNA purity can be determined by measuring absorption at 260 and 280 nm. Protein-free double-stranded DNA should give a 260/280 ratio close to 1.8, and single-stranded DNA should give a ratio nearer 2.0.
2.2.8 Digestion of DNA with restriction endonucleases.

Endonuclease cutting of DNA was typically performed in volumes of between 20 and 100 µl. These contained the requisite amount of DNA (usually 1–10 µg) and the appropriate Boehringer Mannheim restriction buffer at 1x concentration. The restriction enzyme was usually present in a two to fivefold excess, i.e. 2–5 units per microgram of DNA. The digests were made up to their final volume using distilled water. The complete restriction digests were incubated at the recommended temperature (usually 37°C) for 1–3 hours. The products of the reaction were either directly analysed by agarose gel electrophoresis, or phenol extracted, ethanol precipitated and dissolved in a suitable volume of TE buffer for further manipulations.

2.2.9 Partial digestion of DNA.

For partial digestion of DNA, ten two-fold serial dilutions of restriction enzyme were added to fixed amounts of DNA, with 0.5 units of enzyme per µg DNA representing the highest enzyme:DNA ratio. The digests were incubated at the appropriate temperature for 1 hour and terminated by addition of tracking dye (Section 2.2.12). The products of the reactions could then be analysed by agarose gel electrophoresis.

2.2.10 Ligation of DNA.

Ligations of DNA were typically performed in a final volume of 10 µl. These contained between 0.5 and 1 µg total DNA with insert DNA in a 2 to 20-fold molar excess over the vector DNA, 1x Boehringer Mannheim ligation buffer and T4 DNA ligase. Ligase (0.2 units) was used for the ligation of cohesive DNA termini, and 1 unit of the enzyme for the ligation of blunt-ended molecules. The reactions were incubated for at least 12 hours at 16°C. Between 5 and 10 µl of the reaction mixture was then used to transform competent cells of an appropriate strain of *E. coli*.

2.2.11 'Filling in' of recessed 3' termini.

DNA polymerase I Klenow fragment was used to fill-in the recessed 3' termini generated by various restriction enzymes to give blunt-ended DNA molecules. Reactions were performed in a final volume of 20 µl containing 1µg DNA, 1x Klenow buffer, all four dNTPs, each at a concentration of 20 µM and 2 units of Klenow enzyme. The reactions were incubated at 16°C for 45 min. The reactions were stopped
and the unincorporated nucleotides removed by increasing the reaction volume to 200 μl with TE, phenol extracting and ethanol precipitating the DNA.

2.2.12 Agarose gel electrophoresis.

Agarose gel electrophoretic analysis of DNA was always performed using TAE buffer. The gels were made up by melting the appropriate amount of agarose (usually between 0.8 and 1.5%) in 1x TAE buffer using a microwave oven. Gels were cast in 11 x 14 cm Pharmacia gel trays, and once set the DNA samples containing 1x tracking dye (6x tracking dye is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H2O) were loaded into the wells at one end of the tray. Gels were run in Pharmacia gel electrophoresis tanks with their surfaces only just immersed in 1x TAE buffer. Electrophoresis was usually performed overnight at a constant current of 25 mA. After completion of electrophoresis, gels were stained in water containing 2 μg/ml ethidium bromide for about 1 hour with constant shaking, and subsequently destained in fresh water for 30 min. The gels could then be photographed using Polaroid film and UV transillumination.

2.2.13 Isolation of DNA from agarose gel slices.

To isolate DNA from agarose gels one of two methods was generally employed: (i) the Geneclean method or alternatively (ii) centrifugation of the DNA through siliconised glasswool.

(i) Geneclean II® is a product of Bio101 and utilizes a silica matrix (glassmilk®) which binds DNA in high-salt but not in low-salt solutions. The appropriate DNA band was located in an ethidium bromide-stained gel under UV transillumination and cut out using a clean razor blade in as small a volume of agarose as possible. Gel slices were transferred to Eppendorf tubes, the weight of the slice determined and 3 volumes of saturated sodium iodide solution added. These were incubated at 50°C until the gel slice had dissolved. 5 μl of glassmilk was added, the suspension mixed well and put on ice for 5 min. Tubes were briefly spun in a microcentrifuge and pellets washed three times in 0.25–0.5 ml of Geneclean II New-Wash® solution (an alcohol-based washing solution supplied with the kit), centrifuging and resuspending the pellets each time. After the final wash, all traces of the wash solution were removed using a Pasteur pipette and the pellets were then suspended in 5 μl TE buffer. These were incubated at 50°C for 2–3 min, centrifuged for 30 seconds and the DNA-containing supernatant transferred to a fresh tube. A further 5 μl of TE buffer
was added to the glassmilk pellets and the procedure was repeated to give a final DNA-containing solution with a volume of 10 μl. This DNA solution could be directly used for further manipulations.

(ii) The band containing the desired DNA fragment was cut out of a gel as described under (i). A 0.5 ml Eppendorf tube was punctured at the bottom with a narrow-bore needle and half filled with siliconised glasswool. Care was taken not to pack this too tightly. The gel slice was placed on top of the glasswool and the 0.5 ml Eppendorf tube was placed inside a 1.5 ml Eppendorf tube. The tubes were placed inside a micro-centrifuge and spun at 7000 rpm for 10 min, resulting in separation of the DNA solution (which was collected in the larger Eppendorf tube) from the agarose (which was retained on the glasswool). The DNA solution was transferred to a fresh Eppendorf tube, extracted with phenol/chloroform and ethanol precipitated before further use.

2.2.14 Labelling DNA fragments by random-priming.

The DNA to be labelled was gel-purified as in Section 2.2.10. This was done twice for plasmid fragments to avoid contamination with other fragments present on the gel. One round of gel purification was deemed sufficient for PCR products.

The DNA solution was made up to 33 μl with distilled water, placed in a boiling water bath for 3 min and then equilibrated at 37°C for 10 min. The random-priming was set up in an Eppendorf tube as follows and then incubated overnight at room temperature:

oligo-labelling buffer (OLB) 10 μl
bovine serum albumin (10 mg/ml) 2 μl
[α-32P] dCTP (10 mCi/ml) 5 μl
probe DNA 33 μl
DNA polymerase I Klenow fragment 2 Units

OLB consists of a 1:2.5:1.5 mixture of solutions A:B:C as follows:

Solution O 1.25 M Tris-HCl
0.125 M MgCl₂ (pH 8.0)
2.2.15 Southern blotting procedures.

(i) Transfer of DNA from agarose gels to nylon membranes. Chromosomal DNA was digested with the desired restriction enzymes, electrophoresed through agarose, stained and photographed as has been described above. The DNA fragments were depurinated by soaking the gel in 2 volumes of 0.25 M HCl with gentle agitation for 15 min. This was repeated with fresh 0.25 M HCl for a further 15 min. The gel was then rinsed with distilled water and the DNA denatured by soaking in 2 volumes of 0.5 M NaOH/1.5 M NaCl with gentle agitation for 15 min. This was repeated with fresh denaturation solution for a further 15 min. The gel was then soaked in a neutralising solution containing 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA for at least 30 min. The gel was finally rinsed in distilled water.

A Boehringer Mannheim nylon membrane and eight sheets of blotting paper were cut to the same size as the gel. Two sheets of blotting paper (saturated with 20x SSC) were placed on a clean glass plate in a plastic tray. The tray contained 20x SSC and the pieces of blotting paper were arranged so that they hung over the edge of the plate to act as wicks. Three more saturated sheets of blotting paper and the pretreated gel were placed on top of this. The nylon membrane was placed on top of the gel, taking care to exclude any air bubbles; this was followed by a further three sheets of saturated blotting paper. On top of this a 2–4 cm thick wad of dry paper towels was
placed, followed by another glass plate. The whole structure was weighted to provide
even pressure and maintain good contact between the gel, nylon and paper towels;
transfer was allowed to continue overnight. The extracted gel was stained with
ethidium bromide to check that transfer had been successful, while the nylon
membrane was rinsed in 20x SSC, blotted dry, and UV irradiated to cross-link DNA
to the filter. This was done in a Stratagene UV Stratalinker™ at 1200 µjoules, 254 nm.

(ii) Hybridisation of labelled probe to nylon membranes. All steps were carried
out using Techne hybridisation bottles in a Techne Hybridiser HB-1D oven. An
adaptation of the method of Church and Gilbert (1984) was used. The nylon
membrane was pre-hybridised in 20 ml Hybridisation Buffer for at least 2 hours at
65°C: this was to prevent non-specific hybridisation of the probe to the membrane.
The labelled probe (Section 2.2.11) was denatured by placing in a boiling water-bath
for 5 min. This was added to 10 ml of fresh Hybridisation Buffer and incubated
overnight with the membrane at 65°C to allow hybridisation. The temperature could be
reduced depending on the desired stringency conditions. The probe was decanted and
stored at −20°C for possible re-use and the filter was washed with 10 ml Wash
Solution for 10 min at 42°C. This was repeated twice. The membrane was checked
with a radiation monitor and if the background radioactivity was considered high it
was washed for 10 min with 10 ml Wash Solution at 65°C. The membrane was placed
in an air-tight polythene bag, put into an autoradiography cassette with Cronex® X-ray
film and stored at −70°C. The time for which the film was exposed to the filter was
dependent upon the strength of the signal.

| Hybridisation Buffer       | 1 mM EDTA (pH 8.0) |
|                           | 0.5 M sodium phosphate buffer (pH 7.2) |
|                           | 7% (v/v) SDS       |

| Wash Solution              | 1 mM EDTA (pH 8.0) |
|                           | 40 mM sodium phosphate buffer (pH 7.2) |
|                           | 5% (v/v) SDS       |

2.2.16 Site-directed mutagenesis after Taylor et al. (1985).

Site-directed mutagenesis of DNA was in one experiment performed using the
Amersham Oligonucleotide-directed in vitro Mutagenesis System. This system is
based on the method of Taylor et al. (1985) and results in very high yield of mutated
DNA sequences. The method involves a strand-specific selection step, which
eliminates the unwanted non-mutant sequences in vitro, generating a pure homoduplex mutant DNA sequence and thus avoiding host-mediated repair systems. The Amersham system also incorporates a nitrocellulose filtration step which removes any contaminating single-stranded template DNA, which could cause high levels of non-mutant background.

In the procedure, a mutagenic oligonucleotide is annealed to a single-stranded M13 template and is extended by Klenow enzyme in the presence of T4 DNA ligase to generate a mutant heteroduplex. Selective removal of the non-mutant strand is made possible by the incorporation of a thionucleotide into the mutant strand during in vitro synthesis. This makes the DNA resistant to the restriction enzyme NciI in that the enzyme can only nick phosphorothioate-containing DNA on the chemically normal strand. Such nicks are sites for the single-stranded exonuclease III, resulting in the digestion of the non-mutant (non-phosphorothioate) strand of the cloned target sequence. The mutant strand is then used as the template to reconstruct the double-stranded closed-circular molecule, thus creating a homoduplex mutant molecule. This is then used to produce M13 phage plaques on a suitable M13-sensitive strain of E. coli, which can then be used for verification of the intended mutation. The mutagenesis procedure is summarised below.

Single-stranded M13 DNA was prepared as before, and adjusted to give a concentration of 1 μg/μl with TE buffer. Oligonucleotides (generally 20-mers) were purchased from the Oswel DNA Service at Edinburgh University and phosphorylated as described in Section 2.2.17; concentrations were adjusted to approximately 1.6 pmol/μl. To anneal the mutant oligonucleotide to the single-stranded template the following were added to an Eppendorf tube on ice:

- single-stranded DNA template: 5 μl
- phosphorylated mutant oligonucleotide: 2.5 μl
- Buffer 1 (Amersham kit): 3.5 μl
- water: 6 μl
- Total: 17 μl

The tube was then placed in a 70°C water bath for 3 min followed by 30 min in a 37°C water bath. The tube was then placed on ice. To synthesise the mutant strand and ligate the resulting heteroduplex the following were added to the annealing reaction:
### Materials and Methods

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>annealing reaction</td>
<td>17 µl</td>
</tr>
<tr>
<td>MgCl₂ solution</td>
<td>5 µl</td>
</tr>
<tr>
<td>nucleotide mix 1</td>
<td>19 µl</td>
</tr>
<tr>
<td>water</td>
<td>6 µl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>6 Units</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>6 Units</td>
</tr>
</tbody>
</table>

The contents of the tube were then mixed well and placed in a 16°C water bath overnight. The next stage was to remove unreacted single-stranded DNA by centrifuging the mixture through a nitrocellulose filter unit. In 500 mM NaCl, nitrocellulose binds ssDNA but not dsDNA. To the above reaction, 170 µl of water and 30 µl of 5M NaCl were added. This was then mixed well and transferred to the top-half of a disposable filter unit supplied in the kit and centrifuged at room temperature in an HB-4 swing-out rotor at 1500 rpm for 10 min. 100 µl of 0.5 M NaCl was added to the top-half of the unit and it was respun for a further 10 min. The double-stranded DNA was then precipitated by adding 28 µl of 3 M sodium acetate and 700 µl of cold 100% ethanol to the filtrate. This was placed on ice for 15 min and then centrifuged for 15 min. The supernatant was carefully removed and the pellet washed in 1 ml of 70% ethanol. This was respun, the supernatant removed and the pellet dried in a vacuum desiccator. The pellet was then resuspended in 25 µl of Buffer 2, and of this 15 µl were stored at —20°C. To the remaining 10 µl of sample 65 µl of Buffer 3 was added along with 5 units of NciI. This was incubated at 37°C for 90 min in order to nick the DNA. The next stage was to digest away the nicked non-mutant strand with exonuclease III. To the above reaction the following were added on ice:

- NaCl (500 mM) 12 µl
- Buffer 4 10 µl
- exonuclease III (25 Units/µl) 2 µl

The tube was then incubated at 37°C for 30 min. Fifty units of exonuclease III will digest about 3000 nucleotides in 30 min, which is ample since the closest NciI site 3' of the cloned insert is only 600 nucleotides away. The tube was then placed in a 70°C water bath for 15 min to destroy the enzymes. The final step was the repolymerisation of the gapped DNA.
To the reaction mix above the following were added on ice:

- Nucleotide mix 2: 13 µl
- MgCl₂ solution: 5 µl
- DNA polymerase I: 3 Units
- T4 DNA ligase: 2 Units

This mixture was placed in a 16°C water bath for 3 hours. 20 µl of this product was then used to transfect an M13-sensitive strain of *E. coli* and the plaques generated were screened for the incorporation of the expected mutation by direct sequencing of the phage DNA. (See Section 2.3.1 for the preparation of competent *E. coli* for DNA transformation.)

### 2.2.17 Site-directed mutagenesis after Kunkel (1985)

As an alternative to the method described in Section 2.2.16 mutations were introduced using an adaptation of the method described by Kunkel (1985). This method uses single-stranded template DNA in which some of the residues have been replaced by uracil. This is achieved by growing the M13 in an ung dut host strain. In this work BW313 was used for this purpose. The dut mutation leads to a deficiency in dUTPase (which normally serves to convert dUTP into dUMP). The result is that in strains carrying this mutation, uracil is incorporated into the DNA at some sites normally occupied by thymine. The ung mutation affects an enzyme (uracil-N-glycosylase) which removes uracil residues which have been misincorporated into DNA. In an ung host therefore the uracil remains in the DNA. M13 which is repeatedly grown in such a strain can contain up to 30 uracil residues per genome. When these M13 phages subsequently infect an ung⁺ strain, the uracil residues are rapidly removed from the DNA, generating sites which will block replication and are susceptible to cleavage by specific endonucleases. This in turn leads to a decrease in infectivity of approximately 10⁵ fold.

This method of mutagenesis takes advantage of the selection against uracil-substituted DNA. Template DNA is prepared by repeatedly growing a recombinant M13 phage, which carries the target sequence, in BW313 (ung, dut). Usually four rounds of infection gave M13 phages with a 10⁵-10⁶ fold reduced infectivity in an ung⁺ strain. ssDNA is used as a template to which an oligonucleotide containing the desired mutations is annealed (generally two or three mutations were present on an oligonucleotide of approximately 20 bases in length). Oligonucleotides were purchased from Oswel DNA Services, Edinburgh University. They were
phosphorylated as follows: 100 pmoles of mutagenic oligonucleotide in 16.5 μl of water was incubated with 4 Units of bacteriophage T4 polynucleotide kinase in a buffer consisting of 0.05 M Tris-HCl (pH 7.6), 0.01 M MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine HCl, 0.1 mM EDTA and 0.5 mM ATP. Incubation took place at 37°C for one hour. The enzyme was then inactivated by heating to 68°C for 10 min.

The oligonucleotide was annealed to the target DNA as follows: 0.5 pmole ssDNA was added to 10 pmoles of oligonucleotide in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl and 1 mM dithiothreitol. This was put in a boiling water-bath for 10 min before letting cool to room temperature.

The oligonucleotide was used as a primer for the in vitro synthesis of a uracil-free DNA strand complementary to the template strand. To the annealing mixture (10 μl) were added 5 Weiss Units of bacteriophage T4 ligase, 2.5 Units of Klenow fragment of DNA polymerase I and each of the dNTPs at a final concentration of 0.1 mM. These were incubated (in the following buffer, at a final volume of 20 μl: 20 mM Tris-HCl, 10 mM MgCl₂, 25 mM NaCl, 5 mM dithiothreitol and 0.5 mM ATP) for 15 hours at 16°C.

The product of the reaction was used to transfect an M13-sensitive ung⁺ strain of E. coli (generally TG1; see Section 2.3.1). The plaques obtained were screened for incorporation of the mutation by sequencing ssDNA or where appropriate by restriction analysis of dsDNA.

2.2.18 DNA sequencing techniques.

DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based on the chain-terminating dideoxynucleotide sequencing method developed by Sanger et al. (1977). In the original procedure, primer extension was catalysed by the Klenow fragment of E. coli DNA polymerase I. In the kit, however, the Klenow enzyme has been replaced by T7 DNA polymerase, which has the advantage of creating longer chain-terminated fragments with a more even distribution of label between fragments. The major practical difference in using T7 DNA polymerase is that the primer extension reactions are performed in two stages, a labelling reaction and a termination reaction. The two stages are required because the enzyme uses dideoxynucleotides very readily, and therefore in order to allow the synthesis of long chain-terminated fragments, dideoxynucleotides are excluded from the first stage of the reaction, being added for the second. Even so, the time required for the reactions using the T7 enzyme is considerably less than those using Klenow enzyme.

(i) Annealing of primer to single-stranded template. The DNA templates used
MATERIALS AND METHODS

in the sequencing reactions were all single-stranded M13 DNAs and were purified as mentioned previously. The concentration of the template was adjusted to 1 μg/μl in TE. In most cases the Universal Primer supplied in the kit was found to be suitable. This primer is 17 bp long and is at a concentration of 0.80 μM. If another oligonucleotide is used it should be adjusted to the same concentration.

The following was added to an Eppendorf tube on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>template DNA (1μg/μl)</td>
<td>2 μl</td>
</tr>
<tr>
<td>primer (0.80 μM)</td>
<td>2 μl</td>
</tr>
<tr>
<td>annealing buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>water</td>
<td>8 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>14 μl</td>
</tr>
</tbody>
</table>

The contents of the tube were mixed well and incubated at 60°C for 10 min. The tube was then left at room temperature for at least 10 min; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at −20°C until required.

(ii) Sequencing reaction. For each template to be sequenced, four Eppendorf tubes or wells of a microtitre plate were labelled 'A', 'C', 'G' and 'T' respectively and 2.5 μl of the corresponding dideoxynucleotide mix added to each tube or well. To the tube containing the annealed template and primer the labelling mix, (dCTP, dGTP and dTTP in solution), T7 DNA polymerase and labelled dATP were added as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>annealed Template and Primer</td>
<td>14 μl</td>
</tr>
<tr>
<td>labelling mix</td>
<td>3 μl</td>
</tr>
<tr>
<td>[α-35S] dATPαS</td>
<td>1 μl (=10 μCi)</td>
</tr>
<tr>
<td>diluted T7 DNA polymerase (1.5 units/μl)</td>
<td>2 μl</td>
</tr>
</tbody>
</table>

This labelling reaction was incubated at room temperature for 5 min. While this was proceeding the previously dispensed sequencing mixes were incubated at 37°C for one minute in a water bath. After the 5 minute incubation of the labelling reaction, 4.5 μl was added to each of the prewarmed sequencing mixes and returned to the water bath for a further 5 min to allow chain-termination to occur. Finally, 5 μl of Stop Solution was added to each reaction, which could then be stored at −20°C until required for electrophoresis. When the samples were needed for loading onto the sequencing gel they were heated to 80°C for 2 min to denature the DNA. Immediately after this incubation 1.5 to 2.5 μl of each sample was loaded onto the gel.

97
(iii) DNA sequencing gel electrophoresis. DNA sequencing was performed on a 6% polyacrylamide gel using a 30 x 40 cm BRL sequencing apparatus. The glass sequencing gel plates were thoroughly cleaned with ethanol and chloroform, assembled using 0.2 mm spacers and taped together carefully to minimise the possibility of leakage.

The gel was prepared by adding together the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>bis-acrylamide (filtered, 40% w/v)</td>
<td>15 ml</td>
</tr>
<tr>
<td>urea</td>
<td>43 g</td>
</tr>
<tr>
<td>water</td>
<td>35 ml</td>
</tr>
<tr>
<td>10x TBE</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

This was allowed to dissolve with the aid of magnetic stirring. Once dissolved, 1 ml of a 10% ammonium persulphate solution was added followed by 35 µl of TEMED. This was stirred slowly for a few seconds and was then poured between the sequencing plates. The flat edge of a 60-well shark-tooth comb was pushed between the plates to layer the top of the gel. Clingfilm was wrapped round the exposed areas of the gel and each edge of the gel was clamped with bulldog clips. The gel was then set aside for at least 10 min to allow polymerisation. Once set, the bulldog clips, tape and comb were removed and distilled water was squirted along the top of the gel. The shark-tooth comb was then replaced with the points downwards just touching the surface of the gel. The gel was then clamped into the sequencing apparatus and 1x TBE solution poured into the top and bottom reservoirs. The gel was then pre-run at about 66 W (~1500 V) for 1 hour. After this the gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order G, A, T and C immediately after denaturing the DNA (see above). The gel was then electrophoresed at 66 W until the blue dye-front ran off the end of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus and the top plate very carefully removed. The bottom plate (with the gel attached) was placed in a fixing bath containing 10% methanol and 10% acetic acid in water for 20 min. The plate and gel were then removed and two damp sheets of blotting paper placed on top of the gel followed by two sheets of dry blotting paper. Even pressure was applied and the papers were peeled off the glass plate taking the gel with them. The gel and paper sandwich was then dried in a vacuum gel-drier for 1 hour at 80°C. When dry the gel was placed in an autoradiography cassette and allowed to develop at ambient temperature. In most cases a good signal was achieved after 24 hours.
2.2.19 Amplification of DNA using the Polymerase-Chain-Reaction.

Specific regions of DNA were amplified using the Polymerase-Chain-Reaction (PCR). Either chromosomal or plasmid DNA was used as a template and specific oligonucleotide primers were obtained commercially (Oswel DNA Service, Edinburgh University). A typical reaction mixture for a plasmid template was as follows (chromosomal template was used at 10x the concentration of plasmid DNA):

- 10x Thesit Buffer III: 5 µl (or Vent polymerase buffer)
- dNTP mix (1 mM for each dNTP): 5 µl
- oligonucleotide primer 1 (50 µM): 1 µl
- oligonucleotide primer 2 (50 µM): 1 µl
- template DNA (10 ng/µl): 1 µl
- Promega Taq polymerase (5 Units/µl): 1 µl (or Vent polymerase)
- distilled water: 36 µl

100 µl of mineral oil was layered on top of the reaction mixture and then reactions were carried out in a Hybaid™ Thermal Reactor programmed according to the length of the desired product and the approximate melting temperature of the primer/template duplex. Generally the DNA was heated at 94°C for 30 sec (denaturation), annealed for 1 min at the appropriate temperature (depending on the GC content of the primer) and then polymerisation took place at 72°C for 1 min/kb of template.

Thesit Buffer III (10x) (Ponce and Micó, 1992)
- 300 mM Tricine pH 8.4
- 20 mM MgCl₂
- 50 mM β-mercaptoethanol
- 0.1% gelatin
- 1% Thesit
2.2.20 DNase I footprinting.

For preparation of end-labelled fragments plasmid DNA was first digested with one restriction enzyme to generate the end to be labelled, then incubated at ambient temperature for 30 min with $\alpha^{32}\text{P}-\text{dCTP}$ and $\alpha^{32}\text{P}-\text{dGTP}$ (30 to 50 $\mu$Ci of labelled deoxynucleoside triphosphate per restriction site, 3000 Ci/m mole), unlabelled deoxynucleoside triphosphates (20 $\mu$M) and 1.5 Units of bacteriophage T4 polymerase. Polymerase was inactivated at 70°C for 10 min and the DNA was ethanol precipitated, resuspended in the appropriate buffer and cleaved with a second restriction enzyme.

The labelled DNA fragment (at a concentration of approximately 1 nM) was incubated with various concentrations of purified DnaA protein ($0<[\text{DnaA}]<8 \mu\text{g/ml}$) in a final volume of 25 $\mu$l. DnaA protein was obtained from W. Messer's laboratory at the MPI für Molekulare Genetik in Berlin. The reaction mixture contained 2 mM ATP, 40 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM magnesium acetate and 1 mM dithiothreitol. Binding occurred during 5 min incubation on ice, followed by 10 min incubation at ambient temperature. Limited DNase I cleavage was then achieved by adding 1 $\mu$l containing 0.3 Units of DNase I (the optimum concentration had been determined by titration) to the reaction mixture and incubating at ambient temperature for precisely 1 min. The reaction was then stopped by adding 4 $\mu$l of 0.2 M EDTA and 35 $\mu$l of phenol. The samples were then ethanol precipitated.

The resulting DNA fragments were analysed by electrophoresis on a 6% polyacrylamide sequencing gel followed by autoradiography (see Section 2.2.18).

2.2.21 Gel retardation.

A 490 bp PCR product was used in the assay. This was diluted to 0.5 ng/$\mu$l. 3 $\mu$l of DNA solution was incubated with various amounts of purified DnaA protein. The molecular ratio of DNA:DnaA ranged from 1:50 to 2:1. Incubation took place in a total volume of 7 $\mu$l in binding buffer (identical to that described in Section 2.2.20). The samples were electrophoresed on a 2% agarose gel and the DNA was visualised using the SYBR-GREEN staining method developed by Molecular Probes.
2.3 Bacterial techniques

2.3.1 Preparation of competent cells and transformation with plasmid DNA.

To prepare competent E. coli cells the method of Chung et al. (1989) was employed. 5 ml of L-broth was inoculated with a single colony of the appropriate bacterial strain and incubated overnight with shaking at a suitable temperature. This culture was diluted 1 in 100 into fresh L-broth and grown, with good aeration, to an OD$_{600}$ of between 0.3 and 0.4. The culture was chilled on ice, transferred to a universal bottle and centrifuged at 4000x g for 10–15 min. The supernatant was removed and the bacterial pellet resuspended in 0.1x the original volume of ice-cold TSS buffer. At this point the cells could be frozen at −70°C, or could be used immediately for transformation. Freshly prepared cells always gave the highest transformation efficiency. The plasmid DNA (typically 1–100 ng in <10 μl) was added to 0.1 ml aliquots of the competent cells, mixed gently and stored on ice for 15–30 min. After this time between 0.4 and 0.9 ml of LBG (L-broth + 20 mM glucose) was added and the cells incubated at an appropriate temperature for 1 hour to allow expression of plasmid encoded antibiotic-resistance genes. 200 μl of this mixture was then spread onto antibiotic-containing plates and incubated until bacterial colonies appeared. Whenever a transformation was performed an aliquot of competent cells lacking plasmid DNA was used as a control.

Cells can be transformed with replicative form M13 DNA using this method. In this case the LBG step is omitted and 0.25 ml of M13-sensitive plating cells added to the transformed cells. The mixture is added to 3 ml of molten L-top agar, mixed gently and poured onto an L-agar plate which, once set, is incubated at 37°C overnight, after which M13 plaques should be clearly visible.

<table>
<thead>
<tr>
<th>TSS buffer:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco Bacto Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Difco Yeast Extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>PEG 3350</td>
<td>100 g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>20 mM</td>
</tr>
<tr>
<td>DMSO</td>
<td>50 ml</td>
</tr>
<tr>
<td>PIPES Buffer pH 6.5</td>
<td>10 mM</td>
</tr>
<tr>
<td>distilled water to 1 litre</td>
<td></td>
</tr>
</tbody>
</table>
2.3.2 Transformation by electroporation.

For plasmids that were transformed poorly by the TSS method electroporation was used. 5 ml of a fresh overnight culture of the strain to be transformed was inoculated into 1 l of LB. This was grown at the appropriate temperature with vigorous shaking to an OD$_{600}$ of 0.5 to 1.0. The cells were chilled on ice and then harvested by centrifugation at 5000 rpm in a Sorvall Superspeed centrifuge, using a GSA rotor for 15 min at 4°C. The pelleted cells were resuspended in a total of 1 l distilled water; this was repeated using volumes of 500 and 100 ml distilled water. The cells were then resuspended in 20 ml of 10% (w/v) glycerol in a 30 ml glass Corex tube, centrifuged as above using an SS34 rotor and resuspended in a final volume of 2 ml of 10% (w/v) glycerol. Cells were then either aliquotted and stored at −70°C or used directly for electroporation.

For electroporation, 80 µl aliquots of cells were chilled (or thawed) on ice. DNA was then added (generally 1 to 50 ng in 5 µl of TE) and the cells were incubated on ice for a further minute. The cells and DNA were then transferred to a pre-chilled electroporation cuvette. Electroporation was performed using a Gene-Pulser™ fitted with a Pulse Controller (Bio Rad Laboratories Ltd.). The 25 µF capacitor was charged to a potential of 2.5 kV and the Pulse Controller set to 200 Ω. The cuvette was pulsed once for a time constant of 4.5–5 ms (field strength 12.5 kV/cm). The cuvette was then removed from the chamber and the cells were immediately resuspended in 1 ml of SOC. This cell suspension was incubated at an appropriate temperature for 1 hour to allow expression of antibiotic resistance (if necessary) and then dilutions were plated onto the appropriate media.

SOC: 2% Bactotryptone
0.5% Bacto yeast extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl$_2$
10 M MgSO$_4$·7H$_2$O
20mM glucose

2.3.3 Frozen storage of bacterial strains.

It was found that strains of *E. coli* could be conveniently stored at −70°C without suffering a dramatic loss of viability; this included strains harbouring plasmids that might otherwise be lost. A fresh 5 ml overnight culture was prepared with
antibiotic selection if required. This was centrifuged at 4000 rpm for 10–15 min, the
supernatant discarded and the cells resuspended in 0.1x the original volume of Frozen
Storage Buffer. The cells were then left on ice for several hours before storing at
−70°C.

Frozen storage buffer:  50% bacterial buffer
                         50% glycerol (v/v)

2.3.4 F-factor mating of E. coli.
An overnight culture of the donor strain was diluted 20-fold into fresh LB
medium and left without shaking (to avoid damaging the pili) until an OD₆₀₀ of
approximately 0.1 was reached. The recipient strain was grown to similar OD₆₀₀ with
agitation. 100 μl of each strain were mixed together in an Eppendorf tube and
incubated at 37°C for 15 min. 800 μl of LBG was then added to the mixture followed
by vigorous vortexing to disrupt mating pairs. The mixture was then incubated at 37°C
to allow expression of antibiotic resistance. 200 μl aliquots were then plated out on
suitable media which selected against the original donor and recipient strains, but not
against exconjugants. The plates were incubated at an appropriate temperature.

2.3.5 Selection for the loss of tetracycline resistance.
The technique for selection for the loss of tetracycline resistance is that
reported by Maloy and Nunn (1981). (As explained in Section 4.13.1, however, this
method was found unsatisfactory.) A dilution of a culture containing 10⁶–10⁷ cells
was plated out and incubated for 24 to 48 hours on medium of the following
composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>agar</td>
<td>15 g/l</td>
</tr>
<tr>
<td>tryptone broth</td>
<td>5 g/l</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5 g/l</td>
</tr>
<tr>
<td>chlorotetracycline hydrochloride</td>
<td>50 mg/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g/l</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>10.4 g/l</td>
</tr>
<tr>
<td>fusaric acid</td>
<td>12 mg/l</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>

The method is based on the observation that tetracycline resistant cells are
hypersensitive to lipophilic chelating agents.
2.3.6 Sizing and counting of bacterial cells.

A Coulter Counter ZB (Coulter Electronics Ltd., Harpenden, England) and Coulter Channelyser model C-1000 were used for determining the size and number of bacterial cells. Cultures were grown in filtered L-broth or Nutrient broth and 100 µl samples were added to an equal volume of filtered Fixing Solution. The sample could then be stored indefinitely in a sealed tube but cell-size and number determination were routinely carried out within three to five days of sample preparation. For measurements a noted volume of the sample was diluted with 6 ml of filtered Counting Buffer and mixed in a glass vial. The electrode was lowered into the vial and the sample read. The Counter counts the number of cells in a fixed volume (usually samples were chosen to contain in the range of 10000 to 90000 cells per counted volume) and the Channelyser distributes the cells into separate channels according to their sizes. From this distribution the mode and the median cell-size of a culture could be determined.

