Ribonuclease III processing of *Escherichia coli* rpoBC messenger RNA

Richard Anthony Malloch

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Department of Molecular Biology
University of Edinburgh
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Abstract

The polycistronic rplKAJL rpoBC mRNA of E. coli, encoding ribosomal proteins and the RNA polymerase subunits $\beta$ and $\beta'$, is subject to RNase III-dependent cleavage between the rplL and rpoB coding sequences. Under steady-state exponential growth conditions RNase III processing in this region is apparently unimportant for the regulation of rpoBC gene expression. However during a challenge with the drug rifampicin, the stimulation of $\beta\beta'$ synthesis is markedly delayed in a RNase III-deficient strain, as compared with the wild-type control. This is shown to be a post-transcriptional effect, which is probably normally concealed during steady-state growth by autogenous translational control. The construction of a rpoB-lacZ fusion plasmid, and RNase III-target site deletion mutant derivatives, allows a more direct analysis of the role of processing in rpoB expression. The results of detailed studies using these plasmids have led to the proposal of a model wherein deletions of the RNase III target site alter the translational efficiency of rpoB mRNA by altering the equilibrium between conformations at the translation initiation site. The evidence for, and implications of, this model are discussed.
Declaration

I hereby declare that I alone have written this thesis and that, except where indicated, the work presented is my own.
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Abbreviations

AMPS ammonium persulphate
Ap ampicillin
bp base pair(s)
BSA bovine serum albumin
butyl-PBD 2-(4'-tert-buty1phenyl)-5-(4''biphenylyl)-1,3,4-oxadiazole
conc. concentrated
cDNA complementary DNA
CETAB cetyltrimethyl ammonium bromide
ddNTP dideoxynucleoside 5'-triphosphate
dNTP deoxynucleoside 5'-triphosphate
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DEP diethylpyrocarbonate
DTE dithioerythritol
DTT dithiothreitol
dH2O distilled water
EDTA diamino ethane tetraacetic acid
EtBr ethidium bromide
g acceleration due to gravity
IPTG isopropyl-β-D-thiogalactoside
L-broth Luria broth
MOI multiplicity of infection
MOPS 3-[N-morpholino] propane sulphonic acid
mRNA messenger RNA
nt nucleotide(s)
ONPG 0-nitrophenyl-β-D-galactopyranoside
PEG polyethylene glycol
RF replicative form
RNA ribonucleic acid
rRNA ribosomal RNA
RNase ribonuclease
SS single-stranded
TEMED N,N,N',N'-tetramethyl ethylene diamine
Tris tris (hydroxymethyl) aminomethane
tRNA transfer RNA
ts temperature sensitive
UV ultraviolet light
v:v volume to volume
w:v weight to volume
w:w weight to weight
X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactoside
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CHAPTER 1

1.1 **E. coli** Ribonucleases: Introduction

Since my research work has focussed on regulation of the *rpoBC* operon by RNase III processing, I will give particular emphasis to the role of this enzyme in the cell's general RNA metabolism. However it should be remembered that the *E. coli* cell contains a battery of ribonucleases. The *in vivo* function of some of these enzymes is reasonably well defined, whereas for others it can only be guessed at from information obtained through *in vitro* studies.

I will discuss the metabolism of each RNA species in turn: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA), and detail the ribonucleases involved in their processing. Of course, in the cell all species of RNA and all ribonucleases co-exist. Against that background the highly specific interaction between enzyme and substrate RNA is quite remarkable. Also noteworthy is the interplay between different ribonucleases involved in the cell's overall RNA metabolism and the way in which wholesale RNA degradation is avoided. Such considerations have led to the notion that RNases may be compartmentalised within the cell. For instance, a non-specific degradative enzyme, such as RNase I may, it is argued, be isolated from susceptible
RNA molecules by its localisation in the periplasmic space. Regulatory elements, or events, might then trigger its release into the cytoplasm and lead to RNA degradation (Deutscher 1988).

Similarly the intriguing idea of a "processome" has been raised (Miczak et al 1989). Apparently the processing enzymes RNase III, RNase E and RNase P are "primarily located" in the E. coli inner membrane. Whether they are present there independently or are colocalised (in some processing complex) is not known. Obviously the idea of colocalisation of these enzymes in a processing complex ("processome") is an interesting one in that this might confer greater processing efficiency.

As well as being efficient, these enzymes are often highly specific as is clear from the range of precisely defined RNA processing reactions carried out in the cell. These reactions can be broadly divided into two classes. Firstly, there are those involved in degradation or turnover of RNA. The majority of mRNA processing reactions I will deal with are of this nature. The second type are those which are responsible for the conversion of a precursor RNA into its mature, functional form. Most stable RNA (i.e. rRNA and tRNA) processing, which I will discuss, is to this end.
1.2 MESSENGER RNA PROCESSING AND STABILITY

The cellular, steady-state level of any message is a balance between its synthesis and decay rates. Therefore regulating mRNA stability, and hence messenger levels, could be a fundamental way of controlling gene expression in the cell. How messenger degradation is controlled is unclear, although there is a growing body of work on the subject. The number of ribonucleases known to be involved in the process is, to date, quite small. Of the six known *E. coli* exoribonucleases only two play a significant part in mRNA metabolism: RNase II and polynucleotide phosphorylase (PNPase) (Donovan and Kushner 1986). A third enzyme RNase R, whilst displaying the requisite exonuclease activity *in vitro* (Kasai et al 1977) has no identified role in messenger turnover *in vivo*. RNase II and PNPase – like all known *E. coli* exoribonucleases – have processive 3' → 5' activities (No 5' → 3' exoribonucleases have yet been detected in *E. coli*). Mutants which lack both RNase II and PNPase are inviable and accumulate mRNA fragments between 100 and 1500 nucleotides in length. Surprisingly, strains lacking only one of these enzymes appear to degrade mRNA normally suggesting that, despite differences between the enzymes themselves, they are functionally complementary (Donovan and Kushner 1986). There is no evidence that these enzymes are targeted to specific messages; they appear, rather, to be involved
in bulk mRNA degradation. Therefore, the susceptibility of the 3' end of a message to attack by these exonucleases could be a crucial factor in determining its stability. Studies with RNase II and PNPase suggest that RNA secondary structures provide some resistance to these processive exonucleases (Gupta et al 1977, Deutscher 1985). The finding of multiple, highly conserved, inverted repeats throughout the genomes of *E. coli* and *Salmonella typhimurium*, which have the potential to form stable, stem-loop structures in mRNA was therefore highly interesting (Higgins et al 1982, Stern et al 1984, Gilson et al 1984). These sequences—called either R.E.P. (repetitive extragenic palindromic) (Stern et al 1984) or P.U. (palindronic unit, Gilson et al 1984)—are remarkably abundant: about 25% of all *E. coli* transcription units contain a REP/PU sequence (Newbury et al 1987a).

In those operons which have been studied, the REP/PU sequences are either located in a transcribed intergenic region or in a 3' untranslated region upstream of the terminator. Both locations are, of course, consistent with the REP/PU sequence acting as a protective barrier against 3' - 5' exonucleolytic attack. It has been shown that *in vitro* (Higgins et al 1988) and *in vivo* (Newbury et al 1987a) REP/PU sequences can perform such a function. Whether examined in their normal, chromosomal locations (e.g. the *Salmonella typhimurium his JQMP*
operon) or artificial sites, created by cloning into multicopy plasmids, REP/PU sequences cause the stabilisation and accumulation of upstream RNA. Interestingly this stabilised RNA is in a translationally active form, thus hinting at the possibility that REP/PU sequences may play a physiologically significant role in controlling gene expression.

Also suggestive is the observation that a Mu phage insertion at the distal end of the *E. coli* glyA gene reduces its expression. (Plamann and Stauffer 1985). This insertion separates the glyA gene from a 3' REP/PU sequence (Newbury *et al* 1987a, Gilson *et al* 1987). Plamann and Stauffer (1985) suggest that this separation exposes the message at its 3' end, reducing its stability and hence lowering glyA expression in the mutant. Similarly deletion of a 3' REP/PU sequence reduces *E. coli* gdh mRNA stability (Merino *et al* 1987) and gene expression (Becerril *et al* 1985).

Direct evidence for an *in vivo* role for REP/PU sequences in controlling gene expression is provided by the analysis of the malEFG operon in *E. coli*. This operon contains two REP/PU sequences between malE and malF. Normally the specific malE transcript has a longer half-life than the full-length malEFG message and correspondingly the MalE protein is present in excess compared with MalF and MalG (Newbury *et al* 1987a).
Deletion of the REP/PU sequences from the intergenic region on the chromosome destabilises the upstream transcript, leading to reduced \textit{malE} message accumulation. Moreover the deletion also causes a reduction in the level of MalE protein in the cell (Newbury \textit{et al} 1987b).

Clearly these REP/PU sequences are responsible for the differential mRNA stability in this operon which in turn is partly responsible for determining the relative levels of gene expression. The authors (Newbury \textit{et al} 1987b) are careful to note, however, that other factors, such as translational efficiency, may also play a role in the differential gene expression from this operon, since the ratio of MalE to MalF proteins is 20 - 40 : 1 whereas differential mRNA stability accounts for only a nine-fold difference in expression.

Despite the ability of REP/PU sequences to stabilise upstream RNA and in some cases influence relative gene expression, the overriding opinion (Gilson \textit{et al} 1987, Higgins \textit{et al} 1988) is that this is not the primary function of these sequences. It is felt that this alone would not impose the selective pressure to maintain the high degree of sequence conservation between REP/PU sequences. Alternative roles in chromosomal organisation, rearrangements and as "selfish DNA" are discussed in the two reviews.
Other stem-loop structures (not of the REP/PU family) can also act as barriers to 3' - 5' exonucleases. The mature 3' end of *E. coli* tryptophan operon mRNA has such a structure. Correct 3' end formation *in vivo* is mainly a result of 3' - 5' exonuclease processing from a distal rho-dependent terminator (trpt') being blocked at this stem-loop structure (trpt). trpt has features normally associated with a rho-independent terminator. As expected, RNase II *in vitro* processes this transcript to the trpt hairpin, and no further. (Mott *et al* 1985). Therefore the 3' end location of such secondary structures may not only signify a role in transcriptional termination but also in messenger stability. This has been shown to be so for ribosomal protein S20 messenger RNA, where deletion of the rho-independent terminator accelerates the decay of upstream S20 mRNA (Parsons and Mackie 1983, Mackie 1987). Also the terminator region of the cry gene from *Bacillus thuringiensis*, when cloned downstream of foreign genes, stabilises their message (by preventing exonuclease digestion) and increases their expression (Wong and Chang 1986). Stem-loop structures inserted at the 3' end of φX174 gene B or gene D stabilise their specific mRNAs, causing increased production of these proteins. Exactly how this is achieved is unclear because mutations which disrupt the stem base-pairing do not always abolish this stabilising effect (Hayashi and Hayashi 1985).
Interestingly a bacteriophage T7 RNase III site, when cloned downstream of a foreign gene, also confers message stability on the upstream transcript. It is proposed that a single cleavage of the RNase III target sequence does not destroy its secondary structure and hence produces a transcript with a 3' terminal hairpin (Panayotatos and Truong 1985).

Hairpin-loop structures (other than REP/PU) also appear to stabilise upstream message when they are located within a polycistronic transcript. The proximal portion of the *Rhodobacter capsulatus* operon messenger RNA is nine times more abundant than the distal mRNA. These regions are separated by a stem-loop sequence which protects the upstream RNA from 3' - 5' exonucleolytic attack. The half-life of the upstream message (encoding *pufBA*) is about 20 minutes whereas the longer transcript (encoding *pufBALMX*) has a half-life of about 3 - 5 minutes (Belasco *et al* 1985). The *puf* operon also contains an upstream gene *pufQ* (Bauer *et al* 1988). If *pufQ* is part of the same transcription unit, there must be a very rapid endonucleolytic cleavage to separate it from *pufB* since no *pufQBALMX* full, operon-length transcript has been observed (Chen *et al* 1988, Belasco and Chen 1988).
Deletion of the *pufA* - *pufL* intercistronic sequence leads to instability of the upstream RNA and consequently alters the pattern of gene expression from the *puf* operon (Klug *et al* 1987). However, insertion of this sequence at the 3' end of the *puf* operon fails to stabilise the *pufMLX* segment of mRNA (Chen *et al* 1988). In other words, the intercistronic stem-loop is necessary to stabilise *pufBA* mRNA but is, in itself, insufficient to confer message stability when located elsewhere. Therefore, presumably, there are other features of *pufBA* and *pufMLX* messages which contribute to the longevity of the former species and relatively brief life of the latter. Chen *et al* (1988) suggest that the *pufMLX* message is processed endonucleolytically and are currently investigating this process (Belasco and Chen 1988, Chen and Belasco 1989). They argue that this would trigger degradation of this segment of RNA by 3' - 5' exonucleases which would then be blocked on reaching the hairpin-loop structure between *pufA* and *pufM*. Different susceptibilities of *pufBA* and *pufMLX* mRNA to this rate-limiting endonuclease could thus help explain their different stabilities. As a general model for mRNA stability this one of interacting endonucleases and exonucleases determining the rate of degradation is more attractive than the simple one whereby protection against 3' - 5' exonucleases is the sole determinant; and indeed the more complex model has long been preferred.
Certainly complete degradation of mRNA requires the combined activities of endo- and exo-ribonucleases. Donovan and Kushner (1986) observed that without the activities of the exoribonucleases RNase II and PNPase, mRNA fragments accumulate, demonstrating not only that these two enzymes are the major exoribonucleases involved in mRNA turnover, but also that no endonuclease is capable of extensive degradation.