<table>
<thead>
<tr>
<th>Fixing Solution</th>
<th>80% Bacterial Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% formaldehyde (v/v)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Counting Buffer</th>
<th>0.85% NaCl (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.08% sodium azide (w/v)</td>
</tr>
</tbody>
</table>
2.4 Phage techniques

2.4.1 Preparation of λ plate lysates.

Cells were grown in L-broth + 20 mM MgSO4 and maltose at 0.2% until mid-
exponential phase. 200 μl aliquots were then mixed with 10^6 phage, incubated at 37°C for 5 min, and 3 ml of L-top agar containing 20 mM MgSO4 and 0.2% maltose added. This was poured onto an L-agar plate, left to set and incubated at 37°C overnight or until visible lysis occurred. 5 ml of phage buffer was then added to the plate and the layer of top agar scraped off into a sterile 250 ml beaker. A few drops of chloroform were added and the beaker was incubated at room temperature with gentle swirling for 20 min. The contents of the beaker were poured into a universal bottle and centrifuged at 4500x g for 10 min. The supernatant was transferred to a fresh half-ounce bottle and stored over a few drops of chloroform at 4°C.

2.4.2 Preparation and selection of λ lysogens not conferring antibiotic resistance.

A lawn of the bacterial strain to be lysogenized was made by mixing 0.2 ml of a mid-exponential phase L-broth culture and 3 ml of L-top agar with 20 mM MgSO4 and 0.2% maltose added. To this approximately 200 λ phage particles were added, and the mixture poured on a fresh L-agar plate. Once set the plate was incubated overnight at 37°C. This should result in the appearance of isolated λ plaques. The centre of a plaque was then touched with a sterile toothpick and streaked out onto another plate, which again was incubated overnight. The resulting single colonies could be tested for the presence of the λ phage. A lysogenized bacterium will now be resistant to lysis by phages with the same immunity as the one used to lysogenize the strain, but will be sensitive to λ phages that are virulent, or carrying a different immunity. This was initially tested using cross-streaks on an L-agar plate, and positives from this test were checked more fully by trying to make isolated plaques as above. Strains that seemed to be lysogens were then finally tested by examining supernatants from L-broth cultures to see if they were producing the correct phage.

2.4.3 Selecting kanR λ lysogens.

Section 3.8.1 describes the kanR λ phages, based on those constructed by R. W. Simons (Simons et al., 1987), used widely throughout this work. To isolate recombinants of fusions constructed with the kanR vector λRS45, 100 μl of an overnight culture of an appropriate lac^- E. coli strain (grown in L-broth + 20mM...
MATERIALS AND METHODS

MgSO₄) was infected with 100 µl of various dilutions of the phage lysate to achieve a multiplicity of infection between 10⁻⁶ and 10⁻². The phage were left to adsorb at 30°C without agitation after which 2 ml of L-broth + 20 mM MgSO₄ were added and the mixture was incubated with vigorous agitation for 1 hour. Aliquots of 200 µl were plated on selective (kanamycin) medium containing 30 µg/ml X-gal to indicate β-galactosidase synthesis from the phage. Generally colonies were picked for further work from those plates corresponding to the lowest multiplicity of infection.

2.4.4 UV induction of λ lysogens.

Lysogenic bacteria were grown in L-broth + 20 mM MgSO₄ at 37°C with vigorous agitation until an OD₆₀₀ of 0.3 was achieved. The cells were harvested by centrifugation and resuspended in 7 ml of 20 mM MgSO₄. This was transferred to a sterile glass petri dish and the cells were exposed to 600 ergs/mm²/sec of UV irradiation before being diluted five-fold in fresh L-broth + 20 mM MgSO₄. This culture was grown at 37°C with vigorous shaking until lysis occurred. A few drops of chloroform were added, and the lysate clarified by centrifugation prior to titration.

2.4.5 Induction of λ lysogens with mitomycin C.

Lysogenic bacteria were grown in L-broth + 20 mM MgSO₄ at 37°C with vigorous agitation until an OD₆₀₀ of 0.5 was reached. 5 µg/ml of mitomycin C was added and the culture continued to be incubated in the dark at 37°C until lysis occurred, usually two to three hours. The cell debris was removed by centrifugation in a bench top centrifuge and the λ lysate was stored at 4°C. Chloroform was added to prevent microbial growth.

2.4.6 Purification of bacteriophage λ particles from liquid lysates.

DNase and RNase were added to the liquid lysate to a final concentration of 1 µg/ml and incubated at room temperature for 30 min, swirling occasionally. NaCl was then added to the liquid lysate to a final concentration of 1 M, dissolved gently and incubated for 1 hour on ice (or left at 4°C overnight). The lysate was then centrifuged at 10000 rpm for 10 min at 4°C (Sorvall centrifuge, GSA rotor) to remove bacterial debris that had been liberated by DNase and RNase digestion and the high NaCl concentration. The supernatant was decanted into a clean flask, PEG 6000 was added to a final concentration of 10% (w/v) and dissolved gently at room temperature. The
suspension was incubated on ice for 4–6 hours and then centrifuged at 10000 rpm for 15 min at 4°C (Sorvall centrifuge, GSA rotor). The supernatant was discarded and the centrifuge bottle allowed to drain for 10 min. The pellet was resuspended at room temperature in 5 ml of phage buffer by gentle shaking for 1 hour. The suspension was transferred to a glass bottle and a further 1 ml phage buffer used to rinse the inside of the centrifuge bottle; this was added to the suspension. 7 ml of chloroform was added to the suspension and vortexed gently for 10 sec. The phases were separated and the PEG 6000 pelleted by centrifugation at 5000x g for 10 min in a bench centrifuge. The aqueous layer was transferred to a fresh bottle and stored at 4°C.

A CsCl step gradient was prepared by successively underlaying 2.5 ml of 31.2%, 2.0 ml of 45.4% and 1.5 ml of 56.2% CsCl solutions in a 13 ml Beckman Ultra-Clear™ tube using a Pasteur pipette. The phage suspension was then carefully loaded on top of the CsCl solution layers to within 2 mm of the top of the tube. The step gradients were balanced to within 10 mg and centrifuged at 35000 rpm for 35 min at 20°C (Sorvall ultracentrifuge, TH64 titanium swing-out rotor). Two bands could be seen in visible light: the lower consisted of phage particles and the upper of debris. The phage band was collected through the side of the tube using a wide-bore needle and syringe.

The band from the step gradient was dialysed against 2 l of phage buffer for 2 hours at 4°C. The dialysed suspension was then loaded onto a second CsCl step gradient, which was prepared as before. In order to minimise phage loss, the inside of the dialysis tubing was rinsed with 1 ml of phage buffer; this was also loaded onto the CsCl step gradient. The tubes were balanced and centrifuged as before. Normally, only the lower (phage) band was visible after the second round of centrifugation. This was collected as before using a needle and syringe, and stored at 4°C.

2.4.7 Preparation of phage P1 plate lysates.

Preparation of phage P1 plate lysates was as for phage \( \lambda \) except that \( 10^6 \) phage were added to 1 ml of late-log phase cells and this was incubated at 37°C for 30 min prior to addition to the top agar. The maltose was omitted, and the \( \text{MgSO}_4 \) was replaced with 2.5 mM CaCl₂. The phage buffer was also replaced with the same volume of L-broth + 2.5 mM CaCl₂.
2.4.8 Phage P1-mediated transduction.

The recipient strain of *E. coli* was grown up to late-exponential phase in L-broth. The cells were harvested by centrifugation and the bacterial pellet resuspended in 0.1x the original volume of L-broth + 2.5 mM CaCl₂. 100 µl aliquots of this 10x concentrated culture were mixed with either 0.1 ml of phage P1 stock or 0.1 ml of a 10x dilution of the phage stock. These were incubated at 37°C for 15 min. If prototrophic transductants were to be selected, 0.4 ml of phage buffer was added and 0.2 ml aliquots of the cells plated on the appropriate minimal media agar plates. If the selection was the acquisition of antibiotic resistance then 1 ml of phage buffer was added to the cells, the cells were centrifuged and resuspended in 0.6 ml of LBG. This was incubated at 37°C for 1 hour to allow expression of the antibiotic resistance and then 200 µl aliquots were plated out on L-broth agar plates containing the appropriate antibiotic. Plates were incubated at a suitable temperature until colonies appeared.
2.5 β-galactosidase enzyme assay

The method used is essentially that of Miller (1972). For assays of β-galactosidase under control of the dnaA or fisZ promoters, in strains containing a chromosomal copy of the lacZ gene, glucose at 0.2% was added to the medium to minimise transcription from this source (catabolite repression). Chromosomally encoded lacZ expression was undetectable under these conditions. Otherwise cells were grown in the media indicated in the text. The growth of the culture was followed by measuring the OD$_{600}$. Generally, regular dilutions (using pre-warmed media) were made such that the OD$_{600}$ of the culture was kept between 0.05 and 0.2 before sampling. In all experiments strains were maintained in exponential growth for at least four hours before sampling.

All assays were carried out in small glass test-tubes. Samples of either 0.5 ml or 0.1 ml (depending on the β-galactosidase activity of the strain) were taken and added to 0.5 ml or 0.9 ml respectively of Z-Buffer. To permeabilise the cells the Z-Buffer contained 0.005% SDS and in addition 15 μl of chloroform was added to each sample. The sample was vortexed for 10 seconds and could at this point be sealed and stored overnight at 4°C.

To assay, 200 μl of 4 mg/ml ONPG (o-nitrophenyl-β-D-galactoside) in 0.1 M MOPS (pH 7.0) was added to each sample. This sample was incubated at 30°C until sufficient yellow colour (o-nitrophenol) had appeared such that when 0.5 ml of Na$_2$CO$_3$ was added to stop the reaction, the absorbance at 420 nm was between 0.1 and 2.0. Standard curves constructed in this laboratory by T. Paterson had demonstrated that the absorbance measurements are linear within this range. Samples were measured at both 420 nm (o-nitrophenol) and at 550 nm (to correct for light scattering by cell debris). In cases where samples had been taken at high cell-density, the samples were spun in a micro-centrifuge after incubation to remove cell debris; in this case only the absorbance at 420 nm was determined.

Total enzyme activity was calculated as: $1000 \times \frac{(OD_{420} - 1.75 \times OD_{550})}{(T \times V)}$ where $T$ represents the incubation time in minutes and $V$ represents the volume of the sample in millilitres (generally 0.5 or 0.1).

The specific activity is defined as total enzyme activity divided by the OD$_{600}$ of the culture at the time of sampling (i.e. enzyme units per total cell-mass) and is expressed in Miller Units (MU).

Z-Buffer (per liter): 0.06 M Na$_2$HPO$_4$·7H$_2$O 0.001 M MgSO$_4$·7H$_2$O
0.04 M NaH$_2$PO$_4$·H$_2$O 0.01 M KCl
0.05 M β-mercaptoethanol
Chapter III

Autogenous regulation of dnaA gene expression
3.1 Introduction

Due to its fundamental role in initiation of chromosomal DNA replication and likely role in its control, the regulation of dnaA expression has received much attention. This issue was addressed in Section 1.9 and it will be clear that control of dnaA expression is at the moment far from being understood. One subject which has been extensively researched is that of autogenous regulation of the dnaA gene by its product and over the past decade it has become generally accepted that dnaA is autoregulated (Skarstad and Boye, 1994; Messer and Weigel, in press). This regulation appears to be effected through binding of the DnaA protein to a DnaA-box present between the two promoters, dnaA1p and dnaA2p (Figure 1.2). It has indeed been demonstrated that this region is specifically bound by the DnaA protein in vitro (Fuller et al., 1984; Braun et al., 1985; see Section 1.5.1).

The notion of dnaA autoregulation is appealing as it would go some way towards explaining the relative constancy of the initiation mass with respect to growth rate (Donachie, 1968). Assuming that it is the concentration of DnaA protein in the cell which sets the initiation mass, autoregulation could ensure that this concentration would remain constant at different growth rates. As was already stated in Section 1.8, Sompayrac and Maaløe (1973) proposed an autorepressor model for the control of DNA replication in which the initiator protein is expressed from a gene which is located in an operon which also contains a gene autogenously regulating the transcription of the operon. Hansen and Rasmussen (1977) went on to propose that the DnaA protein fulfils both the role of initiator and that of autorepressor based on the observation that more DnaA appears to accumulate in a dnaA18 mutant at the restrictive temperature than at the permissive temperature, implying that active DnaA is required to control its own rate of synthesis.

Evidence for a direct action of the DnaA protein on transcription from the dnaA promoters first emerged from in vivo studies by Braun et al. (1985). The authors constructed a transducing λ phage carrying a 945 bp EcoRI fragment containing the dnaA promoter region and the first twenty one codons of dnaA translationally fused to a lacZ reporter gene (λRB1; Figure 3.1.1). This allowed the expression from the dnaA promoters to be studied in single copy on the chromosome by monitoring the level of DnaA'—'β-galactosidase fusion protein produced by strains which carry λRB1 as a prophage (see Section 2.5).
Figure 3.1.1 The dnaA promoter region and the structure of λRB1.

The structure of λRB1 which contains a translational fusion of the first 21 amino acids of dnaA to the eighth amino acid of lacZ. The 945 bp insert, the structure of the dnaA promoter region and the sequence of the DnaA-box are shown. The construction of λRB1 is described in Braun et al. (1985).

Comparison of a dnaA+ and a dnaA46 λRB1 lysogen showed that β-galactosidase activity of the latter is greater and that this difference in activity becomes considerably more pronounced at the restrictive temperature for the dnaA46 strain. This result was interpreted to mean that as the DnaA46 protein is inactivated at high temperature, it becomes less capable of binding its putative target site between the promoters and no longer functions as a transcriptional repressor.

If DnaA is overproduced in a λRB1 lysogen by introduction of a multicopy plasmid containing the dnaA gene, β-galactosidase activity is reduced compared with a lysogen lacking such a plasmid. This again implies that DnaA is acting as a repressor of its own synthesis. It was further shown that the action of DnaA is likely to be at the transcriptional rather than at the translational level as a transcriptional fusion between the dnaA promoter region and lacZ (λRB9) responds similarly to overproduction of DnaA (Braun et al., 1985). Genetic studies indicate that the site of action of the DnaA protein is located between the promoters. Two deletion derivatives of λRB1 were constructed: λRB16 which lacks dnaA2p, and λRB13 which carries a deletion encompassing dnaA2p, the DnaA-box and the short stretch of DNA between these two sequences (approximately 20 bp). The rate of β-galactosidase synthesis from λRB16
is reduced upon overproduction of DnaA whereas that of λRB13 is not, implying that the presence of the DnaA-box is essential for DnaA to act as a repressor (Braun et al., 1985).

Similar results were obtained by Atlung et al. (1985) using both the tetracycline resistance gene (tet) and lacZ as reporter genes on a plasmid vector. Transcription from both the 945 bp EcoRI fragment as well as a shorter 387 bp ClaI–EcoRI fragment lacking the rpmH promoters was found to be responsive to overproduction of DnaA. Overproduction of DnaA from an inducible promoter resulted in repression of dnaA promoter activity to as little as 15% of its normal value. Again dnaA promoter activity was found to be derepressed in various dnaA18 mutant strains at the restrictive temperature. An extensive set of deletion derivatives of the promoter fusions were constructed with the aim of identifying the site which confers sensitivity to the DnaA protein. It was concluded from studies with these constructs that at most 11 bp upstream of the DnaA-box are required for repression of transcription by DnaA; if the first base pair of the DnaA-box is deleted transcription from the remaining part of the promoter region (containing only dnaA2p) is rendered insensitive to surplus DnaA. This study thus supports that of Braun et al. (1985) in implicating the DnaA-box in autoregulation. Additionally, Atlung et al. (1985) found that a 4 bp insertion in the –35 sequence of dnaA2p abolishes transcriptional repression by DnaA; it is possible that this insertion interferes with the normal interaction of DnaA and RNA polymerase at dnaA2p, the major promoter.

The studies by Braun et al. (1985) and Atlung et al. (1985) are at odds regarding the autoregulation of dnaA1p. In the former report deletion of dnaA2p does not abolish sensitivity to surplus DnaA protein whereas in the latter report transcription from a fusion of dnaA1p to the tet gene is not responsive to DnaA overproduction even though the DnaA-box is still present in the construct. Kücherer et al. (1986) studied autoregulation by looking directly at the transcripts from the two promoters using quantitative S1 mapping. They found that transcription from both promoters is derepressed at high temperature in a dnaA46 or dnaA508 background. Transcription from dnaA1p is increased approximately four-fold and that from dnaA2p approximately two-fold. Overproduction of DnaA from the inducible tac promoter led to repression of both promoters. This is supported by run-off transcription experiments which show that transcription from both dnaA promoters is repressed by purified DnaA protein in vitro (Wang and Kaguni, 1987).

Further evidence for autoregulation comes from work by Hansen et al. (1987). The authors introduced multicopy plasmids containing the oriC region into a strain carrying the dnaA1p2p–lacZ fusion λRB1. This resulted in an elevated β-
galactosidase production from the phage, presumably due to titration of DnaA by the plasmids. Kücherer et al. (1986) looked directly at the dnaA transcripts in a similar experiment and showed that both promoters become derepressed upon introduction of an oriC plasmid; the relative levels of derepression are similar to those found upon inactivation of the temperature-sensitive DnaA mutant protein in a dnaA<sup>ts</sup> background.

Taken together the above studies very strongly suggest that the dnaA gene is subject to autogenous control and that this control is brought about by binding of the DnaA protein to a site between the two promoters, resulting in a decrease in transcription from both promoters. Nevertheless data exist which are in conflict with this model. Masters et al. (1989) discovered that thermal inactivation of the DnaA46 protein at its restrictive temperature does not lead to derepression of transcription from λRB1 in a dnaA<sup>46</sup> strain when this strain is integratively suppressed by an R1 derivative. A similar result was later reported by Polaczek and Wright (1990) using P2 sig5 to integratively suppress a dnaA<sup>46</sup> strain. These authors also further investigated the role of the DnaA-box in the dnaA promoter region using mutational studies. Notably, they found that a deletion of the DnaA-box resulted in a three-fold decrease in the transcriptional activity of dnaA<sup>2p</sup>; this led the authors to conclude that at normal physiological levels the DnaA protein acts as an activator and not as a repressor of this promoter. Only at artificially elevated levels does the protein exercise a repressing effect at this promoter (Polaczek and Wright, 1990). It is interesting to compare these results with those of Atlung et al. (1985) who found that although deletion of the DnaA-box does not decrease expression from dnaA<sup>2p</sup>, it certainly does not lead to an increase as would be expected if the DnaA-box were acting as a binding site for a repressor.

It is the failure of transcription from the dnaA promoters to be derepressed at high temperature in an integratively suppressed dnaA<sup>46</sup> background which is the basis for the work described in this chapter. It was hoped that this would shed light on the unresolved question of dnaA autoregulation.
3.2 Isolation of integratively suppressed derivatives of a dnaA46 strain

3.2.1 Isolation and characterisation of pKN500 integrants.

The work presented by Masters et al. (1989) was carried out with the use of TP88, a version of the dnaA46 ΔlacZ strain ED419 which carries the dnaA1p2p–lacZ fusion λRB1 described above at the λ attachment site (attλ). This strain shows a gradual increase in specific β-galactosidase activity when grown at the restrictive temperature (42°C); after several hours of incubation at this temperature the increase eventually reaches a plateau at roughly five times its initial value at the time of the temperature shift. An integratively suppressed derivative of this strain (TPK88) was constructed which carries the mini-R1 plasmid pKN500 (Molin et al., 1979; Figure 3.2.1) at 89 minutes on the chromosome.

Figure 3.2.1

\[ \text{pKN500 consists of two EcoRI fragments from R1drd-19. The large fragment carries the origin and part of the transfer region of R1, the small one is from the resistance determinant region and carries the \textit{aphA} kanamycin resistance gene and the insertion sequence IS1.} \]

Figure 3.2.2 shows a comparison of the levels of β-galactosidase produced by λRB1 when present in TP88, TPK88 and the isogenic dnaA+ strain TP91 in a temperature-shift experiment. Clearly there is no derepression of lacZ expression in either TP91 or
TPK88, even though in the latter the DnaA46 protein should be inactivated at 42°C. Furthermore, it was shown that this apparent failure of the dnaA promoters to be derepressed at high temperature occurs only when pKN500 is integrated in the chromosome and not when it is present only in the cytoplasm. It was not clear what is responsible for this phenomenon and it was therefore decided to investigate whether it is a general feature of integratively suppressed strains and whether there is any dependence upon the nature of the suppressing replicon or its location on the chromosome.

Figure 3.2.2

Cultures were maintained in exponential growth phase at 30°C in LB medium for several hours before sampling and diluted into LB medium of 42°C at time 0. The cultures continued to be kept in exponential phase. Shown is the β-galactosidase activity per total cell mass (specific activity) plotted against time. Taken from Masters et al. (1989).

My work began with the isolation of further independent pKN500 integrants of TP88. These were isolated by transforming parallel cultures of TP88 with the plasmid and streaking out the transformants resulting from several transformations at 42°C. Transformants typically gave approximately 100-fold more colonies than TP88 after overnight incubation at that temperature (see Table 3.2.1). These possible integratively suppressed derivatives were then restreaked at 42°C; an individual colony
of each was picked and the potential integrants were named TPK1 to TPK12. These strains were then tested for both retention of the dnaA46 allele and for integration of pKN500 by transductional analysis.

**Table 3.2.1** Pseudo-reversion of TP88 carrying various plasmids.

<table>
<thead>
<tr>
<th>strain</th>
<th>cfu/ml 30°C</th>
<th>cfu/ml 42°C</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP88</td>
<td>7.5 · 10⁸</td>
<td>6.3 · 10¹</td>
<td>0.8 · 10⁻⁷</td>
</tr>
<tr>
<td>TP88(F::Tn5)</td>
<td>1.1 · 10⁹</td>
<td>1.1 · 10⁴</td>
<td>1.0 · 10⁻⁵</td>
</tr>
<tr>
<td>TP88(pKN500)</td>
<td>5.9 · 10⁸</td>
<td>8.0 · 10³</td>
<td>1.3 · 10⁻⁵</td>
</tr>
<tr>
<td>TP88(Rldrd-19)</td>
<td>8.0 · 10⁸</td>
<td>9.0 · 10³</td>
<td>1.1 · 10⁻⁵</td>
</tr>
</tbody>
</table>

Overnight cultures were grown under appropriate antibiotic selection at 30°C and the number of colony forming units (cfu) was determined by spreading dilutions onto minimal medium plates which were incubated for two nights at the indicated temperatures.

Retention of the dnaA46 allele in each strain was confirmed by transducing MM18 (dnaA⁺, asnAB) with P1 lysates prepared on TPK1–12 and selecting for the ability to grow on fully supplemented minimal medium plates lacking asparagine. Either transduction of asnA or asnB would result in asparagine prototrophy. However, due to its proximity to oriC transduction of asnA is more efficient than that of asnB (Masters, 1977). asnA is 20% co-transducible with the dnaA gene and therefore the transductants, purified by restreaking on plates lacking asparagine, were subsequently tested for their ability to grow at high temperature. Lysates from each TPK strain were found to co-transduce temperature sensitivity with asnA (see Table 3.2.2) and TPK1–12 therefore seem likely to have all retained the dnaA46 allele.

To test whether the temperature resistant growth of TPK1–12 was due to integrative suppression rather than some other form of extragenic suppression TC182 (dnaA46) was transduced to kanamycin resistance with P1 lysates prepared on each of the potential integrants. If the strains were integratively suppressed by pKN500 temperature resistance would be 100% co-transducible with the kanamycin resistance which is conferred by the plasmid. Transductants were selected on LB plates containing kanamycin both at 30°C and at 42°C. In most cases the same transduction
yielded different numbers of transductants at the two temperatures (see Table 3.2.2). A possible explanation is that pKN500 integrates into the chromosome at various loci by virtue of an IS1 sequence present on the plasmid. It has been shown that IS1 can integrate at a variety of sites on the chromosome (Birkenbihl and Vielmetter, 1989). It is also known that strains integratively suppressed by replicons such as R1 and F can contain these integrants at various positions on the chromosome (Nishimura et al., 1971 and 1973). However, not all sites of integration confer equally effective suppression of the dnaA phenotype, especially when (as in the present case) the cells are grown on rich medium (Louarn et al., 1982; see Section 1.10.8). It is therefore likely that the transductional events which result in equally efficient transduction at both temperatures involve pKN500 integrated at positions which confer efficient integrative suppression. Conversely, inefficient transduction at 42°C may indicate integration of pKN500 at a site which does not result in efficient suppression on rich medium, probably in the proximity of terC (Louarn et al., 1982).

The kanR transductants which were selected at 30°C were then tested for temperature resistant growth by patching on minimal medium plates containing kanamycin and incubating at 42°C. In each case all transductants were found to be capable of growth at that temperature, indicating that TPK1–12 all contain an integrated pKN500 capable of suppressing the dnaA46 phenotype.

TPK1–8 were further tested for integration of pKN500 by Southern hybridisation. Chromosomal DNA was prepared from these strains and digested either with EcoRI or with HindIII. Chromosomal DNA prepared from TP91 and from TP91(pKN500) were treated similarly and served as controls; the former strain does not contain pKN500 and the latter carries only the autonomous plasmid in the cytoplasm. The digested DNA was electrophoresed on agarose gels and transferred to a nylon filter as described in Section 2.2.15. It was then hybridised to a radioactive probe which was prepared from purified entire pKN500 plasmid DNA (see Section 2.2.15).
Table 3.2.2 Transductional data concerning integratively suppressed strains.

<table>
<thead>
<tr>
<th>donor</th>
<th>MM18</th>
<th>recipient</th>
<th>TC182</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no. <em>asn</em>+ transductants tested</td>
<td>no. t*</td>
<td>% t*</td>
</tr>
<tr>
<td>TPK1</td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>TPK2</td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>TPK3</td>
<td>20</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>TPK4</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPK5</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPK6</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TPK7</td>
<td>20</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>TPK8</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TPK9</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPK10</td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>TPK11</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TPK12</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPR1</td>
<td>20</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>TPR2</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPR3</td>
<td>20</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>TPF1</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TPF2</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TPF3</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>TPF4</td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

P1 lysates were prepared from the donor strains listed in the leftmost column and were used to transduce the recipient strains MM18 and TC182. MM18 was transduced to asparagine prototrophy. Transductants were colony purified on -asn plates. Five individual colonies resulting from each of twenty original transductants were tested for temperature-sensitivity by patching at 42°C. If any of the five colonies from a particular transductant were found to be temperature-sensitive the transductant would be scored as such. TC182 was transduced to kanamycin resistance. Selection took place by plating equal aliquots of the transduced culture on kanamycin plates at 30°C and 42°C. The numbers of transductants resulting from each incubation is shown.
The result is shown in Figure 3.2.3. The controls show the expected pattern: in both experiments TP91 shows no hybridisation at all with pKN500 DNA and TP91(pKN500) shows only hybridisation of bands the sizes of which are consistent with those expected from digestion of free pKN500 with the respective restriction enzymes. The patterns observed with the TPK strains are different from the controls in all but one case: TPK2 DNA digested with both EcoRI and with HindIII shows hybridisation of the same bands as TP91(pKN500) DNA digested with the same enzymes. This could indicate that the plasmid is not integrated into the chromosome or alternatively that both an integrated and an autonomous version of pKN500 are present in the strain; in the latter case the plasmid would have to be integrated in a part of the chromosome where digestion of the integrated plasmid with either EcoRI or HindIII results in the formation of three bands of approximately the same size as the internal fragment released by digestion and which are unresolved on the autoradiograms. The transductional data shown in Table 3.2.2 certainly support the idea that the plasmid is integrated. However, this table also shows that TPK2 is a poor donor in transduction of pKN500 to TC182 when selection of kanamycin resistance takes place at 42°C. As described above this may indicate that the site of integration of the plasmid does not confer efficient suppression of dnaA46 and therefore upon repeated growth at high temperature other suppressor mutations may have accumulated in the strain, relieving the necessity for the plasmid to remain integrated. The result of the hybridisation experiment may therefore indicate that TPK2 is not integratively suppressed.
Figure 3.2.3 Southern hybridisation of TPK strains.

Above: chromosomal DNA from the indicated strains digested with HindIII and hybridised to pKN500 DNA. Below: the same chromosomal DNA from the same strains digested with EcoRI. Some hybridisation was found to occur also with the λ size markers (fragment sizes are shown on the right).
The hybridisation patterns yielded by the other TPK strains are consistent with integration of the plasmid. The ISI sequence at which recombination with the chromosome occurs is located on the smaller fragment created when free pKN500 is digested with either EcoRI or HindIII. Thus, upon integration this fragment will be split into two fragments flanked by chromosomal DNA (Figure 3.2.4). Digestion of the integrated plasmid with either enzyme will therefore release a fragment which is equal in size to the larger fragment resulting from digestion of the free plasmid; this "internal" fragment is present in each case (Figure 3.2.3). In addition two other bands will arise which should hybridize to the pKN500 probe and which will vary in size depending upon the site of plasmid integration. Only TPK1 shows less than three bands in both digests. It may be that the size of the other band approaches that of the internal fragment to the extent that the two are not resolved on the gel used.

In conclusion it appears that TPK1-12, with the possible exception of TPK2, are integratively suppressed by pKN500 and that at least TPK1-8 each carries the integrated plasmid at a different location on the chromosome.

**Figure 3.2.4 Structure of integrated pKN500.**

Digestion of the integrated plasmid with either EcoRI (E) or HindIII (H) releases two fragments the size of which depend on flanking chromosomal DNA (as the locations of H_r, H_l, E_r and E_l vary with the site of integration) and one internal fragment of constant size.
3.2.2 Isolation of R1drd-19 integrants.

A number of independent TP88 derivatives integratively suppressed by R1drd-19 were isolated by transfer of this plasmid from MM18(R1drd-19) to TP88 by conjugal transfer. Overnight cultures of both strains were incubated on LB plates for several hours at 30°C and mating was then carried out by cross-streaking the cultures on selective plates; these were minimal medium plates containing ampicillin and the nutritional requirements for TP88 but lacking those for MM18. TP88(R1drd-19) was thus selected. Separate cultures of this strain were streaked out on LB plates and incubated overnight at the restrictive temperature for TP88 (42°C). TP88(R1drd-19) showed a higher frequency of colony formation than did TP88 (see also Table 3.2.1), consistent with the formation of integratively suppressed derivatives. A number of these were purified by restreaking at 42°C and named TPR1 to TPR3.

Retention of the dnaA46 allele in these strains was tested as previously described for the TPK strains by co-transduction of temperature sensitivity with asparagine prototrophy. The results are shown in Table 3.2.2: all three strains had retained the allele and it is thus likely that they are integratively suppressed.

3.2.3 Isolation of F::Tn5 integrants.

A further set of integratively suppressed strains was made by introducing an F plasmid containing Tn5 (F::Tn5; Hansen et al., 1984), which confers kanamycin resistance, into TP88. This was done by conjugal mating between MM38(F::Tn5) and TP88. Overnight cultures were diluted 20-fold into fresh medium and incubated without shaking for six hours. Equal volumes of the strains were mixed and allowed to mate for 15 minutes before this process was interrupted by vigorous agitation. The mixed culture was then plated on selective minimal medium plates containing kanamycin and lacking nutritional requirements for MM38. This yielded TP88(F::Tn5).

Potential integrants were then selected essentially as described above in the case of the pKN500 and R1drd-19 integrants by streaking out at 42°C on minimal medium plates and were named TPF1 to TPF4. Retention of the dnaA46 allele was verified as above by transduction (see Table 3.2.2). Integration of the F factor was tested first by a mating experiment: the structure of F::Tn5 is such that if it is present on the chromosome the kan gene will be transferred last. A short interrupted mating between the TPF strains and a recipient should yield no kanR recombinants. TP88(F::Tn5) and TPF1–4 were mated with MM38 (nalR) for 15 minutes before the
transfer was stopped by the addition of 100 μg/ml nalidixic acid. Recombinants were selected on plates containing kanamycin and nalidixic acid. The results are summarised in Table 3.2.3, TPF1–3 failed to give rise to kanR recombinants, whereas these were obtained from the mating with TP88(F::Tn5) as donor. This could be an indication that the F factor is integrated in the TPF strains. A further test is based upon the observation that F replication is sensitive to a lower dose of the antibiotic acridine orange than replication from oriC (Nishimura et al., 1971). Therefore if the strain is integratively suppressed by the F factor it should be sensitive to 20μg/ml of acridine orange at 42°C, at which temperature chromosomal replication is dependent upon the F origin, but resistant to the drug at 30°C as replication can initiate at oriC at this temperature (Chandler et al., 1977). This was indeed found to be the case for TPF1–4, indicating that F::Tn5 integratively suppresses the dnaA46 mutation in these strains.

### Table 3.2.3 Testing integration of F::Tn5 by mating with MM38.

<table>
<thead>
<tr>
<th>donor</th>
<th>no. of kanR/nalR MM38 recombinants per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP88(F::Tn5)</td>
<td>2.3 × 10⁴</td>
</tr>
<tr>
<td>TPF1</td>
<td>0</td>
</tr>
<tr>
<td>TPF2</td>
<td>0</td>
</tr>
<tr>
<td>TPF3</td>
<td>0</td>
</tr>
<tr>
<td>TPF4</td>
<td>0</td>
</tr>
</tbody>
</table>

An overnight culture of the donor strain was diluted twenty-fold into LB medium and left without agitation for six hours. An aliquot was mixed with an equal amount of MM38 and left for 15 minutes. The mating was interrupted by the addition of 100 μg/ml nalidixic acid. Dilutions were then plated on agar containing kanamycin and nalidixic acid. The numbers of recombinants from each mating are shown on the right.
3.3 β-galactosidase production by λRB1 in integratively suppressed dnaA46 derivatives

3.3.1 TPK strains.

In order to determine whether the newly isolated integratively suppressed strains (TPK1–12), which were described in Section 3.2.1, resemble TPK88 in failing to show derepressed production of λRB1 encoded β-galactosidase at high temperature, β-galactosidase assays were performed on these strains as described in Section 2.5. Overnight cultures of the strains were diluted 100-fold into LB medium and maintained in early exponential growth-phase at 30°C (at an OD600 of between 0.05 and 0.2) by periodic dilution into prewarmed medium. After approximately three to four hours growth under these conditions samples were taken and the cells were then diluted into LB prewarmed to 42°C. Again the cells were kept in early exponential phase and samples continued to be taken at intervals for several hours. Results are shown in Figures 3.3.1 to 3.3.4.

It is apparent in each case that the specific β-galactosidase activity of the integratively suppressed strains at 42°C is lower than that of the non-suppressed strain (TP88) which increases gradually for approximately two hours after the temperature shift. Although the absolute values of the specific activity of a particular strain can vary between experiments (compare for instance, TP88 in Figure 3.3.2 and in Figure 3.3.3) the relative activities of TP88 and its integratively suppressed derivatives varies little. Therefore little, if any, derepression of the cloned dnaA promoters occurs if the strain is integratively suppressed by pKN500 which is present, at least in most cases, at different sites on the chromosome. Only TPK4 and TPK5 show some derepression (see Figure 3.3.2). Its reproducibility was not examined and it is therefore uncertain whether the slight increase in β-galactosidase specific activity at high temperature is significant.
Figure 3.3.1

Autogenous Regulation of DNA Gene Expression

![Graph showing specific activity (MU) over time (min.) at 30°C and 42°C. The graph includes data points for TPK1, TPK2, TP88, and TP91.]}

Figure 3.3.2

![Graph showing specific activity (MU) over time (min.) at 30°C and 42°C. The graph includes data points for TPK3, TPK4, TPK5, and TP88.]
Figure 3.3.3

At 30°C and 42°C, the specific activity (MU) of TPK6, TPK7, TPK8, and TP88 is compared over time (min.).

Figure 3.3.4

At 30°C and 42°C, the specific activity (MU) of TPK9, TPK10, TPK11, TPK12, and TP88 is compared over time (min.).
3.3.2 TPR strains.

The strains integratively suppressed by R1drd-19 (TPR1–3, described in Section 3.2.2) were studied in an experiment similar to the ones described in the previous section. The specific β-galactosidase activities of TPR1–3 were assayed during steady exponential growth at 30°C and at 42°C. TP88 and TP91(dnaA⁺) were again used as controls. TPK7 was included in the assay and served as reference for comparison with the previous experiments. The result is shown in Figure 3.3.5. It is clear that the three TPR strains behave identically to TPK7 and show only a very slight derepression of β-galactosidase activity at high temperature compared with TP91. Integrative suppression by R1drd-19 therefore has the same effect on transcription from the dnaA promoters in a dnaA46 strain as integrative suppression by its derivative pKN500. Although pKN500 carries no DNA which is not present in R1, the two plasmids have in the past been reported to differ in a relevant way. R1 has been reported to be absolutely incapable of replication in the absence of DnaA (Masai and Arai, 1987) whereas pKN500 has been found to be capable of integratively suppressing a dnaA null mutation, albeit only in the presence of an unmapped host mutation known as mad-1 (Hansen and Yarmolinsky, 1986). This apparent difference between the two plasmids, however, seems of no influence on the phenomenon under study here.

Figure 3.3.5

![Graph showing specific activity of β-galactosidase at 30°C and 42°C for different strains](image)
3.3.3 TPF strains.

The TPF strains were studied in a similar fashion. Figure 3.3.6 shows a temperature shift experiment from 30°C to 42°C in LB medium using the strains TPF1 and TPF2. TP88 and TP91 again served as controls. It is clear from this graph that TPF1 and TPF2, although integratively suppressed by F::Tn5 (see Section 3.2.3), behave differently from the TPK and TPR strains discussed above. In TPF1 and TP2 lacZ expression from the dnaA promoters is derepressed at high temperature albeit to a lesser extent than that in TP88.

![Figure 3.3.6](image-url)

It has been reported that replication of the F factor itself displays strain dependent temperature-sensitivity and that in certain backgrounds F does not replicate well at 42°C (Stadler and Adelberg, 1972; Tresguerres et al., 1975). It was therefore a possibility that the failure of the dnaA promoters to remain repressed at high temperature in the TPF strains (unlike the TPK and TPR strains) might be a result of inefficient integrative suppression by the F factor at 42°C. The experiment was repeated again in LB medium, this time shifting the cells to 40°C, a temperature permissive for F replication (Tresguerres et al., 1975). The assay was performed on TPF1, TPF2 and TPF4 with TP88 and TP91 as controls; the result is shown in Figure 3.3.7. Again it is clear that dnaA1p2p dependent lacZ expression in the TPF strains is intermediate in derepression behaviour compared with TP88 and TP91. An indication
that TPF1 and TPF2 are nevertheless better integratively suppressed at 40°C than at 42°C comes from comparison of their growth rates at these two temperatures. Table 3.3.1 shows that the doubling times of both TPF1 and TPF2 are significantly greater at 40°C; TP91 (dnaA+), by contrast, grows at the same rate at both temperatures.

**Figure 3.3.7**

![Graph showing specific activity (MU) over time for different temperatures and strains](image)

**Table 3.3.1** Doubling times of TPF1 and TPF2 at high temperature.

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>TPF91</th>
<th>TPF1</th>
<th>TPF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>24</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>42</td>
<td>24</td>
<td>35</td>
<td>40</td>
</tr>
</tbody>
</table>

The doubling times were determined by monitoring the OD_{600} of cultures growing in LB at the indicated temperatures. Before and during sampling the cultures were maintained in exponential phase by periodic dilution in prewarmed medium.
Pritchard et al. (1975) reported that the number of F particles in the cell falls progressively with increasing growth rate. In LB medium the copy-number of F is approximately half that of the chromosome whereas in poorer medium it will be closer to that of the chromosome. It is therefore a possibility that if chromosomal replication is driven by the F origin at relatively high growth rates, the cell may suffer from underreplication of the chromosome (Tresguerres et al., 1975). This could be envisaged to lead to inefficient integrative suppression even at a temperature permissive for F replication. It was therefore decided to repeat the experiment under conditions even more favourable to integrative suppression by F: a temperature shift experiment from 30°C to 40°C was performed with TPF1, TPF2 and TPF3 in VB minimal medium containing only glucose and essential amino acids. TP88 and TPK1 served as controls; the result is shown in Figure 3.3.8. This figure shows that the derepression of lacZ expression observed from TPF1 and TPF2 is significantly smaller than in previous experiments. In Figures 3.3.6 and 3.3.7 expression from λRB1 increased by between 120% and 150% in both strains at high temperature. In minimal medium the increase at high temperature is approximately 50% (Figure 3.3.8). It must be noted, however, that the increase in enzyme expression observed in the non-integratively suppressed strain, TP88, when grown in rich medium (approx. 200%) is also greater than that when grown in minimal medium (approx. 100%). The ratio of the derepression in the TPF strains to the derepression in TP88 therefore changes from 0.75 (=150/200) in LB medium to 0.5 (=50/100). However, taking this into consideration, there still appears to be a significant effect of growth medium on the relative level of derepression of transcription from the cloned dnaA promoters present in TPF1 and TPF2.

TPF3, which was not studied in the earlier experiments, shows a far greater derepression of lacZ expression than TPF1 and TPF2. Figure 3.3.8 shows that the increase in lacZ expression of TPF3 at high temperature is approximately equal to that of TP88. In order to gain an initial indication of the efficiency of suppression of the dnaA46 mutation in that strain its growth rate at 40°C was compared to those of TPF1 and TPK1. It was found that the growth-rate of TPF3 was only slightly slower than that of TPF1 and TPK1.
Finally, Figure 3.3.8 shows that the basal levels of β-galactosidase specific activity of the strains are higher when they are grown in minimal medium than when grown in LB. Hansen et al. (1991a) report a similar but less pronounced result with strains carrying the λRB1 prophage and found that this was a result of the position of prophage on the chromosome. The gene dosage of the the dnaA gene relative to the dnaA'–'lacZ gene present on λRB1 increases with increasing growth rate; once corrected for gene dosage effects the specific β-galactosidase activities of strains carrying λRB1 were found not to vary with growth rate. However, the differences in the basal specific activities at 30°C in Figures 3.3.7 and 3.3.8 are too great to be solely due to gene dosage effects. If the same correction which was used by Hansen et al. (1991a) is applied to the data in these figures the specific β-galactosidase activities of the various strains do not become identical (Table 3.3.2). An alternative explanation exists which may account for these differences. λRB1 produces a hybrid DnaA'–β-galactosidase fusion protein which has been found to be unstable under the reaction conditions used to assay β-galactosidase (T. Atlung, personal communication). Therefore, if different incubation times are used in different experiments (or even with different samples in the same experiment), apparent differences in specific activities
will in part be due to differential protein degradation (or inactivation) during the assay. This also accounts for the aberrant values for specific β-galactosidase activity often found after dilution of a culture. Due to the decreased cell density of such a sample, the incubation time during the assay will generally be increased, leading to an underestimation of the true specific DnaA'–β-galactosidase activity. As the instability described above was unknown during the experiments done with λRB1, the results obtained were not corrected for its effects.

<table>
<thead>
<tr>
<th>strain</th>
<th>medium</th>
<th>sp. act (30°C)</th>
<th>doubling time (min.)</th>
<th>C (min.)</th>
<th>correction factor</th>
<th>corrected sp. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP88</td>
<td>LB</td>
<td>52</td>
<td>45</td>
<td>43</td>
<td>1.56</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>101</td>
<td>124</td>
<td>71</td>
<td>1.30</td>
<td>131</td>
</tr>
<tr>
<td>TPF1</td>
<td>LB</td>
<td>45</td>
<td>50</td>
<td>45</td>
<td>1.52</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>59</td>
<td>114</td>
<td>68</td>
<td>1.32</td>
<td>78</td>
</tr>
<tr>
<td>TPF2</td>
<td>LB</td>
<td>42</td>
<td>50</td>
<td>45</td>
<td>1.52</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>MM</td>
<td>59</td>
<td>143</td>
<td>78</td>
<td>1.29</td>
<td>76</td>
</tr>
</tbody>
</table>

The specific activities at 30°C were taken from the data in Figures 5.3.6 (LB) and 5.3.8 (MM); average values are shown. The doubling times (tD) of the strains were determined by monitoring the OD600 in the same experiments in which β-galactosidase activities were determined. The chromosome replication time (C) was calculated from the formula: C=0.357 tD + 27 (min.) (Kubitschek and Newman, 1978). The correction factor was the same as that used by Hansen et al. (1991a): 2(0.67C/tD) and was applied to the specific activities to correct for the gene position.
3.4 Efficiency of integrative suppression

3.4.1 Growth on agar.

In the Section 3.3.3 it was shown that lacZ expression from λRB1, in dnaA46 strains integratively suppressed by F::Tn5, is derepressed at high temperature. In this respect it differs from lacZ expression in strains integratively suppressed by R1 or its derivative pKN500. Conditions were sought under which integrative suppression by F would be as efficient as possible, yet some derepression of lacZ expression at high temperature was observed under all growth conditions tested. It is a possibility that this is a consequence of less efficient suppression of the dnaA46 mutation by F, even under the most permissive conditions, than by R1.

A comparison of the efficiency of integrative suppression was initially carried out by studying the growth of representatives of the two classes of integrants on agar plates containing either rich or minimal medium. TP88, TP91, TPK1 and TPF1 were streaked out on LB plates and on glucose minimal medium plates containing VB salts. The LB plates were incubated overnight and the minimal medium plates for two nights at various temperatures. The result is summarised in Table 3.4.1. This shows that in contrast to TPK1, TPF1 is hardly viable at 42°C on rich medium plates (even though it is capable of growth in liquid LB medium). In accordance with Tresguerres et al. (1975) (see Section 3.3.3) suppression by F at 40°C is more efficient; this is even more apparent on minimal medium. Suppression by pKN500 is efficient at all temperatures although the colony-sizes at 42°C on rich medium appear slightly smaller than those of TP91 (dnaA+). These results do not clearly support a correlation between efficiency of integrative suppression and derepression of dnaA promoter activity; at 40°C on minimal medium TPF1 and TPK1 grow equally well, yet showed a difference in β-galactosidase activity at this temperature (Figure 3.3.8).

This conclusion must be qualified. Inferences concerning the efficiency of suppression are made here by comparing colony formation whereas β-galactosidase production was measured during growth in liquid medium, where suppression may be different. Therefore more detailed studies were carried out by comparing cell-sizes of the various strains in liquid medium.
### Table 3.4.1

<table>
<thead>
<tr>
<th>strain</th>
<th>LB agar plates</th>
<th>VB minimal medium plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>37°C</td>
</tr>
<tr>
<td>TP88</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>TP91</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>TPK1</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>TPF1</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Colony sizes were compared by eye to those of TP91 grown under the same conditions. ++++ indicates good growth. +++ indicates a slightly smaller colony-size. ++ indicates the formation of very small single colonies. + indicates growth only in areas on the plate of high cell density (no clear single colonies). - indicates no growth.

### 3.4.2 Cell-size distributions.

A dnaAts strain suffers a block in the division process at non-permissive temperatures and forms filaments. It is not clear what is the molecular basis for this; it seems unlikely that the division block is mediated by an interaction of the SfiA and FtsZ proteins (see Section 1.14) as dnaA mutations do not normally lead to induction of the SOS response (Walker, 1984). The division block does, however, appear to be a direct consequence of cessation of initiation of DNA replication as suppression of dnaAts mutations by various mechanisms results in the relief of this block (see Section 1.10). Not all suppressors are equally effective: bypass suppression through cSDR, for instance, results in slower growth rate of the suppressed strain in comparison with the wild-type. In addition, the cell sizes of the suppressed strain are greater than those of the wild-type (Masters et al., 1989). Integrative suppression by pKN500 located at 89 minutes on the chromosome in the case of TPK88, by contrast, results in growth rate and cell sizes approximating those of a dnaA+ strain (Masters et al., 1989). Therefore, comparison of the cell-size distributions of the integratively suppressed TP88 derivatives under various growth conditions may shed light on the efficiency of suppression in each case.
Figure 3.4.1 shows the cell-size distributions of TP88 (dnaA46) and TP91 (dnaA+) after prolonged growth at 30°C, 40°C and 42°C in LB medium. The strains were kept in early exponential phase by periodic dilution into prewarmed medium and samples were taken after approximately four hours growth. The cell-size distributions were then determined with the aid of a Coulter Counter as described in Section 2.3.6. TP91 shows little variation in cell-size at the different temperatures; TP88 shows a normal cell-size distribution at 30°C and clearly deviates from this at both 42°C and 40°C (see Figure 3.4.4) due to the formation of filaments.

When TP88 contains an integrated copy of pKN500 the cell-size distributions change little with growth temperature (Figure 3.4.2). Figure 3.4.3, by contrast, shows cell-size distributions of TPF1 grown in LB medium at 30°C, 40°C and 42°C. At 42°C TPF1 shows clear filamentation whereas at 40°C there is little, if any, difference of cell-sizes compared with those at 30°C. This supports the notion that incubation at 40°C is significantly more permissive for integrative suppression by F than incubation at 42°C (see Section 3.3.3). TPF2 and TPF4 showed cell-size distributions similar to TPF1 at all temperatures.

Finally, the cell-size distributions of strains in glucose minimal medium were compared at 40°C (Figure 3.4.4). At high temperature TP88 filaments whereas TPK1 shows a more or less normal distribution. TPF1 and TPF2 show a slight broadening of the peak, indicating that suppression by F is not quite as efficient as that by pKN500. TPF3, which showed a greater derepression of dnaA promoter activity at 40°C, (Figure 3.3.8) also shows some indication of greater division problems at this temperature; TPF3 contains a sub-population of cells with twice the size of the main population which is not as apparent in the cases of TPF1 and TPF2.
Cells were maintained in exponential growth phase in filtered LB medium for three hours at the respective temperatures before being taken into formaldehyde for analysis with a Coulter Counter.
Figure 3.4.3

Above: cells were maintained at the respective temperatures in filtered LB medium for three hours before sampling. Below: cells were grown in VB minimal glucose medium at 40°C for four hours after a temperature shift from 30°C.
3.4.3 Discussion.

Masters et al. (1989) showed that a dnaA46 rnh double mutant in which the dnaA mutation is suppressed by cSDR does not grow as well as does an isogenic wild-type strain at high temperature. The growth rate of the mutant is lower and the cell-size distribution is broader compared to its parent strain. Moreover, lacZ expression from λRB1 in the mutant is derepressed at high temperature, in contrast to that in the integratively suppressed strain TPK88 which grows well at high temperature and has a normal cell-size distribution. The experiments described in the previous two sections were performed to establish whether derepression of the dnaA promoters is a general feature of strains in which dnaA mutations are poorly suppressed.

The data in Sections 3.3 and 3.4.2 indicate some correlation between the efficiency of integrative suppression and the level of dnaA promoter derepression. This is certainly the case with the strains integratively suppressed by F, as shown by the difference in filamentation at 42°C in LB and 40°C in minimal medium and the greater dnaA promoter derepression under the former conditions. In addition, TPF3 shows a greater degree of dnaA promoter derepression at 40°C than TPF1 and TPF2 and a lesser degree of suppression. Figure 3.4.4 shows that the cell-size distribution of the TPF strains is slightly more affected at 40°C than is that of TPK1, which unlike the TPF strains, shows no dnaA promoter derepression.

It is important to note, however, that the dnaA promoter in strains integratively suppressed by pKN500 is less sensitive to the effects of growth conditions which affect cell-size than is found in the TPF strains. Comparison of Figures 3.4.2 and 3.4.3 shows that a shift from 30°C to 42°C has a similar effect on the cell-size distribution of TPK3 as a shift from 30°C to 40°C on that of TPF1. Yet, the effect on promoter derepression is far more pronounced in TPF1 than in TPK3 (compare Figures 3.3.2 and 3.3.7) indicating that factors in addition to the efficiency of suppression may be influencing dnaA promoter activity.
3.5 The effect of integrative suppression of various dnaA alleles on dnaA promoter activity

3.5.1 Construction of integratively suppressed dnaA<sub>18</sub> derivatives of TP91.

Possible explanations for the effect of integrative suppression by pKN500 on dnaA promoter activity were suggested by Masters et al. (1989). The authors proposed that the plasmid may encode a substitute for the DnaA protein which is capable of binding the dnaA promoter and consequently preventing derepression when the DnaA46 protein is no longer active. Alternatively, the plasmid may encode a factor which restores activity to the defective DnaA46 protein. In either case these putative plasmid encoded products would be expressed only when the plasmid is integrated in the chromosome as pKN500, when maintained in the cytoplasm, does not prevent derepression of the cloned dnaA promoters. If pKN500 exerts its influence through a product which interacts with the mutant DnaA protein it would seem likely that such an interaction would be allele specific. Atlung et al. (1985) reported that the rate of lacZ expression from λRB1 is increased in a number of dnaA mutants relative to that in the wild-type at 42°C; dnaA46, dnaA167, dnaA601, dnaa204 and dnaA508 all showed varying degrees of derepressed dnaA promoter activity at high temperature. These alleles respond differently to various suppressors of their initiation phenotype such as rpoB and seqA mutations and overproduction of GroELS (see Sections 1.6.1, 1.10 and Table 1.1), showing that the nature of the dnaA defect is different in each case. Here it is investigated whether integration of pKN500 in other dnaA mutants leads to the same failure of the dnaA promoter to be derepressed at 42°C as it does in a dnaA46 background. To this end a number of dnaA alleles were transduced to the TP91 background and pKN500 was then transduced from TPK88 into the resulting strains as described below.

Table 3.5.1 shows the series of MM18 derivatives used to donate the dnaA alleles. These strains all carry the bgl<sup>+</sup> allele which is normally not expressed. bgl is involved in the metabolism of salicin; in order to use salicin as a sole carbon source E. coli requires a mutation at the bgl locus which is thought to change the local superhelical density of the DNA resulting in expression of the gene (DiNardo et al., 1982). Such mutations, often insertions, arise spontaneously at relatively high frequency and can be selected by requiring growth on minimal medium containing 0.4% salicin as the carbon source. Possible bgl mutants of the MM18 derivatives were isolated (MMB181 to MMB190) in order to co-transduce the dnaA alleles with this selectable marker. For unknown reasons the bgl<sup>+</sup> mutation is not selectable in pro mutants and for this reason TP91 was transduced to pro<sup>+</sup> (TP92). TP92 was
transduced to \textit{bgl} with P1 lysates prepared on the MMB strains and transductants were then purified by restreaking and screened for temperature-sensitivity. This strategy was successful in all but four cases (see Table 3.5.1). The temperature-sensitive \textit{bgl} TP92 derivatives were named TP182 etc. (Table 3.5.1). It is possible that MMB181, MMB183, MMB184 and MMB186 did not contain a mutation at the \textit{bgl} locus but a transductionally unlinked mutation elsewhere. This was not tested.

\textbf{Table 3.5.1} Construction of \textit{dnaA}\textsubscript{ts} derivatives of TP91 and integratively suppressed versions thereof.

<table>
<thead>
<tr>
<th>\textit{dnaA} mutants</th>
<th>spontaneous \textit{bgl} derivatives</th>
<th>\textit{dnaA}\textsubscript{ts} TP92 derivatives</th>
<th>\textit{dnaA}\textsubscript{ts} TP93 derivatives</th>
<th>int. suppr. by pKN500</th>
<th>\textit{dnaA} allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM181</td>
<td>MMB181</td>
<td>MMBT181</td>
<td></td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>MM182</td>
<td>MMB182</td>
<td>TP182</td>
<td>TPK182</td>
<td>5</td>
<td></td>
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<tr>
<td>MM183</td>
<td>MMB183</td>
<td>MMT183</td>
<td>TP183</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>MM184</td>
<td>MMB184</td>
<td>MMBT184</td>
<td></td>
<td>508</td>
<td></td>
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<tr>
<td>MM185</td>
<td>MMB185</td>
<td>TP185</td>
<td>TPK185</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>MM186</td>
<td>MMB186</td>
<td>MMT186</td>
<td>TP186</td>
<td>203</td>
<td></td>
</tr>
<tr>
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<td>MMB187</td>
<td>TP187</td>
<td>TPK187</td>
<td>602</td>
<td></td>
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<tr>
<td>MM188</td>
<td>MMB188</td>
<td>TP188</td>
<td>TPK188</td>
<td>601</td>
<td></td>
</tr>
<tr>
<td>MM189</td>
<td>MMB189</td>
<td>TP189</td>
<td>TPK189</td>
<td>604</td>
<td></td>
</tr>
<tr>
<td>MM190</td>
<td>MMB190</td>
<td>TP190</td>
<td>TPK190</td>
<td>606</td>
<td></td>
</tr>
</tbody>
</table>

The table shows the strains used in the construction of integratively suppressed \textit{dnaA} derivatives of TP91. The original donor strains are shown in the leftmost column and the \textit{dnaA} alleles carried by them in the rightmost column. See text for details.

To construct the remaining strains a different strategy was used. TP91 carries Tn7, which confers resistance to trimethoprim, 40\% linked to the \textit{dnaA}\textsuperscript{+} allele (and located between \textit{dnaA} and \textit{oriC}). MMB181, MMB184, MM183 and MM186 were transduced to \textit{tmp}\textsuperscript{R} using TP91 as a donor. The resulting transductants were screened for temperature-sensitivity. The temperature-sensitive \textit{tmp}\textsuperscript{R} derivatives were named
MMBT181/184 and MMT183/186 and contain Tn7 linked to the dnaA<sup>ts</sup> alleles. The intention was to co-transduce these alleles with trimethoprim resistance and therefore a tmp<sup>S</sup> derivative of TP91 was required: TP88 was transduced to temperature resistance using TP91 as a donor. Tmp<sup>S</sup> colonies were isolated by screening. This dnaA<sup>+</sup> derivative of TP88 was named TP93. This strain was subsequently transduced to tmp<sup>R</sup> using MMBT181, MMBT184, MMT183 and MMT186 as donors and temperature-sensitive colonies were again isolated by screening the transductants. This was only successful using lysates prepared on the MMT183 and MMT186 donors. This result appears to suggest that the bgI<sup>+</sup> allele is not tolerated in the TP8503 background in combination with the dnaA167 and dnaA508 alleles; this was not investigated further. The resulting strains are shown in Table 3.5.1.

The dnaA<sup>ts</sup> TP91 derivatives were transduced to kan<sup>R</sup> using TPK88 as donor. All became temperature-resistant and should have pKN500 integrated at 89 minutes on the chromosome. During the course of this work it was shown by Hansen <i>et al.</i> (1992) that certain pairs of dnaA alleles carry identical mutations (see Table 1.1): TP(K)183/186, TP(K)187/188 and TP(K)189/190 should therefore represent pairs of identical strains.
3.5.2 lacZ expression from λRBI in integratively suppressed dnaA5, dnaA601, dnaA604 and dnaA204 strains.

Temperature shift β-galactosidase assays as previously described in Section 3.3.1 were performed on TP182, TP183, TP188, TP189 and their integratively suppressed derivatives. The results are shown in Figure 3.5.1 and it can be seen that dnaA promoter activity is repressed to the same extent in each of the integratively suppressed strains. Especially in the case of TP183 (dnaA204) this is interesting as the other alleles tested are all reversible and are all suppressed by the rpoB902 mutation and by overproduction of GroELS. dnaA204 is different in all respects and yet shows the same lack of derepression of the dnaA promoters when integratively suppressed. This shows that it is unlikely that the failure of the dnaA promoters to derepress in integratively suppressed strains is due to modulation of the activity of the thermally inactivated DnaAs protein by way of a specific protein/protein interaction with a factor produced by pKN500.

The presence of the dnaA204 allele in TP183 was partly verified by transformation of this strain and of TP88 with pND5, a multicopy plasmid carrying the groE operon. TP88(pND5), but not TP183(pND5), was capable of growth at 42°C as expected (see Table 1.1).

Figure 3.5.1
3.6 Sensitivity of the dnaA promoters to DnaA overproduction in an integratively suppressed strain

Atlung et al. (1985) showed that β-galactosidase production from λRB1 is inhibited upon overproduction of the DnaA protein from a plasmid which carries the dnaA gene under the inducible λpL promoter. Kücherer et al. (1986) showed that transcription of the chromosomal dnaA gene is similarly repressed by overproduction of DnaA. These experiments suggest that in a normal strain DnaA appears to be a repressor of its own synthesis. In previous sections it was shown that in a strain which is integratively suppressed by pKN500, the cloned dnaA promoters do not respond to inactivation of this putative autorepressor. It was therefore examined whether they are responsive to overproduction of DnaA in such a strain.

Table 3.6.1 Overproduction of DnaA in TPK1.

<table>
<thead>
<tr>
<th>strain</th>
<th>sample time after addition of IPTG (min.)</th>
<th>specific β-galact. activity (MU)</th>
<th>average specific activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPK1</td>
<td>105</td>
<td>20.4</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>24.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>TPK1(pLSK5)</td>
<td>110</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>TPK1(pBR328)</td>
<td>110</td>
<td>24.6</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>27.9</td>
<td></td>
</tr>
</tbody>
</table>

The cultures were grown in LB medium at 37°C in the presence of ampicillin (except for TPK1) and maintained in early exponential phase by periodic dilution into prewarmed medium. After several hours 2μg/ml IPTG was added to the medium (for each culture) and the cells were kept under inducing conditions. Samples were taken at the indicated times after initial IPTG addition and β-galactosidase specific activities were determined.

TPK1 was transformed with pLSK5 (Kücherer et al., 1986). This plasmid carries the dnaA gene under control of the inducible tac promoter of the expression
vector pJF118. TPK1(pLSK5) was diluted from an overnight culture and maintained in exponential growth in LB medium containing ampicillin for approximately three hours. 2μg/ml IPTG was then added to the medium and the culture was again maintained in early exponential phase for a further two hours before samples were taken. Table 3.6.1 shows the specific β-galactosidase activity of this strain after overproduction of DnaA from pLSK5.

β-galactosidase synthesis is clearly repressed in the strain carrying pLSK5 and not in the control strain which carries pBR328. It appears therefore that integrative suppression by pKN500 does not abolish sensitivity of the dnaA promoter to repression by elevated intracellular levels of the DnaA protein.
3.7 Integrative suppression by the R1 minimal replicon

3.7.1 Attempt to clone the R1 minimal origin of replication using PCR.

Integrative suppression of dnaA46 by pKN500 and F do not both lead to the same level of repression of the dnaA promoter at high temperature. In Section 3.5.1 the possibility was raised that pKN500 may produce a factor which either substitutes for the DnaA autorepressor function or prevents its thermal inactivation; in the same vein F may produce a similar but less effective factor.

pKN500 is approximately 25 kb long and hence has the coding capacity for approximately twenty five proteins; thus the formal possibility exists that one or more of its products may have the properties of one of the putative factors described above or may be otherwise involved in influencing dnaA regulation. For this reason it was decided to construct a smaller derivative which should contain only the sequences necessary to promote replication and to enable integration into the chromosome. Previously an attempt had been made to use the plasmid pGW71 (which carries only the R1 basic replicon) to study the effects of integrative suppression by the R1 minimal origin. This failed as the plasmid, unlike pKN500, does not contain an IS1 sequence and as a consequence does not readily integrate into the host chromosome.

pKN500 consists of two EcoRI fragments from Rldrd-19: one of ca. 18 kb and one of ca. 6.7 kb. The smaller fragment contains the IS1 sequence and the kanamycin resistance gene. It was attempted to ligate this 6.7 kb fragment to a 2.9 kb EcoRI fragment containing the R1 origin of replication and incompatibility factors (Molin et al., 1979; Clerget et al., 1981). The 2.9 kb fragment was obtained by PCR amplification of the pKN500 origin region using primers containing EcoRI restriction sites. For the sequence of the fragment see Ryder et al. (1982). Although a fragment of this size was produced by the reaction, repeated attempts to ligate it to the 6.7 kb fragment were unsuccessful.

Amplification by PCR is generally an error prone process. In this case the Thermococcus litoralis DNA polymerase (marketed by New England Biolabs as Vent polymerase) was used, which possesses an editing function and should therefore minimise misincorporation of nucleotides (Matti la et al., 1991). This and the fact that the ligation was attempted with products from separate reactions makes it unlikely that the PCR product contained mutations that inactivated origin function in each case. Therefore the failure to ligate to the 6.7 kb fragment was likely to be due to other causes: the PCR product may not have been the desired one in spite of its size or the primers used to amplify it may not have contained the correct restriction sites. For this reason it was then attempted to clone the PCR product into a vector purchased from
Invitrogen, known as pCRT™1000. This cloning system takes advantage of the activity of thermostable polymerases to add single adenine residues to the 3’ end of PCR products in a non-template dependent manner (Clark, 1988). Cleavage of pCRT™1000 with HphI creates an insertion site which contains single 3’ thymine residue overhangs; this should allow direct ligation of any PCR product. However, repeated attempts to ligate the 2.9 kb R1 origin fragment to this vector failed. A possibility is that the R1 origin is unstable in this vector due to copy-number effects. It was decided to employ a different strategy to construct a mini-R1 plasmid.

3.7.2 Construction of pGW1S.

pGW71 is a 5 kb plasmid which contains the R1 minimal replication origin and the bla gene which confers ampicillin resistance (Bernander et al., 1989; Figure 3.7.1). The 3 kb HindIII fragment from pKN500 containing the IS1 sequence (Figure 3.2.1) was cloned into this plasmid as described below.

**Figure 3.7.1**

pGW71 carries the R1 basic replicon and the bla gene which confers ampicillin resistance. Constructed by E. G. H. Wagner (Bernander et al., 1989).
pH\(\Omega 1\) is a 12.7 kb plasmid containing the \(\Omega\) fragment. This is a 2 kb fragment which contains the streptomycin and spectinomycin resistance gene flanked on either side by transcriptional and translational terminators in both orientations. In pH\(\Omega 1\) the \(\Omega\) fragment is flanked by \textit{HindIII} sites adjacent to and inside \textit{PstI} sites (Figure 3.7.2). pH\(GW71\) contains four \textit{PstI} sites: one within the \textit{bla} gene, one within the \textit{repA} gene, one beside \textit{copB} and one just outside the origin. pH\(GW71\) was partially digested with \textit{PstI} and the \(\Omega\) fragment, released by digestion of pH\(\Omega 1\) with the same enzyme, was ligated to the \(GW71\) \textit{PstI} product corresponding to full length linearised plasmid. Transformation of the ligation products yielded amp\(R\), spc\(R\) colonies which contained the recombinant plasmid at the same copy-number as the parent (judged from miniprep DNA yields). These must contain the \(\Omega\) fragment inserted in one of the possible orientations into the \textit{PstI} site located outside the replication control region, as insertion elsewhere would not yield a viable amp\(R\) product with the correct copy-number, \textit{repA} being indispensable for plasmid replication and the \textit{copA/copB} region being involved in copy-number control. The resulting plasmid was named pH\(GW\Omega\).