Pin-pointing the location of the endonucleolytic cut in the message is vital to explaining its role in mRNA degradation. For instance, an endonucleolytic cleavage near the 3' end of a message invariably exposes that RNA molecule to exonuclease attack. This is the model proposed by Chen et al (1988) (discussed above) for the degradation of the pufMLX mRNA segment. It has also been noted that in mutants where RNase II and PNPase are inactivated, S20 mRNA is cleaved by an endonuclease toward the 3' end of the transcript. This causes the downstream message to be stabilised relative to the upstream portion (Mackie 1989). Presumably those mutants have invoked some alternative pathway of mRNA degradation whereby the initial endonucleolytic processing triggers upstream decay, since in wild-type strains, S20 mRNA decays without accumulation of discrete intermediates.
Internal processing may also mediate differential gene expression from the multifunctional $rpsU - dnaG - rpoD$ operon (see Fig. 1.3). Cleavage of this single transcript at the $dnaG - rpoD$ intercistronic region separates $rpoD$ message from the upstream RNA and could expose the 3' end of the $dnaG$ message to exonucleases. This degradation may then be halted by a terminator structure $t_1$ at the 3' end of $rpsU$. In this way low-level $dnaG$ expression relative to $rpsU$ and $rpoD$ could be explained in terms of differential mRNA stability (Burton et al 1983). A similar mechanism could explain the regulation of *E. coli* pilin gene expression. Uropathogenic *E. coli* produce Pap pili (that facilitate adhesion to the urinary tract) from the *pap* gene cluster. Two genes of this cluster, *papB* and *papA*, are co-transcribed to produce a 1300 nucleotide long *papBA* transcript (Baga et al 1985). Cleavage of the transcript, between the two genes, results in the production of a highly stable and abundant 800 nucleotide *papA* message, and rapid degradation of the upstream 500 nucleotide *papB* segment (Baga et al 1988). A potential stem-loop structure at the 3' end of the *papA* message may contribute to its long half-life of approximately 27 minutes (Baga et al 1988).

One of the best known examples of processing destabilising upstream message is that of $\lambda$ int mRNA. The $\lambda$ int gene encodes a protein involved in site-
specific recombination. Its expression is regulated differentially during successive phases of the \( \lambda \) life cycle. \( \lambda \) int mRNA can be produced from two promoters: \( P_1 \) or \( P_L \). In a lysogenic response to infection \( \lambda \) cII protein stimulates transcription initiation from \( P_1 \) (Schmeissner et al 1981). This transcript terminates 277 nucleotides beyond \( int \) at a rho-independent terminator \( T_1 \) (Schmeissner et al 1984a), creating a relatively stable transcript from which high levels of Int proteins are made. This is yet another example of a terminator structure also serving to stabilise upstream message.

In a lytic response, the anti-termination activity of \( \lambda N \) protein allows \( P_L \) initiated transcripts to pass through several genes including \( int \). Whilst this transcript can allow production of numerous polypeptides both proximal and distal to \( int \), it is incapable of directing the synthesis of Int protein. This is due to action at a regulatory element named \( sib \) located at the 3' end of the \( int \) gene transcript (Guarneros et al 1982): a process termed retroregulation (reviewed in Gottesman et al 1982). \( Sib \) is in fact an RNase III target site which is cleaved by the enzyme \textit{in vitro} and \textit{in vivo} (Schmeissner et al 1984b), thus explaining the earlier connections made between RNase III and \( int \) expression (Belfort 1980, Schindler and Echols 1981, Guarneros et al 1982). \( P_1 \) initiated transcripts do not contain this
RNase III target site as they terminate just upstream at T1. PL derived messages read through T1 (due to \(\lambda\) N action) and hence contain the entire RNase III target site. Cleavage at this site is followed by 3' - 5' exonuclease degradation which is then blocked, at a region of potential mRNA secondary structure, within the int coding region. (Plunkett and Echols 1989). This neatly explains why the int gene alone is not expressed from PL derived transcripts.

Interestingly, expression of the \(\lambda c\) II protein may also be regulated by RNase III processing at a site distal to the gene. Krinke and Wulff (1987) have proposed an elaborate model whereby anti-sense OOP RNA hybridises with complementary sequences at the 3' end of \(\lambda c\) II message to produce a double-stranded RNA structure which is sensitive to RNase III cleavage (Krinke and Wulff 1987, Krinke and Wulff 1989). Once cut, the assumption is that the 3' end of cII mRNA is attacked by exonucleases, leading to transcript degradation and the consequent inhibition of \(\lambda c\)II gene expression.

RNase III also processes the major leftward "early" (Anevski and Lozeron 1981) and "delayed early" (Lozeron et al 1977, Wilder and Lozeron 1979) (including int mRNA) and the "late" major rightward (Lozeron et al 1983, Daniels et al 1988) RNAs of bacteriophage \(\lambda\). Little is known about the significance of most of these cuts; many
may be superfluous. RNase III cleavage near the terminator of the N-antiterminated N gene mRNA, destabilises the upstream message (Wilder and Lozeron 1979, Lozeron et al 1983). The position of the cut site in the "late" major rightward, Q antiterminated, 6S RNA is also near a terminator, although there is no evidence to suggest that cleavage here alters the message stability (Daniels et al 1988).

Internal cleavage of mRNA near the 5' end has been known about for some time. *E. coli* trp messenger RNA is degraded in a net 5' - 3' direction with 5' end turnover occurring even before transcription of the entire operon is complete (Morikawa and Imamoto 1969, Morse et al 1969). Since it is widely held that no 5' - 3' exonuclease activity exists in *E. coli*, this implies that endonucleolytic cleavage must occur to liberate fragments which are then processed by the existing 3' - 5' exonucleases, giving rise to an apparent wave of degradation in the 5' - 3' direction (Morikawa and Imamoto 1969, Morse et al 1969, Apirion 1973).

Discrete endonucleolytic cleavage has been observed near the 5' end of trp operon mRNA (Kano and Imamoto 1979) and, importantly, along its length (Lim and Kennell 1980). Rather than defined sequential fragmentation from the 5' end (Schlessinger et al 1977) trp mRNA can be processed at specific internal sites in a less ordered
fashion (Lim and Kennell 1980). Nonetheless the general apparent directionality of degradation (5' - 3') is maintained. Specific internal cleavages also occur in the E. coli gal operon messenger RNA (Achord and Kennell 1974) and Bacillus subtilis cat mRNA (Ambulos et al 1987). Since neither 5' nor 3' terminal sequences determine the stability of E. coli cat mRNA, it too is assumed to initiate degradation via one or more endonucleolytic cleavages (DeFranco and Schottel 1989).

Detailed analysis of this type of message decay has concentrated on the E. coli lac operon. This contains three genes: the lacZ gene (encoding β galactosidase), the lacY gene (encoding lactose permease) and the lacA gene (encoding galactoside acetyl transferase). They are produced from a single polycistronic mRNA in the order lacZYA, which is cleaved internally along its length (Blundell and Kennell 1974, Lim and Kennell 1979, Cannistraro et al 1986, Subbarao and Kennell 1988). The proximal lacZ message is twice as stable as the distal lacY and lacA portions of the transcript (Cannistraro et al 1986, Blundell et al 1972) and is degraded in a net 5' - 3' direction (Cannistraro and Kennell 1985a).

A careful study using S1 mapping and direct sequencing of the discrete RNA fragments produced during messenger decay showed the intergenic lacZY region, and considerable amounts of sequence on either side, to be
peppered with distinct cleavage sites (Cannistraro et al 1986, Subbarao and Kennell 1988). These cleavage sites show some sequence similarity, the tetramer UUAU appearing to be especially vulnerable (Cannistraro et al 1986). Recently a novel *E. coli* endoribonuclease was discovered (RNase M) which has the capability to cut at such sites (i.e. it cleaves at pyrimidine - adenosine bonds) (Cannistraro and Kennell 1989).

It is tantalising to draw a parallel between this tetramer (UUAU) and the pentamer AUUUA which is present in the 3' untranslated region of many labile eukaryotic mRNAs (Shaw and Kamen 1986, Brawerman 1987). This pentameric sequence is part of a larger AU rich region which, when cloned into the 3' untranslated region of stable messages, can render them unstable (Shaw and Kamen 1986). Conversely, the removal of this region confers greater stability upon messages produced from transfected plasmid constructs (Greenberg et al 1986, Wilson and Triesman 1988). A cytosolic protein (termed adenosine - uridine binding factor AUBF) has been identified, which specifically binds to RNA molecules with this AUUUA sequence (Malter 1989).

The finding of Cannistraro et al (1986) that *E. coli lacZ* mRNA is endonucleolytically cleaved at specific sites may be of general relevance to bacterial messenger breakdown. Internal cleavage is clearly not a random
process. This is consistent with work on several different transcripts which has demonstrated that bacterial message stability is not related to transcript length, but rather is due to some specific sequence or region of the message (Gorski et al 1985, Belasco et al 1986, Petersen 1987, Belasco and Chen 1988). In contrast, there appears to be a general inverse relationship between mRNA length and stability in *Saccharomyces cerevisiae* (Santiago et al 1986), although this correlation does not apply to all the transcripts tested. The authors (Santiago et al 1986) therefore propose that some other major factor(s) is/are involved in determining messenger stability in yeast.

The specific nature of bacterial mRNA endonucleolytic cleavage has led to speculation about the enzyme(s) involved. The number and variety of cut sites in *lac* mRNA suggests that an enzyme (or enzymes) of broad specificity (such as RNase M) may be at work. Perhaps secondary structure also plays a role in the definition of the target site.

RNase E has been reported to be essential for the processing of bacteriophage T4 gene 32 mRNA (Mudd et al 1988, Carpousis et al 1989). This cleavage renders gene 32 transcript stable, while the upstream messages decay (Belin et al 1987). Gorski et al (1985) provide evidence which suggests that binding of a bacteriophage
T4 factor (or factors) at the 5' end of gene 32 mRNA may also confer stability to the transcript. This region of gene 32 mRNA is proposed to form a 'pseudoknot' structure which is part of the autogenous regulatory site on gene 32 mRNA (McPheeters et al 1988).

Besides RNase M and RNase E, the only other identified E. coli mRNA endoribonuclease is RNase III. RNase III certainly has the ability to cut lacZ mRNA and also trp mRNA in vitro (Shen et al 1981, Shen et al 1982, Schlessinger et al 1983) but there is no evidence that it actually attacks either transcript in vivo; and since lac message is still inactivated in an RNase III-deficient strain (Talkad et al 1979) it seems clear that other endonucleases play the major role in initiating lac mRNA decay. RNase III has target sites in the intercistronic region of the E. coli rpoBC operon (Barry et al 1980), the ribosomal protein S15 operon (Regnier and Portier 1986) and the rnc operon (Bardwell et al 1989), as well as multiple sites in several bacteriophage transcripts, all of which are processed in vivo. Whilst RNase III processing of the rpoBC operon has been clearly demonstrated, the functional significance of this event remains in doubt and will be dealt with, in depth, later.
What is established is that RNase III processing of the S15 and rnc operon messenger RNAs does play a major role in controlling the expression of the encoded genes.

The S15 operon is comprised of two genes: rpsO (encoding r-protein S15) and pnp (encoding polynucleotide phosphorylase). pnp mRNAs are synthesised from two promoters (P1 and P2 - see Fig. 1.1). All transcripts which encode PNPase are normally processed at the RNase III target site which lies between P2 and the pnp coding region. A portion of the transcripts initiated at P1 (coding for S15) are also endonucleolytically cleaved downstream of rpsO (close to t1) at a site M. This processing is clearly not RNase III-mediated, since it is seen in rnc105 mutant strains. (Regnier and Portier 1986). The identity of the endonuclease which cuts at M is not known.

The importance of RNase III cleavage at its target site in the S15 operon on expression of the distal pnp gene was first demonstrated by comparative studies on the rnc105 mutant (BL321) and its isogenic rnc+ partner (BL322) (Portier et al 1987). Similar studies were also made of an rnc105 mutant (N2077) and its rnc+ partner (N2076). (Takata et al 1987). Differences in the magnitude of observed effects reported in these two papers may be due to the strain variations, or could be because the BL321/BL322 cells were grown in LB medium.
Structural organisation of the S15 operon. *rpsO* encodes r-protein S15. *pnp* encodes polynucleotide phosphorylase. P1 and P2 are promoters; t1 and t2 are terminators. *rns* is the RNase III target site, M is a maturation site. Open boxes represent the structural genes.
Fig 1.1
whilst the N2076/N2077 cells were grown in M9 minimal medium. These workers observed that the rnc105 mutants had either two or three times (N2077) or eleven times (BL321) as much pnp mRNA as their respective rnc+ partners. This increased level is due to an increase in the stability of pnp mRNA in the rnc105 mutants. Portier et al (1987) showed that RNase III-processed pnp mRNA has an estimated half-life of 1.5 minutes, whilst that of the unprocessed message is over 40 minutes. Takata et al (1987) initially reported mRNA half-lives of 1.2 minutes in their rnc+ strain and 2.5 minutes in the rnc partner. However, in a recent detailed study of the degradation of pnp mRNA they revise their estimate of the half-life of unprocessed pnp mRNA to 8 minutes. (Takata et al 1989). In no case was the upstream rps0 mRNA stabilised.

The stable, unprocessed message is functional, giving rise to a tenfold overexpression of PNPase in BL321 (relative to BL322) and a 4.4fold overexpression of PNPase in N2077 (relative to N2076).

The common conclusion from these papers is that RNase III processing is normally involved in the functional inactivation of pnp mRNA, and triggers the destabilisation of the pnp message. RNase III cleavage gives the message a half-life of approximately 1.5 minutes which is uniform along the length of pnp mRNA.
In contrast unprocessed mRNA has an estimated half-life of 8 minutes which is not uniform along the length of the mRNA. Sequences near the 5' end of the transcript are more stable than those in the middle, which are in turn more stable than those towards the 3' end of the mRNA. These observations are most easily explained by a mechanism involving progressive 3' - 5' exonuclease decay of the pnp mRNA. The RNase III endonucleolytic cleavage obviously greatly enhances the rate at which degradation of the entire pnp message can proceed. It is, however, not known exactly how this is achieved, particularly since the RNase III cleavage is at the 5' end of pnp mRNA.

The effect of RNase III processing on messenger stability in the rnc operon is very similar. This operon most likely contains three genes. Certainly two of them: rnc (encoding RNase III) and era (encoding Era protein) are co-transcribed from a single promoter upstream of rnc (Bardwell et al 1989). They are so closely linked that the termination codon of rnc overlaps the initiation codon of era (Ahnn et al 1986). Downstream of era lies a third gene: recO (Morrison et al 1989). Genetic studies suggest that all these are in a common operon since mutations in rnc have polar effects on era expression and era mutants similarly affect recO expression (Takiff et al 1989). Transcripts are processed by RNase III upstream of the rnc gene,
producing an \textit{rnc} - \textit{era} transcript with a half-life of 0.75 minutes. Failure to process, in an \textit{rnc}105 mutant, leads to the accumulation of \textit{rnc} - \textit{era} message with a half-life of 4.8 minutes (Bardwell \textit{et al} 1989). This in turn is responsible for the \textit{rnc}105 mutant producing RNase III and Era proteins 3 - 5 times more rapidly than the \textit{rnc}+ partner. Thus, as with \textit{pnp} message, RNase III cleavage near the 5' end is responsible for accelerated turnover of the entire transcript. Besides apparently sharing a common mechanism of messenger decay, these operons are also related in that they both encode ribonucleases. RNase III controls its own level of expression, and that of polynucleotide phosphorylase, raising the intriguing possibility that ribonucleases can form a regulatory network in \textit{E. coli}.

This possibility has also been suggested by Claverie-Martin \textit{et al} (1989). They have cloned the altered message stability (\textit{ams}) gene of \textit{E. coli} - the product of which is involved in mRNA decay (Arraino \textit{et al} 1988). Whether this gene encodes a ribonuclease or a regulator of nuclease activity is still unclear.

Site-specific endonucleolytic cleavages at the 5' noncoding region of \textit{ompA} mRNA also control the stability of that entire transcript. However in this case (unlike \textit{rnc} and \textit{pnp}) the identity of the endonuclease is unknown. Interestingly these endonucleolytic cleavages seem to be

One factor so far omitted from this discussion, which could play a major role in bacterial mRNA stability \textit{in vivo}, is the translating ribosome. The association of ribosomes on mRNA may sterically protect the transcript from endonucleolytic attack. Conversely endonucleolytic cleavage at the 5' end of a message may prevent ribosome association with the transcript, rendering it functionally inactive, and hence alter message stability. A summary of this complex and controversial subject follows.

(which prevents translation initiation) or puromycin (which blocks translocation, releasing the ribosome from the message) could expose mRNA, whilst chloramphenicol-induced slowing (or blockage) of ribosomes on the message may actually increase the protection against endonucleases, particularly as only relatively short, specific segments of the transcript may be determinants of messenger stability.

*E. coli trp* polar mutants (Yanofsky and Ito 1966) show accelerated degradation of messenger RNA distal to the nonsense codon (Morse and Yanofsky 1969, Hiraga and Yanofsky 1972). This was interpreted as demonstrating that the loss of ribosome association with this region of *trp* mRNA rendered it vulnerable to attack from degradative ribonucleases, which was initially assumed to be the primary mechanism of the polar effect. However an alternative explanation, now in favour, is that polarity is due primarily to premature transcription termination (Imamoto *et al* 1970) at a normally cryptic rho-dependent site(s), after the loss of ribosome association with the mRNA. (Richardson *et al* 1975, Ratner 1976, Korn and Yanofsky 1976 reviewed in Galloway and Platt 1986). Consistent with this explanation is the finding that, just as N-modification of RNA polymerase leads to antitermination in λ, so too it abolishes polarity by causing transcriptional readthrough of the rho-dependent terminators. (Franklin 1974, Segawa and
Moreover, polarity-suppressor mutations were isolated and found to be rho mutants (Ratner, 1976). The hyper-labile trp mRNA detected in polar mutants (Morse and Yanofsky 1969, Higara and Yanofsky 1972) is relatively short in length. It has been proposed that it represents the truncated transcript generated by rho-dependent termination and is unstable because it has an exposed 3' end vulnerable to 3' – 5' exonucleases. (Yanofsky and Crawford 1987). If this exonucleaseolytic degradation continued through the gene carrying the nonsense mutation and into the next upstream gene, it could also explain the phenomenon of "anti-polarity". This term refers to the process whereby some polar mutants inhibit the expression of the gene just upstream (Yanofsky and Ito 1967). In summary, however, the role of messenger degradation in polarity seems limited and of secondary importance to the role of premature transcription termination.

As predicted by the above model for nonsense polarity inefficient translation initiation of lacZ mRNA (and hence increased ribosome spacing on the transcript) can also lead to rho-mediated transcription termination at a site in the lacZ gene (Stanssens et al 1986).

In one case mutational inhibition of translation does seem to alter message stability without causing any premature transcriptional termination. Introduction of
a translation stop signal at codon position 29 of the chromosomally located \textit{ompA} gene results in a ten-fold increase in the rate of \textit{ompA} mRNA degradation (Nilsson et al. 1987). However this same study produced contradictory results with a different transcript (see later).

The effect of translation blockage on the stability of messenger RNA can be studied in wild-type cells without the addition of drugs. Message "protection" afforded by translating ribosomes can be lost by mRNA-protein interaction which inhibits ribosome loading or passage. For example, ribosomal protein messenger RNAs (r-protein mRNA) are subject to translational regulation by a specific repressor r-protein, which interacts with the transcript to the exclusion of ribosomes. (For general reviews of translational regulation of r-protein synthesis see:- Nomura et al. 1984, Lindahl and Zengel 1986, Nomura 1986 and Jinks-Robertson and Nomura 1987). Because r-protein genes are often clustered in polycistronic operons, translational repression can be used to control the synthesis of several r-proteins simultaneously. This is achieved by a process of "translational coupling", whereby the translation of distal genes in a polycistronic mRNA is reliant on prior translation of upstream genes. Binding of a repressor r-protein to the messenger RNA of a proximal gene in a polycistronic operon inhibits translation of that gene
directly, and, through the dependence on translational coupling, indirectly shuts down synthesis of distal r-proteins. The exact mechanism of translational coupling is not known, although the hypothesis is that the translation initiation sites of the downstream genes may be masked by structural features of the polycistronic mRNA. Translation of the upstream gene may be required to 'unmask' the initiation site of the distal gene and allow its translation. This process could be repeated along the entire length of the polycistronic message.

Translational coupling is the main way of linking the expression of proximal and distal genes in r-protein operons. However another effect of translational repression of proximal genes is that distal messenger RNA becomes exposed and destabilised, due to the loss of ribosome association and consequent attack by ribonucleases. This may provide a way of tightening r-protein operon regulation if translational coupling is not entirely efficient.

These processes are at work in regulating the expression of the genes in the "L11 operon". This "operon" contains the r-protein genes L11 and L1. (i.e. it is the proximal portion of the large rpoBC operon which codes for L11, L1, L10, L7/L12, β and β' proteins - see later). L1 is a repressor r-protein which binds to its target site at the 5' end of L11 mRNA (Dean and
Nomura 1980, Yates et al 1980, Yates and Nomura 1981) and, due to translational coupling, represses synthesis of both proteins (Baughman and Nomura 1983). Translational repression does not inhibit transcription of this "operon", and hence the process is unrelated to transcriptional polarity. Mutation of the target site abolishes feedback regulation and results in mRNA with an increased half-life; 1.8 minutes for unregulated L11 mRNA as opposed to 0.37 minutes for mRNA regulated by L1 binding. A double mutation which abolishes feedback regulation and translation initiation results in a transcript with a very short half-life (between 6 and 15 seconds) (Cole and Nomura 1986). These results demonstrate that loss of ribosome association resulting from L1 binding (and not some specific, perhaps nucleolytic, effect of L1 binding) destabilises the message. In other words, ribosomes proceeding along the message stabilise this transcript.

In an alternative approach, a strain carrying a mutation in the gene for r-protein S4 (rpsD), which results in a reduced translational repressor activity at its target on α operon mRNA (Jinks-Robertson and Nomura 1982), was shown to contain α messenger RNA with increased stability (Singer and Nomura 1985).

Altered mRNA stability resulting from translational repression also plays a significant role in controlling
expression from the spc operon. This is a large operon encoding ten r-protein genes. The r-protein S8, encoded by the fifth gene of the operon, is a translational repressor (Yates et al 1980, Dean et al 1981). It binds to the message near the translation initiation site of the third gene (encoding L5), directly inhibiting L5 translation and, via translational coupling and increased mRNA degradation, indirectly controlling the expression of all the distal r-protein genes in the operon. (Cerretti et al 1988, Mattheakis and Nomura 1988). Surprisingly by binding at the same mRNA target site, S8 also depresses synthesis of the first two proteins of the operon (Mattheakis et al 1989). This is achieved by a process of retroregulation. The model proposed to explain this is that S8 binding, to the exclusion of ribosomes, exposes an endonuclease target site in the naked, distal mRNA. Cleavage at this site (by an as yet unidentified enzyme) produces a 3' end which is sensitive to exonucleolytic digestion. If one assumes that the S8-mRNA interaction is reversible and that as S8 protein levels increase so the probability of S8 protein being bound to the message is raised, then at relatively low S8 protein concentrations it is possible that the 3'-5' exonuclease could proceed past the S8 target site on the mRNA and degrade the message encoding the first two genes in the operon (L14 and L24). Only by making these assumptions can one reconcile this result (Mattheakis et al 1989) with an earlier observation, that over-
production of S8 in trans (from an inducible plasmid) inhibits synthesis of L5, and distal proteins, but not of L14 and L24. (Dean et al 1981). Mattheakis et al (1989) have demonstrated that shortly after induction of S8 synthesis from the plasmid (low S8 levels) retroregulation works and L14/L24 synthesis is inhibited. However as induction proceeds (high S8 levels) retroregulation is lost, presumably because bound S8 protein is now blocking the progress of the 3' - 5' exonuclease along the message to the L14-L24 genes.

The stabilities of other messenger RNAs encoding r-protein genes, such as the monocistronic S20 mRNA, are also altered by translational repression. (Parsons et al 1988). However this may not always be a universal consequence of feedback control. For instance, r-protein L4 represses the expression of genes from the S10 operon at the level of translation (Lindahl and Zengel 1979, Yates and Nomura 1980, Zengel et al 1980). Effects seen at the messenger RNA level (after sudden perturbations) are primarily due to L4-mediated transcription attenuation upstream of the first gene in the operon; although a minor contribution from decreased mRNA stability cannot be entirely excluded (Lindahl et al 1983, Lindahl et al 1989).
As I mentioned earlier, bacteriophage T4 gene 32 protein binding to the 5' end of its own mRNA results in stabilisation of the transcript (Gorski et al 1985).

In *Bacillus subtilis* the *ermC* gene (one of the erythromycin-resistance genes) encodes an mRNA which is stabilised in the presence of subinhibitory levels of erythromycin (Shivakumar et al 1980). Such levels of erythromycin induce *ermC* expression by a mechanism called "translation attenuation". The *ermC* coding region is preceded by a translatable leader sequence which when being translated normally (in the absence of erythromycin) can be folded into a secondary structure that renders the *ermC* Shine and Dalgarno sequence unavailable for translation initiation (Weisblum 1983). Erythromycin addition causes stalling of erythromycin-bound ribosomes during translation of the leader sequence. This in turn "opens up" the *ermC* translation initiation site, and leads to increased *ermC* expression. This induction is accompanied by a 15 - 20fold increase in *ermC* mRNA stability which is not due simply to ribosome "protection" of the *ermC* coding mRNA. Rather it is the stalled ribosome in the leader mRNA which is responsible for conferring stability on the downstream transcript (Bechhofer and Dubnau 1987). The stalled ribosome protects the message from a progressive wave of 5' - 3' degradation. This is either due to an endonuclease activity which "tracks" in this direction
(and is rapidly followed by 3' - 5' trimming exonucleases) or *Bacillus subtilis* contains its own (as yet unidentified) 5' - 3' exonuclease activity (Bechhofer and Zen 1989). The same mechanism is proposed to explain erythromycin induction of *Staphylococcus aureus* *ermA* and *ermC* genes (Sandler and Weisblum 1989, Mayford and Weisblum 1989).

One intricate method by which mRNA stability might be altered is through the interaction of anti-sense RNA. The large majority of naturally occurring prokaryotic anti-sense RNAs function at the level of translation (see review by Inouye 1988). This can involve anti-sense RNA binding at, or upstream of, the translation start site of the targeted transcript, causing translation initiation to be physically impeded (Mizuno *et al* 1984, Dempsey 1987, Liao *et al* 1987, Wu *et al* 1987). This could then influence mRNA stability secondarily, although that has not yet been tested.

Alternatively the RNA-RNA duplex created by sense- anti-sense RNA hybridisation, could serve as a signal for endonucleolytic cleavage, which could trigger messenger decay. RNase III has been proposed to cut such a duplex at the 3' end of λcIII mRNA (as previously discussed). (Krinke and Wulff 1987). Similarly it has been suggested that RNase III cleavage of a duplex created by base-pairing of transposon *Tn10* transposase mRNA and its

It has still not been widely demonstrated that preventing translation (whether by protein or anti-sense RNA interference) results in messenger instability. Actually there are several lines of evidence which would contradict this theory. For instance, the untranslated portion of *ompA* mRNA is no less stable than the translated region (von Gabain et al 1983). Also, mutation of the Shine and Dalgarno sequence of the *Bacillus subtilis* *ermC* gene, which prevents translation of that message, has no effect on *ermC* mRNA stability (Bechhofer and Dubnau 1987). Even depriving a messenger segment of its usual complement of ribosomes does not necessarily render that transcript more sensitive to degradation (Stanssens et al 1986, Petersen 1987, Nilsson et al 1987). Using several mutants of the *bla* gene Nilsson et al (1987) observed that *bla* message stability seems to be dependent on efficient translation of a 5' portion of the transcript. Since specific cleavages have been observed in the 5' noncoding region of this message (Nilsson et al 1988), it is possible that ribosomes either protect this transcript against cleavage or against the degradative effects of endonucleolytic processing in this region.
The presence of the translating ribosome on mRNA is not a general mechanism for wholesale protection of entire transcripts from ribonuclease attack. Rather ribosome 'protection' seems relevant only if it serves to mask the particular site (or sites) on the transcript where messenger degradation is initiated - or to block further degradation.