Figure 3.7.2

\[\text{Diagram of pH}\Omega 1\]

\(\text{pH}\Omega 1\) carries the 2 kb omega fragment which contains the \textit{aadA} gene conferring both spectinomycin and streptomycin resistance. For details of construction see Mc\textsc{Lennan} (1993).
Digestion of pGWΩ with *HindIII* allowed exchange of the \( \Omega \) fragment with the 3 kb *HindIII* fragment from pKN500, which contains the IS\( 1 \) sequence. Initial screening of the ligation products by small-scale plasmid DNA isolation and digestion with *HindIII* showed that all twelve colonies tested contained plasmids which displayed the predicted restriction pattern. However, five out of twelve plasmid isolations yielded substantially more DNA than the others, suggesting that approximately half of the ligation products have a higher copy-number than the rest. It is possible that transcription originating in the 3 kb fragment is interfering with copy-number control due its location close to the replication origin of the plasmid. The fragment may be present in either of two orientations and it may be that only its presence in one particular orientation influences plasmid copy-number. Such a change in copy-number was not observed after cloning the \( \Omega \) fragment into pGW71; this could be due to the fact that this fragment is transcriptionally isolated as it contains transcriptional terminators at either side. A clone with low copy-number (identical to that of the parent plasmid, pGW71) was used for further experiments and named pGW'IS (Figure 3.7.3).

**Figure 3.7.3**

|BglII/BamHI| BglII| PstI
|---|---|---
|PsiI| stu| copB
|bla (amp)| copA| repA
|pGW'IS| oriR| 8.0 kb

pGW'IS contains the R1 basic replicon from pGW71 and the 3 kb *HindIII* fragment containing the IS\( 1 \) sequence from pKN500. For details of construction see text. Note that the orientation of the *HindIII* insert has not been determined and may therefore be opposite to the one shown.
3.7.3 Integrative suppression of dnaA46 with pGW'IS.

In order to establish whether pGW'IS is capable of integratively suppressing the dnaA46 mutation, TP88 was transformed with this plasmid. Various dilutions of a culture of TP88(pGW'IS) which had been grown overnight at 30°C were plated out and incubated at either 30°C or 42°C. Table 3.7.1 shows that TP88(pGW'IS) gives rise to an approximately sixty-fold greater frequency of temperature-resistance than either TP88 or TP88(pGW71). It appears therefore that this plasmid is capable of integrative suppression.

Table 3.7.1 Integrative suppression of TP88 by pGW'IS.

<table>
<thead>
<tr>
<th>strain</th>
<th>cfu/ml at 30°C</th>
<th>cfu/ml at 42°C</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP88</td>
<td>1.44 · 10⁹</td>
<td>0.03 · 10⁵</td>
<td>0.02 · 10⁻⁴</td>
</tr>
<tr>
<td>TP88(pGW71)</td>
<td>0.97 · 10⁹</td>
<td>0.03 · 10⁵</td>
<td>0.03 · 10⁻⁴</td>
</tr>
<tr>
<td>TP88(pGW'IS)</td>
<td>1.07 · 10⁹</td>
<td>1.70 · 10⁵</td>
<td>1.59 · 10⁻⁴</td>
</tr>
</tbody>
</table>

Cultures of the strains were incubated overnight in selective medium where appropriate. Dilutions were then plated out on LB and incubated at either 30°C or 42°C. The numbers of colonies which appeared were counted and colony forming units per ml calculated from this. The ratio of cfu/ml which are temperature-resistant to the total is shown.

Five independent possible integrants of pGW'IS in TP88 were then isolated by selecting for growth at 42°C of TP88(pGW'IS). Retention of the dnaA46 allele was verified by co-transduction with asparagine prototrophy to MM18 as described in Section 3.2.1. All showed between 20% and 40% co-transduction with temperature-sensitivity. These temperature-resistant derivatives of TP88 were designated TPG1 to TPG5. In order to assess whether these strains are integratively suppressed ED419(dnaA46) was transduced to ampR with P1 lysates prepared on the TPG strains. AmpR transductants were obtained although repeatedly only at low frequency; these transductants were all viable at 42°C although poorly (this is a common
observation when transducing an integratively suppressing plasmid to a dnaA recipient and will be discussed in Section 3.9). This indicates that the TPG strains are integratively suppressed. A control experiment showed that MM18 was transduced to asparagine prototrophy at a 10–100% greater frequency than to ampR. AmpR transduction appears to be inefficient for unknown reasons; it is possible that the pGW'IS plasmid is not stably maintained in its integrated state.

To study the behaviour of dnaA'–lacZ expression from λRB1 a temperature-shift β-galactosidase assay was performed as described earlier. The result is shown in Figure 3.7.4. The TPG strains all show the same level of repression of lacZ expression as TPK1, which was used as a control. This experiment shows that most of R1 is dispensible in preventing derepression of the dnaA promoters.

**Figure 3.7.4**

![Graph showing the repression of lacZ expression in different strains.](image-url)
3.8 Construction and characterisation of \(\lambda\)RWS945

3.8.1 Construction of \(\lambda\)RWS945.

In all experiments so far described, the behaviour of the \(dnaA\) promoters has been studied by assaying the rate of synthesis of the DnaA'—\(\beta\)-galactosidase fusion product produced from \(\lambda\)RB1. The synthesis rate of this protein appears to be a faithful measure of \(dnaA\) promoter activity as it has in the past been shown to respond in the same way to intracellular levels of DnaA as the rate of chromosomal \(dnaA\) transcription determined directly by quantitative S1 mapping (Kücherer et al., 1986). For several reasons, however, it was decided to construct a new but similar reporter system using the pRS551 and \(\lambda\)RS45 vectors described by Simons et al. (1987). Firstly, to determine whether the DnaA-box located in the \(dnaA\) promoter region was involved in the repression in an integratively suppressed strain, it was decided to mutate the DnaA-box and to study the effect of the mutation in a transcriptional reporter gene fusion (Section 3.12.6); for comparison a similar construct would be necessary containing the wild-type DnaA-box. Secondly, studying the effect of integrative suppression in a transcriptional fusion would eliminate the possibility that it acts at the level of \(dnaA\) translation rather than at the level of transcription. Thirdly Simons' system has the advantage over \(\lambda\)RB1 that it contains a strong transcriptional terminator immediately upstream of the cloned promoter \(lacZ\) fusion. This eliminates the possibility that transcripts originating elsewhere, either in the vector or on the chromosome, interfere with or contribute to \(lacZ\) expression.

Simons et al. (1987) describe a two component system which consists of a phage, \(\lambda\)RS45, and a plasmid, pRS551. \(\lambda\)RS45 contains the \(lacY\) and \(lacA\) genes downstream of a \(lacZ\) gene which has a deletion at its 5' end. The phage also contains the 5' half of the \(\beta\)-lactamase gene (\(bla\)). pRS551 contains \(lacY\) and \(lacA\) as well as the complete \(lacZ\) gene. Upstream of \(lacZ\) there are four tandem copies of the \(rrnB\) T1 transcriptional terminator and a limited number of unique restriction sites in which to insert the desired promoter sequence. On the other side of the terminators there is a copy of the \(aphA\) gene from Tn903, which confers kanamycin resistance and a complete copy of the \(bla\) gene, which confers ampicillin resistance. Hence, pRS551 carries homology to \(\lambda\)RS45 on either side of the cloning site and double recombination between these homologous sequences will yield a phage carrying any promoter sequence which was inserted into pRS551 downstream of a strong transcriptional terminator. Additionally, the recombinant phage will carry the \(aphA\) gene and confer kanamycin resistance, a property which facilitates selection of lysogens of the
recombinant phage. It will, however, not confer ampicillin resistance as the recombination event will not result in a complete copy of the $bla$ gene on the phage.

The 945 bp EcoRI fragment containing the dnaA promoter region was taken from pJM87 (March, 1988) and cloned into the unique EcoRI site in pRS551. The orientation of the insert in the resulting clones was determined by digestion with BamHI and EcoRV. The plasmid containing the dnaA promoter transcribing towards $lacZ$ was named pRWS945 (Figure 3.8.1). Even though the dnaA translational start is present in the construct, $lacZ$ will only be translated from its own translational start as stop codons are present in all reading frames between dnaA' and $lacZ$.

**Figure 3.8.1**

![Diagram](image)

 Above: pRWS945, see text for details of construction. Below: $\lambda$RWS945 created by *in vivo* recombination between pRWS945 and $\lambda$RS45. The insert in the phage is drawn to scale, the $\lambda$ arms are not. T denotes four tandem repeats of the rrnB T1 terminator.
pRWS945 was transformed into NM621/λRS45 which carries λRS45 as a prophage, to allow in vivo recombination between the plasmid and the phage. Recombinants were selected by inducing the transformed strain with mitomycin C and infecting TP8503 with the resulting lysate as described in Section 2.4.3. Lysogens of the recombinant phage gave rise to kanR colonies. As the lysate prepared from NM621/λRS45(pRWS945) contains preponderantly unrecombined phage the chance is great that many of the kanR lysogens will contain λRS45 in addition to recombinant phage. Therefore a new lysate was prepared by induction of a kanR lysogen and used to infect TP8503 at various low multiplicities of infection (10^{-6}<moi<10^{-2}). KanR lysogens were selected and eight colonies were taken from the plate containing lysogens from the infection at the lowest multiplicity of infection. The β-galactosidase activity of these strains was determined to distinguish single from multiple lysogens. Lysogens with the lowest activity were considered to be single lysogens; one of these was used for further experiments and named TP945 (for the structure of XRWS945 see Figure 3.8.1). DNA was prepared from a λ lysate prepared by induction of this strain as described in Sections 2.4.6 and 2.2.5. Digestion with EcoRI showed that the phage contains the 945 bp EcoRI fragment present in pRWS945.

3.8.2 Characterisation of λRWS945.

Initially the behaviour of λRWS945 in a dnaA46 strain was investigated by lysogenising ED419 with the phage in the same way as described above. A temperature-shift β-galactosidase assay was performed. Figure 3.8.2 shows the specific β-galactosidase activities of λRWS945 in TP945 (dnaA+), ED945 (dnaA46) and TPC945 (dnaC325) at 30°C and 42°C.

Firstly, it is apparent that the activity is much greater than that of λRB1 in the same background. This does not appear to be due to multiple lysogeny as care was taken to isolate lysogens with the lowest possible activity, indeed, repeated infections never yielded lysogens with lower activity. It is also not due to multiple insertions of the 945 bp dnaA promoter fragment in pRWS945 as this possibility was refuted by restriction analysis. It is possible that the translational efficiency of lacZ in this construct is greater than that of the dnaA'−lacZ fusion of λRB1. In addition it is possible that the stability and/or specific activity of β-galactosidase is greater than that of the DnaA'−'β-galactosidase fusion. Braun et al. (1985) also report that their transcriptional fusion in λRB9 showed greater activity than λRB1; the difference they found was, however, smaller than that between λRWS945 and λRB1.
Secondly, lacZ expression in ED945 is derepressed at high temperature; the level of derepression is similar to that typically observed with TP88. In TP945 the specific β-galactosidase activity drops at 42°C. This had also been observed with λRB1 but to a lesser extent. The reason for this drop in activity is unclear as the activity of RNA polymerase has been reported to increase due to an increase in the polymerisation rate of the enzyme (Ryals et al., 1982). It may be that the stability of β-galactosidase or of its mRNA is affected at high temperature.

Thirdly, Figure 3.8.2 shows that lacZ expression from λRWS945 is not derepressed in a strain carrying the dnaC325 (initiation temperature-sensitive) allele, TPC945. This was previously shown for λRB1 (Masters et al., 1989) and indicates that the derepression seen in a dnaA strain is not due to an initiation defect per se.

Hansen et al. (1987) report that lacZ expression from λRB1 is derepressed in strains carrying pBR322 derivatives containing various oriC sequences presumably due to titration of DnaA by the oriC DnaA-boxes. The activity of λRWS945 was compared to that of λRB1 in a strain carrying pOC161; this is a pBR322 derivative containing 1541 bp from the origin region including DnaA-boxes R1–R4 and the DnaA-box present in the mioC promoter. Figure 3.8.3a shows that the presence of the plasmid leads to a derepression of approximately 30% at 30°C and 20% at 42°C. Figure 3.8.3b shows that the derepression in TP91 is similar. This is lower than the derepression reported by Hansen et al. (1987) for a plasmid carrying the same DnaA-boxes. The reason for this difference is unclear.
Figure 3.8.3a

![Graph showing temperature effect on specific activity](image)

Figure 3.8.3b

![Graph showing temperature effect on specific activity](image)
The behaviour of λRWS945 in an integratively suppressed dnaA46 strain was investigated by isolating two independent pGW’IS integrants of ED945 as described before for TP88. These strains were named EDG1 and EDG2. Retention of the dnaA46 allele and integration of pGW’IS were verified by transduction as described before. The result of a temperature-shift β-galactosidase assay is shown in Figure 3.8.4: λRWS945 shows no derepression in these integratively suppressed strains.

Figure 3.8.4

The response of λRWS945 to growth-rate was tested by comparing the β-galactosidase activities of TP945 grown in VB minimal glucose medium and in LB medium supplemented with 0.4% glucose. Table 3.8.1 shows that the specific β-galactosidase activity is virtually identical at both growth rates. In addition, β-galactosidase activity was measured during growth of TP945 from exponential phase into stationary phase. Figure 3.8.5 shows that the specific activity remains relatively constant. These results are in agreement with those of Hansen et al. (1991a) who report that the specific activity of λR81 is insensitive to growth-rate. The specific activity is not a good measure of the rate of β-galactosidase production in stationary phase as the increase over time of both total protein synthesis and β-galactosidase is small and slight relative changes in the rates of synthesis will not be perceptible. A clearer indication of relative changes in protein synthesis is given by plotting the β-galactosidase activity per volume of culture against the total protein or cell mass per
volume (expressed in OD$_{600}$ units). Figure 3.8.6 shows that the differential rate of β-galactosidase synthesis from λRWS945 in stationary phase decreases, in accordance with Polaczek and Wright (1990) who found that the dnaA2p promoter activity decreases in stationary phase.

From the work described above it can be concluded that the behaviour of λRWS945 is qualitatively the same as that of λRB1 under the conditions tested and it was therefore considered an adequate system for further study of the dnaA promoters.

**Figure 3.8.5**

Shown are the OD$_{600}$ (left scale) and the specific activity (right scale) of TP945 at each time point.
The differential rate of β-galactosidase synthesis in TP945 is given by the first derivative (slope) of the graph. This is a clearer indication of small changes in enzyme synthesis than the plot of the specific activity. If A is enzyme units (activity) per volume and M is mass of culture per volume the differential rate of enzyme synthesis will be given by dA/dM and the specific activity by A/M. In steady state the two will be equal but if a change in enzyme synthesis occurs dA/dM will change immediately whereas A/M will only slowly approach the new equilibrium value.

Table 3.8.1 Growth-rate response of TP945 specific β-galactosidase activity.

<table>
<thead>
<tr>
<th>medium</th>
<th>doubling time (min.)</th>
<th>sp. act. (MU)</th>
<th>average sp. act. (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB (0.4% glucose)</td>
<td>25</td>
<td>165</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>glucose min. med.</td>
<td>60</td>
<td>176</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>178</td>
<td></td>
</tr>
</tbody>
</table>

TP945 was grown in LB supplemented with 0.4% glucose and in VB salts minimal glucose medium and maintained in exponential phase (0.03<OD600<0.15) for six hours before sampling. Two samples were taken with a twenty minute interval. Values of the specific β-galactosidase activities and their average are shown.
3.9 The influence of secondary mutations

3.9.1 Additional mutations accumulate in integratively suppressed strains.

It has been observed that for integrative suppression to be efficient, integration of the plasmid into the chromosome may not be the sole requirement. This was first noticed in the case of suppression by the F factor. Tresguerres et al. (1975) report that recombination between an Hfr recipient and a dnaA46 donor does not result in suppression of temperature-sensitivity in the resulting strain. An additional mutation, believed to effect relief of a block in F replication, is necessary for integrative suppression to occur. In addition, it has been reported that, although suppression by F is normally only efficient on minimal medium, colony formation at 42°C on rich medium can occur after additional unidentified mutations (Tresguerres et al., 1975). A puzzling observation which was reported by Wechsler and Gross (1971) suggests that facilitating mutations can occur in an integratively suppressed strain at loci other than the integrated F factor. A dnaA strain was integratively suppressed by F' ts62 lac, which can be spontaneously cured at 42°C. After curing, however, the dnaA strain "reverted" to temperature-resistance at an elevated frequency. This suggests that when integratively suppressed, the strain had acquired a mutation which promoted further mutation to temperature-resistance even after loss of the integrated F factor.

During the course of this work it was noticed that transduction of pKN500 from an integratively suppressed strain to a dnaA46 recipient allows growth of the resulting strain at 42°C but that this growth is poor compared to that of the donor strain. Patching at high temperature often results in only partial growth of the patch or growth of isolated colonies, which again suggests that other events in the cell (which are not transductionally linked to the integrated plasmid) must take place for full viability at high temperature. Repeated growth of an integratively suppressed strain at high temperature does indeed result in improved growth.

The difference between integrative suppression by R1 and F in repression of the dnaA promoters may be due to a difference in efficiency of integrative suppression although this was not unambiguously demonstrated (Section 3.4). If, however, the repression of the dnaA promoters is not due to integrative suppression per se but is rather a consequence of additional mutations which accumulate in an integratively suppressed strain, then if different "co-suppressing" mutations are acquired by the TPK strains than are acquired by the TPF strains this may account for the different behaviour of the dnaA promoters in those strains at high temperature. This hypothesis was prompted by the possibility that TPK2 may not be integratively suppressed (Section 3.2.1), and that integration of pGW'TS may be unstable (a possible
3.9.2 Curing of pKN500 from an integratively suppressed strain.

An experiment was designed which would test the hypothesis that subsequent mutations rather than plasmid integration prevents dnaA derepression. If an integratively suppressed strain were allowed to accumulate mutations which increase viability at high temperature and were subsequently cured of the integrated plasmid, the behaviour of the dnaA promoters before and after curing could be compared to see whether mutations unlinked to pKN500 are responsible for preventing derepression of dnaA at 42°C.

To this end pKN500 was transduced from TPK88 to the strain NF279 (dnaA46, metB); kanR transductants were all temperature-resistant and 23% had become metB⁺ as would be expected from the location of pKN500 on the chromosome of TPK88 (89 min.). Nine transductants which had retained the metB⁻ allele were repeatedly streaked out on kanamycin plates at 42°C to allow selection for mutants with full viability at that temperature. Large colonies were picked and restreaked until the strains showed good growth upon patching at 42°C. The nine strains with improved viability at high temperature were named NF#31 to NF#39.

It was attempted to remove pKN500 from the chromosome of the NF# strains by transducing them to metB⁺ using a lysate prepared on TP8503. Both unpurified and colony purified metB⁺ transductants were tested by patching for kanamycin and temperature-resistance as shown in Table 3.9.1. Surprisingly, kanS temperature-sensitive transductants only appeared at a very low frequency, suggesting that either pKN500 is no longer 23% linked to metB or that for some other reason pKN500 cannot be removed.

It was decided to verify the transductional linkage of pKN500 and metB in NF#31 and NF#32 by a back-cross; pKN500 was transduced from these strains to ED419 (dnaA46, metB⁺). In both cases approximately 20% of kanR transductants had become metB⁻, as would be expected if pKN500 were integrated at 89 min. It was also tested if pKN500 could be moved from the metB⁺ derivatives of NF#31 by co-transduction with metB. P1 lysates were prepared on nine purified metB⁺ kanR temperature-resistant derivatives of NF#31, named NF#A to NF#I, and used to transduce NF279 to metB⁺. Transductants were patched to test for co-transduction of pKN500 (kanR, tempR). Table 3.9.2 shows that in all cases pKN500 is co-transduced.
but at varying frequencies: NF#B and NF#H show particularly poor co-transduction. A possibility is that pKN500 in these strains is no longer stably maintained on the chromosome but is cytoplasmically maintained. It is, however, unclear why these strains are then still temperature-resistant.

Table 3.9.1 Transduction of NF279::pKN500 to \textit{metB}^+.

<table>
<thead>
<tr>
<th>\textit{metB}^+ deriv. of</th>
<th>unpurified transductants</th>
<th>purified transductants</th>
<th>% \textit{kan}^{S_t}$S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no. tested</td>
<td>no. \textit{kan}^{S_t}$S</td>
<td>no. tested</td>
</tr>
<tr>
<td>NF#31</td>
<td>10</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>NF#32</td>
<td>10</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>NF#33</td>
<td>110</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#34</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#35</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#36</td>
<td>110</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#37</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#38</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NF#39</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\textit{metB}^+ transductants of the strains listed on the left were tested for kanamycin and temperature-resistance by patching on LB plates at 30 and 42°C and on LB kan plates at 30°C. Transductants were purified by streaking out on minimal medium plates lacking methionine, five colonies from each original transductant were then patched. If any of the colonies were \textit{kan}^{S} or \textit{t}^{S}, the transductant was scored as such.

NF#A to NF#I were transformed with the R1 derivative pGW71 (Bernander \textit{et al.}, 1989), which confers ampicillin resistance and which is incompatible with pKN500, to attempt to cure the strains of this plasmid. \textit{Amp}^R transformants were obtained and repeatedly streaked out on ampicillin plates. A number of large colonies from each transduction were tested for kanamycin and temperature-resistance (Table 3.9.3). It appears that in some cases that the cells have become cured of pKN500 and that in others it has been maintained but is no longer capable of efficiently replicating the chromosome at high temperature, presumably due to the presence of pGW71. A
strain which appeared cured of pKN500, NF#B(pGW71), was used for further experiments.

It is still not clear why the transductions to metB+ of NF#31 to NF#39 did not result in temperature-sensitivity. The curing of pKN500 from the strains and concomitant reversion to temperature-sensitivity shows that these strains have not accumulated secondary mutations which allow growth at 42°C in the absence of an integrated pKN500 plasmid.

3.9.3 dnaA promoter activity in NF279 and NF#B(pGW71).

In order to test the dnaA promoter activity at high temperature in the cured strain both NF#B(pGW71) and its original parent strain NF279 were infected with λRWS945 and lysogens were selected as described before. A temperature-shift β-galactosidase assay was performed (Figure 3.9.1). This shows that dnaA promoter activity is derepressed in both strains to an equal extent at 42°C. Therefore the hypothesis that mutations accumulating during growth at high temperature of an integratively suppressed strain are responsible for the failure of the dnaA promoter to derepress is incorrect.

Table 3.9.2 Co-transduction of pKN500 and metB from kanR tempR NF#31 derivatives.

<table>
<thead>
<tr>
<th>donor strain</th>
<th>NF279 (metB+)</th>
<th>% co-transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>transductants tested</td>
<td>no. kanR tempR</td>
</tr>
<tr>
<td>NF#A</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>NF#B</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>NF#C</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>NF#D</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>NF#E</td>
<td>120</td>
<td>20</td>
</tr>
<tr>
<td>NF#F</td>
<td>70</td>
<td>12</td>
</tr>
<tr>
<td>NF#G</td>
<td>70</td>
<td>17</td>
</tr>
<tr>
<td>NF#H</td>
<td>70</td>
<td>8</td>
</tr>
<tr>
<td>NF#I</td>
<td>70</td>
<td>12</td>
</tr>
</tbody>
</table>

NF279 was transduced to metB+ with lysates prepared on the strains listed on the left. Transductants were then patched on LB plates at 30 and 42°C and on LB kan plates at 30°C.
Table 3.9.3 Curing experiment.

<table>
<thead>
<tr>
<th>strain</th>
<th>kan (30°C)</th>
<th>amp (30°C)</th>
<th>amp (42°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF#A(pGW71)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#B(pGW71)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#C(pGW71)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#D(pGW71)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#E(pGW71)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#F(pGW71)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#G(pGW71)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#H(pGW71)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NF#I(pGW71)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The transformants shown on the left were repeatedly streaked out on LB amp plates. Large colonies from each transformant were tested under the conditions indicated by patching.

Figure 3.9.1
3.10 The behaviour of the dnaA promoters on a plasmid in an integratively suppressed strain

So far in this work the failure of the dnaA promoters to derepress at high temperatures under integratively suppressed conditions has only been studied in phage borne systems. To ensure that this is not an artefact of such a system the experiment was repeated using the plasmid pRWS945, which carries the dnaA1p2p–lacZ fusion described in Section 3.8.1.

pKN500 was transduced into ED419 using a P1 lysate prepared on TPK88. The resulting integratively suppressed strain was named EDK419 and transformed with pRWS945. A temperature-shift β-galactosidase assay was performed on this strain. Figure 3.10.1 shows that the specific β-galactosidase activities of EDK419(pRWS945) and TP8503(pRWS945) remain repressed compared to that of ED419(pRWS945). It must be borne in mind that the derepression observed with the latter strain is an underestimate due to the fact that the copy-number of pBR322 derived plasmids is reduced in dnaA strains at high temperature due to indirect effects resulting from cessation of chromosomal DNA replication (Chiang et al., 1991; Section 1.12.1). This shows that the failure of the dnaA promoters to derepress in an integratively suppressed background is not limited to a λ borne system.

Figure 3.10.1

![Figure 3.10.1](image_url)
3.11 The effect of extragenic suppression of dnaA on dnaA promoter activity

3.11.1 Isolation of spontaneous pseudo-revertants of ED945.

To investigate whether suppression of dnaA per se leads to repression of the dnaA promoters at high temperature, the effects of forms of suppression other than that through an integrated replicon were examined. First a number of independent spontaneous temperature-resistant "revertants" of ED495 were isolated. Five separate cultures of this strain were grown up overnight and plated out at 42°C. Each plate yielded circa 50 colonies, one of which was taken in each case and streaked out at 30°C and 42°C. The various revertants vary in viability and colony-size at both temperatures; the largest colonies was taken from the plate at 42°C and restreaked at 30°C and 42°C (Table 3.11.1). A representative colony was then taken from the 42°C plate and used (i) to prepare a P1 lysate and (ii) for a β-galactosidase assay. The revertants were named EDrev1 to EDrev5.

Table 3.11.1 Characterisation of spontaneous "revertants" of ED945.

<table>
<thead>
<tr>
<th>strain</th>
<th>growth on LB plates (1st round)</th>
<th>growth on LB plates (2nd round)</th>
<th>co-transduction of asnA and dnaA46</th>
<th>rifR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>42°C</td>
<td>30°C</td>
<td>42°C</td>
</tr>
<tr>
<td>EDrev1</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDrev2</td>
<td>+/−</td>
<td>++</td>
<td>+/−</td>
<td>++</td>
</tr>
<tr>
<td>EDrev3</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDrev4</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>EDrev5</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Growth characteristics: + denotes small colonies; ++ denotes colonies similar to those of the wild-type; +/− denotes heterogeneous colony-sizes; − denotes no growth. Co-transduction of asnA and dnaA was determined by patching purified asn+ transductants at 30°C and 42°C. Five colonies from each transductant were streaked out. If any were rifR, the transductant was scored as such.
PI lysates prepared on the five revertants were used to transduce MM18 to asparagine prototrophy to ascertain whether the dnaA46 allele was retained in these strains as described earlier. Table 3.11.1 shows that EDrev1, EDrev2 and EDrev3 co-transduced temperature-sensitivity with asnA and therefore must contain extragenic suppressors. EDrev4 and EDrev5 failed to transduce temperature-sensitivity but are unlikely to be true revertants as EDrev4 is cold-sensitive at 30°C and growth of EDrev5 is less good than that of TP8503 (dnaA+) at 42°C. The revertants were not characterised further other than by testing for rifampicin-resistance. It has been shown that rifR rpoB mutants are easily isolated (Bagdasarian et al., 1977; Atlung, 1984). None of the revertants isolated here are rifR.

A β-galactosidase assay was performed to test the behaviour of the dnaA promoters at high temperature in the EDrev strains. Due to problems with cold-sensitivity the EDrev strains were grown throughout the entire experiment at 42°C. ED945 and TP945 were grown at 30°C and shifted to 42°C at the time of the first sample. Figure 3.11.1 shows that the revertants show identical β-galactosidase activity to that of TP8503 which is not derepressed at 42°C, unlike that of the parent strain ED945.

![Figure 3.11.1](image-url)
3.11.2 Behaviour of λRWS945 in other suppressed strains.

A rifR temperature-resistant derivative of ED945 was isolated by growing the strain on rifampicin plates at 42°C. It was shown that temperature and rifampicin resistance were 100% co-transducible to the parent strain ED945. The transduced strain was named EDR945. It is likely that this strain contains a suppressing mutation in rpoB identical or similar to the rpoB902 mutation isolated by Atlung (1984) though this was not tested. Figure 3.11.2 shows that EDR945 shows no derepression of dnaA promoter activity at 42°C. Even at 30°C there is a significant difference in promoter activity between the suppressed strain and its parent.

![Figure 3.11.2](image)

Overproduction of GroEL and GroES suppresses the dnaA46 mutation but renders the cells cold-sensitive. ED945 and TP945 were transformed with the plasmid pND5 which carries the groELS operon to study the effect of this on dnaA promoter activity at 42°C in the resulting strains. Table 3.11.2 shows that the β-galactosidase activity of both the dnaA+ and the suppressed strains are similar. The specific activity in both cases is considerably lower than that observed in other experiments with λRWS945; the reason for this is unclear.

It has been shown in this section that a variety of suppressors of dnaA result in failure of the promoters to derepress at high temperature and that this is therefore not solely a property of integrative suppression.
Table 3.11.2 Specific β-galactosidase activity of λRWS945 in a strain suppressed by GroE.

<table>
<thead>
<tr>
<th>strain</th>
<th>sample time (min.)</th>
<th>sp. activity (MU)</th>
<th>average sp. act. (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED945(pND5)</td>
<td>350</td>
<td>35</td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>355</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>TP945(pND5)</td>
<td>350</td>
<td>52</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td>355</td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

The strains were grown at 42°C under antibiotic selection for approximately six hours before sampling. Two samples were taken and the values for the specific β-galactosidase activities as well as the average are shown.
3.12 Involvement of the DnaA-box


It has been concluded from numerous in vivo and in vitro studies that the DnaA-box located in the dnaA promoter region is the site mediating repression of the promoter by its product (see Section 3.1). In order to investigate whether the DnaA-box is involved in the failure of derepression of the dnaA promoters observed at high temperature in a variety of suppressed dnaA strains it was inactivated by site-directed mutagenesis.

The 945 bp EcoRI fragment carrying the dnaA promoter region was taken from pJM87 and cloned into the phage M13mp19. This phage allows isolation of single-stranded DNA, a property which is commonly exploited in site-directed mutagenesis procedures. The DnaA-box was mutated using the method developed by Kunkel (1985); see Section 2.2.17. Because the DnaA-box is located within a promoter region the GC:AT ratio of the sequence was not changed to avoid possible changes in the efficiency of transcription due to such a change. The base changes are shown in Table 3.12.1; the mutations introduce base changes which would be extremely detrimental to DnaA-box activity according to Schaefer and Messer (1991). Figure 3.12.1 shows the sequence resulting from the mutagenesis procedure verified by dideoxy nucleotide sequencing (Section 2.2.18).

The mutated 945 bp fragment was then ligated into the EcoRI site of pRS551 and the orientation of the cloned fragment was determined as described in Section 3.8.1. The resulting plasmid was named pRWS945M which, apart from the DnaA-box mutations, is identical to pRWS945 (Figure 3.8.1). This was recombined in vivo with λRS45 and single lysogens of the resulting phage, λRWS945M, in the strains TP8503 and ED419 were selected as described in Section 3.8.1. The lysogens were named TP945M and ED945M.
Table 3.12.1 Mutagenesis of the DnaA-box in the dnaA promoter region.

<table>
<thead>
<tr>
<th>Consensus (Fuller et al., 1984)</th>
<th>T T A T C C A C A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus (Schaefer and Messer, 1991)</td>
<td>T T A T A C A A A C C T G C C T</td>
</tr>
<tr>
<td>DnaA-box sequence (dnaA promoter)</td>
<td>T T A T C C A C A</td>
</tr>
<tr>
<td>Mutated DnaA-box sequence</td>
<td>A T A T C G A G A</td>
</tr>
</tbody>
</table>

Comparison of the wild-type and mutated DnaA-box sequences to the published consensus sequences. Sequences are shown 5' to 3'. Mutations are shown in bold print.

Figure 3.12.1 Sequencing gel of the mutated DnaA-box.