The precision of endonucleolytic cleavage is a common theme in bacterial mRNA degradation. Studies on lacZ message decay show that, at least for this mRNA, these specific internal cleavage sites are abundant. Therefore rapid decay of this message in an apparent 5' - 3' direction could be achieved by cleavage at these internal sites immediately after the passage of the last translating ribosome. The oligonucleotides produced could then be destroyed by the existing 3' - 5' exoribonucleases.

Secondary structures may offer protection against non-specific degradative exonucleases, but the controlled turnover of many messages is initiated by a specific internal cleavage (or cleavages). Masking of internal cleavage sites may be one way of controlling message decay.
1.3 RIBOSOMAL RNA PROCESSING

There are seven rRNA operons in E. coli (rrnA, rrnB, rrnC, rrnD, rrnE, rrnG, rrnH). Their positions have been mapped on the chromosome and they have been sequenced. All seven have the same basic organisation (summarised in Morgan 1982) and are transcribed in the order promoter - 16S - tRNA(s) - 23S - 5S, as a single 30S transcript. The existence of at least one tRNA between the 16S and 23S genes is common to all seven operons. Three also contain distal tRNA genes (3' of the 5S gene) and rrnD has a second 5S rRNA gene (with a tRNA sandwiched between the two 5S genes) (Duester and Holmes 1980). The mature species in these operons are separated by spacer sequences which are removed by processing. Long inverted repeats bracket both the 16S (Young and Steitz 1978) and 23S genes (Bram et al 1980). These have the potential to form long double-stranded stems which would "loop out" the genes. Such loops containing 16S and 23S sequences have been visualised by electron microscopy of 30S precursor RNA prepared from RNase III-deficient strains (Klein et al 1985a). The stems are the major targets for RNase III cleavage of 30S RNA, which yields the direct precursors of rRNA. Thereafter the maturation pathways for the different rRNA molecules are extremely complex and involve multiple cleavages at the 5' and 3' ends of each species. For
this reason I will consider the maturation of each species individually, where possible.

1.3.1 **16S rRNA**

An intermediate RNA molecule, pre-16S, is the first product of RNase III cleavage at the stem formed by pairing of complementary sequences flanking the 16S gene. The pre-16S RNA is larger than the mature species, with 115 extra nucleotides at the 5' end and 33 extra at the 3' end. The cut sites produce a staggered break in the target duplex, leading to suggestions that cleavage there is a two-step process, involving separate reactions for each strand. The processing reactions are very rapid, with a reaction half-time *in vivo* of between 12 and 30 seconds (in rich medium at 37°C) (King and Schlessinger 1983). The speed of these reactions suggests that processing may occur at the 5' end of a 30S molecule before transcription of the entire operon is complete.

Detailed studies of 23S rRNA formation using rifampicin to block transcription initiation (King and Schlessinger 1983) produced more surprising results. Cleavage of the 23S rRNA precursor occurs at the 5' site before the 3' site of the same molecule has even been transcribed. This could be explained in at least two ways. Firstly, the formation of the large stem could involve intermolecular pairing between the 5' sequence of
one 23S precursor undergoing transcription and the 3' complementary region of another (completed) transcript. In this way the absolute secondary structural requirement of an RNase III target site could be met, and the 5' site cleaved, prior to transcription of the 3' end of the same molecule. Alternatively, two independent structures may form at the 5' and 3' ends of 23S rRNA, each constituting a separate RNase III target site. Thus interaction between the 5' and 3' ends would not be necessary for cleavage. This would, of course, challenge the model of RNase III cleavage at two staggered sites on a single large stem - formed by the intramolecular pairing of complementary sequences at the 5' and 3' ends of the 23S rRNA precursor and would therefore challenge the physiological significance of this stem formation (which has been visualised by electron microscopy).

In contrast the evidence from kinetic studies suggests that no such arguments need be raised to explain cleavage at the 16S rRNA precursor. These results suggest that the full 16S precursor sequence is transcribed prior to RNase III processing and therefore the accepted model of large stem formation, and cleavage therein, remains unchallenged for the 16S case.

The RNase III cleavage of 30S precursor RNA observed in vivo can be precisely mimicked in vitro using purified enzyme and naked precursor rRNA - although the efficiency
of cleavage is highly dependent on ionic strength (Birenbaum et al 1978).

The significance of RNase III cleavage has largely been assessed by studying rRNA formation in mutants deficient in the enzyme (King and Schlessinger 1983, Srivastava and Schlessinger 1989a). Such cells contain no RNase III-cleaved species, yet do produce mature 16S rRNA molecules. Hence the intermediate pre-16S rRNA - normally produced by RNase III processing - has been bypassed and is clearly not essential for mature 16S rRNA formation. These results imply that other enzymes involved in 16S rRNA maturation can function independently of RNase III cleavage.

A mutant, the BUMMER strain, accumulates a novel RNA species called 16.3S RNA containing an extra 66 nucleotides at the 5' end (Dahlberg et al 1978). Extracts from its parental strain can convert this 16.3S precursor to 16S rRNA by an endonucleolytic cleavage. This partially characterised activity is called RNase M16. RNase M16 will not cleave naked 16.3S RNA but requires it to be associated with ribosomal proteins in 30S subunits or 70S ribosomes. The BUMMER strain shows normal kinetics for RNase III cleavage of 16S precursors. However, final maturation of 16S rRNA in this strain is almost three times slower than in wild-type strains (King and Schlessinger 1983).
These results suggest that the BUMMER strain is deficient in the activity responsible for 16S rRNA 5' maturation; that the reaction depends to some extent on ribosome assembly; and that it is an endonucleolytic cleavage of the intermediate molecule already made by RNase III processing of 30S precursor.

Srivastava and Schlessinger (1989a) also present evidence which indicates that 16S rRNA 5' maturation may be achieved by a single endonucleolytic cleavage and suggest a similar mechanism for 3' end formation (Srivastava and Schlessinger 1989b). However 16S rRNA 3' maturation is less well characterised. A partially purified enzyme activity has been identified which converts a precursor RNA-protein complex into a 16S rRNA-protein complex (Hayes and Vasseur 1976). This is achieved by trimming the 3' end of the precursor and may be endonucleolytic - since no intermediates containing multiple 3' ends (indicative of exonuclease processing) have been detected.

An alternative, yet complementary, approach to investigating the processing of 16S rRNA has been the attempt to define the sequences surrounding, or even inside the gene which are required for maturation. Proper 5' end formation clearly does not require the presence of the large double-stranded stem, which
brackets the gene, since mutants devoid of this secondary structure still produce 16S rRNA with the correct 5' terminus. (Krych et al 1987, Srivastava and Schlessinger 1989b). Similarly 16.3S RNA, which has a mature 3' end and only 66 extra nucleotides at the 5' end, can be converted to mature 16S rRNA by cell extracts. (Dahlberg et al 1978). Equally, correct 16S rRNA 3' ends are made without the large stem being present (Srivastava and Schlessinger 1989b). Deletions within part of the 16S gene - even of only a single base - can lead to a failure of maturation (Gourse et al 1982, Stark et al 1982, Stark et al 1984). However it should be stressed that in a series of mutants where changes were localised to the 3' end of the 16S gene, no effect on processing or ribosome assembly was seen. (Jemiolo et al 1985).

Partial, or entire, deletion of the downstream, spacer tRNA impairs RNase III processing at the 16S rRNA target (Szymkowiak et al 1988). Why this should be so is not obvious. Unfortunately this work sheds no light on the importance of the spacer tRNA as regards maturation, of 16S rRNA, because the mutants used also had extensive deletions within the 16S gene.

The importance of the spacer tRNA sequences is more effectively tackled by Srivastava and Schlessinger (1989b). They have constructed plasmids with deletions
in the distal spacer region: between 16S and the spacer tRNA. As predicted these deletions have no effect on 5' end formation. Correct 3' end formation appears to require an intact spacer tRNA positioned more than 24 nucleotides downstream of the 16S gene. One hypothesis regarding the role of tRNA in 3' end formation is that it acts as a guide for RNase P, directing it towards the 16S rRNA 3' end. However since 16S rRNA matures normally in a temperature-sensitive RNase P strain at the non-permissive temperature (Apirion and Gegenheimer 1981) the enzyme's role (if any) is obviously not an essential one. This has been demonstrated more directly by Srivastava and Schlessinger (1989b). They were able to show that after 3' maturation the RNA released had not been processed by RNase P, RNase III or any other enzyme. No prior cleavage is needed at the 3' end, before 16S maturation can begin.

Why then could this correctly placed spacer tRNA be required for 3' maturation?

There are suggestions that the conformation of 16S RNA in the precursor transcript is different from that in the mature molecules of 30S ribosomal particles (Klein et al 1985b). Moreover reconstitution of 30S ribosomal subunits in vitro occurs with a much lower activation energy if the precursor form of 16S RNA is used instead of the mature form. (Mangiarotti et al 1975, Traub and
The theory, then, is that the tRNA sequences may be necessary to achieve, or retain, the correct conformation of pre-rRNA required for both processing and ribosome assembly. This could explain why partial, or entire, deletion of this tRNA restricts RNase III processing of the 16S rRNA precursor (Szymkowiak et al. 1988). Loss of part, or all, of the tRNA may alter the precursor conformation to such an extent as to impair RNase III cleavage.

1.3.2 23S rRNA

Precise definition of the reactions involved in maturation of 23S rRNA is difficult because, besides the predominant 5' and 3' termini, other termini can also exist in wild-type cells (Sirdeshmukh and Schlessinger 1985). However, it is known that, like 16S rRNA, RNase III processing liberates an intermediate species (pre-23S RNA) from the 30S precursor transcript by cleavage at target sites which bracket the gene. In both cases this initial reaction is then followed by a series of maturation cleavages.

One important difference between 16S and 23S rRNAs is that whilst maturation of the former can proceed without prior RNase III cleavage, such processing is absolutely required to allow the production of mature 23S rRNA. In RNase III deficient strains, failure to
process 30S precursor transcript results in the complete inability to produce mature 23s rRNA (King et al 1984). Instead these strains contain various species of 23S rRNA which have between 20 and 97 extra nucleotides at the 5' end and extensive additional 3' sequence. Intriguingly some (or all) of these unprocessed pre-23S RNA molecules must form ribosomes which are capable of protein synthesis, since the RNase III-deficient strains are viable. Thus, although RNase III cleavage is essential for 23S rRNA maturation, it would appear that maturation itself is not absolutely essential to make 23S RNA functional. This is in stark contrast to 16S rRNA, where maturation is required for 30S ribosome formation.

The unprocessed 23S RNA (derived from RNase III deficient strains) can be cut in vitro by purified RNase III (Sirdeshmukh and Schlessinger 1985). Major cleavage occurs at two distinct sites at the 5' end, and a single position at the 3' end, yielding two types of intermediate pre-23S rRNA molecule (with three or seven extra nucleotides at the 5' end, and eight extra at the 3' end). There also appears to be a minor RNase III cleavage site at the 5' end. Processing here generates a molecule which is four nucleotides shorter at the 5' end than the 'accepted' mature molecule. The functional significance of this is not known (Sirdeshmukh et al 1985).
Removal of the extra nucleotides from pre-23S RNA in vitro to generate mature termini, requires a wild-type cell extract which is capable of in vitro protein synthesis (Sirdeshmukh and Schlessinger 1985). These authors suggest that maturation of the 5' end occurs endonucleolytically, whilst correct 3' end formation depends on an exonuclease activity. Neither activity has been identified.

1.3.3 5S rRNA

The pattern of *E. coli* 5S rRNA processing is similar to that of its 16S and 23S RNA molecules. An initial endonucleolytic cleavage produces an intermediate species, which is further processed and trimmed to make the mature molecule. In this case the primary endonuclease is RNase E. Mutants deficient in this enzyme (*rne*-) accumulate a 9S molecule which contains 5S rRNA sequences running from the RNase III target site at the 3' end of 23S rRNA to the 3' terminus of the rRNA operon (Apirion and Lassar 1978, Ghora and Apirion 1979, Misra and Apirion 1979). This 9S RNA molecule can be processed *in vitro*, using cell extracts from an *rne*+ strain, to produce a pre-5S rRNA molecule (Ghora and Apirion 1978) which is six nucleotides longer than the mature 5S rRNA. Pre-5S rRNA contains three extra nucleotides each at the 5' and 3' ends (Roy *et al* 1983). How these are removed during maturation is not known.
Species carrying one, two or all three additional nucleotides at the 5' end accumulate in cells wherein protein synthesis has been shut down (Feunteun et al 1972). They have also been detected shortly after pulse-labelling of exponentially growing cells. Their disappearance then coincides with the emergence of mature 5S rRNA (Jordan et al 1970). This work suggests that these molecules are true precursors of 5S rRNA. These precursors have been found in polysomes, indicating not only that they can probably function in protein synthesis, but also that this is where final maturation occurs (Feunteun et al 1972). There appear to be no insights into 5S rRNA 3' maturation.

5S rRNA processing in Bacillus subtilis is less complicated. In this case the endonuclease RNase M5 generates both mature 5' and 3' termini by a single cleavage (Meyhack et al 1977). RNase M5 is comprised of two protein components: one, the α subunit, is required for catalysis; the other is a ribosomal protein apparently needed to attain the correct orientation for cleavage. (Stahl et al 1984, Pace et al 1984). Interestingly RNase M5 seems to recognise sequences of the mature 5S RNA within the precursor, since addition of synthetic sequence to the 5' or 3' end of the mature 5S RNA gene does not affect the proper processing of such a molecule (Pace 1984). No similar activity has been identified in E. coli.
Despite the ability of some cleavages to occur \textit{in vitro} on naked pre-RNA, a strong common theme of ribosomal RNA maturation seems to be the requirement of ribosomal protein interaction. Some of the final processing reactions even seem to require the pre-ribosome to be engaged in protein synthesis.

Once made, mature ribosomal RNAs suffer only limited turnover. The intact ribosome is highly resistant to cytoplasmic nucleases, but is sensitive to the periplasmic RNase I (Raziuddin \textit{et al} 1979). Release of RNase I from the periplasm, under adverse conditions, may trigger the degradation of rRNA in the cell.
1.4 TRANSFER RNA

Fifty three tRNA genes have been located in the *E. coli* chromosome. They are distributed throughout the genome in operons of diverse organisation (Fournier and Ozeki 1985). 19% are in monocistronic tRNA operons, 42% in multicistronic tRNA operons, 26% are in rRNA operons, and 13% are in complex operons with protein coding genes (Altman et al 1981, Hudson et al 1981, Ishii et al 1984, Rossi et al 1981).

Every tRNA gene transcript - even those from monocistronic operons - requires 5' and 3' processing to produce its mature functional tRNA molecule. Maturation of the 5' end of all *E. coli* tRNA species is carried out by the endonuclease RNase P (Kole and Altman 1981, Deutscher 1984). A particularly interesting feature of the ribonucleoprotein RNase P is that the RNA component (MIRNA) is the catalytic subunit of the enzyme. (Guerrier-Takada et al 1983). Since all tRNA molecules are eventually cleaved at the 5' end by RNase P it is theoretically possible that the same enzyme separates the individual tRNA precursors out of the multicistronic transcript. This does happen in bacteriophage T4, where a dimeric tRNA precursor (containing two RNase P target sites) can be separated by RNase P cleavage. However it should be noted that the rapidity of cutting is not equal for both sites (Schmidt and McClain 1978). This point
is consistent with the idea that the initial separation of multicistronic tRNA precursors is carried out instead by one or more alternative endonucleases. Where the multicistronic transcript is a poor substrate for RNase P cleavage, cutting by enzymes such as RNase P2, RNase F or RNase O has been postulated. (Note that RNase P2, RNase F and RNase O may be one and the same enzyme). (King and Schlessinger 1987). There is in fact evidence that some multicistronic tRNA operons are cleaved by RNase O prior to RNase P maturation (Sakano and Shimura 1975). In the case of one bacteriophage T4 tRNA, (tRNA^{Gln}), RNase III is required to cut at the 5' end prior to RNase P maturation (Fukuda and Abelson 1980, Schmidt 1984, King et al 1986). RNase PC also makes several cuts in the primary transcript of the phage T4 tRNA operon to separate the tRNA precursors, prior to final processing (Goldfarb and Daniel 1980).

Recently it has been shown that RNase III cleavage at the 3' end of a tRNA (metY : the first gene of the complex polycistronic operon metY - nusA - infB) releases it from the full-length operon transcript and simultaneously initiates degradation of the downstream mRNA (Regnier and Grunberg-Manago 1989). 5' maturation of this tRNA, presumably mediated by RNase P proceeds with or without prior 3' RNase III cleavage. 3' maturation is probably carried out by exonucleases at a very rapid rate (as below).
Also, interestingly, RNase P (Nomura and Ishihama 1988) and RNase E (Ray and Apirion 1981) have been shown to make endonucleolytic cleavages near the 3' end of certain tRNA molecules as a preliminary step in 3' end maturation. Mature 3' ends are generated by exonuclease activity. Several ribonucleases have been identified which have the capability to remove nucleotides from the 3' end of tRNA molecules, namely: RNase D, RNase II, RNase BN and RNase T. However RNase D mutants grow normally and are not impaired in tRNA biosynthesis (Blouin et al 1983). Likewise a mutant devoid of the exonucleases RNase D, RNase BN and RNase II also appears to have unaffected growth and tRNA production (Zaniewski et al 1984). It seems that these exonucleases are functionally redundant and that new nucleases for 3' processing may remain to be discovered. With regard to this latter point, the ability of a mutant devoid of all four exonucleases to correctly process the 3' terminus in vitro has been shown to be due to a phosphorolytic exonuclease activity (Cudny and Deutscher 1988).

The apparent functional interchangeability of exonucleases has made the precise mechanism of 3' end formation difficult to elucidate, though certain clues have been uncovered. Cudny and Deutscher (1980) compared the 3' processing activities of purified RNase II and RNase D preparations. RNase D, which is a non-
processive exonuclease, managed to process a mixture of tRNA precursors properly generating correct amino acid acceptor activity in the mature molecules. Processing slows down greatly at the CCA 3' terminal sequence, thereby allowing aminoacylation and highlighting the careful nature of RNase D trimming.

RNase II, which is a processive exonuclease, removes precursor nucleotides but also one to two extra nucleotides and so loses amino acid acceptor activity. For one particular molecule (tRNA\textsuperscript{Tr}r precursor) RNase II degradation is extensive. This evidence suggests that RNase D is an important (albeit nonessential) tRNA 3' maturation exonuclease whilst RNase II is a nonspecific degradative enzyme.

Studies on mutants lacking both RNase D and RNase II led to the discovery of RNase BN (Asha et al 1983) another exonuclease involved in tRNA 3' trimming. RNase BN seems to be required for the processing of some phage encoded tRNA transcripts but not those of \textit{E. coli}. The important difference between these molecules is that whilst all \textit{E. coli} precursor transcripts contain the entire mature species, some phage transcripts require the post-transcriptional addition of a 3' CCA. (Fukuda and Abelson 1980). Consequently these 3' processing reactions are different in that the additional precursor nucleotides must be removed from the phage transcript
prior to the CCA addition. The existence of an RNase BN mutant strain which is unable to process these particular phage transcripts helped pinpoint the enzymes role in this particular reaction (Seidman et al 1975). However since no E. coli tRNA precursor requires this type of processing, the exact function of RNase BN in the uninfected cell remains unclear.

Unclear also is the importance of the reaction mediated by RNase T. This non-processive exonuclease removes the 3' terminal AMP from mature tRNA molecules (Deutscher et al 1984). It can be added back by tRNA nucleotidyl transferase (Deutscher et al 1977a). The cyclic nature of these enzyme reactions has led to the process being called tRNA end-turnover. As I have said, the importance of this process is unknown, although tRNA nucleotidyl transferase (cca) mutants do show accumulation of defective tRNA molecules (Deutscher and Hilderman 1974) and a consequent decrease in growth rate (Deutscher et al 1977b). RNase T deficient derivatives of such mutants (i.e. cca, RNase T double mutants) show less accumulation of defective tRNAs and exhibit a faster growth rate than their RNase T+ parents. The amount of defective tRNAs in cells therefore appears to be determined by the balance of these two enzymes. However since single RNase T mutant strains (lacking as much as 70% of wild-type RNase T activity) display normal growth, the physiological significance of end-turnover remains a
mystery (Deutscher et al 1985). Perhaps the major function of RNase T lies elsewhere.

Once the processing and trimming of precursor tRNAs is complete, the mature molecule is remarkably resistant to further nuclease attack. This is due to the highly compact, stable structure adopted by mature tRNAs. Mutations which disrupt this structure often render the molecule sensitive to degradation (Smith 1974).
1.5 *E. coli* RNA polymerase

1.5.1 Introduction

*E. coli* RNA polymerase is a large and complex molecule comprised of four subunits: α, β, β' and, in the major holoenzyme, σ^70^ (Burgess 1969). These are encoded by the genes *rpoA, B, C* and *D* respectively (nomenclature: Hayward and Scaife 1976). The DNA sequence of these genes allows one to predict proteins of the following molecular weights: α (36Kd) (Post and Nomura 1979, Post et al 1980, Meek and Hayward 1984, Bedwell et al 1985), β (150Kd) (Ovchinnikov et al 1981), β' (155Kd) (Ovchinnikov et al 1982) and σ^70^ (70Kd) (Burton et al 1981). The gene *rpoZ*, for an associated factor: the omega (ω) factor, has also been cloned and sequenced (Gentry and Burgess 1986). From this DNA sequence one can predict a protein of molecular weight 10Kd. Normal assembly and function of RNA polymerase is not dependent on the association of omega factor. It will be discussed, separately, later.

The *rpoA, B, C* and *D* genes are present in the cell in three different operons: the α operon, the *rpoBC* operon and the MMS operon (which includes *rpoD*). Assembly of the polymerase subunits to form an active enzyme proceeds as follows:
\[ \alpha + \alpha \rightarrow \beta \rightarrow \alpha_2 \beta \rightarrow \alpha_2 \beta' \rightarrow \sigma^{70} \]

This pathway was elucidated from the results of work with assembly mutants (Ito et al. 1975, Ishihama et al. 1976, Taketo and Ishihama 1976).

The core enzyme \((\alpha_2 \beta')\) displays a non-specific DNA binding ability which can be converted, upon addition of \(\sigma^{70}\), to a highly specific, stable interaction with transcriptional promoters. The core enzyme plus sigma is the holoenzyme. Genuine transcription initiation can now be achieved. The sigma factor is released from the polymerase molecule shortly after mRNA chain elongation starts. Other factors may then interact with polymerase at, or before, the stage of transcription termination. These processes will be touched on during my discussion of the RNA polymerase subunits and their genes. I will also focus on the regulation of gene expression in the \(rpoBC\) operon which - although well studied - still poses some very interesting questions relevant to the control of gene expression in general.

1.5.2 \(\alpha\) SUBUNIT

The \(\alpha\) subunit of RNA polymerase is encoded by the \(rpoA\) gene, which is cotranscribed with the ribosomal protein genes S13, S11, S4 and L17 in an operon at 73
minutes on the *E. coli* chromosome (Jaskunas *et al* 1975a, 1975b, 1975c, 1977, Jaskunas and Nomura 1977, Bachmann 1983). Transcription extends from the upstream *spc* operon into the α operon, essentially occluding the α promoter (Ceretti *et al* 1983). This is akin to the situation in the *rpoBC* operon where transcription from *PL11* may partially occlude *PL10* (see later).

α dimers are produced in about 10% molar excess over β and β' (Iwakura *et al* 1974, Hayward and Fyfe 1978a). The regulation of the *rpoA* gene is intriguing. It resides between the genes for two ribosomal proteins (S4 and L17, see Fig. 1.2), yet it appears to be regulated independently of them and of the other r-proteins in the α operon. Whilst the synthesis of the r-proteins - S13, S11, S4 and L17 - is subject to stringent control (Dennis and Nomura 1974), α subunit synthesis is not (Reeh *et al* 1976, Blumenthal *et al* 1976). Also, α subunit production is enhanced during a challenge with rifampicin, whereas S4 and L17 syntheses are not similarly induced (Nakamura and Yura 1976, Hayward and Fyfe 1978b, Pedersen *et al* 1978).

*S1* nuclease analysis of α operon mRNA has shown that there is no unique transcription initiation or termination within the α operon, nor any cleavage events which could dissect the message and so easily explain the independent regulation (Thomas *et al* 1987). It thus
**Fig. 1.2**

Structural organisation of the α operon. Pa is the partially occluded promoter of the α operon. Open boxes represent the structural genes.

**Fig. 1.3**

Structural organisation of the MMS operon. rpsU encodes r-protein S21, dnaG encodes primase and rpoD encodes sigma 70. P1, P2, P3, Pa, Pb and Phs are promoters; t1 and t2 are terminators. Open boxes represent the structural genes.
appears that the differential expression occurs at the translational level.

All four r-proteins of the α operon are translationally regulated by the repressor r-protein S4 (Jinks-Robertson and Nomura 1982) which binds at a single target site upstream of the S13 operon (Deckman and Draper 1985, Thomas et al 1987). Mutational and nuclease analyses have demonstrated that this mRNA site forms a complex "pseudoknot" structure (Deckman et al 1987, Deckman and Draper 1987, Tang and Draper 1989) which may form to bring sites into close proximity in the RNA tertiary structure (Draper 1989). S4 binding at this site represses the synthesis of r-proteins from the α operon without affecting α subunit synthesis (Thomas et al 1987).

The function of α is unclear. Its major role may be to hold the core enzyme together (Chenchick et al 1981). Temperature-sensitive mutations in rpoA can alter the transcriptional fidelity of RNA polymerase in vitro, although these mutants show no indication of erroneous transcription in vivo (Ishihama et al 1980).

The reconstitution of active RNA polymerase from its isolated subunits has an absolute requirement for α (Heil and Zillig 1970), α2β being an intermediate in the RNA polymerase assembly pathway. It is therefore not
surprising that a temperature-sensitive α subunit mutant blocks assembly at an early stage (Kawakami and Ishihama 1980).

1.5.3 β SUBUNIT

The β subunit is encoded by the *rpoB* gene. This is part of the large and complex *rpoBC* operon which I will discuss later. This subunit appears to be involved in a range of functions in all areas of transcription. Mutations conferring resistance to the antibiotic rifampicin - a drug known to block transcription initiation - are localised to the β subunit of RNA polymerase (Heil and Zillig 1970). Some rif* β subunits have an altered mobility with respect to rif* β subunits (Rabussay and Zillig 1969).

The connection between β and transcription initiation is strengthened by the work of Glass *et al* (1986a, 1986b). They isolated a deletion mutant which, although displaying no obvious phenotype in vivo, showed altered promoter selectivity in vitro (Glass *et al* 1986a). This could be an indirect effect since the region deleted in the mutant may be one which is involved in sigma binding (Glass *et al* 1986b). Other studies on *rpoB* mutants also suggest a role for β subunit in transcription initiation (Tessman and Petersen 1976,

The β subunit also binds the antibiotic streptolydigin (Iwakura et al 1973) - a drug which inhibits both initiation and elongation. This indicates a possible role for β in elongation too. The amino-terminal region of the β subunit has been shown to be involved in transcriptional elongation (Nikiforov et al 1983, 1985) and affinity labelling of RNA polymerase with uridine triphosphate analogues demonstrated the location of the substrate binding site on the β subunit (Frischauf and Scheit 1973, Armstrong et al 1976).