Left: the sequence of the DnaA-box in M13pdnaA1 (M13mp19 with the 945 bp EcoRI insert carrying the wild-type dnaA promoter region). Right: the sequence of the DnaA-box after mutagenesis (the clone shown is M13pdnaA8). The order of the lanes is shown.
3.12.2 Characterisation of λRWS945M in a dnaA46 strain.

The behaviour of λRWS945M in both a dnaA+ and a dnaA46 strain was compared to that of λRWS945 in a temperature-shift β-galactosidase assay. Figure 3.12.2 shows that the two phages behave identically. This is not what would be expected if the DnaA-box were the operator site for a repressor of transcription. However, the result is in agreement with work presented by Polaczek and Wright (1990) who found that inactivation of the DnaA-box has no effect on dnaA expression if both promoters are present in tandem. They investigated the effect of a DnaA-box deletion on each promoter cloned separately and found that dnaA1p activity increased seven-fold upon deletion of the DnaA-box but that dnaA2p activity decreased three-fold. The lack of any effect when both promoters are present was proposed to be due to compensation of the decreased transcription from dnaA2p for the increased transcription from dnaA1p. The quantitative data presented by Polaczek and Wright (1990) fit such an explanation.

Figure 3.12.2

[Graph showing the behaviour of XRWS945M in both a dnaA and a dnaA46 strain, compared to that of XRWS945 in a temperature-shift β-galactosidase assay.]
3.12.3 Activity of ARWS945M in stationary phase.

Polaczek and Wright (1990) showed that transcription from dnaA2p decreases with decreasing growth rate. The relative activities of the two promoters (dnaA2p/dnaA1p) consequently changes from 10:1 in exponential phase in rich medium to 3:1 in stationary phase. As a result the contribution of the minor promoter, dnaA1p, which is repressed by DnaA becomes greater in stationary phase and an increase in transcription from this promoter upon inactivation of the DnaA-box should no longer be compensated by a decrease in transcription from dnaA2p. This was tested by comparing the β-galactosidase activities of TP945 and TP945M in stationary phase. Figure 3.12.3 shows that the differential β-galactosidase synthesis of both strains is identical. Therefore, contrary to expectation, inactivation of the DnaA-box has no perceptible effect on dnaA promoter activity in stationary phase.

![Figure 3.12.3](image)

3.12.4 Overproduction of DnaA.

If the DnaA-box mediates repression by DnaA, then overproduction of DnaA should have no effect on β-galactosidase production from λRWS945M. This was investigated by introducing the plasmid pLSK5, which carries the dnaA gene under control of the inducible tac promoter (see Section 3.6), into TP945 and TP945M. DnaA production from the plasmid was induced using either 2 μg/ml or 100 μg/ml
IPTG and β-galactosidase production from λRWS945 and λRWS945M was followed for several hours after induction. Figure 3.12.4 shows that both phages respond identically to DnaA overproduction.

**Figure 3.12.4a**

![Graph 1](image1.png)

**Figure 3.12.4b**

![Graph 2](image2.png)

The strains were grown up for approximately four hours before addition of IPTG, which was also added to the strains not containing pLSK5.
3.12.5 Titration of DnaA.

The effect of titration of intracellular DnaA was tested as previously described in Section 3.8.2, by introduction of the oriC pBR322 hybrid plasmid pOC161. Figure 3.12.5a shows that lacZ expression from both \(\lambda\)RWS945 and \(\lambda\)RWS945M responds slightly to the presence of the plasmid as shown before in the case of \(\lambda\)RB1. A different oriC plasmid, pH4, which had previously been shown to lead to greater derepression of lacZ expression from \(\lambda\)RB1 (T. Owen-Hughes, unpublished results) was also introduced into TP945 and TP945M. Figure 3.12.5b shows that lacZ expression from both phages increases dramatically in the presence of the plasmid. Derepression from \(\lambda\)RWS945M is at least twice as great as that from \(\lambda\)RWS945 in this experiment; the reason for this is unclear. It has not been tested whether this is repeatable.

**Figure 3.12.5a**
The strains in Figures 3.12.5a and b were maintained in exponential phase for approximately four hours before sampling.

3.12.6 Integrative suppression.

Five independent integrants of pGW'IS in ED945M were isolated as described before. All grew well at 42°C and had retained the dnaA46 allele. The strains were named EDG1M to EDG5M. Figure 3.12.6 shows that in all cases but one the dnaA promoters are repressed at high temperature. This indicates that the DnaA-box is not involved in this form of repression.

The nature of EDG5M, which shows intermediate derepression of the dnaA promoters, was further investigated. Its growth-rate is identical to that of EDG2M which does not show any derepression. pGW’IS was then transduced from EDG5M and from EDG2M to ED945 to see if partial derepression is a property of the host strain or of the integrated plasmid. The resulting strains were named EDG945(#5) and EDG945(#2). Figure 3.12.7 shows that EDG945(#5) does not exhibit the same level of β-galactosidase activity as EDG5M and shows only slightly more lacZ expression at high temperature than EDG945(#2). It appears therefore that the increased lacZ expression observed in EDG5M may be a property of the host and perhaps of λRWS945M in that strain. This was not further investigated.
Figure 3.12.6

Autogenous Regulation of DNA A Gene Expression

Figure 3.12.7
3.12.7 Effects of overproduction of Dam methylase.

Mutation of the DnaA-box appears to have had no consequence for regulation of the dnaA gene in the experiments so far described. These have all involved aspects of possible autoregulation. The recent discovery that the dnaA promoter is sequestered through attachment to the cell membrane (Campbell and Kleckner, 1990) prompted an experiment to investigate whether the dnaA promoter activity is affected under circumstances where the sequestration period is reduced. This can be achieved by overproduction of Dam methylase (Boye and Løbner-Olesen, 1990; Section 1.8.3). The multicopy plasmid pdam118, carrying the dam gene, was introduced into TP945 and TP945M and β-galactosidase activities were compared with those of the parent strains. The presence of pdam118 raises the intracellular Dam concentration approximately five-fold (W. Messer, personal communication). Table 3.12.2 shows that dnaA promoter activity is slightly reduced by the presence of the plasmid. This is contrary to expectation as a reduction in the sequestration period would be thought to result in a greater promoter activity. The result may indicate that this plasmid does not produce sufficient Dam protein to elicit an effect or alternatively that the dnaA promoter is only sequestered when present at its normal location and not when it is present at attL. (Very recently, however, Hansen and Atlung (1995b) showed that transcriptional activity of the dnaA promoters is reduced during replication, even when they are present at attL.) Again the DnaA-box mutations have no effect under these circumstances.

**Table 3.12.2 Overproduction of Dam methylase.**

<table>
<thead>
<tr>
<th>strain</th>
<th>TP945</th>
<th>TP945M</th>
<th>TP945 (pdam118)</th>
<th>TP945M (pdam118)</th>
</tr>
</thead>
<tbody>
<tr>
<td>specific activity (MU)</td>
<td>221</td>
<td>226</td>
<td>198</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>214</td>
<td>231</td>
<td>187</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>224</td>
<td>222</td>
<td>187</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>232</td>
<td>226</td>
<td>191</td>
<td>201</td>
</tr>
<tr>
<td>average (MU)</td>
<td>223</td>
<td>226</td>
<td>191</td>
<td>197</td>
</tr>
</tbody>
</table>

The strains were grown in LB medium with appropriate antibiotics at 37°C and maintained in exponential phase for four hours before sampling. Samples were taken at five minute intervals. Specific activities and their average value are shown.
3.12.8 Resequencing of the DnaA-box mutation in λRWS945M.

In summary, it has been shown that the dnaA promoter activity is not influenced by triple mutation of the DnaA-box. As this was an unexpected result it was deemed important to again verify the mutation in λRWS945M before drawing further conclusions. To this end λ DNA was prepared from TP945M as described in Sections 2.4.6 and 2.2.5. The 945 bp EcoRI dnaA promoter fragment present in the phage was ligated into M13mp18. Single-stranded DNA was prepared from the resulting phage and was sequenced as described in Section 2.2.18. The resulting sequence is shown in Figure 3.12.8 and is that of the mutant.

Figure 3.12.8 Resequencing of the insert in λRWS945M.

The 945 bp EcoRI insert was cloned from λRWS945M into M13mp18 and sequenced. The sequence of the DnaA-box region is shown. The region carries the mutations shown in Table 3.12.1. The order of the lanes is shown above.
3.13 Binding of DnaA to the mutant DnaA-box in vitro

3.13.1 DNase I footprinting.

Because the mutant dnaA promoter region behaves identically to the wild-type an unlikely possibility, which nevertheless had to be eliminated, was that the mutant DnaA-box is capable of binding DnaA protein. In vitro footprinting and gel-retardation were applied to refute this possibility.

The footprinting experiment was performed on a 385 bp BssHII–HinfI fragment from pRWS945M containing the mutant DnaA-box and on the corresponding fragment from pJM87 containing the wild-type sequence. Figure 3.13.1 shows the labelling strategy. Aliquots of the labelled fragment were incubated with various amounts of purified DnaA protein and then digested with an appropriate concentration of DNase I as described in Section 2.2.20. The samples were then fractionated on a denaturing polyacrylamide gel. The result is shown in Figure 3.13.2. A region corresponding to the position of the DnaA-box is protected in the fragment carrying the wild-type DnaA-box but the corresponding mutated region shows no protection. The DNase I digestion patterns in this region differ due to the base changes in the mutant.

3.13.2 Gel retardation.

The footprinting experiment was supported by a gel retardation assay carried out by Christoph Weigel at the Max-Planck-Institut für molekulare Genetik in Berlin. My own part in this work was merely to supply the pJM87 and pRWS945M plasmids. Aliquots of 490 bp PCR products from these plasmids containing the DnaA-box region were incubated with various amounts of purified DnaA protein and electrophoresed on an agarose gel. Figure 3.13.3 shows that the mutant fragment binds DnaA only at the highest concentrations (no competitor was included in the experiment). At lower concentrations there is very little retardation of the mutant fragment compared with that of the wild-type.

Although the wild-type and mutant DnaA-box behave identically in the in vivo experiments described in Section 3.12, their behaviour in in vitro DnaA binding assays is markedly different and confirms that the mutant sequence is not effective at binding DnaA.
The 621 bp EcoRV–EcoRI fragment was cut from pRWS945M and from pJM87 and gel-purified. This was then digested with BssHII which results in a 5' overhang with the sequence: CGCG. The fragments were labelled using the T4 DNA polymerase and α-32P-dCTP and α-32P-dGTP. Although this should not label the EcoRV (blunt) and EcoRI (AATT overhang) ends, the possibility existed that some label would be non-specifically incorporated there. For this reason the fragments were digested with HindIII to create fragments labelled only at one end. The footprinting reaction was carried out on the resulting mixtures of fragments. The major labelled fragments were 384 bp and 89 bp in length. Asterisks denote radioactively labelled ends.
Figure 3.13.2 *In vitro* DNase I footprint of the DnaA-box region.

Approximately 50 ng of DNA was incubated with various amounts of DnaA protein (shown above). Numbering down the left denotes distance in bp from the labelled BssHII end. The position of the DnaA-box is indicated. The wild-type fragment shows some protection from and also some enhanced sensitivity to DNase I at the location of the DnaA-box; the mutant shows no change in the DNase I cleavage pattern after incubation with DnaA. Braun *et al.* (1985) report protection at sites other than that of the DnaA-box alone; the footprint shown here is too faint to determine whether such protection is occurring.
Figure 3.13.3 Gel retardation of the dnaA promoter region.

0.2 pmol of DNA fragment was incubated with various amounts of DnaA protein in the same binding buffer used for the footprinting experiment and electrophoresed on a 2% agarose gel. The bands were visualised by staining with SYBR-GREEN (by Molecular probes). The molar ratio of DnaA:DNA is shown above each lane. Lanes 1 to 7: wild-type fragment from pJM87; lanes 8 to 13: mutant fragment from pRWS945M.
3.14 Behaviour of λRWS945 and λRWS945M in a dnaA0 strain


It has been established that the failure of the dnaA promoters to be derepressed at high temperature in a dnaAts strain is not unique to integratively suppressed strains but is also a concomitant of other suppressing mutations which allow growth at high temperature (Section 3.11). It therefore seemed plausible that derepression in a dnaAts strain at the non-permissive temperature is not a result of decreased DNA binding capacity of the mutant protein but rather a consequence of its deficiency in initiation of chromosomal replication. A means to test this hypothesis is provided by the existence of strains which lack active DnaA protein but which replicate by virtue of an integrated pKN500 plasmid (Hansen and Yarmolinsky, 1986); thus, the behaviour of the dnaA promoters can be studied in a strain in which the putative role of DnaA as autoregulator is uncoupled from its role in replication. EH3791 is integratively suppressed and carries a TnlO (tetR) insertion within the dnaA gene, rendering it inactive. In addition the strain carries an unidentified mutation, mad-1, which allows the strain to grow on rich medium and which also relieves the cold-sensitivity exhibited by integratively suppressed dnaA0 mutants.

EH3791 was infected with both λRWS945 and λRWS945M and lysogens were selected (EH945 and EH945M). Both lysogens were then transduced to dnaA+ by co-transduction with Tn7 (tmpR) using TP8503 as a donor. Transductants were screened for tetracycline-sensitivity as an indication that the dnaA::TnlO gene had been replaced by dnaA+. TetS tmpR derivatives were named EHA945 and EHA945M respectively.

The mode of replication in the EH and EHA strains is different. The strains lacking active DnaA are forced to initiate replication at the pKN500 origin (oriR) whereas the dnaA+ strains can initiate replication at either oriC or oriR. Therefore oriC in EHA945 was replaced by the oriC deletion of CM1843 by transduction, selecting for tetracycline-resistance (CM1843 carries TnlO in the origin region). The resulting strain was named EHA945A.

The mutations in the various strains were verified by Southern hybridisation. Replacement of the dnaA::Tn10 mutation was tested by probing chromosomal HindIII digests with a labeled HindI dnaA fragment from pJM87. Figure 3.14.1a shows that the probe hybridises to two fragments in the case of EH945 due to the fact that Tn10 contains a HindIII site. In the cases of EHA945 and EHA945A only one band, corresponding to the 3.5 kb chromosomal HindIII fragment, hybridises to the probe. The deletion of oriC was tested by probing chromosomal PstI digests with a PstI oriC
fragment from pOC142-R which is internal to the deletion in CM1843. Figure 3.14.1b shows that the probe hybridises to the 887 bp chromosomal oriC PstI fragment in the cases of EH945 and EHA945 but not in the case of EHA945Δ.

Figure 3.14.1 Southern hybridisation confirming dnaA replacement and oriC deletion.

Chromosomal DNA of the indicated strains was prepared and hybridised to a dnaA and to an oriC probe. Right panel (HindIII digest): EH945 shows hybridisation of two main bands to dnaA due to the introduction of a HindIII site by the Tn10 interruption. The other strains show hybridisation of only one principle band showing that the dnaA gene has been restored. There is a minor band at the top of the gel; its nature is unclear. Left panel (PstI digest): EH945 and EHA945 show hybridisation to the oriC probe, EHA945Δ does not, showing that it carries a deletion of that region. Two separate clones of EHA945Δ were used. No molecular weight standards were included.

The specific β-galactosidase activities of λRWS945 and λRWS945M were determined in the various isogenic strains described above (Table 3.14.1). lacZ expression from both phages is elevated in the dnaA0 strain compared to the dnaA+ strains. This is not a consequence of the mode of replication as EHA945 and EHA945Δ show similar levels of β-galactosidase activity. It must be concluded that the presence of DnaA has consequences for dnaA promoter activity which are not linked to its role in replication and which are not mediated by the DnaA-box. It is interesting to note that the difference in lacZ activity of λRWS945 between the dnaA0 strain and an isogenic dnaA+ is less than half of that between a dnaA46 strain and a dnaA+ strain at high temperature.

Table 3.14.1 Specific β-galactosidase activities of λRWS945 and λRWS945M in isogenic dnaA+ and dnaA0 strains.

<table>
<thead>
<tr>
<th>strain</th>
<th>EH945 (dnaA::Tnl0)</th>
<th>EHA945 (dnaA+)</th>
<th>EHA945Δ (dnaA+ ΔoriC)</th>
<th>EH945M (dnaA::Tnl0)</th>
<th>EHA945M (dnaA+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>specific activity (MU)</td>
<td>532</td>
<td>229</td>
<td>259</td>
<td>454</td>
<td>165</td>
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<td>494</td>
<td>180</td>
<td>177</td>
<td>408</td>
<td>164</td>
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<tr>
<td>average (MU)</td>
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<td>187</td>
<td>210</td>
<td>401</td>
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<td>487</td>
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<tr>
<td></td>
<td>502</td>
<td>201</td>
<td>223</td>
<td>423</td>
<td>160</td>
</tr>
</tbody>
</table>

The strains were grown in LB medium at 37°C and maintained in exponential phase for six hours. Samples were then taken at five minute intervals. The specific activity calculated from each sample as well as their average value are shown.
3.15 Discussion

3.15.1 Thermal inactivation of DnaAts protein; DnaA as a transcriptional regulator.

The idea that dnaA is autoregulated was in part derived from experiments performed with temperature-sensitive dnaA mutants as described in Section 3.1. The work presented in this chapter using the dnaA1p2p transcriptional and translational lacZ fusions λRB1, λRWS945 and pRWS945 show that dnaA promoter activity is increased at the restrictive temperature in a number of dnaAts backgrounds and thus confirms previous data. Hansen and Rasmussen (1977) predicted such results from the increased initiation capacity of reversible dnaA mutants when returned to the permissive temperature after prolonged incubation at the restrictive temperature. They proposed that this is due to a failure of the thermally inactivated protein to regulate its own synthesis and furthermore that this regulation takes the form of autorepression.

Regulation of transcription by protein factors is a common phenomenon and is generally thought to occur through binding of the regulator at an operator site at or in the vicinity of a promoter sequence (Sections 1.2.3 and 1.2.4), from which site it influences RNA polymerase/DNA interactions. The DnaA-box sequence, which is located between the two dnaA promoters and which specifically binds DnaA protein in vitro, appeared to define such an operator site. There is no doubt that transcription from the dnaA promoters, both when studied in various reporter gene fusions and when studied directly, is sensitive to inactivation, depletion and overproduction of DnaA in a manner which suggests that it is autoregulated. The work presented here shows that, although dnaA may be autoregulated, this cannot be inferred from experiments using dnaA1s mutants. In addition it has been shown that, although a likely candidate, the DnaA-box is not an operator site mediating responsiveness of dnaA transcription to intracellular levels of the DnaA protein in the system employed.

The discovery that the dnaA promoters are not derepressed at high temperature in a strain integratively suppressed by the minimal R1 origin shows that inactivation of the DnaA1s protein alone is insufficient to elicit derepression. It seems unlikely that the plasmid is producing a substitute for the inactivated DnaA protein or a factor which restores activity to it as suggested by Masters et al. (1989). It has also been ruled out that secondary mutations arising during growth of an integratively suppressed strain at high temperature are responsible for failure of the dnaA promoters to be derepressed (Section 3.9).

It seems unlikely that the other dnaA suppressors which were examined (Section 3.11) would restore promoter binding activity to the inactivated DnaA1s protein each to the same extent. All suppressors were selected for their ability to
initiate replication at high temperature and do so by different mechanisms. In the case of GroE suppression it could be envisaged that this occurs through an interaction which changes protein conformation to resemble that of non-denatured DnaA and that this would concomitantly restore affinity of the protein for the promoter. The rpoB mutation, however, is believed to suppress by facilitating strand separation at the origin through transcriptional activation (Section 1.7.5). Although the allele specificity of suppression through rpoB mutations suggests that this involves an interaction with DnaA, it is not immediately apparent why a similar interaction at the dnaA promoter would restore repression. Finally, it has not been shown that thermal inactivation of DnaA46 in fact diminishes its DNA binding capacity. Indeed the contrary appears to be the case; DnaA46 does not bind DNA well in vitro at low temperature but its reduced binding capacity is not further diminished at higher temperatures (Hwang and Kaguni, 1988b).

3.15.2 Indirect effects of DnaA inactivation on dnaA promoter activity.

It has been argued that the effect of thermal inactivation of a DnaA\textsuperscript{ts} protein on dnaA promoter activity is slow, unlike its effect on initiation of replication which is immediate (Bremer and Churchward, 1991). As this conclusion is based on studies using \( \lambda \)RB1, where protein (\( \beta \)-galactosidase) synthesis serves as a measure of promoter activity, such an argument can only be validated if there are reliable data concerning the rate of DnaA'\-'\( \beta \)-galactosidase protein synthesis from the phage. These data are unfortunately not available. However, Atlung \textit{et al.} (1985) state that in a temperature-shift experiment the rate of (pulse-labelled) reporter protein synthesis does not increase until fifteen minutes after the shift to 42°C. This does indeed suggest (but not prove) that derepression of transcription may well also be delayed. This in turn would indicate that it is not inactivation of the protein directly but another aspect of cell physiology, which is altered as a consequence, which leads to increased promoter activity. Two lines of evidence may indicate that it is cessation of chromosomal replication which is responsible, either directly or indirectly, for derepression of transcription in a dnaA mutant: (i) there appears to be a correlation between dnaA\textsuperscript{1p2p} derepression and the efficiency of integrative suppression (Sections 3.3 and 3.4 show that in integratively suppressed strains derepression of dnaA\textsuperscript{1p2p} only occurs when DNA replication is less efficient than normal); (ii) a variety of other dnaA suppressors which have been tested, and which should function differently to achieve suppression, each give rise to a failure of the dnaA promoters to be derepressed at high temperature (Section 3.11).
Polaczek and Wright (1990) as well as Bremer and Churchward (1991) propose that the dnaA promoter derepression is a consequence of an imbalance between RNA polymerase levels and DNA concentration: inactivation of DnaA leads to gradual cessation of DNA synthesis, whereas protein synthesis is not immediately affected, resulting in a gradually increasing excess of RNA polymerase availability per promoter. They further propose that as dnaA2p has the characteristics of a growth-rate sensitive promoter, it may be particularly sensitive to elevated intracellular levels of RNA polymerase and therefore shows increased activity after DnaA\textsuperscript{ts} inactivation. This is supported by the observation that the dnaA2p promoter in isolation, without the neighbouring DnaA-box, is still derepressed upon inactivation of DnaA\textsuperscript{ts} protein (Polaczek and Wright, 1990). It also adequately explains the lack of derepression in a suppressed strain in which efficient chromosomal replication is permitted to continue in the absence of fully active DnaA protein. The observation by Masters et al. (1989) that an rnh mutation does not prevent some derepression from occurring is not necessarily in conflict with the above model as it is known that rnh mutants are sensitive to rich medium (Kogoma and von Meyenburg, 1983), which could be an indication that DNA replication in such mutants does not efficiently keep pace with mass increase. Similarly, the partial derepression in strains integratively suppressed by the F factor may be due to a similar deficiency in replication. Tresguerres et al. (1975) noted that integratively suppressed Hfrs exhibit rich medium sensitivity. Although the TPF strains used in this study showed virtually normal cell-size distributions in LB medium at 40°C, they did show better suppression on minimal plates than on LB plates (Table 3.4.1), indicating a degree of rich medium sensitivity. It would remain, however, to be clarified why they still show some dnaA promoter derepression when grown in minimal medium. It is possible that even under those circumstances the efficiency of suppression is not adequate to maintain the proper balance between replication and RNA polymerase availability.

It is interesting to note that dnaA promoter derepression through introduction of an oriC plasmid may be explicable by the above model. Section 3.12.5 showed that the level of derepression caused by pH\textsc{p}4 is far greater than that caused by pOC\textsc{161}. The former plasmid also causes a great reduction in growth rate and may therefore be interfering with chromosomal replication. It could be that such interference, by cloned sequences in the vicinity of oriC or otherwise, in combination with DnaA titration or alone is causing dnaA promoter derepression by an indirect route similar to that proposed for DnaA\textsuperscript{ts} inactivation.

The above model is, however, incompatible with the fact that the dnaB\textsc{252} and the dnaC\textsc{325} mutations, which like dnaA display a slow-stop replication phenotype,
do not lead to dnaA promoter derepression at high temperature (Masters et al., 1989; Section 3.8.2). Moreover, Section 3.15 shows that an integratively suppressed dnaA0 mutant exhibits increased dnaA promoter activity compared with an isogenic dnaA+ strain and furthermore that this is not due to possible inefficient replication from pKN500 as a ΔoriC strain, which is also reliant on pKN500 for initiation of replication, does not show increased dnaA promoter activity. Thus, the above model must be rejected as cessation of chromosomal replication does not always lead to increased dnaA promoter activity and conversely, two strains which should replicate with the same efficiency show different levels of dnaA promoter activity.

As an alternative model, it could be speculated that the membrane sequestration process may be involved (Campbell and Kleckner, 1990; Lu et al., 1994). In a dnaAts strain at high temperature the sequestration process fails due to cessation of replication, resulting in increased dnaA promoter activity. If replication is restored, the dnaA promoters will again periodically become hemimethylated and will be sequestered for part of the cell-cycle. In the case of integrative suppression or suppression by cSDR in an rnh mutant sequestration may in some cases not be efficient due to effects such as the nature and location of the active origin of replication. This may be the cause of the intermediate derepression seen in F integrants and rnh mutants (Masters et al., 1989). If oriC plasmids are introduced into the cell, they may compete with the dnaA promoter for the sequestration machinery resulting in a shorter sequestration period and higher overall dnaA promoter activity. A model attributing the derepression of the dnaA promoters in a dnaAts strain at the restrictive temperature to a failure of the sequestration process would, however, again not explain the lack of derepression in a dnaCts mutant at high temperature. In addition, introduction of a plasmid carrying the dam gene into TP945 showed little effect on dnaA promoter activity in λRWS945 (Section 3.12.7). Introduction of this plasmid would be expected to raise intracellular levels of Dam methylase and thus lead to a disruption of the sequestration process (Boye and Løbner-Olesen, 1990); according to the above model this should lead to increased dnaA promoter activity.

Comparison of Table 3.14.1 and Figure 3.8.3 shows that dnaA promoter activity is increased to a far greater extent (approximately three to six-fold) in a dnaAts strain at restrictive temperature compared with a dnaA+ strain than in a dnaA0 strain (approximately two-fold). This could be interpreted to indicate that dnaA is involved in two separate processes, both of which together normally maintain dnaA promoter activity at a certain basal level. Thermal inactivation of DnaA1ts disrupts both processes and leads to a large increase in promoter activity. Restoration of DNA replication can in most cases restore both processes leading to normal control of the dnaA promoters,
on the condition that a form of DnaA (albeit thermally inactivated) is present in the cell. If no DnaA is present the ability to replicate only restores one process so that dnaA promoter activity is left at a partially elevated level. Although restoration of the ability to replicate the chromosome can restore either one or both processes, a block in replication itself (dnaC) is not the cause of their disruption. Processes with the characteristics required in this model have not yet been described.

The failure of the dnaA promoters to be derepressed has only been shown in systems where the dnaA promoter activity is studied indirectly using a lacZ reporter gene (λRB1, λRWS945 and pRWS945). It cannot be excluded that it is an artefact of such systems. However, other studies have shown that the effects of DnaA inactivation, overproduction and depletion lead to very similar changes in the expression of other reporter genes (galK and tet) and, more importantly, in the level of transcription from the chromosomal dnaA promoters (Atlung et al., 1985; Polaczek and Wright, 1990; Kücherer et al., 1986). Atlung and Hansen (1993) have also shown that the effect of DnaA overproduction on lacZ expression from λRB1 is similar to its effect on actual DnaA protein levels in the cell. There appears therefore to be little reason to believe that the failure of the dnaA promoters to be derepressed in an integratively suppressed strain is limited to the systems used in this study.

3.15.3 Mutation of the DnaA-box.

A surprising result from the work described in this chapter is the fact that the DnaA-box between the two promoters appears to have no role in mediating the response of the promoter to levels of DnaA in the cell. This is to some degree in accordance with results by Polaczek and Wright (1990) who found that introduction of multiple point mutations in the DnaA-box does not increase promoter activity in a dnaA+ strain, nor does it abolish the response to thermal inactivation of DnaA18s protein. They did not test their point mutants for their response to DnaA overproduction. However, they did test a fragment which contains dnaA2p and sequences downstream to the EcoRI site in the dnaA gene but no sequences upstream of dnaA2p; transcription from this fragment, which completely lacks the DnaA-box, does not respond to overproduction of DnaA. This would suggest that the part of the promoter region which confers DnaA sensitivity to transcription must be located upstream of dnaA2p. Atlung et al. (1985) carried out deletion studies and concluded that sequence extending to at most 11 bp upstream of the DnaA-box could be involved in response to overproduction of DnaA. Removal of any sequences further upstream
does not abolish sensitivity to DnaA. A deletion extending from upstream into the DnaA-box, even by only one base pair abolishes DnaA sensitivity. These studies are summarised in Figure 3.15.1. and clearly delimit the DnaA responsive site to the DnaA-box and its very limited vicinity, see also Braun et al. (1985).

The studies presented in this chapter are surprising in the light of the above but it must be stressed that the experiments were carried out several times with the same stock of TP945M which was used to verify the mutations by resequencing. Here, the effect of point mutations and not of a deletion has been investigated. However, these point mutations abolish DnaA binding \textit{in vitro} (Section 3.13) and would also be expected to do so \textit{in vivo}. \textit{dnaA} promoter activity in LRWS945M and in LRWS945 are equally responsive to DnaA overproduction from an inducible plasmid even shortly after induction (Section 3.12.4); this would not be expected if the response of LRWS945M was due to non-specific binding of DnaA. The results, in combination with the deletion studies by other investigators described above, thus indicate that it is the presence of the DNA between the promoters and not the integrity of its sequence which renders \textit{dnaA} transcription sensitive to DnaA \textit{in vivo}.

A possible consequence of this hypothesis is that although the DnaA-box binds DnaA protein efficiently \textit{in vitro}, it may not do so \textit{in vivo}. The effect of high levels of DnaA protein on transcription of the \textit{dnaA} gene may be mediated by an RNA polymerase/DnaA interaction rather than through a DnaA-box/DnaA interaction. It has long been accepted that RNA polymerase and DnaA interact as shown by allele specific suppression of \textit{dnaA} mutations by mutations in \textit{rpoB}. In addition, it has been reported that high levels of intracellular DnaA may lead to an alteration of RNA polymerase activity, as indicated by the occurrence of rifampicin-resistant initiation of replication upon vast overproduction of DnaA (Atlung and Hansen, 1993). It may be that such an alteration of RNA polymerase activity is somehow responsible for the altered \textit{dnaA} promoter activity and that for this reason it is not abolished by mutation of the DnaA-box. As a consequence it would be expected that this effect would not be unique to transcription of \textit{dnaA}, but that transcription in general would be reduced if DnaA is present in excess. Indeed, it is shown in the next chapter that transcription from the \textit{ftsZ} promoters is reduced if DnaA is overproduced and preliminary experiments in our laboratory show that this is also the case with the \textit{pcnB} promoter.
Figure 3.15.1 Summary of deletion analyses determining the DnaA responsive site in the promoter region.

Summarised are various studies examining the effect of DnaA overproduction in vivo on the activity of either dnaA1p or dnaA2p; in each case this was monitored with the aid of a reporter gene (lacZ, tet or galK). Arrowheads represent promoters. Horizontal lines represent cloned fragments of the dnaA promoter region fused to reporter genes. Vertical lines indicate the end-points of the cloned sequences with respect to the dnaA promoter region shown above. The various data are summarised at the bottom of the figure by the thick horizontal line which indicates the minimal element necessary to confer DnaA sensitivity. Taken from Atlung et al. (1985), Braun et al. (1985) and Polaczek and Wright (1990).

3.15.4 Is dnaA autoregulated?

Although the mechanism remains unclear, it is beyond doubt that the dnaA promoters are responsive to variations in the intracellular levels of the DnaA protein and in this sense the gene is autoregulated. The question of whether it is autoregulated in a manner which would be expected to keep the concentration of its product constant under physiological conditions is far more complicated. Atlung and Hansen (1993) studied the response of the dnaA promoters as well as the response of DnaA production to increased intracellular levels of plasmid encoded S. typhimurium DnaA protein and found that the promoters only begin to respond when the total intracellular DnaA concentration is greater than twice its normal level, a concentration at which DNA replication becomes seriously affected, and as such under non-physiological conditions. This study suggests that the promoters do not respond to slight changes in
intracellular levels of DnaA as would be expected if the gene were under tight autoregulation mediated by a repressor. The matter is, however, complicated by the fact that DnaA may be an activator of dnaA2p at physiological levels, thus cancelling any repressing effect on the weaker promoter dnaA1p (Polaczek and Wright, 1990). If this is true then autorepression would only occur under circumstances where dnaA2p is inactive. Polaczek and Wright (1990) propose that this is the case in stationary phase. Abolition of the DnaA-box, however, elicits no increased transcription under these conditions (Section 3.12.3). Taken together the data from various investigations indicate that dnaA is not autorepressed. If it is autoregulated, which has not been refuted, it is not regulated in a manner which would compensate for slight or even moderate alterations in DnaA concentration.