Interestingly, a transcriptional proofreading mechanism has been proposed whereby RNA polymerase could monitor the fidelity of transcription (Kahn and Hearst 1989, Libby et al 1989). The reduced accuracy displayed by an rpoB mutant suggests that the β subunit is involved in this proofreading mechanism (Libby et al 1989).

A whole 'bank' of mutations conferring rifampicin resistance is located in the middle of the rpoB gene. Different alleles affect termination and/or antitermination of transcription (Jin and Gross 1988, Jin et al 1988a, 1988b). In addition, several suppressed amber mutants located in the β gene allow the growth of λ phage defective in N-mediated antitermination, also
suggesting the participation of β subunit here (Nene and Glass 1984).

Recently, two mutations which were selected to suppress the termination defects of a rho mutant (Guarente 1979) were mapped to the distal half of rpoB (Jin and Gross, 1989).

Thus there is strong genetic evidence for the involvement of β in termination.

1.5.4 β' subunit

The gene rpoC, part of the rpoBC operon, encodes the β' subunit of RNA polymerase. It is the only subunit of RNA polymerase which can bind to DNA by itself (Zillig et al 1970, 1971, Fukuda and Ishihama 1974). However this binding seems to be non-specific, since β' is highly basic and binds to other polyanions such as heparin (Zillig et al 1970). This property of β' presumably contributes to the non-specific DNA binding ability of RNA polymerase core enzyme. A temperature-sensitive β' mutation affects the DNA binding properties of RNA polymerase (Panny et al 1974).

Mutational studies have also implicated β' subunit in the process of promoter selection (Gross et al 1976,
Nomura et al 1984) (as with β) although, as noted below, this is primarily the responsibility of σ subunit.

Finally, it has been suggested that β' participates in termination since one of the polymerase mutants responsible for suppression of the termination defects of a rho mutant (Guarente 1979) maps in rpoC (Jin and Gross 1989).

1.5.5 σ SUBUNIT

In E. coli the primary sigma factor, responsible for the majority of cellular transcription initiation, is σ70, encoded by the gene rpoD. This gene is located in an unusual operon with genes whose products are involved in translation initiation (rpsU) and priming of DNA replication (dnaG) (Burton et al 1983, Lupski et al 1983). Since these genes are involved in initiating the syntheses of the cell's major macromolecules, the operon has been called the macromolecular synthesis (MMS) operon (Lupski and Godson 1984).

Transcription in the MMS operon can initiate at six known promoters (see Fig. 1.3). These have different strengths, as judged by their relative abilities to drive expression of the CAT gene in plasmid constructs (Lupski et al 1984). Promoter occlusion by P1 appears to operate at P2 and P3 (Lupski et al 1984). Transcription
from the heat-shock promoter (P_hs), present in the coding region of dnaG, is thermoinducible and dependent on the rpoH gene product, σ^{32} (Taylor et al 1984, Grossman et al 1984). The terminator T_1 is more than 90% efficient in stopping transcripts initiated at P_1, P_2 or P_3 from passing into the dnaG gene (Burton et al 1983, Lupski et al 1983, 1984). As mentioned earlier, an RNA processing event in the dnaG - rpoD intergenic region may expose the 3' end of dnaG message to exonuclease attack. Degradation could then be blocked at the T_1 terminator structure. This would help explain the relatively low levels of dnaG expression (Burton et al 1983).

Antitermination at T_1, either by an endogenous E. coli antiterminator protein (Lupski et al 1983, Peacock et al 1985) or by λ N gene product (Nakamura and Yura 1975) could lead to the overexpression of the downstream genes dnaG (see Wold and McMacken 1982) and rpoD. Selective, increased expression of the rpoD gene product (σ^{70}) has been detected during λ infection (Nakamura and Yura 1975). Further differential expression may be achieved at the translational level, by differences in translation efficiency and codon usage between the three genes (Lupski and Godson 1984).

A "dissection" of sigma protein into functional regions and sub-regions is presented in a review by Hellman and Chamberlin (1988). This allocates sigma's
distinct functional activities, such as core binding, promoter recognition, and DNA melting to specific regions within the protein. These are often conserved between species and amongst the alternative sigma factors.

The sub-region "2-4" appears to be involved in recognising the "-10 region" of promoters (consensus sequence TATAAT for $\sigma^{70}$) by making direct contact with the DNA (Siegele et al 1989, Zuber et al 1989) whilst residues in sub-region "4-2" of the major sigma appear to be in direct contact with nucleotides in the "-35 region" of bacterial promoters (consensus sequence TTGACA) (Gardella et al 1989, Siegele et al 1989).

Besides $\sigma^{70}$, *E. coli* has four other $\sigma$ factors (so far identified) each directing core enzyme to promoters with their own unique recognition sequences: - i) $\sigma^{32}$ (encoded by rpoH) which transcribes heat-shock genes from their special promoters (Grossman et al 1984) ii) $\sigma^{54}$ (rpoN), a factor required for the transcription of nitrogen-regulated (and other types of positively-regulated) genes (Kustu et al 1986). iii) $\sigma^F$ which appears to be responsible for transcription of the flagellar, chemotaxis and motility genes in *E. coli* (Arnosti and Chamberlin 1989). iv) $\sigma^E$ which activates transcription from rpoH P3 (Wang and Kaguni 1989, Erickson and Gross 1989), the only one of the $\sigma^{32}$ gene promoters which is functional at 50°C (Erickson et al 1987). $\sigma^E$ also
activates the promoter of the *htrA* gene, the product of which is required for viability at temperatures above 42°C (Lipinska et al 1988, 1989). Thus σE seems to be involved in high-temperature gene expression.

*Streptomyces coelicolor* and *Bacillus subtilis* have batteries of sigma-factors, some of which are involved in the control of differentiation and developmental regulation of gene expression in these organisms (Buttner 1989, Losick et al 1986).

1.5.6 **ω SUBUNIT**

The omega subunit is a factor which is associated with RNA polymerase (Burgess 1969) but which is not essential for normal RNA polymerase function. It is encoded by the gene *rpoZ* (Gentry and Burgess 1986). Interestingly this is located in the same operon as *spoT* (Gentry and Burgess 1989), a gene whose product is involved in ppGpp metabolism (Cashel and Rudd 1987). *In vitro* studies suggest that factor is involved in the ppGpp-mediated alteration of promoter selectivity, associated with the "stringent response" during conditions of amino acid limitation (Igarashi et al 1989).
1.6 CONTROL FEATURES OF THE \textit{rpoBC} OPERON

1.6.1 PROMOTERS

Transcriptional regulation of the \textit{rpoBC} operon is complex. Figure 1.4 details the major (PL\textsubscript{11}) and secondary (PL\textsubscript{10}) (Linn and Scaife 1978, Yamamoto and Nomura 1978, Post \textit{et al} 1979) and minor (PL\textsubscript{7}, P\textsubscript{8}, P\textsubscript{9'}) promoters of the operon. Detailed S\textsubscript{1} nuclease analysis has shown that PL\textsubscript{11} is the predominant promoter \textit{in vivo}, from which all six genes are transcribed, and that there is also significant transcription initiation at PL\textsubscript{10} (Downing and Dennis 1987). It may be that PL\textsubscript{11} is the predominant promoter \textit{in vivo} due to promoter occlusion of PL\textsubscript{10} (Bruckner and Matzura 1981) although the exact mechanism of this is unknown.

There is no indication of initiation at any of the proposed weak promoters in the intact \textit{rpoBC} operon. These minor promoters have been detected by indirect transcriptional analyses \textit{in vivo} of several lambda and plasmid partial-operon clones (Ma \textit{et al} 1981, Barry \textit{et al} 1979, An and Friesen 1980, Ralling and Linn 1984). Their strengths have been estimated at 1-2\% of those of the major promoters, and hence their possible contribution to normal operon expression is insignificant. Whether they are evolutionary relics "uncovered" by \textit{in vitro} manipulation (which normally play
Structural organisation of the *rpoBC* operon. *rplK*, *rplA*, *rplJ* and *rplL* encode r-proteins L11, L1, L10 and L7/L12 respectively. *rpoB* and *rpoC* encode the β and β' subunits of RNA polymerase respectively. *PL11* and *PL10* are strong promoters; *PL7*, *Pβ* and *Pβ'* are much weaker promoters. *tL7* and *tβ* are respectively, partial and strong terminators. *rns* is the RNase III target site. Open boxes represent the structural genes.
Fig 1.4

Diagram showing gene locations:
- PL11
- rplK
- rplA
- PL10
- rplJ
- PL7
- rplL
- rpoB
- rpoC
no role \textit{in vivo}), or whether they are functionally significant during, for example, extreme growth conditions, is unknown.

1.6.2 TERMINATORS

There are two terminators in the \textit{rpoBC} operon: \textit{tL7} and \textit{t\beta}. (see Fig. 1.4).

\textbf{tL7}

\textit{tL7} is a partial terminator (or attenuator) of transcription. Approximately 80\% of all transcripts which emanate from \textit{PL11} and \textit{PL10} are terminated at \textit{tL7}. The remaining 20\% of mRNA molecules continue into the \textit{rpoBC} genes. Prior to the physical identification of an attenuated transcript by S1 nuclease and direct RNA sequence analyses (Barry \textit{et al} 1980), and remarkably by recent electron microscopy studies (French and Miller 1989), the existence of an attenuator had been reasoned on the basis of several observations.

Lindahl \textit{et al} (1977) suggested that since \textit{rplK} and \textit{rpoBC} are cotranscribed and since there is a roughly fivefold excess of ribosomes over RNA polymerase molecules in the cell, then a regulatory mechanism such as transcriptional attenuation might exist to attain such differential expression. Transcription studies
confirmed that this ratio exists at the mRNA level and strengthened the argument for the existence of an attenuator (Dennis 1977). Sequence analysis of the rpoBC operon (Post et al 1979) - since revised and extended (Morgan et al 1984, Delcuve et al 1980, Ovchinnikov et al 1981, 1982) - also indicated that there was a simple terminator in the rplL - rpoB intercistronic region. tL7 is assumed to be a factor-independent terminator, although one report does suggest that rho increases, and that both NusA and the sfrB gene product decrease, termination efficiency in the rplL - rpoB intergenic region (Ralling and Linn 1987).

The efficiency of this terminator is fixed over a range of growth rates (Ralling et al 1985). However, it can be altered. One of the earliest indications of this was the response of partial diploids (rif/rifs containing a mixture of drug-resistant and drug-sensitive RNA polymerase) to a challenge with rifampicin. Against the background of a decrease in total protein synthesis caused by the drug, the RNA polymerase subunits β and β' showed transiently increased rates of synthesis (Hayward et al 1973). Such induction was then shown to occur for all four of the RNA polymerase subunits in merodiploids (Hayward and Fyfe 1978b) and also in sensitive haploid strains treated with subinhibitory levels of rifampicin (Nakamura and Yura 1976).
In contrast, streptolydigin - which also binds to the β subunit - does not produce this effect (Tittawella and Hayward 1974, Nakamura and Yura 1976). Thus it appears that the effect is a consequence of rifampicin binding, rather than just generalised drug inactivation of polymerase.

Direct analysis of mRNA after rifampicin induction revealed a marked increase in the levels of rpoBC message relative to those of rplKAJL (Blumenthal and Dennis 1978, Bass et al 1979). There is no evidence that rifampicin stimulates any of the promoters in the rpoBC operon (Howe et al 1982, Newman et al 1982, Fukuda and Nagasawa-Fujimori 1983, Morgan and Hayward 1985, Morgan and Hayward 1987), whereas several papers have shown, both indirectly and directly, that rifampicin promotes transcriptional readthrough at the attenuator tL7 (Howe et al 1982, Newman et al 1982, Fukuda and Nagasawa-Fujimori 1983, Morgan and Hayward 1987). However, this does not reflect some specific regulatory feature of the tL7 attenuator in the rpoBC operon. Rifampicin also causes readthrough of all rho-independent and rho-dependent terminators tested (Howe et al 1982, Newman et al 1982, Cromie and Hayward 1984). The likeliest explanation seems to be that a direct action of the drug on RNA polymerase (via the β subunit) alters the efficiency of transcription termination in general. This is consistent with the findings that rifampicin-

One possibility suggested by Tittawella (1976) is that rifampicin induced acceleration of ββ' synthesis first requires the production of a positive control protein (π) made during the first four minutes of drug treatment. This would then presumably act at tL7 mediating readthrough in some fashion. No such positive control protein has yet been identified, biochemically or genetically.

Studies with RNA polymerase subunit mutants also suggested that the efficiency of tL7 could be specifically modulated. Using a poorly suppressed amber mutation of rpoB or an rpoB t.s. mutation at the non-permissive temperature, the synthesis of ββ' subunits was shown to be stimulated (Glass et al 1975, Dmitriev et al 1976). Temperature-sensitive mutants of rpoC, which are assembly-defective, also over-produce these subunits at their non-permissive temperature (Kirschbaum et al 1975, Taketo et al 1976, Taketo and Ishihama 1976, Khesin et al 1976, Taketo and Ishihama 1977). Stimulation of ββ' synthesis in both cases could be due to transcriptional readthrough at the attenuator (Dennis 1977b, Kirschbaum 1978, Little and Dennis 1979, Little and Dennis 1980,
Little et al 1981). This does not necessarily imply a specific modulation of tL7 efficiency in response to the lowered levels of active RNA polymerase. Instead it is possible that at the higher-temperature the mutant enzyme is generally defective in transcription termination. This is comparable with the "rifampicin-bound" situation discussed above. Certain t.s. rpoB mutants show a decrease in the rate of ββ' synthesis when shifted to the non-permissive temperature (Kirschbaum et al 1975, Taketo and Ishihama 1976, Taketo et al 1976). Thus (as discussed above) depending on the mutation in rpoB, the ability of RNA polymerase to function at termination may be increased or decreased. As a consequence the readthrough of the rpoBC attenuator (tL7) would be altered, as would ββ' expression, not as a specific, direct response to the changed levels of active RNA polymerase, but non-specifically and indirectly.

However, as a counter argument, one interesting study with an rpoD t.s. mutant strain maintains the possibility that the rpoBC attenuator is a site for specific autogenous regulation of transcription. At the non-permissive temperature this t.s. sigma mutant produces RNA polymerase which is inefficient at transcription initiation. Under these conditions increased production of ββ' is observed (Blumenthal and Dennis 1980). Since, during transcription, the sigma subunit normally disengages shortly after RNA chain
elongation starts, it is unlikely to be involved in readthrough of terminators. Therefore the model of non-specific readthrough of tL7 to explain increased ββ' production in the rpoD t.s. mutant, is not easily applicable here. It is possible that this work demonstrates a specific regulatory mechanism which "compensates" for the decrease in levels of initiation-competent RNA polymerase by increasing the transcription of rpoBC through tL7 attenuator readthrough.

It is interesting, on the other hand, to note that sequences around prokaryotic promoters can affect the efficiency of termination at downstream sites (Telesnitsky and Chamberlin 1989, Goliger et al 1989). Telesnitsky and Chamberlin (1989) suggest that this might be due to sequence-mediated alterations in the conformation of the transcribing RNA polymerase, which could alter its ability to terminate at simple (factor-independent) terminators. One could imagine that rpoD t.s. mutants might also contribute to altering the conformation of the transcribing RNA polymerase, rendering it inefficient at termination at tL7, and thereby explaining the increase in ββ' production.

The transcripts which read through tL7 (to encode rpoBC) are finally terminated at tβ'. (Squires et al 1981).
1.6.3 *rpoBC* MESSENGER RNA PROCESSING


*rplA* - *rplJ*

This intergenic space contains the PL10 promoter, but lacks any recognisable rho-independent termination signals. S1 nuclease mapping shows the 5' end of the putative PL10 - initiated *in vivo* transcripts to be extremely heterogeneous (Morgan and Hayward 1985, Downing and Dennis 1987). This may reflect some sort of progressive processing at the 5' end of the PL10 initiated transcript. Discrete processing sites, both 5' (Downing and Dennis 1987) and 3' (Bruckner and Matzura 1981, Morgan and Hayward 1985, Downing and Dennis 1987) of the putative PL10 initiation site, have been detected. Two major endonucleolytic sites at positions 1500 and 1595 (scale of Post *et al* 1979) are detected in three separate studies (Bruckner and Matzura 1981, Morgan and Hayward 1985, Downing and Dennis 1987). The identity of the ribonuclease (or ribonucleases) which makes these
specific cleavages is not known, although it is clear that RNase III is not involved (Downing and Dennis 1987). Equally unclear is the functional significance of these cleavages.

**rplL** - **rpoB**

RNase III does process *rpoBC* mRNA in this region. This was first observed during S1 nuclease mapping studies of the *rpoBC* operon (Barry *et al* 1980). Plasmids were used to increase the level of mRNA specific for this region, by increasing the copy number or further by fusion to the inducible arabinose promoter. Total RNA was extracted from cells, hybridised to appropriate DNA probes, treated with S1 nuclease and the resultant nuclease-resistant DNA-RNA hybrids were analysed on acrylamide gels. A hybrid corresponding to the tL7 - attenuated transcript was observed, but no full-length, readthrough message could be detected. Instead an array of discrete, smaller bands was seen, which were interpreted as being processing products of the readthrough transcript. The cleavage sites occur in the vicinity of a potential large stem-loop structure, which resembles known RNase III sites. S1 nuclease analysis of RNA prepared from an RNase III-deficient strain showed no indication of processing at this position. The exact location of cleavage has proved difficult to define. The combined data of these detailed studies suggest that
the RNase III target site is cut on both "arms" of the large stem. On the 5' arm, cleavages has been mapped at positions 2770 (±3) (Railing and Linn 1987), 2776 (±1) (Downing and Dennis 1987) and 2785(±3) (Fukuda and Nagasawa-Fujimori 1983, Downing and Dennis 1987, Railing and Linn 1987); and on the 3' arm, at 2864 (Fukuda and Nagasawa-Fujimori 1983) and 2860 (Barry et al 1980) (scale of Post et al 1979). The indication from these papers is that processing at the 5' arm is perhaps more efficient than cleavage at the 3' arm. On the whole, processing at this site is normally inefficient: approximately 50% of transcripts are cleaved by RNase III in vivo (Morgan and Hayward 1987) at a variety of growth conditions and growth rates (Downing and Dennis 1987). In vivo, mRNA derived from plasmids containing the rplL - rpoB intergenic region seems to be processed at the RNase III site with much higher efficiency (Barry et al 1980). This may be a general feature of plasmid-borne RNase III target sites (Panayotatos and Truong 1985). Transcripts made in vitro do not seem to be well processed at this site (Fukuda and Nagasawa-Fujimori 1983).

The functional significance of RNase III cleavage of rpoBC mRNA is debatable. No differences were detected in the rate of rpoBC transcription or the synthesis rates of β and β' proteins in an rnc105 mutant (N2077) and its isogenic wild type partner (N2076) under steady-state growth, in one study (Dennis 1984). However Portier et
al (1987) present preliminary evidence that β and β' proteins are overexpressed in an *rnc105* mutant (BL321) relative to its isogenic wild type partner (BL322).

A study employing plasmid-borne deletions of the RNase III target site suggests that sequences in this region are involved in determining the translational efficiency of *rpoB* mRNA, but that processing *per se* is not involved in this (Dennis 1984). However another possible conclusion one could draw from this work is that the observed reduction in *rpoB* translational efficiency from these plasmids is predominantly due to effects on autogenous translational repression. By comparison the observed, deletion-mediated alterations in intrinsic translational efficiency are fairly minor. Clearly this topic requires further investigation.

1.6.4 **TRANSLATIONAL REGULATION**

All six genes of the *rpoBC* operon are subject to translational regulation. The four ribosomal genes exhibit an autogenous control which seems to be common for ribosomal proteins generally. As discussed earlier, the r-protein genes L11 and L1 are regulated by the binding of repressor r-protein L1 to its target site at the 5' end of L11mRNA (Dean and Nomura 1980, Yates *et al* 1980, Yates and Nomura 1981). As I also mentioned, translational coupling ensures that this event represses
the synthesis of both proteins simultaneously (Baughman and Nomura 1983). This translational repression is accompanied by an increased instability of this message (Cole and Nomura 1986).

In common with all the known ribosomal translational repressor proteins, L1 recognises a specific site at its target mRNA and also binds directly to rRNA (reviewed in Noller 1984).

The sites in L1 protein and L11 mRNA involved in the repressor-mRNA interaction are conserved in other bacterial species. L1 protein from *Serratia marcescens* and *Proteus vulgaris* both regulate the *E. coli* L11 operon (Sor and Nomura 1987). The L11 mRNA target site sequence conservation between these three species is highlighted in Fig. 1.5 (taken from Draper 1989). Furthermore, *E. coli* L1 protein can bind to a segment of *Dictyostelium discoideum* 26SrRNA which is homologous to the *E. coli* rRNA binding site (Gourse *et al* 1981), thus indicating that there is also significant conservation of this site. The identical nucleotides are also marked in Fig. 1.5.

The conserved bases focus attention on a consensus L1 binding site present in both L11mRNA and 23SrRNA (Branlant *et al* 1981, Gourse *et al* 1981, Draper 1989). This site displays both primary and secondary structure
Fig. 1.5A
Binding site for L1 protein on *E. coli* L11mRNA. Base numberings are from the 5' terminus of the mRNA transcript. 'Tick' marks are located every 10 bases. Bases in bold face are conserved in L1 binding sites from *Serratia marcescens*, *Proteus vulgaris* and *E. coli* messages. Boxed sequence is conserved in primary and secondary structure between *E. coli* L11 mRNA and 23SrRNA. Underlined bases indicate the initiator AUG and Shine-Dalgarno sequence.

Fig. 1.5B
Binding site for L1 protein on *E. coli* 23SrRNA. Base numberings are from the 5' end of *E. coli* 23SrRNA. 'Tick' marks are located every 10 bases. Bases in bold face are conserved in L1 binding sites from *Dictyostelium discoideum* 26SrRNA and *E. coli* 23SrRNA. Boxed sequence is conserved in primary and secondary structure between *E. coli* 23SrRNA and L11 mRNA.

Fig. 1.6A
Binding site for L8 complex on *E. coli* L10 mRNA. Base numberings are from the 5' terminus of the mRNA transcript. 'Tick' marks are located every 10 bases. Boxes outline nucleotides similar in primary and secondary structure between L10 mRNA and 23SrRNA.

Fig. 1.6B
Binding site for L8 complex on *E. coli* 23SrRNA. Base numberings are from the 5' end of *E. coli* 23SrRNA. 'Tick' marks are located every 10 bases. Boxes outline nucleotides similar in primary and secondary structure between 23SrRNA and L10 mRNA.
conservation. Mutations which relieve translational regulation occur in this conserved region (Thomas and Nomura 1987) and destruction of the potential secondary structure by site-directed mutagenesis (Baughman and Nomura 1984) abolished translational regulation. Similarly site-directed mutations within the 23SrRNA "consensus" site also effect L1 binding (Said et al 1988).

The ability to interfere specifically with translational control allowed its significance to be elucidated. A chromosomal mutation which abolishes translational regulation of L11mRNA also abolishes growth-rate-dependent regulation and stringent control of L11 and L1 proteins (Cole and Nomura 1986b). As expected, the regulation of other r-proteins, not under L1 control, is unaffected by this mutation. This work demonstrated the physiological importance of translational regulation of r-protein synthesis. Growth-rate-dependent regulation of r-protein synthesis in other operons was already known not to involve altered promoter activity (Miura et al 1981); therefore such a translational control mechanism may be widespread amongst r-protein genes.

Stringent control of ribosomal protein synthesis was previously thought to be achieved at the transcriptional level, for all r-protein operons. Aminoacyl tRNA
limitation causes a rapid reduction in rRNA and r-protein synthesis (called the "stringent response": for a review see Cashel and Rudd 1987). It seems clear that rRNA synthesis is directly affected by stringent control, but less clear how r-protein synthesis is regulated under these conditions. The promoter for the S10 r-protein operon appears to be responsible for the stringent control of r-protein production from this operon (Freedman et al 1985). However neither PL11 nor PL10 (of the rpoBC operon) is subject to stringent control in vivo (Wright et al 1988, Morgan and Hayward 1985). This is in agreement with the conclusion of Cole and Nomura (1986b) that stringent control of the 'L11 operon' is mediated at the translational level. Such indirect control could be achieved as follows. Stringent control would directly lower the levels of rRNA, which would lead to free L1 protein accumulation. This could then repress translation of rplKA. This may be a common model for the stringent control of the synthesis of r-proteins, but it is clearly not a universal one (see above). Acceptance of it as a general model requires one to explain away apparently contradictory data.

For instance, the observation that PL10 is sensitive to ppGpp in vitro (Kajitani and Ishihama 1984) suggests that it should be stringently regulated in vivo. This must be countered with the direct evidence against it (Morgan and Hayward 1985, Wright et al 1988) and with the
argument that these \textit{in vitro} experiments detect a phenomenon which is distinct from the physiological stringent control \cite{Baracchini1988}. Also, the evidence that transcription of the \textit{rplKALJL} genes is reduced during partial amino acid starvation \cite{Maher1977} must be explained in terms of increased messenger instability. Translational repression of L11 and L1 sharply decreases the half-life of their mRNA \cite{Cole1986}. Therefore stringent control of r-protein synthesis from this operon - mediated by translational repression - could also decrease the stability of \textit{rplKALJL} transcripts.

This model is also attractive because it explains why the cotranscribed \textit{rpoBC} genes do not exhibit stringent control \cite{Maher1977}, without the need to invoke some specific modulation of tL7 efficiency during amino acyl tRNA limitation.

The other two r-proteins of the \textit{rpoBC} operon (L10 and L7/L12) are similarly regulated at the translational level. A protein complex of L10-L7/L12 (termed L8) binds to the leader sequence of L10mRNA, blocking translation of both L10 and L7/L12 \cite{Yates1981}. The L8 complex also has a specific binding site on 23SrRNA \cite{Beauclerk1984}. These two binding sites are compared in Fig. 1.6 \cite{Draper1989}. Nuclease protection studies indicate that the L8 complex binds at
a site more than 100 nucleotides upstream of the rplJ ribosome binding site (Johnsen et al 1982). Analysis of genetically selected mutations in the rplJ leader (Fiil et al 1980, Friesen et al 1983) combined with the results of site-directed mutagenesis of this region (Christensen et al 1984) support the idea that long range RNA secondary structure interactions are responsible for the translational regulation of the 'L10 operon', presumably by bringing the L8 and ribosome binding sites into close proximity. By analogy with the 'L11 operon', it is presumed that growth-rate-dependent regulation and stringent control of L10 and L7/L12 syntheses are mediated at the translational level.

The β and β' subunits of RNA polymerase are also translationally regulated. An early indication of this was that over-expression of rpoBC mRNA, either from an inducible λrif418 lysogen or from a multicopy plasmid, did not result in equivalent increases in ββ' syntheses (Kirschbaum and Scaife 1974, Dennis and Fiil 1979).

In vitro studies in which purified RNA polymerase holoenzyme, or its assembly intermediates, were added to in vitro transcription-translation systems indicated that α2β and holoenzyme could both act as translational repressors of ββ' synthesis (Fukuda et al 1978, Kajitani et al 1980). This translational repression can be lifted in vitro by rifampicin – presumably by direct
interference, since the drug binds to the β subunit of RNA polymerase (Lang-Yang and Zubay 1981, Fukuda and Nagasawa-Fujimori 1983).