Does the cell require dnaA to be autoregulated? Løbner-Olesen et al. (1989) studied a strain in which dnaA was constantly expressed from the lac promoter. At a certain level of induction of the promoter, the strain showed characteristics indistinguishable from that of the wild-type. Initiation mass was normal and initiation was synchronous. Whether the initiation mass would have changed were the strain to be grown at a different growth rate was unfortunately not tested. Until such an experiment has been performed the need for autoregulation cannot be discussed. Even so, the result of such an experiment would be open to ambiguity, as other elements of dnaA growth-rate control, such as possible sensitivity to ppGpp (Chiaramello and Zyskind, 1990) could also be affected.

The fact that DnaA is regulated at a functional level through binding of ATP has implications in assessing the need for autoregulation as it can be argued that it is the level of active DnaA-ATP, rather than that of total DnaA, which sets the initiation mass. If the level of active DnaA-ATP at different growth rates is a constant proportion of total DnaA then it would seem likely that the cell requires dnaA to be autoregulated. If the level of initiation competent DnaA is independently controlled the cell may not require total DnaA synthesis to be tightly regulated. It must also be borne in mind when considering this issue that autoregulation may not be required to avoid premature reinitiation in the cell when DnaA levels are high as the sequestration process independently ensures that the dnaA promoters will temporarily be shut down. At present there is simply not sufficient information available concerning the relative contributions of regulatory mechanisms influencing DnaA activity to allow a judgment other than through an empirical approach. This would require the construction of a strain in which the dnaA gene at its normal chromosomal location is placed under the control of a promoter which is insensitive to intracellular levels of DnaA, but yet which is subject to all other forms of control which influence dnaA promoter activity.
If this were to prove possible, examination of parameters such as synchrony and initiation mass under various conditions would permit the importance of autoregulation to be assessed.
Chapter IV

The role of DnaA in transcription of \( ftsZ \)
4.1 Introduction

4.1.1 Regulation of transcription of ftsZ.

ftsZ lies towards the distal end of a cluster of contiguous genes, known as the mra operon, at 2 minutes on the genetic map (Figure 4.1.1). This cluster comprises genes involved in peptidoglycan synthesis and genes which are essential for cell division. The arrangement of genes in the mra operon is such that the coding capacity of the DNA is almost fully utilised leaving little intergenic space. Research into the transcription of the ftsQ, ftsA and ftsZ genes has shown that these genes are all transcribed from promoter sequences which are present within the coding regions of upstream genes (Robinson et al., 1984 and 1986) and in this sense the mra operon differs from a "true" operon such as lacZYA in which the constituent genes are solely transcribed co-ordinately from a common promoter. However, as the mra operon has been shown to contain no transcriptional terminators in the direction of transcription some degree of co-ordinate control of the genes present appears likely.

Early studies showed that an element in the ftsA gene, which lies immediately upstream from ftsZ, is necessary for ftsZ expression (Lutkenhaus and Wu, 1980). Two separate sites with promoter activity were subsequently identified within ftsA (Sullivan and Donachie, 1984) and the DNA sequence revealed several putative promoters corresponding to these sites (Robinson et al., 1984). It is estimated that these promoters together account for 80% or more of ftsZ transcription (Yi et al., 1985; Garrido et al., 1993). For complementation of an ftsZ null mutation by a single copy vector carrying ftsZ the presence of DNA further upstream of the cloned gene, extending beyond ftsW, is absolutely required indicating that essential ftsZ transcripts may be initiated over 6 kb upstream of the ftsZ gene (Dai and Lutkenhaus, 1991). A number of ftsZ proximal promoters have been mapped, four within ftsA (ftsZ1p to ftsZ4p) and two within the ddl gene (ftsQ1p and ftsQ2p) (Aldea et al., 1990). ftsZ1p has recently been reported to be not a promoter but a cleavage site for RNase E which has been suggested to be involved in post-transcriptional control of ftsZ expression (Cam et al., 1994).

The fact that a large number of promoters appear to contribute to ftsZ transcription would allow for the possibility of various transcriptional regulators exercising an their influence on this process. Only one such regulatory factor has so far been clearly identified. Wang et al. (1991) showed that the product of the sdiA
Figure 4.1.1 The *mra* operon.

Above: the *mra* operon; genes involved in cell division are shaded. Below: enlargement of the distal end of the operon. Restriction sites which were used in this work are indicated for reference (B=BamHI; Bg=BglII; C=ClaI; E=EcoRI; H=HindIII; P=PstI). Black arrowheads denote promoter sequences; white squares denote DnaA-boxes.
gene activates transcription from $ftsQ2p$; overproduction of the SdiA protein leads to an increase in transcription from $ftsQ2p$ specifically and deletion of the $sdiA$ gene reduces transcription from this promoter by approximately 50%. Deletion of $sdiA$, however, shows no division defect and its purpose in gene regulation is therefore still obscure.

Transcription from some of the $ftsZ$ promoters has been shown to be growth-rate sensitive. Aldea et al. (1990) showed that $ftsQ1p$ is induced at low growth-rates by studying both the behaviour of transcriptional fusions and by measuring mRNA from a plasmid carrying the $ddlB$ region under different growth conditions. Dewar et al. (1989) showed that the level of transcription from the 1.8 kb EcoRI-HindIII fragment containing $ftsQ$ and part of $ftsA$ (Figure 4.1.1) is also inversely correlated to growth-rate. The same appears to be true of the $ftsZ$ proximal promoters (Smith et al., 1993; Chapter V). Although this indicates another form of regulation of $ftsZ$ expression, the factors which are responsible for this have not yet been identified. Growth-rate sensitivity of $ftsZ$ expression will be discussed further in Chapter V.

4.1.2 DnaA as a possible transcriptional regulator of $ftsZ$.

Work in our laboratory gave a preliminary indication that the DnaA protein may be acting as a negative regulator of $ftsZ$ transcription (Masters et al., 1989). Three putative DnaA binding sites were identified upstream of $ftsZ$, two within the $ftsA$ gene and one within $ftsQ$. Each has one mismatch to the DnaA-box consensus defined by Fuller et al. (1984) but all conform to the less stringent definition of a DnaA-box by Schaefer and Messer (1991) (Table 4.1.1). The DnaA-box in $ftsA$, nearest to the $ftsZ4p$ promoter is identical to the DnaA-box found in the $mioC$ promoter region, which is known to bind DnaA protein in vitro. The possible effect of DnaA on transcription was studied using the $fts$-$lacZ$ fusion phage $\lambda$I100 which was constructed by J. F. Lutkenhaus (Donachie, 1984) (Figure 4.1.2). This phage contains a transcriptional fusion between the 1.8 kb EcoRI–HindIII $ftsQA'$ fragment and the W205 fusion of $trpA$, $B$ and $lacZ$ (Mitchell et al., 1975). $\beta$-galactosidase activity should reflect the level of promoter activity in the cloned $fts$ fragment as neither $trp$ nor $lac$ promoters are present. The $fts$ fragment contains the $ftsZ3p$ and $ftsZ4p$ promoters as well as a possible weak promoter present in $ftsQ$, designated $ftsAp$ (Robinson et al., 1984). The putative DnaA-boxes lie between $ftsAp$ and $ftsZ4p$ and are all present in the same orientation relative to transcription.
Masters et al. (1989) measured β-galactosidase production from λJFL100 in a dnaA46 strain (TP30) upon a transfer from the permissive to the restrictive temperature and found an immediate increase in the rate of enzyme synthesis after the temperature shift. The specific β-galactosidase activity eventually reached a value of nearly four times that at the permissive temperature. The equivalent experiment using a dnaA+ strain (TP48) showed no such derepression of promoter activity (Figure 4.1.3). A tentative conclusion therefore was that DnaA may be acting as a negative transcriptional regulator of ftsZ as the thermal inactivation of its mutant form (DnaA46) leads to an increased activity of at least some of the promoters present in λJFL100.

**Figure 4.1.2** The structure of λJFL100.

It will be clear from Chapter I that the DnaA protein has many reported functions and its inactivation may have a pleiotropic effect. A possibility is that cessation of DNA replication may have some indirect effect on transcription from λJFL100 and for this reason the behaviour of the phage was studied in the temperature-sensitive dnaB252 and dnaC325 mutants which like dnaA46 are deficient in initiation of DNA replication (Zyskind and Smith, 1977). A shift to the non-permissive temperature in the dnaB252 and dnaC325 mutants carrying λJFL100 as a prophage (TPB48 and TPC48 respectively) again leads to a derepression of transcription from the cloned promoters but this derepression is not equivalent to that observed in the dnaA46 strain (Figure 4.1.3). In the dnaB (not shown) and dnaC mutants the specific activity of β-galactosidase begins to increase nearly an hour after that in a dnaA mutant and also shows a lower final value (a 1.5-fold
The role of DNA in transcription of \( ftsZ \) derepression compared to a 3 to 4-fold derepression in the \( dnaA46 \) strain. It therefore seems that inability to initiate replication has consequences for transcription from \( \lambda JFL100 \) but the possibility cannot be excluded that inactivation of DnaA also plays an additional direct role in regulating \( ftsZ \) transcription. In order to test this further, experiments were performed in a system which would allow inactivation of the DnaA protein without arresting replication. In \( dnaA46 \ rnh \) double mutants replication can be initiated at high temperature from alternative origins, a process known as stable DNA replication (cSDR, see Section 1.10.6). In such a strain transcription from \( \lambda JFL100 \) was derepressed upon a shift from permissive to restrictive temperature albeit to a lesser degree than in a \( dnaA46 \) single mutant. Although replication in such a strain is inefficient, this may indicate that DnaA exercises an effect on \( ftsZ \) transcription other than through replication alone. Surprisingly, a similar experiment using an integratively suppressed \( dnaA46 \) strain (TPK30) carrying \( \lambda JFL100 \) showed no derepression at all at the restrictive temperature. The reason for this was unclear and apparently contradicted the result obtained with the \( rnh \) strain. Therefore it remained unclear what is the direct cause for the derepression of transcription from \( \lambda JFL100 \) in a \( dnaA46 \) strain.

**Figure 4.1.3** Specific \( \beta \)-galactosidase activity of \( \lambda JFL100 \) in various strains.

Cultures were maintained in exponential growth phase at 30°C for several hours before sampling and diluted into medium of 42°C at time 0. The cultures continued to be kept in exponential phase. The graph shows the specific \( \beta \)-galactosidase activity plotted against time. Taken from Masters *et al.* (1989).
An attempt was made to show in vitro binding of DnaA to the \textit{ftsQA}' fragment by a filter binding assay. This proved difficult as non-specific binding of the protein to DNA was a problem under the conditions used; binding appeared to be a function of the length of the DNA fragment rather than of its DnaA-box content (T. Owen-Hughes, personal communication).

Rather than to begin by optimising the conditions for the filter binding assay to show in vitro binding it was decided to investigate the \textit{in vivo} role of the DnaA-boxes in \textit{ftsQ} and \textit{ftsA} by mutating them using a site directed mutagenesis procedure. The aim was to study the effect of the mutations both in transcriptional fusions similar to \textit{\lambda}JFL100 and also to study their effect when introduced into the chromosome by replacing the wild-type \textit{ftsQA} sequence with the mutant. The nature of the mutations should be such that the activity of the DnaA-boxes would be minimised. A measure for this was developed by Schaefer and Messer (1991) by comparing the efficiency with which various putative DnaA-boxes promote transcription termination (Section 1.5.1). The three DnaA-boxes, which are the subject of this study, all lie within the coding sequences of \textit{ftsQ} and \textit{ftsA} and for this reason it was important to choose the mutations so that the FtsQ and FtsA amino acid sequences would not be altered in view of the aim to eventually study the mutated DnaA-boxes in their natural environment. The planned mutations are shown in Table 4.1.1.
Table 4.1.1 Sequences of the DnaA-boxes in the *ftsQ* and *ftsA* genes and their mutated derivatives.

<table>
<thead>
<tr>
<th>DnaA-box</th>
<th>Sequence</th>
<th>Consensus (Fuller et al., 1984)</th>
<th>Consensus (Schaefer and Messer, 1991)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ftsQ</em> (691)</td>
<td>TTAATCAAA</td>
<td>TTAATCAAA</td>
<td>CCGCCTT</td>
</tr>
<tr>
<td><em>ftsA</em> (986)</td>
<td>TTAATCATT</td>
<td>TTAATCAAA</td>
<td>CCGCCTT</td>
</tr>
<tr>
<td><em>ftsA</em> (1175)</td>
<td>TTCTCACA</td>
<td>TTCTCACA</td>
<td>CCGCCTT</td>
</tr>
<tr>
<td><em>ftsQ</em> (691) mutated</td>
<td>CTCGACAA</td>
<td>CTCGACAA</td>
<td>CCGCCTT</td>
</tr>
<tr>
<td><em>ftsA</em> (986) mutated</td>
<td>TTGTCCTG</td>
<td>TTGTCCTG</td>
<td>CCGCCTT</td>
</tr>
<tr>
<td><em>ftsA</em> (1175) mutated</td>
<td>TTCTCGAC</td>
<td>TTCTCGAC</td>
<td>CCGCCTT</td>
</tr>
</tbody>
</table>

Comparison of the wild-type and mutated DnaA-box sequences to the published consensus sequences. Sequences are shown 5' to 3'. Mutations are indicated in bold print. a Location of the sequence with reference to Robinson *et al.* (1984). b Identical to the DnaA-box in the mioC promoter region.
4.2 Mutation of the DnaA-box in ftsQ

Initially the mutation of a single DnaA-box was studied. The procedure devised by Taylor et al. (1985) (Section 2.2.16) was used to introduce the mutations shown in Table 4.1.1 into the ftsQ DnaA-box. The 1.8 kb EcoRI-HindIII fragment containing ftsQA' from pSU200 (a plasmid containing this fragment upstream of lacZ) was cloned into the M13 mp18 polylinker, yielding M13#1. Single stranded DNA was prepared from this as described in Section 2.2.4 and site directed mutagenesis was performed. The mutation introduces a Taq I restriction site into the sequence and the presence of the mutation was verified by restriction analysis (Figure 4.2.1). M13M1 was used for further work.

Figure 4.2.1 Verification of the ftsQ DnaA-box mutation by TaqI digestion.

Double-stranded M13 DNA of M13#1 and of six possible mutants (M13M1 to M13M6) was prepared, digested with TaqI and electrophoresed on a 1.5% agarose gel. The restriction pattern of the wild-type sequence is shown by M13#1. The mutations in the ftsQ DnaA-box introduce a TaqI site, cleavage of which results in the loss of the 1.56 kb wildtype fragment. This is replaced by a 1.24 kb and a 0.32 kb band. The digestion patterns show that all the possible mutants tested are indeed mutated. Molecular weight markers (created by HindIII digestion of λ DNA) are shown on the left and their sizes are indicated in kb.
In order to obtain an indication of the effect of the mutation on transcription from this region both the mutated 1.8 kb \textit{ftsQA}' fragment and the corresponding wild-type sequence were cloned into the transcriptional fusion plasmid pTL61T (Linn and St. Pierre, 1990). This plasmid contains the \textit{lacZ} reporter gene downstream of a polylinker. An RNase III processing site is present just upstream of \textit{lacZ} and this ensures independent translation of the \textit{lacZ} mRNA and thus avoids possible post-transcriptional interference by cloned sequences. In addition there is a strong transcriptional terminator upstream of the polylinker so that levels of β-galactosidase produced by the plasmid should be a direct measure of transcription initiating in the cloned sequence. The resulting plasmids were pTLM1, which contains the mutant \textit{ftsQ} sequence, and pTLQA, which contains the wild-type sequence (Figure 4.2.2). These plasmids were introduced into both a \textit{dnaA46} strain (ED419) and an isogenic \textit{dnaA}+ strain (TP8503) and levels of β-galactosidase were measured in a temperature shift experiment (30–42°C) as described in Section 2.5 (Figure 4.2.3).

\textbf{Figure 4.2.2}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.2.2.png}
\caption{pTLQA carries the 1.8 kb \textit{ftsQA}' fragment from pSU200 (obtained from S.J. Dewar). pTLM1 is identical apart from the mutations in the \textit{ftsQ} DnaA-box. Black squares indicate DnaA-boxes; white arrows indicate relevant promoters.}
\end{figure}
If the DnaA-box in *ftsQ* were acting as an operator site for a transcriptional repressor it would be expected that β-galactosidase levels from TP8503 (pTLM1) would be higher than those from TP8503 (pTLQA). The specific activities of both strains, however, are identical. In ED419 (*dnaA*46) transcription from both plasmids shows an identical derepression upon a shift from 30–42°C, indicating that if it is failure of the mutant protein to bind sequences within *ftsQA* at high temperature which is responsible for this derepression, the *ftsQ* DnaA-box is not an essential part of its binding site.

**Figure 4.2.3**

The derepression in ED419 seen in Figure 4.2.3 is likely to underrepresent the true derepression of transcription as the copy-number of ColE1 plasmids drops upon inactivation of the DnaA46 protein. Plasmid accumulation has been reported to cease completely one to two hours after the temperature shift (Chiang *et al.*, 1991). pTLQA and pTLM1 also show a derepression of transcription in TP8503 after the temperature shift; this may be due to copy-number effects although it has been reported that plasmid copy-number in a *dnaA*+ strain is unaffected by a temperature shift (Chiang *et al.*, 1991). Copy-number was not examined. pTLM1T, the parent vector, contains an unidentified sequence present near the polylinker with weak but measurable transcriptional activity (Linn and St. Pierre, 1990). An increase in β-
galactosidase synthesis proportional to that from pTLQA and pTLM1 is observed from this plasmid when present in TP8503, indicating that increased lacZ expression at high temperature is not specific to that promoted by the ftsQA sequence.

In conclusion it appears that mutation of the DnaA-box in ftsQ has no effect on transcription from ftsQA' in this system. A strong possibility was that DnaA binds to the three DnaA-boxes present in this region in a manner analogous to its binding to the four oriC DnaA-boxes and that abolition of a single box has little effect on the resulting protein/DNA structure (Section 1.7.3). Therefore it was decided to construct a triple mutant and to examine its effect.
4.3 Construction of new transcriptional fusions of fisZ promoters and lacZ

Because of the difficulty in directly comparing transcriptional effects in a plasmid system in dnaA+ and dnaAts strains due to the effect of dnaA mutations on plasmid copy-number it was decided to study the effect of the triple mutation in a single copy λ system similar to λJFL100. Several such systems have been developed which allow the construction of transcriptional fusions with a lacZ reporter gene. Linn and St. Pierre (1990) reported the construction of a phage, λTL61, which like pTL61T contains lacZ downstream of several unique restriction sites to facilitate the cloning of promoter sequences into this vector. It has the advantage of an RNase III cleavage site upstream of the reporter gene but unlike pTL61T does not contain a transcriptional terminator upstream of the the cloned sequence. This is an important consideration in the light of work presented by Robin and D’Ari (1993). These authors attempted to find mutations in the fisQA’ sequence leading to increased fisZ transcription using the λJFL100 phage, described earlier, by selecting for increased lacZ expression from this phage. Although mutants showing greater lacZ expression were isolated, none of the mutations were found to reside in the phage and the authors proposed the possibility that transcription initiating outside the cloned sequence could be responsible for the increased lacZ expression. Although this has not yet been proven (R. D’Ari, personal communication), it cannot be discounted that under certain circumstances lacZ is transcribed from promoters other than those present in the fisQA’ fragment, either from promoters residing on phage DNA or from chromosomal promoters in the vicinity of the λ attachment site (attλ). The translational start for fisQ in λJFL100 lies very near to attλ and, although no promoters for fisQ have been found on the 1.8 kb EcoRI–HindIII fis insert, the phage has been shown to express fisQ (Kenan, 1985); this is further evidence to suggest that the insert is not isolated from outside promoters. In order to avoid potential complications arising from transcription from outside promoters, it was decided to use a vector system which does contain transcriptional terminators upstream of the cloned sequence. Simons et al. (1987) describe a two component system which consists of a phage (λRS45) and a plasmid (pRS551) which introduces cloned promoters downstream of four tandem copies of the T1 terminator of rrnB. This system was described in Section 3.8 and was chosen for further work.

With λJFL100 only fisZ3p, fisZ4p and fisAp were available for study. In addition to repeating experiments done on λJFL100 with the same insert in λRS45, it was decided to study the possible effects of DnaA on a larger fragment also containing the fisZ proximal promoter fisZ2p. To this end the 2.3 kb EcoRI fragment
containing \textit{ftsQ} and \textit{ftsA} from pSZ24 (Dewar et al., 1989) was inserted into the unique \textit{EcoRI} site in pRS551. Transformation of TP8503 with the ligation products using the method designed by Chung et al. (1989) repeatedly failed to yield transformants. Two reasons for this could be envisaged: the resulting plasmid would be relatively large (14.7 kb), a quality which does not favour efficient transformation (Hanahan, 1983) and more importantly, the plasmid would introduce multiple copies of \textit{ftsA} into the cell. Overproduction of the \textit{ftsA} gene product is not well tolerated (Dewar et al., 1990) and this could also negatively influence the transformation efficiency. The first problem was addressed by using electroporation of the cells to transform them with the ligation product; this procedure is generally more efficient than the transformation procedure previously used. It was also decided to use an \textit{ftsA}^{ts} strain (TOE13) as a recipient, in order to exploit the fact that the recombinant plasmid expresses \textit{ftsA} as a selective advantage. It was hoped that overexpression of \textit{ftsA} might be more readily tolerated in such a background. This procedure did yield a number of transformants, all of which contained the 2.3 kb \textit{EcoRI} fragment. The orientation of the insert was tested by digestion with BamHI; in the correct orientation this should release a fragment of 2.2 kb. The resulting plasmid was named pRWS100 (Figure 4.3.1a).

A plasmid containing the 1.8 kb \textit{EcoRI}–\textit{HindIII} fragment was also constructed. Two cloning strategies were used simultaneously: in the first pRS551 was digested with \textit{EcoRI} and pSZ24 was digested with \textit{EcoRI} and \textit{HindIII}. The 5' overhangs were then filled in using the Klenow fragment of DNA polymerase I, resulting in two blunt ended fragments which were then ligated. The orientation of the insert in the resulting plasmid was again tested by digestion with BamHI. The plasmid promoting \textit{fts} transcription towards \textit{lacZ} was named pRWS200 (Figure 4.3.1a).

The second strategy was a directional cloning of the \textit{EcoRI}–\textit{HindIII} fragment from pSZ24 into pRS551 which was digested with \textit{EcoRI} and BamHI. A double stranded \textit{HindIII}–\textit{BamHI} adaptor from New England Biolabs was used. The three fragment ligation yielded pRWS201 (Figure 4.3.1a).

To effect \textit{in vivo} recombination of the fusions into the single copy \textit{\lambda} vector the plasmids were introduced into NM621/\textit{\lambda}RS45 and lysogens of recombinant phage in TP8503 and ED419 were selected as described in Section 3.8.1. The phages and resulting lysogens were name \textit{\lambda}RWS100 (TP100, ED100), \textit{\lambda}RWS200 (TP200, ED200) and \textit{\lambda}RWS201 (TP201, ED201) after the respective plasmids (Figure 4.3.1b).
DNA was prepared from these phages and subjected to restriction analysis to verify the inserts. In addition, λRWS100 was introduced into the fisA ts strain TOE13 to verify whether it could complement its temperature-sensitive phenotype. TOE13/λRWS100 grew at 42°C whereas TOE13/λRWS200 did not, indicating that λRWS100 expresses fisA, as expected.

**Figure 4.3.1a**

Above: pRWS100 contains the 2.3 kb EcoRI fisQAZ fragment. Below: pRWS200 and pRWS201 contain the 1.8 kb fisQA EcoRI-HindIII fragment (pRWS200 has lost both the EcoRI and the HindIII sites, pRWS201 only the latter). Both plasmids carry transcriptional fusions of these regions with lacZ. See text for details of construction. Relevant promoters are indicated by open arrows.
Structure of the the single copy transcriptional fusion vectors derived from the plasmids shown in Figure 4.3.1a. The inserts in the phages are drawn to scale, the λ arms are not. Promoters are represented by arrows. T denotes four tandem copies of the rrnB T1 transcriptional terminator. λRWS201 and λRWS20M contain a HindIII–BamHI linker between the fts DNA and lacZ. λRWS20M carries the triple DnaA-box mutation. lacZ will be translated from its own ribosome binding site as the constructs contain stop codons in all reading frames in the DNA present between the cloned insert and lacZ.
4.4 Behaviour of λRWS100, λRWS200 and λRWS201 in a dnaA46 host

Figure 4.4.1 shows a comparison of the behaviour of λJFL100 and λRWS201 in ED419 and in TP8503 in a temperature shift experiment. The lysogens were grown for approximately four hours at 30°C in LB medium and kept in exponential growth phase (between an OD600 of approximately 0.03 and 0.15) by periodic dilution in prewarmed medium to ensure that the cultures were growing at a constant rate. Samples were taken over 100 minutes and the cells were then shifted to 42° by diluting into medium of that temperature. Samples continued to be taken over a 150 minute period in which the cells were again kept in exponential growth by dilution into prewarmed medium. In Figure 4.4.1 the specific β-galactosidase activity is plotted as a function of time.

![Figure 4.4.1](image)

The response to the temperature shift of both phages is similar in TP8503 (dnaA+). In contrast to the effect of the temperature shift when the fts promoters are present on a plasmid (Figure 4.2.3) the specific activity decreases over the first 80 minutes after the shift to 42°C. It is likely that the increased growth-rate of the cells at the higher temperature is responsible for the decrease in promoter activity.
Growth-rate sensitivity of these promoters has been reported and will be discussed in the following chapter.

In a dnaA46 background, ED419, λJFL100 and λRWS201 behave differently when the host strain is shifted to high temperature. The derepression of lacZ expression seen from λJFL100 is greater than ten-fold whereas that from λRWS201 is less than two-fold. This could be due to the fact that the terminators which are present upstream of the cloned sequence in λRWS201 are indeed blocking dnaA responsive transcription from outside the fts fragment which is responsible for the greater transcriptional derepression from λJFL100. An unlikely alternative would be that the transcriptional fusion of the fts fragment with lacZ in λRWS201 using the HindIII–BamHI adaptor has created a sequence which influences transcription through it in a dnaA dependent manner.

Figure 4.4.2 shows a similar experiment with λRWS200 which contains the same 1.8 kb fts insert as λJFL100 and λRWS201 but which was constructed in a different manner from the latter and does not contain foreign DNA between the insert and the lacZ gene. When present in TP8503, λJFL100 and λRWS200 behave similarly to λRWS201 at both temperatures. In ED419, λJFL100 again shows a far greater derepression of transcription than λRWS200 indicating that it is not the
nature of the ligation of \( fts \) fragment to the reporter gene which is accountable for the difference in transcription at high temperature. Comparison of Figures 4.4.1 and 4.4.2 shows that the derepression of transcription from \( \lambda JFL100 \) after the temperature shift is less in the latter than in the former experiment. This illustrates that there is some degree of variation in \( \beta \)-galactosidase activity between experiments. It is not clear what the reasons are for this but as the \( fts \) promoter activity is very sensitive to growth-rate it is possible that slight variations in experimental conditions such as medium composition or temperature of the incubator are responsible.

Comparison of Figures 4.4.1 and 4.4.2 also shows that the specific \( \beta \)-galactosidase activity of \( \lambda RWS201 \) is higher than that of \( \lambda RWS200 \). In an experiment where the two phages are studied simultaneously this difference is repeatable (Table 4.4.1). TP201 has a 1.5 fold higher activity than TP200. As the phages in these strains are identical apart from the \( fts-lacZ \) junction, it seems that the nature of this junction influences transcription or translation of \( lacZ \) for unknown reasons. It is unlikely that a difference in multiplicity of lysogeny between TP200 and TP201 is responsible for the difference in activity as repeated infection of various strains with \( \lambda RWS201 \) failed to give rise to lysogens with lower activity (see Section 4.7).

**Table 4.4.1** Comparison of \( \beta \)-galactosidase activities of TP200 and TP201.

<table>
<thead>
<tr>
<th>Specific Activity (MU)</th>
<th>TP200</th>
<th>TP201</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.7</td>
<td>52.3</td>
<td></td>
</tr>
<tr>
<td>31.7</td>
<td>55.2</td>
<td></td>
</tr>
<tr>
<td>30.8</td>
<td>54.1</td>
<td></td>
</tr>
<tr>
<td>33.3</td>
<td>55.9</td>
<td></td>
</tr>
<tr>
<td>31.0</td>
<td>55.9</td>
<td></td>
</tr>
<tr>
<td>33.0</td>
<td>57.2</td>
<td></td>
</tr>
<tr>
<td><strong>Average (MU)</strong></td>
<td>31.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55.1</td>
<td></td>
</tr>
</tbody>
</table>

The strains were maintained in exponential phase in LB medium at \( 37^\circ C \) for approximately three hours before sampling. Samples were then taken at five minute intervals. The specific \( \beta \)-galactosidase activities and their average values are shown.
Figure 4.4.3 shows that transcription from λRWS100 is derepressed two to three-fold in ED419 at 42°C. Although not clearly apparent from this figure the specific β-galactosidase activity of λRWS100 in TP8503 is reduced at 42°C as was observed with λRWS200, λRWS201 and λJFL100 under similar conditions. Again this could be due to changes in growth-rate. The specific activity of λRWS100 is five-fold higher than that of λRWS200 as would be qualitatively expected, due to the presence of an additional promoter, fisZ2p. Sullivan and Donachie (1984) found that the promoter activity of a fragment carrying the fisZ3p and 4p promoters is equal to that of a fragment carrying the fisZ2p promoter. Surprisingly, a fragment carrying all three promoters showed an activity which was nearly twice the sum of that of the two separate fragments. This indicates that the promoters act co-operatively. The relative β-galactosidase activities of λRWS100 and λRWS200 are in agreement with the relative promoter activities found by Sullivan and Donachie (1984).

In conclusion λRWS100, λRWS200 and λRWS201 show increased lacZ synthesis at 42°C when present in a dnaA46 background compared with their β-galactosidase activity in a dnaA+ strain under the same conditions. However, the derepression of lacZ expression upon a shift from 30°C to 42°C in a dnaA46 strain is less than that observed with the phage λJFL100.

Figure 4.4.3
4.5 Behaviour of λRWS100 and λRWS200 in a dnaC325 host

λJFL100 shows a slow increase in lacZ expression in dnaB252 and dnaC325 initiation mutants at the non-permissive temperature (Masters et al., 1989). Figures 4.5.1 and 4.5.2 show the behaviour of λRWS100 and λRWS200 in a dnaC325 background. TPC100 and TPC200 were made by transduction of the dnaC325 allele from TPC91 to TP100 and TP200 respectively by co-transducing the temperature-sensitive dnaC allele with Tn10 (telR). Both TPC100 and TPC200 show a higher level of lacZ expression than TP100 and TP200 at both the permissive and the non-permissive temperatures. At 42°C the specific β-galactosidase activities of the dnaC strains are slightly greater than those of the dnaA mutants but the increase in activity after the temperature-shift is greater in the latter due to the higher basal level of specific activity in the dnaC strains at 30°C.

The higher basal activity of the phages in a dnaC host may be a consequence of the slower growth-rate of such a strain compared with that of a dnaA strain. Under the growth conditions used for the β-galactosidase assay in the above experiments the dnaC and dnaA strains have generation times of 57.5 min. and 49.7 min. respectively at 30°C.

Figure 4.5.1

![Graph showing the behaviour of λRWS100 and λRWS200 in a dnaC325 host. The graph shows the specific activity of the phages at 30°C and 42°C over time. The specific activity is measured in Miller Units (MU). The graph includes data for TP100, ED100, and TPC100. The data points are marked with various symbols and connective lines. The x-axis represents time in minutes, and the y-axis represents specific activity in Miller Units. The graph shows an increase in specific activity with temperature for all strains.]
Figure 4.5.2

![Graph showing the role of DNAA in transcription of FTSZ]
4.6 Mutagenesis of the ftsA and ftsQ DnaA-boxes

4.6.1 Mutagenesis.

The M13 clone containing the 1.8 kb EcoRI–HindIII fragment, with a mutated ftsQ DnaA-box (M13M1, described in Section 4.2), was used for oligonucleotide directed mutagenesis by the method developed by Kunkel (1985). Mutagenesis of the two other DnaA-boxes was attempted simultaneously by performing the mutagenesis reaction with two oligonucleotides carrying the mutations. This initially yielded only a double mutant in the ftsQ DnaA-box and in the ftsA DnaA-box at position 1175 (Table 4.1.1). A second mutagenesis reaction was required to alter the third DnaA-box. The resulting M13 clone with three mutated DnaA-boxes was designated M131.8/m3/6. The new mutations are shown in Figure 4.6.1.

Figure 4.6.1 Sequencing gels of the ftsA DnaA-box mutations.

![Sequencing gels of the ftsA DnaA-box mutations.](image)

Wild-type and mutated sequences of the ftsA DnaA-boxes. Lanes from left to right: CTAG. Above: the DnaA-box at position 986. Below: the DnaA-box at position 1175. The wild-type sequences (on the left) are of M13M1, the mutated sequences (on the right) are of M131.8/m3/6.
4.6.2 Construction of pRWS20M and λRWS20M.

The mutant EcoRI–HindIII fragment from M13(1.8m3/6) was cloned into pRS551. This was simultaneously attempted in two ways, previously described for the cloning of the equivalent wild-type fragment (Section 4.3): (i) a strategy involving blunting of the restriction fragment and ligating to the blunted EcoRI site in pRS551 and (ii) a three fragment ligation of the restriction fragment into the EcoRI and BamHI sites of pRS551 using a BamHI–HindIII adaptor. In this case only the latter strategy was successful yielding a plasmid (pRWS20M) with the same structure as pRWS201 (Figure 4.3.1).

This plasmid was recombined in vivo with λRS45 as previously described (Section 3.8.1) and gave the phage λRWS20M. Lysogens of ED419 and TP8503 were selected and named ED20M and TP20M.
4.7 Behaviour of λRWS20M in a dnaA46 host

Figures 4.7.1 shows a comparison of the specific β-galactosidase activities of λRWS201 and λRWS0M in dnaA46 and dnaA+ host at 30°C and 42°C. It is clear that TP201 and TP20M behave identically. ED201 and ED20M have the same activity at 42°C but repeatedly showed differences in activity at 30°C. This appears not to be due to growth-rate as ED201 and ED20M had equal generation times in both experiments. A possible explanation is that the mutant and wild-type DnaA-boxes bind the wild-type DnaA protein with equal affinity at 30°C but that they show a difference in their affinity for the DnaA46 protein. An alternative is that other differences in the host strains may be responsible for differences in activity. Although the host strain in both cases is ED419 the possibility exists that the particular clones of the strain used for lysogenising the two phages had undergone subtle changes which account for the difference in activity of the two phages. To examine this possibility one clone of ED419 was relysogenised with both phages, yielding ED201#2 and ED20M#2. These strains were then transduced to dnaA+ using TP8503 as a donor using Tn7 (tmpR) to co-transduce the dnaA gene. The dnaA+ derivatives were named TP201#2 and TP20M#2.

Figure 4.7.1
Figure 4.7.2 shows that λRWS20M does have an elevated level of lacZ expression in both hosts at both temperatures. This is very unlikely to be a result of multiple lysogeny as three separate infections of ED419 failed to yield clones with lower activity; a total of 18 clones were tested. The higher activity of TP20M#2 could be considered to be consistent with DnaA acting as a repressor of ftsZ transcription by binding to the DnaA-boxes. At 42°C in a dnaA46 strain, however, one would expect the difference in activity to diminish as the protein no longer binds even the wild-type DnaA-boxes. The fact that the difference in activity persists even at non-permissive temperature suggests that the higher basal level of lacZ expression in ED20M#2 is due to increased transcription from the cloned region due to other reasons than diminished affinity for DnaA and that this transcription is equally sensitive to changes in growth-rate as that in ED201#2.

Figure 4.7.2
The role of DnaA in transcription of \textit{ftsZ}

4.8 Overproduction of DnaA.

The effect of overproduction of DnaA on transcription from \textit{ftsQ} and \textit{ftsA} was examined using the plasmid pLSK5 which carries \textit{dnaA} under control of the inducible \textit{tac} promoter (Section 3.6). Figure 4.8.1 shows that the specific \(\beta\)-galactosidase activities of \(\lambda\)RWS100, \(\lambda\)RWS201 and \(\lambda\)RWS20M remain unchanged upon induction of DnaA expression from pLSK5. This would indicate that transcription from the cloned region is insensitive to elevated levels of DnaA. However, induction of pLSK5 causes a decrease in the growth-rate of the cells and this would be expected to result in increased activity of the \textit{ftsZ} promoters (Dewar \textit{et al.}, 1989; Smith \textit{et al.}, 1993; Chapter V). The fact that this was not seen may indicate that a decrease in expression of \textit{lacZ} due to DnaA overproduction is being obscured by an opposite and equal effect due to decreased growth-rate. Therefore the experiment was repeated using a greater concentration of IPTG (100 \(\mu\)g/ml) to induce \textit{dnaA} expression from pLSK5. Figure 4.8.2a shows that \(\lambda\)RWS100 does exhibit up to 30\% decreased activity upon high level overproduction of DnaA. Figure 4.8.2b shows that \(\lambda\)RWS201 and \(\lambda\)RWS20M both respond in the same way to strong DnaA overproduction: \textit{lacZ} expression is again decreased by approximately 30–40\%. As there are no accurate quantitative data available concerning the exact correlation between variations in growth-rate and \textit{ftsZ} expression the decrease in expression resulting from DnaA overproduction alone cannot be calculated.

In conclusion, both the construct containing the \textit{ftsZ4p3p} promoters (\(\lambda\)RWS201) and the construct containing an additional \textit{ftsZ2p} promoter (\(\lambda\)RWS100) respond similarly to DnaA overproduction, indicating that \textit{ftsZ3p4p} transcription and \textit{ftsZ2p} transcription are affected in equal proportions by excess DnaA. As \(\lambda\)RWS20M behaves similarly to \(\lambda\)RWS201 it appears that the DnaA-boxes are not mediating the effect of DnaA overproduction.
Figure 4.8.1

![Graph showing specific activity (MU) over time (min.) with different concentrations of IPTG and one of the three DNA constructs (TP100, TP201, TP20M).]

Figure 4.8.2a

![Graph showing specific activity (MU) over time (min.) with 100 µg/ml IPTG and one DNA construct (TP100) and its corresponding plasmid (pLSK5).]
Figure 4.8.2b

![Graph showing the role of DNA A in transcription of ftsZ. The graph displays the specific activity (MU) over time (min.) with different conditions and strains.](image-url)
4.9 Introduction of oriC plasmids

It was shown in Section 3.12.5 that introduction of the oriC pBR325 chimeric plasmid pOC161 into a strain carrying λRWS945 caused a slight increase in dnaA promoter activity which can be interpreted as being due to titration of intracellular DnaA protein. The effect of the presence of this plasmid on ftsZ promoter activity was examined in TP100, TP201#2 and TP20M#2. Table 4.9.1 shows that introduction of this plasmid does not lead (at least in two of the strains) to an increase in activity; the opposite may even be the case.

Table 4.9.1 Effect of pOC161 on activity of the cloned ftsZ promoters.

<table>
<thead>
<tr>
<th>strain</th>
<th>specific β-galactosidase activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP100</td>
<td>387</td>
</tr>
<tr>
<td>TP100(pOC161)</td>
<td>314</td>
</tr>
<tr>
<td>TP201#2</td>
<td>73</td>
</tr>
<tr>
<td>TP201#2(pOC161)</td>
<td>82</td>
</tr>
<tr>
<td>TP20M#2</td>
<td>128</td>
</tr>
<tr>
<td>TP20M#2(pOC161)</td>
<td>110</td>
</tr>
</tbody>
</table>

The experiment was repeated using a different oriC plasmid, pH4, which was shown in Section 3.12.5 to have a greater effect on the dnaA promoters than pOC161. Figure 4.9.1 shows the specific β-galactosidase activities of TP100 and TP201#2 carrying this plasmid. λRWS100 again shows no increased activity, λRWS201 on the other hand shows a clear three-fold derepression of lacZ expression in the presence of pH4. As with DnaA overproduction the cause of this is uncertain as TP8503(pH4) has a generation time nearly twice that of the plasmid-free strain and this could also elicit an increase in ftsZ promoter activity. That such an increase is not observed with λRWS100 may be an indication that the presence of
pHP4 has a negative effect on transcription from the cloned promoters which cancels the growth-rate effect. Whatever the case, there is no clear indication that titration of DnaA by the oriC plasmids is derepressing ftsZ transcription.

Figure 4.9.1
4.10 Integrative suppression

As it is a strong possibility that the increased activity of λRWS100 at high temperature in a dnaAts host is due to decreasing growth-rate rather than a direct effect of inactivation of the DnaA ts protein, the activity of the phage was examined in a strain which is integratively suppressed by the R1 derivative pGW'IS described in Section 3.7.2. In such a strain replication can continue in the absence of active DnaA and the growth-rate of the suppressed strain is virtually equal to that of a dnaA + strain. Two independent integrants of pGW'IS in ED100 were selected as described previously (Section 3.7.3) and retention of the dnaA + allele was verified by co-transduction of temperature-sensitivity with asnA. Figure 4.10.1 shows that in both integratively suppressed derivatives (EDG101 and EDG102) lacZ expression does not increase at high temperature and is in fact decreased as in TP100 under similar conditions (compare Figure 4.4.3). This is a strong indication that inactivation of DnaA ts protein exerts its influence on ftsZ transcription indirectly. This had already been shown for the ftsZ3p and 4p promoters present in λJFL100 (Masters et al., 1989).

Figure 4.10.1
4.11 Behaviour of the ftsZ promoters in a dnaA0 strain

The study of the *dnaA* promoters in EH3791 (*dnaA0*) was discussed in Section 3.14.2. A similar examination was carried out of the *ftsZ* promoter activity by introducing the λRWS100 and λRWS201 phages into EH3791 (EH100 and EH201) and making *dnaA*+ derivatives of the resulting strains (EHA100 and EHA201). ΔoriC derivatives of EHA100 and EHA201 (EHA100Δ and EHA201Δ) were then constructed as described in Section 3.14.1. Table 4.11.1 shows the specific β-galactosidase activity of each of these strains. *lacZ* expression from both phages is elevated in the *dnaA0* host compared with the *dnaA+* host. Interestingly the same increased activity is seen in the *dnaA*+ ΔoriC strains strongly suggesting that it is not the absence of active DnaA in EH100 and EH201 which is causing increased transcription. Rather it would seem that the mode of replication is responsible as both the *dnaA*::Tn10 strains and the *dnaA*+ ΔoriC strains are forced to initiate DNA replication at the integrated pKN500 origin and consequently have greater generation times than EHA100 and EHA201. In LB medium at 37°C the generation times of EH3791, EHA100 and EHA100Δ are 32, 22 and 35 min. respectively. Thus, the *dnaA0* and the ΔoriC strains appear to show a correlation between *lacZ* expression and growth-rate.

<table>
<thead>
<tr>
<th>strain</th>
<th>EH100</th>
<th>EHA100</th>
<th>EHA100Δ</th>
<th>EH201</th>
<th>EHA201</th>
<th>EHA201Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>relevant genotype</td>
<td><em>dnaA</em>::Tn10</td>
<td><em>dnaA</em>+</td>
<td><em>dnaA</em>+ ΔoriC</td>
<td><em>dnaA</em>::Tn10</td>
<td><em>dnaA</em>+</td>
<td><em>dnaA</em>+ ΔoriC</td>
</tr>
<tr>
<td>specific activity (MU)</td>
<td>380</td>
<td>248</td>
<td>351</td>
<td>182</td>
<td>81</td>
<td>174</td>
</tr>
</tbody>
</table>

The cultures were maintained in exponential phase in LB medium at 30°C for approximately five hours before sampling. Four or five samples were then taken at five minute intervals and their average specific β-galactosidase activity is shown. In the case of EH100 and EHA100Δ the data come from separate experiments; the values were normalised to that of EHA100 which was assayed in both experiments.
4.12 Influence of the DnaA-boxes on transcription from ddlB

4.12.1 Construction of pTLJZ4D and pTLJMA.

As the experiments described above indicate that DnaA does not directly regulate ftsZ transcription from the promoters present in λRWS100 by binding to the ftsQ and ftsA DnaA-boxes an experiment was designed to examine whether the presence of the DnaA-boxes influences transcription from upstream. As explained in Section 1.11.3 DnaA has been implicated in termination of transcription (Schaefer and Messer, 1988 and 1989) probably by binding to two DnaA-boxes which have the same orientation relative to the direction of transcription. The sequences of the three DnaA-boxes in the ftsQA region shown in Table 4.1.1 are all located on the coding strand of the ftsQA DNA and any pair of these could in principle be acting as a terminator of transcription from the ftsQ or ddlB promoters. To investigate this a construct was made carrying the 2 kb BamHI–BglII fragment with the ddlB promoter, the ftsQ1p and 2p promoters and the putative ftsA promoter upstream of a lacZ reporter gene. The cloned fragment contains the ftsQ and the ftsA (pos. 986) DnaA-boxes but not the one in ftsA at position 1175. An equivalent construct was made which carries the mutated ftsQ and ftsA (986) DnaA-boxes.

The constructs described above were made in several stages. First the 4.3 kb PstI–ClaI fragment from pZAQ (Ward and Lutkenhaus, 1985) was directionally ligated into the PstI and ClaI sites in the polylinker of the vector pT7-3 yielding pT7/ZAQ. The cloned fragment contains the complete ftsQ, ftsA and ftsZ genes, as well as a 3' part of ddlB and the 5' end of envA (Figure 4.12.1a). pT7/ZAQ was found, as expected, to be capable of complementing the temperature-sensitive ftsA allele in TOE 13.

To construct an equivalent plasmid containing the mutated DnaA-boxes the 1.7 kb BamHI–HindIII fragment from pT7/ZAQ was exchanged with the equivalent fragment from M13(1.8m3/6) (Section 4.6.1). This required partial digestion of pT7/ZAQ as it contains an additional BamHI site in the polylinker. The procedure yielded pT7/ZAM; the resulting plasmid was subjected to restriction analysis using TaqI as the mutations in the ftsQ and ftsA (1175) introduce TaqI restriction sites at these two locations.

In order to introduce the rest of ddlB and its promoter sequence into the constructs the 0.8 kb BamHI fragment from pT7/ZAQ and pT7/ZAM was exchanged for the 1.1 kb BamHI fragment from pT7-4ddl (obtained from M. M. Khattar). This fragment contains the entire ddlB gene (Figure 4.1.1). The orientation of the cloned fragment was checked using a PstI/BglII digestion which should release a 1.75 kb
band if the *BamHI* fragment has been ligated in desired orientation. The plasmids containing the correct insert were then again verified with a *TaqI* digestion. The plasmid with the wild-type insert was named pT7/ZAD, the one carrying the mutant DnaA-boxes pT7/MAD (Figure 4.12.1b).

To transcriptionally fuse parts of the inserts in pT7/ZAD and pT7/MAD to *lacZ* the vector pTL61T (Linn and St. Pierre, 1990; described in Section 4.2) was chosen because of the suitability of the restriction sites in its polylinker. pTL61T was partially digested with *SacI* and a fragment corresponding to the linearised plasmid was then digested with *BamHI*. The fragment corresponding in size to pTL61T cut at the *SacI* and *BamHI* sites in the polylinker was ligated to the 2.3 kb *SacI*–*BglIII* fragments from pT7/ZAD and pT7/MAD. These fragments contain the complete *ddlB* and *ftsQ* genes with the 5' end of *ftsA* carrying the DnaA-box at position 986. The resulting plasmids were named pTL/ZAD and pTL/MAD (Figure 4.12.2).

**Figure 4.12.1a**

---

pT7/ZAQ contains the 4.3 kb *PstI*–*ClaI* fragment from pZAQ. pT7/ZAM is identical but contains the triple DnaA-box mutation. See text for details of construction.
4.12.2 lacZ expression from pTL/ZAD and pTL/MAD.

TP8503 was transformed with pTL/ZAD and pTL/MAD. The specific β-galactosidase activities of both strains were determined and are shown in Figure 4.12.3. If DnaA were acting as a transcriptional regulator by causing DnaA-box mediated termination of transcripts from ddlB or ftsQ, pTL/MAD would be expected to show a higher degree of lacZ expression than pTL/ZAD. This is clearly not the case and indicates that the DnaA-boxes present in pTL/ZAD are not involved in transcriptional regulation. The possibility, however, exists that the two DnaA-boxes present in pTL/ZAD are only active in the presence of the third DnaA-box at position 1175; the fact that this DnaA-box is not present in this construct does for this reason not permit a definite conclusion concerning the possibility that DnaA may be acting as a terminator. A similar experiment including all three DnaA-boxes remains to be performed.
pTL/ZAD and pTL/MAD contain the 2.1 kb BamHI–BglII fragments from pT7/ZAD and pT7/MAD respectively. Two of the ftsQA DnaA-boxes are present on pTL/ZAD and mutated on pTL/MAD.

Shown is a diagrammatic representation of the inserts in pTL/ZAD and pTL/MAD. TP8503 containing either plasmid was maintained in exponential phase with antibiotic selection for the plasmid for four hours before sampling. Shown are the average value of three samples.
4.13 Replacement of the chromosomal ftsQ and ftsA genes with those carrying mutant DnaA-boxes

4.13.1 First strategy.

The work described above failed to give an indication of any function of the ftsQ and ftsA DnaA-boxes in transcription from the distal part of the mra operon. In order to examine whether the DnaA-box mutations affect cell physiology it was decided to replace the chromosomal ftsQ and ftsA genes with the mutated versions. The mutations introduced into the DnaA-boxes were chosen to be silent, leaving the amino-acid sequences of the encoded proteins unaltered, thus permitting such a replacement.

Figure 4.13.1

Gene replacement strategy: DnaA-boxes are represented by squares (black = mutated). ftsA is represented by a shaded rectangle, the tet resistance gene by a black rectangle. The ftsA::tet gene was to be introduced as a means to select clones with the DnaA-box mutations. The strain to be used is a derivative of SHA5 which carries a chromosomal duplication of the mra operon.
At first the replacement was attempted with the hope of being able to use a selection procedure for tetracycline-sensitivity (Bochner et al., 1980). The process is outlined in Figure 4.13.1. The aim was to construct a strain which carries an $ftsA$ allele interrupted with the tetracycline gene and then to replace this allele with a DNA fragment carrying the $ftsQ$ and $ftsA$ genes with the mutated DnaA-boxes by homologous recombination.

The initial aim was to introduce, by homologous recombination, the $ftsA::tet$ allele into a strain which carries a chromosomal duplication of a 12 minute region containing the $mra$ operon. This strain (SHA5) was isolated by S. G. Addinall in our laboratory (Addinall, 1994). Interruption of one $ftsA$ gene would be tolerated in this strain as it would be complemented by the other allele present. The $ftsA$ interruption was constructed on a plasmid as follows. A 1.4 kb fragment expressing the tetracycline resistance gene was prepared by amplifying the appropriate region from pBR322 by PCR. The primers used for the reaction each differ by one base from their target sequences on the pBR322 template and were chosen to create $BglII$ sites at either end of the amplified product. The resulting product was then ligated into the unique $BglII$ site within the $ftsA$ gene in the plasmid pT7/ZAQ described in Section 4.12.1. The resulting plasmid, pT7/tet, was indeed found to confer resistance to tetracycline and to be unable to complement the temperature-sensitivity of TOE13 ($ftsA^{ts}$).

A derivative of SHA5 with the following genotype was obtained from D. S. Boyle in our laboratory: DSB1, $recD::tet$, $leu::cat$. The $recD$ inactivation facilitates transformation of linear DNA fragments into the strain as the absence of the exonuclease ExoV (which is in part encoded by $recD$) reduces linear DNA degradation; it was planned to introduce the $ftsA$ alleles by linear transformation. The chloramphenicol-resistance gene ($cat$) present in the leucine locus (2 min.) would serve as a marker for eventual co-transduction of the mutated $ftsA$ and $ftsQ$ alleles to other strains. For introduction of the $ftsA::tet$ allele the $tet^R$ gene in $recD$ would have to be removed as its presence would prevent selection for the interruption of a chromosomal $ftsA$ gene. It was attempted to select spontaneous tetracycline-sensitive mutants of DSB1 in the hope that some of these would be $recD$ deletions. The selection procedure described by Bochner et al. (1980) and even the improved method of Maloy and Nunn (1981), both based on the sensitivity to fusaric acid of tetracycline-resistant strains, proved inadequate. Although many fusaric acid resistant colonies were isolated none proved to be tetracycline-sensitive. As this selection procedure was planned to be used eventually for the selection of tetracycline-sensitive cells carrying the mutant replacement of the $ftsA::tet$ allele.
(Figure 4.13.1) it was decided to attempt a different strategy for the gene replacement. A second reason for the abandonment of this procedure was that the \textit{ftsA::tet} allele was shown to express tetracycline-resistance only very poorly when integrated into the chromosome (S. G. Addinall, personal communication). In combination with a poor selection system this was thought to render the above project unfeasable.

\textbf{4.13.2 Gene replacement using pMAK705.}

A different strategy proved more successful. For this I am grateful to Sean McAteer in our laboratory who carried out the cloning and replacement procedures described below. The method made use of the pSC101 derivative pMAK705 (Hamilton \textit{et al.}, 1989) which is unable to replicate at 44°C due to a mutation in RepA. The 4.7 kb \textit{BamHI–ClaI} fragments carrying \textit{ddlB} and the \textit{ftsQAZ} genes from pT7/ZAD and pT7/MAD were ligated into the corresponding sites in the pMAK705 polylinker and the resulting constructs (pMAK/ZAD and pMAK/MAD) were introduced into W311A (a derivative of W3110 carrying the temperature-sensitive \textit{ftsA12} allele obtained from N. F. McLennan). Chromosomal integrants of these plasmids were selected by growing W311A(pMAK/ZAD) and W311A(pMAK/MAD) at the non-permissive temperature for pMAK replication. Integration occurs through a single recombination event between the insert present in the plasmid and the homologous chromosomal sequence. The \textit{ftsA+} genes present on the integrated plasmids will complement the \textit{ftsA12} allele at that temperature and thus allow growth of the strains. By returning the strains to the permissive temperature the plasmids were permitted to resolve from the chromosome; this can occur in two ways (Figure 4.13.3). The reverse of the recombination event which led to integration would result in the plasmid returning to the autonomous state with its original insert. An alternative, which would be expected to occur with an equal frequency (since the DnaA-boxes are central in the homologous regions), is that the resolved plasmid had exchanged its insert for the equivalent chromosomal sequence. In W311A(pMAK/ZAD) the latter recombination event would lead to exchange of \textit{ftsA12} for \textit{ftsA+}; in W311A(pMAK/MAD) the same event would result in replacement of the chromosomal \textit{ftsQ} and \textit{ftsA12} genes with the alleles carrying the mutant DnaA-boxes. By then returning the strains once more to the non-permissive temperature the resolved pMAK plasmids will be lost and only cells which have undergone exchange of \textit{ftsA12} for \textit{ftsA+} will form colonies. As the reversion frequency of \textit{ftsA12} is relatively high the possible recombinants were analysed as
The Role of DNA Transcription with P53

The role of DNA transcription with P53

Transcription of P53: The DNA strand is cleaved at the 3' end by an enzyme called DNA polymerase. The RNA polymerase then synthesizes a complementary strand of RNA complementary to the DNA template.

The DNA strand is then cleaved again at the 5' end by another enzyme called DNA helicase. The RNA polymerase then synthesizes a complementary strand of RNA complementary to the DNA template.

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The DNA strand is then cleaved again at the 3' end by another enzyme called DNA polymerase. The RNA polymerase then synthesizes a complementary strand of RNA complementary to the DNA template.
Figure 4.13.3

pMAK/MAD contains the 4.6 kb BamHI-ClaI insert from pT7/MAD carrying the triple DnaA-box mutation (represented by black squares). At 44°C the chloramphenicol resistant cells must contain an integrated copy of the plasmid as this temperature is restrictive for autonomous replication; the ftsA gene in the integrated plasmid will complement the ftsA12 chromosomal mutation. At 30°C the plasmid can resolve from the chromosome in either of two ways (A or B). Upon a return to the restrictive temperature, without chloramphenicol selection, only cells which have exchanged the ftsA12 allele (represented by ts) for the mutated "ftsA++" gene will survive. Clones can then be verified by screening for chloramphenicol sensitivity and presence of the mutated DnaA-boxes can be tested by TaqI digestion of an appropriate PCR product. T denotes the positions of relevant TaqI sites.
4.13.3 Initial characterisation of the chromosomal DnaA-box mutations.

The fact that the construction of strains carrying the mutated DnaA-boxes in \textit{ftsQ} and \textit{ftsA} is possible immediately proves that these sequences perform no essential function in \textit{E. coli}. To ascertain whether cell-growth is in any way impaired the growth-rates of W3110/MAD and TP/MAD were compared with those of W3110/ZAD and TP/ZAD. No difference was observed in exponential phase in LB or in minimal medium at 30°C, 37°C or 44°C. Cell-size distributions are also unaltered by the mutations (Figure 4.13.4).

To determine whether the mutations would place the strain at a disadvantage during prolonged growth a competition experiment was performed. Derivatives of TP/ZAD and TP/MAD were constructed which carry a kanamycin resistance marker at 94 minutes on the chromosome. The marker was introduced by P1 transduction from W311K (obtained from N. F. McLennan). TP/ZAD(\textit{kan}) and TP/MAD were diluted from overnight cultures and equal numbers of cells from the two cultures were mixed and allowed to grow for 24 hours in minimal medium at 37°C. The numbers of viable cells of each strain in the culture were then determined by plating out equal volumes on kanamycin plates and on non-selective plates. The culture was diluted $10^6$-fold and again allowed to grow for 24 hours. The process was repeated twice. The result of this experiment and that of the reciprocal (competition between TP/MAD(\textit{kan}) and TP/ZAD) is shown in Table 4.13.1. No clear growth advantage of either strain is apparent under the conditions tested; further and more extensive study is, however, desirable.

### Table 4.13.1 Competition experiment between TP/MAD and TP/ZAD.

<table>
<thead>
<tr>
<th>strain</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>kan$^R$</td>
<td>ratio</td>
<td>total</td>
</tr>
<tr>
<td>ZAD(\textit{kan}) vs. MAD</td>
<td>7.99</td>
<td>4.71</td>
<td>1.43</td>
<td>7.95</td>
</tr>
<tr>
<td>ZAD vs. MAD(\textit{kan})</td>
<td>6.63</td>
<td>4.21</td>
<td>0.57</td>
<td>6.44</td>
</tr>
<tr>
<td>ZAD(\textit{kan}) vs. MAD</td>
<td>3.32</td>
<td>2.36</td>
<td>2.46</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Cell numbers are expressed in 10$^8$ cfu/ml. Ratio refers to TP/ZAD(\textit{kan}):TP/MAD and TP/ZAD:TP/MAD(\textit{kan}). These were determined by plating equal aliquots on selective and non-selective medium.
Cell-size distributions of the chromosomal replacements (mutated and wild-type DnaA-boxes) in W3110 compared with that of the parent strain. Samples were taken into formaldehyde for analysis using a Coulter Counter after six to seven hours growth at the respective temperatures. Cells were maintained in exponential phase throughout and were grown in glucose minimal medium supplemented with casamino acids.
4.14 Discussion

4.14.1 DnaA is not a regulator of ftsZ transcription.

The aim of the work described in this chapter was to investigate the putative role of DnaA as a transcriptional regulator of the ftsZ gene. This work was prompted by the observation by Masters et al. (1989) that transcription from the phage λJFL100 increases at the non-permissive temperature in a dnaA46 host. One possible explanation for this observation is that DnaA is acting as a negative regulator of transcription originating at any or all of the promoters present in λJFL100 (ftsZ3p, ftsZ4p and ftsAp) and that thermal inactivation of the DnaA46 protein abolishes this activity. The fact that three nine base-pair sequences which fit the DnaA-box consensus sequence are present within the cloned fts DNA makes this model attractive. This model could also be consistent with the growth-rate sensitivity displayed by the ftsQ1p and ftsZ4p, 3p and 2p promoters (Aldea et al., 1990; Dewar et al., 1989; Smith et al., 1993). The activity of these promoters shows an inverse correlation with growth-rate. Chiaramello and Zyskind (1989) reported that the intracellular DnaA concentration increases with growth-rate (although this has been disputed by Hansen et al., 1991a) and this could in the above model be responsible for the decreasing levels of ftsZ promoter activity with increasing growth-rate.

Evidence exists which implicates DnaA as a negative regulator of transcription of other promoters which functions either by repressing promoter activity or by acting as a transcriptional terminator (Sections 1.11.1 and 1.11.3). In the former case DnaA is thought to act by binding to a DnaA-box in the immediate vicinity of the promoter sequence; Figure 4.1.1, however, shows that none of the putative DnaA-boxes present in ftsQ and ftsA are so located. Thus, if DnaA were to be acting as a transcriptional repressor its mechanism of action would presumably be different from that at other DnaA-regulated promoters. If it were to be acting as a transcriptional terminator only transcription originating from ftsAp would be subject to its action. ftsAp is a very weak promoter (Robinson et al., 1984) and its existence has even been disputed (Aldea et al., 1990). For this reason it would seem unlikely that alleviation of transcriptional termination alone would result in the increase in transcription of lacZ observed from λJFL100 (Figure 4.1.3).

Section 4.3 describes the construction of new transcriptional fusions between the 1.8 kb ftsQA' fragment and lacZ (λRWS200 and λRWS201). Although these phages carry the same insert as λJFL100 they differ from this phage in that they carry a strong transcriptional terminator isolating the cloned sequence from transcription originating elsewhere. In a dnaA46 host λRWS200 and λRWS201
show much less increase in lacZ expression after a shift to 42°C than does λJFL100 (Section 4.4). This may indicate that the lacZ gene in λJFL100 is being expressed at least in part from an additional promoter, which is not present in λRWS200 and λRWS201, and which is either directly or indirectly responsive to inactivation of DnaA. Some evidence exists that transcripts originating outside the cloned fts insert may be entering this region and may contribute to lacZ expression, at least under some conditions (Kenan, 1985; Robin and D’Ari, 1993; see Section 4.3); therefore it is possible that the increase in lacZ expression from λJFL100 at high temperature in a dnaA46 host is to a large extent due to transcription not originating within the cloned ftsQ and ftsA sequences. This was not further investigated. An alternative explanation is that some other difference between λJFL100 and the λRWS200/201 phages is responsible for the difference in lacZ expression from the phages upon inactivation of DnaA. λJFL100 contains the W205 fusion of trpA, trpB and lacZ (Mitchell et al., 1975) whereas the λRWS200/201 phages contain the same fusion junction but carry only 75 bp of trp DNA. Simons et al. (1987) report that trpA contains a weak promoter and it is possible, as its exact location is not known, that this promoter is present in the trp sequence in λJFL100; if this is so it may account for the greater lacZ expression from λJFL100. However, for it to do so would require it to be responsive to inactivation of the DnaA protein. No sequences fitting the DnaA-box consensus (Schaefer and Messer, 1991) were found within the trp sequence present in λJFL100 but this does not exclude the possibility that a promoter within this sequence is sensitive to changes in cell physiology due to inactivation of DnaA.

In Section 4.4 it was demonstrated that λRWS200 (and λRWS201) show a two to four-fold greater level of lacZ expression at high temperature in a dnaA46 host than in a dnaA+ host. In this respect they are qualitatively similar to λJFL100 and show that the response of the promoters within the ftsQA' fragment to inactivation of DnaA is not limited to λJFL100. λRWS100, which contains an additional promoter (ftsZ2p), also shows a three-fold greater lacZ expression at high temperature in a dnaA46 strain than in a dnaA+ strain. This indicates that ftsZ2p is induced as a response to inactivation of DnaA to the same extent as ftsZ3p and 4p. During the course of this work Garrido et al. (1993) studied the behaviour of a phage which is identical in all respects to λRWS100 (λTGV15). They found no difference in β-galactosidase activity from this phage at high temperature in a dnaA46 and dnaA+ host, although their experiment was apparently identical to the one described in Section 4.4 (Figure 4.4.3), with the exception of the host strains used. This may
indicate that the derepression observed in this work is not due to the dnaA mutation but to other differences between ED419 and Garrido's dnaA46 strain GC2018.

Masters et al. (1989) showed that the derepression of lacZ expression from λJFL100 occurs not only in a dnaA18 strain at high temperature but also in other conditional initiation mutants. In a dnaB252 and in a dnaC325 host lacZ expression is also increased at the non-permissive temperature but to a lesser extent than in a dnaA host. Section 4.5 describes the behaviour of λRWS100 and λRWS200 in a dnaC325 strain and shows that the level of lacZ expression at the non-permissive temperature is similar to that found in a dnaA46 strain. This argues in favour of a model where ftsZ transcription is not regulated directly by DnaA but is sensitive to cellular changes in response to cessation of chromosomal replication. This is strongly supported by the fact that inactivation of DnaA in an integratively suppressed strain does not cause derepression of the ftsZ promoters (Section 4.10). A possibility is that the increased transcription under conditions where replication is prevented is a consequence of the same (unknown) mechanism which renders ftsZ transcription sensitive to growth-rate. This is consistent with the fact that the growth-rates of both the dnaA and dnaC mutants at the non-permissive temperature must eventually become identical but that they are different at the permissive temperature; this is reflected by a difference in lacZ expression at the permissive, but not at the restrictive temperature. Sensitivity to growth-rate, but not to DnaA directly, is also supported by the fact that the elevated ftsZ promoter activity in an integratively suppressed dnaA0 strain is equal to that in an integratively suppressed ΔoriC, dnaA+ strain with the same reduced growth-rate (Section 4.11).

To determine whether the DnaA-boxes in the ftsQA sequence mediate a possible regulatory activity of DnaA they were mutated. Section 4.7 shows that the mutations lead to a higher level of transcription from the 1.8 kb ftsQA' fragment but that transcription is still equally sensitive to inactivation of DnaA; this is not consistent with the idea that DnaA acts as a repressor of ftsZ transcription by binding to the DnaA-boxes. Furthermore, overproduction of DnaA decreases lacZ expression in both λRWS201 and in λRWS20M to an equal extent (section 4.8). The repressing effect of DnaA overproduction is therefore not mediated by the DnaA-boxes.

It is unclear how excess DnaA might influence ftsZ transcription. Garrido et al. (1993) report that overproduction of DnaA from pLSK5 does not repress lacZ expression from λTGV15 (which is identical to λRWS100). This is consistent with the results in Section 4.8 as they used a low concentration of IPTG to induce dnaA expression from pLSK5. An effect of DnaA overproduction only becomes apparent at a higher level of induction. It is possible that this reflects non-specific binding of
DnaA to the *ftsZ* promoters. However, DnaA overproduction causes a decrease in the growth-rate of the cells. This in turn would be expected to increase *ftsZ* promoter activity and may cancel a decrease in activity at low levels of DnaA overexpression. If this is the case, the experiments in Section 4.8 may support an indirect regulatory role of DnaA (as λRWS20M is equally sensitive to DnaA overproduction), perhaps via a product which is itself directly regulated by DnaA. A consequence of this hypothesis would be that the growth-rate sensitivity of the *ftsZ* promoters under normal circumstances cannot be due to possible growth-rate dependent variations in DnaA concentration (Chiaramello and Zyskind, 1989; see above). It must be noted here that experiments presented elsewhere in this thesis indicate that repression of cloned promoters by excess intracellular DnaA may be a general feature of the *lacZ* fusion system employed (Section 3.12.4 and 3.15.3). This urges caution in drawing conclusions from the experiments with pLSK5.

Mutation of two of the putative DnaA-boxes does not alter transcription from upstream promoters in *ddlB*. The two DnaA-boxes studied do not appear therefore to be part of a transcriptional termination signal, although it cannot be discounted that they require additional DNA for their function which is not present in the construct used to test them (Section 4.11).

Finally, mutation of the DnaA-boxes at their normal chromosomal location does not lead to any obvious phenotypic change. The lack of a role for the DnaA-boxes is supported by a recent study conducted by Zhou and Helmstetter (1994). *ftsZ* transcription is not constant but oscillates during the cell-cycle. If DnaA were exerting an effect on transcription by binding to the DnaA-boxes this effect would be expected to change also during the cell-cycle as *dnaA* expression is not constant. The authors, however found that transcription originating upstream as well as that originating downstream of the DnaA-boxes shows the same oscillating pattern. As it would be unlikely that DnaA would influence both types of transcription in the same way, it must be concluded that DnaA does not influence either. Of course it cannot be excluded that the DnaA-boxes are non-functional under laboratory conditions but fulfill a role under extreme conditions. Further study of TP/MAD and W3110/MAD would be necessary to define such a role.

In summary, no evidence has been found which suggests DnaA is acting directly as a transcriptional regulator of *ftsZ* transcription. The effects reported by Masters *et al.* (1989) are likely to be a consequence of physiological changes which follow cessation of replication and may be mediated by the same system which mediates sensitivity to growth-rate. The nature of this system has yet to be discovered but is unlikely to involve DnaA. DnaA overproduction does influence
ftsZ promoter activity but it must be borne in mind that a net effect is only obvious at very high levels of intracellular DnaA, and as such under non-physiological conditions.

4.14.2 Importance of ftsZ transcription in the timing of cell division.

As the intracellular concentration of DnaA is one of the factors controlling DNA replication, the work described in this chapter, in a wider context, is an investigation into a possible common regulatory element in both this process and in that of cell division. The survival of dnaA0 strains shows that DnaA is not an essential element in the division process. However, as FtsZ appears to be a limiting factor in septation, it could be argued that a modulating effect of DnaA on ftsZ transcription would place the timing of cell division under control of DnaA. Regardless of the fact that such an effect has not been demonstrated in the study presented here, the above argument can only be true if changes in ftsZ transcription lead to changes in the timing of septation. It is true that overproduction of FtsZ results in an increase in the number of division events per cell-cycle but the "extra divisions" occur only at the cell poles (Ward and Lutkenhaus, 1985). The frequency of medial divisions does not increase significantly even if FtsZ is present in the cell at seven times its normal concentration and division then only occurs slightly earlier in the cell-cycle (Ward and Lutkenhaus, 1985). This indicates that the action of FtsZ is subject to control at the functional level which would be expected to override any minor or even considerable fluctuations in concentration arising from alterations in the rate of ftsZ transcription. This is borne out by the fact that the DnaA-box mutations lead to a greater rate of transcription from the ftsQA' fragment (Section 4.7) but despite this do not result in alterations in the cell-size distribution when replacing the wild-type DnaA-boxes at their normal chromosomal location (Section 4.13). It is therefore unclear why transcription of ftsZ would have to be subject to tight regulation. It is noteworthy in this respect that the only two well defined regulators of FtsZ activity, SfiA and the MinC/D proteins, act at the protein level and not at the level of gene expression. In addition, fluctuations in ftsZ promoter activity during the cell cycle, which have previously been proposed to reflect periodic synthesis to requirement of FtsZ (Dewar et al., 1989; Garrido et al., 1993), may merely be a consequence of decreased promoter activity during replication of the mra operon (Zhou and Helmstetter, 1994).
Chapter V

Growth-rate sensitive transcription of

ftsZ
5.1 Introduction

As described in the previous chapter, ftsZ is transcribed predominantly from promoters within the ftsA and ddiB genes (Figure 4.1.1). Evidence has accumulated to suggest that transcription from these promoters decreases with increasing growth-rate. Aldea et al. (1990) showed that the transcriptional activity of ftsQ1p decreases nearly five-fold between generation times of 240 minutes and 30 minutes. In addition, transcription from this promoter increases five-fold between exponential growth and stationary phase conditions of a culture. This was shown by a transcriptional fusion of this promoter with lacZ and through quantitative analysis of transcripts arising from ftsQ1p on a plasmid carrying part of the ddiB gene. Growth-rate dependent transcription appears to be reflected in increasing levels of FtsZ protein per cell mass with decreasing growth-rates; a four-fold increase in relative FtsZ levels was detected between generation times of 30 and 120 minutes (Aldea et al., 1990).

Analysis of the transcriptional fusion of ftsQ and ftsA' with lacZ present in the phage λJFL100 (Chapter IV; Figure 4.1.2) by Dewar et al. (1989) indicated that the ftsZ3p and/or ftsZ4p promoters also respond inversely to growth-rate. This study was however criticised as λJFL100 lacks transcriptional terminators upstream of the cloned fts DNA (Aldea et al., 1990). Because this criticism does not seem unfounded, as already described in Chapter IV, the study was repeated using the phages λRWS200 and λRWS100, which contain the 1.8 kb EcoRI–HindIII and the 2.3 kb EcoRI fragments from the ftsQAZ region, respectively. These phages were described in Section 4.3 and contain strong transcriptional terminators isolating the fts fragments from upstream transcription. They differ also from λJFL100 in that they carry less trp DNA upstream of lacZ. As the trpA DNA present in λJFL100 may contain a weak promoter (Simons et al., 1987), this is an additional improvement over the system used by Dewar et al. (1989). Results from the work described in this chapter have been published (Smith et al., 1993) and the publication is included as an appendix in this thesis. In the studies using these phage constructs (or other reporter systems) certain assumptions must be made. These include that parameters such as mRNA stability, protein stability and the frequency of translation initiation remain unchanged at different growth-rates.
5.2 Growth-rate sensitivity of the proximal ftsZ promoters

TP100 (which carries the phage λRWS100) and TP200 (which carries λRWS200) were grown in media of various compositions to achieve different growth-rates. Figures 5.2.1 and 5.2.2 show the specific β-galactosidase activities of the strains in exponential growth phase plotted as a function of the generation time. The figures show that there is an approximately linear increase in specific activity with decreasing growth-rate over the range tested. This is true for both strains, indicating that both transcription from ftsZ3p4p and that from ftsZ2p must respond in a similar fashion to changes in growth-rate.

Cell-size increases as a function of growth-rate (Schaechter et al., 1958). This means that at high growth-rates there are less cells per total cell mass than at low growth-rates and consequently fewer septa to be formed. If it is the amount rather than the concentration of FtsZ which is important for septum formation, less FtsZ protein per cell mass would be required at high growth-rates. It is then a possibility that the inverse correlation between ftsZ promoter activity and growth-rate reflects a mechanism to ensure that the amount of FtsZ per cell remains relatively constant under different growth conditions. Figures 5.2.3 and 5.2.4 show comparisons of the amount of β-galactosidase enzyme per total cell mass (specific activity) and the amount of enzyme per cell at different cell volumes (median cell volumes were determined with the aid of a Coulter Counter and Channelyser). Figure 5.2.3 shows that the amount of enzyme per cell expressed from the ftsZ3p4p promoters varies less than the specific activity. The same is true, albeit slightly less apparent in Figure 5.2.4, of enzyme expressed from ftsZ2p3p4p. The amount of enzyme per cell, however, is not completely constant with growth-rate.
The strains were maintained in exponential phase in various media at 37°C for several hours before sampling. Shown are the average values of several samples taken in independent experiments. The media are: LB with 0.4% glucose, Nutrient Broth, VB salts minimal medium with 0.2% glucose, or 0.2% glycerol; CAA indicates VB minimal medium with casamino acids (5mg/ml).
Expression of the *ftsZ* promoters as a function of cell volume (determined electronically). The lower panel in each figure shows the specific \(\beta\)-galactosidase activity, the upper panel \(\beta\)-galactosidase per cell (from sp. act. \(\times\) volume). The data is from the same experiments shown in Figures 5.2.1 and 5.2.2.
5.3 Behaviour of the ftsZ promoters during a nutritional shift-up

Dewar et al. (1989) showed that the rate of transcription from \( \lambda JFL100 \) reflects the frequency of cell division rather than the overall rate of mass increase. The experiment performed by them was repeated with \( \lambda RWS200 \) and \( \lambda RWS100 \) to ensure that their result was not due to additional promoters interfering with \( lacZ \) expression from \( \lambda JFL100 \) and more importantly to investigate whether the same holds true for transcription originating from all the proximal \( ftsZ \) promoters (i.e. \( ftsZ2p3p4p \)).

If a culture is shifted from a poor medium to a richer one the rate of mass increase will immediately change to a greater value. The frequency of cell division, however, will remain at its pre-shift value for a period of time after the transition to rich medium. This is due to the fact that the new doubling rate will not be attained until the C and D periods have elapsed (where C is the time necessary to complete a round of chromosomal replication and D is the period between termination and completion of cell division). The consequence of this is that the cells increase in size. Dewar et al. (1989) found that the rate of \( lacZ \) expression from \( \lambda JFL100 \) increases approximately 40 minutes after the nutritional shift-up. As the frequency of septum initiation also changes 40 minutes after the shift-up (assuming C is equal to 40 minutes), this result indicates that the rate of transcription from the \( ftsZ \) promoters is proportional to the frequency of septum initiation.

TP100 and TP200 were grown for seven hours in VB glucose minimal medium before being diluted into LB medium with 0.4% glucose. Throughout the experiment the cultures were periodically diluted into prewarmed medium in order to maintain them in exponential growth phase. Samples were taken to determine \( \beta \)-galactosidase activity and to determine median cell volume with the aid of a Coulter Counter. Figures 5.3.1 and 5.3.2 show that the \( \beta \)-galactosidase activity per volume of culture shows a delay in reaching its post-shift value. The rate of transcription from the cloned \( ftsZ \) promoters in both experiments appears to mirror the frequency of cell division rather than the rate of mass increase. This is reflected in the amount of enzyme per cell remaining relatively constant before and after the change to richer medium. Thus, the results obtained with \( \lambda RWS100 \) and \( \lambda RWS200 \) are similar to those presented by Dewar et al. (1989).
Effects of a shift-up from poor (minimal VB salts glucose medium, generation time=70 min.) to rich medium (LB with 0.4% glucose, generation time=25 min.). TP100 was maintained in exponential phase by periodic dilution into fresh medium (the data have been corrected for these dilutions). The time of the shift (375 min.) is indicated by a vertical line. The primary measurements are OD600, cells/ml and β-galactosidase/ml. Other values were calculated from these.
Shift-up of TP200 from VB salts minimal glucose medium to LB with 0.4% glucose. Generation times are 70 and 25 minutes respectively. See Figure 5.2.1 for details.
5.4 Behaviour of the ftsZ promoters during entry into stationary phase

As cells enter into stationary phase their rate of mass increase slows down. The frequency of cell division, however, remains unchanged for a period and the cells consequently become smaller. If the rate of ftsZ transcription is linked to the frequency of cell division, the activity of the ftsZ promoters should increase relative to general transcription during the transition from exponential to stationary phase. This should be reflected in an increase in differential lacZ expression from the λRWS100 and λRWS200 phages under those conditions. Figures 5.4.1 and 5.4.2 show β-galactosidase per volume of culture plotted against total cell mass (OD600) for TP100 and TP200 respectively. Samples were taken during stable exponential growth and during entry into stationary phase. Both graphs show that the rate of β-galactosidase accumulation per increase in cell mass is greater at high OD600 values, when mass increase is becoming slower, as expected. It must be noted here that for both axes of the graph logarithmic scales were used in order to accommodate the wide range of values. As a consequence of this type of plot, the slope of the graph does not strictly speaking represent the differential rate of enzyme synthesis (which is defined as d[E/ml]/d[OD/ml]); a change in the slope does however imply a change in this parameter.

The increase in activity in stationary phase is not a consequence of λ induction. λ induction in stationary phase has not been reported but nevertheless it was desirable to eliminate this possibility. For this purpose a dnaK756 mutant strain (MF746) was used; this strain is viable at 30°C but fails to replicate λ at this temperature, DnaK protein being essential for λ replication (Zylicz et al., 1989). λ lysogens of this strain can however be formed. MF746 was lysogenised with λRWS100 and lacZ expression in exponential and stationary phase were compared. A similar result to those obtained with the dnaK+ strains (TP100 and TP200) was found. Thus increased lacZ expression in stationary phase is not a consequence of λ replication.

A sigma factor (σ^S) has been discovered which is required for the transcription of some genes which are preferentially expressed during entry into stationary phase (Lange and Hengge-Aronis, 1991a). σ^S is encoded by the rpoS gene and has been shown to be necessary for the stationary phase induction of bolA (involved in cell morphology), katE (which encodes catalase HPII) and xthA (which encodes exonuclease III) (Lange and Hengge-Aronis, 1991a). Inactivation of rpoS prevents this induction. To examine whether rpoS plays a role in the increased expression of ftsZ upon entry into stationary phase, the rpoS gene in the strains
TP100 and TP200 was replaced with the inactive $rpoS::Tn10$ allele by P1 transduction, resulting in TPS100 and TPS200. These strains show low catalase production in stationary phase and also show a prolonged lag in attaining exponential growth after dilution of an overnight culture into fresh medium, phenotypes consistent with $rpoS$ inactivation. However, TPS100 and TPS200 show no difference in $ftsZ$ promoter-driven $lacZ$ transcription either in exponential or in stationary phase (Figures 5.4.1 and 5.4.2). It appears that $rpoS$ is not required for stationary phase transcription of $ftsZ$. 
Relative increase in β-galactosidase activity per milliliter and OD units per milliliter of batch cultures of TP100, TP100 (rpoS::Tn10) (Figure 5.4.1), TP200 and TPS200 (rpoS::Tn10) (Figure 5.4.2) in VB salts glucose minimal medium with casamino acids. OD was measured with appropriately diluted samples to obtain values in the range where cell mass and OD are proportional and these values were corrected for dilution. Entry into stationary phase occurred at OD>2.
5.5 Discussion

The work described in this chapter confirms the results previously obtained by Dewar et al. (1989) which indicate that the rate of transcription from ftsZ3p and ftsZ4p is sensitive to growth-rate and appears to reflect the frequency of cell division. The study was extended to include also the ftsZ2p promoter which is present in λRWS100 in addition to the ftsZ3p and 4p promoters. As lacZ expression from this construct shows the same proportional inverse correlation with growth-rate as that from λRWS200, it must be concluded that ftsZ2p is regulated in the same or in a very similar manner to ftsZ3p4p. It cannot be deduced from the experiments described in this chapter whether ftsZ3p and 4p both respond to changes in growth-rate or whether only one promoter is induced at lower growth-rate.

The results are consistent with a model where the amount, rather than the overall concentration, of FtsZ in the cell is important. If, as would seem likely, FtsZ interacts with other proteins to form a septum, the intracellular amounts of these proteins may be regulated in a similar manner to that of FtsZ. This may be a reason that the ftsQ, ftsA and ftsZ genes are to some extent co-transcribed. Recently, biochemical evidence has emerged that FtsA and FtsZ interact (Hale et al., 1994). It also known that the ratio of FtsZ and FtsA must be kept at approximately 100:1 for septation to take place (Dai and Lutkenhaus, 1992; Dewar et al., 1992). It is attractive to suppose that the common response of the ftsQ1p and the ftsZ2p3p4p promoters to growth-rate may be to ensure that the ratio of the two proteins remains constant under different conditions. The proportional changes in ftsQ1p, ftsZ2p and ftsZ3p4p transcriptional activity with growth-rate are identical (Aldea et al., 1990; Smith et al., 1993) and, disregarding other possible forms of transcriptional regulation, this would be expected to maintain the ratio of the two proteins at a constant level. The intracellular levels of FtsA protein at various growth-rates have been quantified immunologically in two studies, the results of which are in conflict. Aldea et al. (1990) report that the amount of FtsA per cell is invariant with growth-rate. A later study by Wang and Gayda (1992) indicates that FtsA concentration per total cell mass is constant with growth-rate and as a consequence that the number of molecules per cell increases in proportion to growth-rate. The two studies differ in the source of FtsA. Wang and Gayda (1992) examined the amount of chromosomally encoded FtsA whereas Aldea et al. (1990) quantified plasmid encoded protein. The differences in the two studies could be due to several causes. Firstly, the ftsA gene on the plasmid used by Aldea et al. (1990) is transcribed only from the promoters present in and just upstream of ddlB. The chromosomal copy
may be partly transcribed also from promoters further upstream which may influence growth-rate regulation. An alternative and more likely cause for the discrepancies between the two studies stems from the fact that plasmid copy-number varies with growth-rate. Although this fact is mentioned by Aldea et al. (1990) their data show no correction for this. It would seem therefore that the data of Wang and Gayda (1992) are more reliable and casts in doubt the need for growth-rate sensitive transcription of \( \text{ftsA} \) as this is not reflected in the levels of its product. As \( \text{ftsQ} \) is transcribed to a large extent from the same promoters as \( \text{ftsA} \) it would be interesting to determine the growth-rate dependence of FtsQ concentrations. However, the low level of expression of this protein, which is estimated to result in only 25 molecules per cell, does not facilitate such a study.

Figures 5.2.3 and 5.2.4 show that the level of \( \lambda RWS100/200 \) encoded \( \beta \)-galactosidase per cell varies much less than the level of enzyme per total cell mass. However, the amount of enzyme per cell is not absolutely constant. Vicente et al. (1991) showed that this is the case also with enzyme synthesis driven by the growth-rate sensitive \( \text{ftsQ1p} \) and \( \text{bolA1p} \) promoters. At low growth-rates there is an excess of enzyme synthesis. Both the \( \text{ftsQ1p} \) and the \( \text{bolA1p} \) promoters are located immediately downstream of another promoter which is not sensitive to growth-rate (\( \text{ftsQ2p} \) and \( \text{bolA2p} \) respectively). In contrast to transcription from the \( \text{bolA1p} \) or \( \text{ftsQ1p} \) promoters alone, the rate of transcription from both pairs of promoters results in a constant amount of enzyme per cell at different growth-rates (Vicente et al., 1991). As the \( \text{ftsZ} \) promoters have been studied here in isolation from promoters further upstream (among which is \( \text{ftsQ2p} \)) it may be that the influence of additional transcription from such promoters keeps the intracellular amount of FtsZ constant with growth-rate. This has not yet been investigated, but the possibility that transcription from both growth-rate sensitive and growth-rate insensitive promoters is required could be a clue to the reason for the complex transcriptional arrangement in the \( \text{mra} \) operon.

The mechanism which governs the inverse response of transcriptional activity to growth-rate is unclear. A number of genes, the expression of which increases during the transition from exponential to stationary phase, have been identified. Some of these, \( \text{katE} \), \( \text{xthA} \) and \( \text{bolA} \) have already been mentioned (Section 5.4). In addition, the \( \text{mcbA} \), \( B \) and \( C \) genes, which are responsible for the production of microcin B17, share this property (Hernández-Chico et al., 1986). However, whether the \( \text{katE} \), \( \text{xthA} \) and \( \text{mcbABC} \) gene expression is growth-rate as well as growth-phase sensitive is not known. Therefore it is not certain whether these genes are subject to the same or a similar kind of control as the \( \text{bolA} \) or \( \text{fts} \) genes.
Transcription of these genes has received more attention and it was shown in Section 5.4 that they are not all part of a common regulon. The $rpoS$ gene, which encodes $\sigma^S$, is necessary for the stationary phase induction of $bolA1p$ (Lange and Hengge-Aronis, 1991b) and $ftsQ1p$ (M. Vicente, personal communication). Inactivation of $rpoS$, however, does not prevent stationary phase induction of the $ftsZ2p3p4p$ promoters.

Table 5.5.1 Sequences of growth-rate regulated promoters.

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<tr>
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<td>CGG c a a gT</td>
<td></td>
</tr>
<tr>
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<tr>
<td>consensus</td>
<td>TTG a c a</td>
<td>TA t a a T</td>
<td></td>
</tr>
</tbody>
</table>

A tentative consensus sequence is shown for the gearbox promoters. The consensus sequence for regular promoters is after Harley and Reynolds (1987). Adapted from Aldea et al. (1990) which should be consulted for more extensive promoter sequence information.

The $mcbA$, $bolA1p$ and $ftsQ1p$ promoters show sequence similarities around the $-10$ and $-35$ regions relative to the transcription start point. Furthermore, these sequences differ significantly from those of "standard" $\sigma^{70}$ promoters (Table 5.5.1). Mutation of the $bolA1p$ $-10$ sequence abolished growth-rate sensitivity indicating that this sequence is involved in transcriptional regulation. A consensus sequence was derived and the promoters fitting this were termed "gearbox" promoters (Aldea et al., 1990). It was subsequently discovered that despite the sequence similarities
mcbA, unlike bolA1p and ftsQ1p, does not depend upon rpoS for stationary phase induction. This is therefore another example indicating that there are distinct classes of growth-rate sensitive promoters. It is not yet clear what regulates mcbA in this respect. It has been known for some time that the gene is under the control of OmpR and is dependent upon this protein for expression (Hernández-Chico et al., 1986). OmpR is a regulatory protein which has been reported to be involved in stationary phase induction of ompC which encodes an outer membrane porin (unpublished results cited in Hernández-Chico et al., 1986). Recently it has been shown that the sequences in the mcbA promoter region which respond to OmpR and those which are necessary for growth-phase induction are separable (Bohannon et al., 1991). It appears that OmpR activates mcbA transcription both in exponential and in stationary phase but is not responsible for the differential gene expression under these conditions. Other candidates which could be responsible for growth-rate or growth-phase regulation have not yet been proposed.

In summary, the ftsZ promoters which have been studied here show remarkable similarity in behaviour with bolA1p and ftsQ1p. Despite this they do not share the gearbox promoter motif (Table 5.5.1), nor are they regulated by rpoS. There is evidence which points to the existence of alternative growth-rate sensitive transcriptional regulation. It is likely that the ftsZ2p3p4p promoters are part of such a regulon, the nature of which is unknown. A possible candidate, the influence of which could in future be examined, is ppGpp, which confers growth-rate sensitive control to a variety of genes.
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BIBLIOGRAPHY


BIBLIOGRAPHY


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Appendix

Published work
Cell Division and Transcription of ftsZ

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For normal cell division, the ftsZ gene must be transcribed from a number of promoters that are located within the proximal upstream genes (ddIB, ftsQ, and ftsA). We show that the main promoters have identical responses to changes in growth rate, i.e., under all conditions, the frequency of transcription per septum formed is approximately constant and independent of cell size or growth rate per se. We also show that transcription from these promoters is independent of stationary-phase transcription factor o'

The Mg\textsuperscript{2+}-dependent GTase FtsZ (10, 20, 21) is a key cell division protein in *Escherichia coli* and probably in all subeutaria (8). Five thousand to twenty thousand molecules of FtsZ are distributed throughout the cytoplasm of nondividing cells, but these aggregate into a ring around the center of the cell when division begins (5). During subsequent ingrowth of the peptidoglycan septum, this ring decreases in diameter and finally disappears when the septum is complete (5). FtsZ is essential for cell division (9), but excess FtsZ can inhibit all division (3, 4, 30). Regulation of the level of FtsZ activity is therefore likely to be important to the progress of the cell cycle. In this report, we describe the relationship between growth rate and transcription from two of the three main sets of promoters (lying within upstream genes *ddIB, ftsQ*, and *ftsA*) [Fig. 1] which are together required for full expression of the ftsZ gene (9). This confirms previous reports (2, 12, 15, 16) that, at all growth rates, transcription from ftsZ promoters is approximately constant per cell (although cell size itself increases exponentially with growth rate) and shows that each of the three sets of required promoters shows a similar response. We also show here that this behavior is independent of the stationary-phase sigma factor o' (6, 17, 18).

The procedure of Simons et al. (26) was used to construct two new \( \lambda \) bacteriophages (\( \lambda \)RSW100 and \( \lambda \)RSW200) in which lacZYA is transcribed from promoters within the *ftsQ* and *ftsA* genes. Figure 1 shows that \( \lambda \)RSW100 has all of these promoters (ftsZ4p3p2p1p) within *ftsA* (2, 19, 23, 24, 27, 31) and the very weak promoter within *ftsQ* (11), while \( \lambda \)RSW200 lacks the proximal promoters (ftsZ2p3p4p). The promoters cloned into \( \lambda \)RSW200 are the same as those in the *\lambda*FL100 phage originally used by us (12), but the new phage has four transcriptional terminators immediately upstream of the cloned segment and also lacks all but 70 bp of the *trp* DNA which is located between the promoters and lacZ in *\lambda*FL100. Expression of lacZ in the new phages cannot be due to readthrough from promoters (in the phage or in the chromosome) upstream of the *ftsQA* DNA (as has been suggested as a possible criticism of results obtained with *\lambda*FL100 [2]); it also does not arise in the residual *trp* fragment, because an otherwise identical phage (\( \lambda \)RSW1) which lacks the *ftsQA* DNA does not express lacZ.

\( \lambda \)RSW100 expresses lacZ from all of the *ftsZ* promoters, except those within *ddIB*. A construct similar to \( \lambda \)RSW100 has been briefly described in an earlier report (1) and was reported to show increased transcription in the stationary phase.

We infected a \( \Delta lac-proB \) strain (TP8503 [23]) with each of the ARWS phages and screened for monolysogens (26). These strains were grown in different media (Vogel-Bonner [VB] salts-glycerol, VB-glucose, VB-Casamino Acids, L broth, and L broth-glucose) at 37°C to obtain a range of growth rates and cell sizes. The cells were maintained in exponential-phase growth for several generations before and during the assay period by regular dilution with fresh medium at 37°C. Figure 2 shows the average specific \( \beta \)-galactosidase activities (measured at intervals during exponential-phase growth, when growth rate and cell size were both constant) as a function of average cell size (measured in the same cultures at the same times). Average cell size increases as an exponential function of growth rate (13, 25), and the specific activities (\( \beta \)-galactosidase activity per optical den-
transcription from \( ftsZ4p_{3p} \) (in \( \lambda JFL100 \)) reflected the frequency of cell division rather than the overall rate of mass and protein synthesis during the transition period between one growth rate and another. In consequence, the amount of \( \beta \)-galactosidase per cell did not change during the transition from one growth rate to the next. Figure 4 shows that transcription from \( \lambda RWS100 \) in TP8503 mirrors the increase in cell numbers during the transition from slow to fast growth, so that as in our previous study of \( \lambda JFL100 \), \( \beta \)-galactosidase per cell remains nearly constant. We obtained similar results with \( \lambda RWS200 \) (data not shown).

![Diagram](image-url)

**FIG. 2.** Differential expression of different sets of promoters (E/OD) as a function of cell size (\( \nu \); measured as median cell volume in arbitrary units with a Coulter Particle Analyzer). Estimates of E/OD were made from the differential rates of \( \beta \)-galactosidase activity and OD increase during log-phase growth in cultures in different growth media at 37°C. Symbols: ●, TG8503(\( \lambda RWS100 \)); ○, TG8503(\( \lambda RWS200 \)). Also shown (dashed lines and dots) is the equivalent of the enzyme/cell ratio (\( [E/OD]\times V \)) for both strains.

![Diagram](image-url)

**FIG. 3.** Differential expression of different sets of promoters (E/OD) as a function of estimated average cell size (\( \nu \), calculated from the growth rate (\( R \), doublings per hour) by assuming that \( V = k \cdot 2^R \) (13, 14). Data for TG8503(\( \lambda RWS100 \)) (●; left scale) and TG8503(\( \lambda RWS200 \)) (○; right scale) are the same as in Fig. 2, except that \( V \) was calculated from the growth rate rather than measured directly, to allow comparison of our data with those reported for other promoters (28). Data for the \( ftsQ2p \) promoters (▲, △) were obtained from reference 28 and replotted against \( V = k \cdot 2^R \) on a vertical scale chosen to superimpose these points on those of the two TG8503 strains (representing the behavior of \( ftsZ4p_{3p} \) and \( ftsZ4p_{3p2p1p} \)). The relationship between transcription and cell size (or growth rate) is closely similar for all three sets of promoters. Also shown are datum points for the \( bolA1p \) promoter (■, □) replotted from reference 28 as before. The relationship between transcription and growth rate appears to be different from that of the \( ftsZ \) promoters.
FIG. 4. Effects of a shift-up from poor medium to rich medium on cell growth and division and transcription from the \(ftsZ\) set of promoters. Cells of TG8503(XRWS100) were maintained in the log phase by periodic dilution with fresh medium (minimal medium-glycerol) and then shifted to L broth-glucose (dashed line at 375 min) and maintained thereafter below an OD of 0.2 by periodic dilution with fresh broth. (The datum points have been corrected for these dilutions.) The primary measurements (upper three curves) are OD \((x)\), cells per milliliter \((\Phi)\), and enzyme activity per milliliter \((\Sigma)\). Calculated from these were OD per 10^7 cells \((\Delta)\), enzyme activity per OD unit \((\Lambda)\), and enzyme activity per 10^7 cells \((\Theta)\). Although growth rate, cell size, and enzyme activity per OD unit all changed after the shift, enzyme activity per cell remained almost constant throughout.

In contrast, another report (22), on transcription of \(lacZ\) from \(ftsZ\) in \(\lambda\)JFL100 in a different strain of cells (GC3439), suggested that \(\beta\)-galactosidase per cell increases after a similar medium shift (at least initially). The difference between our results and these appears to be that strain GC3439 shows prolonged inhibition of cell division (i.e., constant cell numbers) after a shift up in growth rate, in contrast to the behavior of most \(E. coli\) strains (25). Perhaps cell separation is delayed after a medium change in strain GC3439, while chromosome replication and septum initiation proceed normally; despite this, the change in E/OD after the shift appears to be very similar to that seen in our strains.

Our results can be summarized by saying that the frequency of transcription of \(ftsZ\) seems to be closely tied to the frequency of cell division (or septum initiation) under most growth conditions, but we still do not know how this is achieved. An extreme condition in which this behavior is seen is during entry into the stationary phase; as the rate of mass increase slows down, the rate of cell division remains unchanged for a period, so that cell size progressively diminishes (25). Under these conditions, the rate of transcription from the set of \(ftsZ\) promoters remains linked to the rate of division, so that the rate of transcription of \(ftsZ\) increases relative to the rate of overall protein synthesis (2, 28; Fig. 5). The \(rpoS\) gene, which codes for a novel sigma factor (\(\sigma^s\)), is required for transcription of some genes (e.g., \(bolA\) [1]) which are preferentially expressed during entry into the stationary phase (18). Moreover, the \(rpoS\) gene itself appears to be expressed at a rate which is inversely proportional to growth rate, in a way similar to that of \(ftsZ\) (18). We therefore examined the effect of inactivation of \(rpoS\) on transcription of \(ftsZ\) in our strains. Inactivation of the \(rpoS\) gene had no detectable effect on growth rates during the log phase (18). We therefore examined the effect of inactivation of \(rpoS\) on transcription of \(ftsZ\) in our strains. Inactivation of the \(rpoS\) gene had no detectable effect on growth rates during the log phase.

![FIG. 4. Effects of a shift-up from poor medium to rich medium on cell growth and division and transcription from the \(ftsZ\) set of promoters.](image1)

![FIG. 5. Relative increase in \(\beta\)-galactosidase activity per milliliter and OD units per milliliter of batch cultures of TG8503(XRWS100) \((\bullet)\), TG8503(XRWS100)\(\Lambda rpoS\) \((\bigcirc)\), TG8503(XRWS200) \((\bigcirc)\), and TG8503 \(\Lambda rpoS\) \((\bigcirc)\) in VB salts-glucose-Casamino Acids.](image2)
at which the increased relative rate of transcription commenced. The only detectable effect of rpoS inactivation is that the E/O D ratio may reach a higher level during the stationary phase. This effect, which is not large, may be correlated with the prolonged division of rpoS::Tn10 cells during entry into the stationary phase (18). No effects of rpoS inactivation on transcription were seen in any of the other growth media (data not shown).

REFERENCES