In vivo experiments have shown that cells compensate for a limitation of β and β' syntheses, produced by poor suppression of amber mutations in rpoB, primarily by increasing the translational efficiency of rpoB mRNA. This compensatory mechanism allows those strains to produce enough RNA polymerase to support a near-normal rate of growth, despite the poorly suppressed mutation (Dennis et al 1985). The existence of such a mechanism suggests that the production of ββ' subunits are autogenously regulated at the translational level. This was directly demonstrated by overexpression of ββ' proteins from a lysogen carrying the rpoBC genes fused to the strong, controllable promoter P_L in phage λ. Induction of the lysogen increases rpoBC mRNA levels by at least tenfold, whilst ββ' oversyntheses is about 2 - 3fold. Overproduced ββ' also regulate the synthesis of β from the chromosome in strains carrying electrophoretic mobility mutations in rpoB. This regulation was shown to be post-transcriptional (Meek and Hayward 1986). Similarly, over expression of polymerase from inducible plasmids is also ultimately compensated for by repression of ββ' syntheses from the chromosome (Bedwell and Nomura 1986).
There is also a suggestion that β and β' syntheses can be regulated independently. Excess β subunit (alone or, more probably, as part of a complex) autogenously regulated β synthesis, but did not affect the rate of β' synthesis (Meek and Hayward 1986). It is possible that alternative regulatory molecules (probably of a very similar nature) control β and β' syntheses.

A recent study (Passador and Linn 1989) has clearly demonstrated the existence of autogenous regulation of β subunit synthesis at the translational level. A series of transcriptional and translational fusions of rpoB to the lacZ reporter gene have been constructed in lambda vectors. In parallel, plasmids carrying rpoBC under the control of a regulatable promoter were also made. A comparison of the results from lysogens carrying either the transcriptional or translational fusion plus the multicopy plasmid showed that whilst ββ' overproduction did not specifically affect the transcription of the rpoB – lacZ fusions, it did have a significant affect on the translation of the fusions. This is a neat and relatively straightforward way of analysing the translational feedback regulation of rpoB.

One of the most intriguing problems has been to locate the precise target site on rpoB mRNA at which the regulatory molecule acts. Deletion studies of the rplL – rpoB intergenic region (Dennis 1984) suggested that
sequences in the vicinity of the RNase III processing site may be important for translational efficiency and regulation. However, autogenous regulation appears to occur when almost all of the intergenic region is removed—right up to 26 base pairs short of the *rpoB* initiation site (Meek and Hayward 1986). Joint consideration of these papers points to the possibility of a complex, long range RNA secondary structure involving the interaction of sequences at the RNase III target site and the site for autogenous regulation. However a surprising result from the work of Passador and Linn (1989) indicates that at least part of the translational regulation site may lie well inside the coding region of *rpoB* (between 126 and 403 base pairs inside *rpoB*). This is intriguing, and may be similar to the translational regulation of the *gnd* gene of *E. coli* which is mediated by the interaction of an internal sequence (located at codons 69 - 74 of the *gnd* structural gene) with the ribosome binding site of the gene, to which it is complementary (Carter-Muenchau and Wolf 1989). This type of interaction has also been postulated for the *trmD* operon of *E. coli* (Wikstrom and Bjork 1989).
1.7 **THIS THESIS**

In this introduction an attempt has been made to survey two broad areas of bacterial molecular biology: ribonucleases and RNA polymerase in *E. coli*. My thesis work has focussed on a point where these topics converge: the role of RNase III processing in controlling expression of the RNA polymerase subunit genes *rpoBC*. This subject has attracted a fair degree of attention but is, as yet, not properly resolved. The following chapters describe work which is, initially, a comparison of *rpoBC* gene expression in *rnc*<sup>+</sup> and *rnc105* strains under steady-state and perturbed growth conditions. Subsequently a more detailed analysis of the role of processing at the RNase III target site in the *rplL* - *rpoB* intergenic space is presented.
2.1 MATERIALS

2.1.1 STANDARD SOLUTIONS

1 x TE : 10mM Tris-HCl, 1mM EDTA; adjusted to pH8.0 with concentrated HCl.
10 x TBE : 0.89M Tris base, 0.89M boric acid, 20mM EDTA pH8.0.
10 x TAE : 0.4M Tris base, 0.2M sodium acetate, 10mM EDTA; adjusted to pH8.3 with glacial acetic acid.
20 x SSC : 3M NaCl, 0.3M sodium citrate; adjusted to pH7.0 with concentrated NaOH.
20 x SSPE : 3.6M NaCl, 0.2M NaH₂PO₄, 20mM EDTA; adjusted to pH7.4 with concentrated NaOH.
50 x Denhardt's Solution : 1% (w:v) Ficoll, 1% (w:v) polyvinylpyrrolidone, 1% (w:v) BSA (Pentax Fraction V); filter-sterilised through a 0.45μm Acrodisc filter and stored in aliquots at -20°C.
RNase A : 5mgml⁻¹. Heated to 100°C for 15 minutes, allowed to cool slowly to room temperature and stored in aliquots at -20°C.
10 X Tris-glycine running buffer : 3% (w:v) Tris base, 14.4% (w:v) glycine; adjusted to pH8.6 with concentrated NH₄OH.
Phenol: Redistilled under nitrogen into TE. For extractions of DNA solutions phenol was equilibrated with 1M Tris-HCl (pH8.0) and then TE buffer. 8-Hydroxyquinoline was added to 0.1% (w:v) and aliquots stored at -20°C. For extractions of RNA solutions phenol equilibrated with dH₂O was used.

Chloroform: Chloroform and iso-amyl alcohol mixed 24:1 (v:v); stored in closed bottles at room temperature.

2.1.2 GROWTH MEDIA

All quantities listed are for 1 litre final volume, unless otherwise stated. Solutions are autoclaved (see section 2.2.2) and stored at room temperature. Antibiotic and amino acid stock solutions were added to media immediately before use.

L-broth (LB): 10g Difco Bacto tryptone, 5g Bacto yeast extract, 10g NaCl; adjusted to pH7.2 with NaOH.

LB-agar: LB plus 15g Difco agar.

BBL-agar: 10g Baltimore Biological Laboratories trypticase, 5g NaCl, 10g Difco agar.

BBL-top agar: as for BBL-agar, but with only 6.5g Difco agar.
Spizizen minimal medium: 2g \((NH_4)_2SO_4\), 14g \(K_2HPO_4\), 6g \(KH_2PO_4\), 1g Na₃citrate.2H₂O, 0.2g MgSO₄.7H₂O with the following supplements as required: 0.2% (w:v) sugars, 2mg vitamin B₁, 20mg amino acids, 5mg nicotinic acid.

Spizizen minimal agar: Spizizen minimal medium plus 15g Difco agar.

Bacterial buffer: 3g \(KH_2PO_4\), 7g \(Na_2HPO_4\), 4g \(NaCl\), 0.2g \(MgSO_4.7H_2O\).

Phage buffer: 3g \(KH_2PO_4\), 7g \(Na_2HPO_4\), 5g \(NaCl\), 10ml 0.1M \(MgSO_4\), 10ml 0.01M \(CaCl_2\), 1ml 1% (w:v) gelatin.

Antibiotics: When required, the media were supplemented with the following antibiotics at the stated final concentrations.

- Ampicillin 100\(\mu g/ml^{-1}\)
- Chloramphenicol 50\(\mu g/ml^{-1}\)
- Streptomycin 200\(\mu g/ml^{-1}\)

2.1.3 BACTERIAL STRAINS

All strains were derivatives of \(E. coli\) K12.
### Table 2.1

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<td>Messing <em>et al</em> (1981)</td>
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<td>TGI</td>
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<td>hsdD5/F' traD36,</td>
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HB101  $F^-, \text{pro}A2, \text{lacY}I, \text{ara}14$, Boyer and Roulland-
$\text{gal}K2, \text{xy}l-5, \text{mt}1-1$,  Dussoix (1969)
$\text{rpsL}(\text{Str}^r), \text{recA}13$,
$\text{hsd}20(\text{rB}-\text{mb}^-)$.

2.1.4  BACTERIOPHAGE

Table 2.2

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### 2.1.5 PLASMIDS

#### Table 2.3

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<td>A. Nicolaidis</td>
</tr>
<tr>
<td>pSKS106</td>
<td>Plac(^+) vector for expression of proteins fused to ( \beta )-galactosidase via multiple cloning sites in the 5' end of lacZ gene. Ap(^r)</td>
<td>Shapira et al (1983)</td>
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<tr>
<td>pRAM1</td>
<td>pSKS106 with 277 bp HindIII -XmaI fragment from M13mp19-5, cloned between the same sites in the multiple cloning region. Produces a ( rpoB' - 'lacZ)-encoded fusion protein. Ap(^r)</td>
<td>This work</td>
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pRAM2 Analogue of pRAMI carrying This work 169 bp *HindIII*-*XmaI* fragment from M13mp19-9. Contains entire deletion of RNase III target site of *rplL*-rpoB region.

Apr

pRAM3 Analogue of pRAMI, carrying This work 174bp *HindIII*-*XmaI* fragment from M13mp19-6. Contains partial deletion of RNase III target site. Apr

pRAM4 Analogue of pRAMI, carrying This work 247bp *HindIII*-*XmaI* fragment from M13mp19-11. Contains partial deletion of RNase III target site. Apr

pMRMI Analogue of pRAMI, carrying M. Martin 271bp *HindIII*-*XmaI* fragment (This work) from M13mp19-10. Deletes two copies of UUU triplet from RNase III target site.

Apr
2.2 GENERAL METHODS

2.2.1 GENERAL GUIDELINES

All procedures were carried out at room temperature, unless otherwise stated.

For RNA work, all materials were handled with gloved hands and all glassware was soaked in 0.1% (v:v) DEP solution at 37°C for 16 hours and baked at 250°C for 16 hours prior to use.

All small-scale procedures were performed in 1.5ml polypropylene microfuge tubes (unless otherwise stated). These were sterilised by autoclaving at 120°C, 15psi for 15 minutes. Large-scale procedures were performed in 15ml or 30ml Corex tubes (sterilised by baking at 250°C for 16 hours) or in 50ml Falcon Tubes (sterile as purchased). Liquids were dispensed using Gilson Pipetman P-20, P-200 and P-1000 automatic pipettors and tips (sterilised as for microfuge tubes) or glass pipettes (sterilised as for Corex tubes).

2.2.2 PREPARATION AND STERILISATION OF SOLUTIONS

All solutions were prepared using dH₂O and (for stock solutions) were filtered through Whatman No. 1. filter paper. Most solutions were sterilised either by
autoclaving at 120°C, 15psi for 15 minutes or by filtration through Acrodisc filter-sterilisation units (0.45μm pore size, Gelman Sciences). Solutions were stored at room temperature, unless otherwise stated.

2.2.3 DEIONISATION OF SOLUTIONS

Where required (as stated in particular methods) solutions were deionised by treatment with 0.1 volume of mixed-bed ion-exchange resin (20-50 mesh, Bio-Rad Laboratories) for 60 minutes at room temperature. The resin was removed by filtration through Whatman No. 1 filter paper.

2.2.4 DIALYSIS

Dialysis tubing (Visking 8/32") was prepared by boiling in a large volume of 2% (w:v) sodium bicarbonate 1mM EDTA for 10 minutes, rinsing thoroughly in dH₂O and boiling for a further 10 minutes in dH₂O. It was stored at 4°C in 50% (v:v) ethanol and was washed thoroughly with dH₂O before use. Tubing was always handled with gloved hands.

2.2.5 DEPROTEINISATION OF NUCLEIC ACID SOLUTIONS

Aqueous nucleic acid solutions were deproteinised by extraction with phenol and chloroform. An equal volume
of phenol/chloroform (1:1, (v:v)) was added to the nucleic acid solution and the phases mixed by vortexing. The phases were then separated by centrifugation in a microfuge (11,600g) or Sorvall SS34 rotor at 10,000 rpm (12,000g) for 3-5 minutes and the upper (aqueous) phase transferred to a fresh tube. Extraction was repeated as required. The procedure was then repeated with chloroform to remove any residual phenol. Nucleic acids were subsequently ethanol precipitated (see section 2.2.6) which removed any residual chloroform.

2.2.6 ETHANOL PRECIPITATION OF NUCLEIC ACIDS

To precipitate nucleic acids from an aqueous solution, 0.1 volumes of 3M sodium acetate (pH5.2) or 0.025 volumes of 4M NaCl and 2-3 volumes of ethanol were added and the mixture incubated at -70°C for 15 minutes or at -20°C for 1 hour. The precipitated nucleic acids were recovered by centrifugation in a microfuge (11,600g) or Sorvall SS34 rotor at 10,000 rpm (12,000g) for > 15 minutes at 4°C. The pellet was washed by resuspension in 70% (v:v) ethanol and recovered by centrifugation, as before. Following the wash step, the supernatant was drained off and the pellet dried under vacuum. The nucleic acid could then be resuspended in the desired volume of the appropriate buffer.
2.2.7 QUANTITATION OF NUCLEIC ACIDS

The A260 and A280 of the nucleic acid solution were determined by UV spectrophotometry. Purity was estimated by the ratio of A260 to A280. Concentration was estimated by assuming that an A260 of 1.0 corresponds to 50μg/ml⁻¹ for double stranded DNA and to 40μg/ml⁻¹ for SS DNA or RNA. Alternatively the concentration of a double stranded DNA solution was estimated by comparison of the test DNA with that of standard (known) DNA solutions in an agarose gel after staining with EtBr (see section 2.9.1).

2.2.9 AUTORADIOGRAPHY

[32P] labelled nucleic acids, whether in gels or on filters, were detected by exposure to X-ray film (DuPont Cronex or Amersham Hyperfilm MP) in light-proof lead-shielded cassettes. Depending on the amount of radioactivity present, one of the following methods of exposure was used (in order of increasing sensitivity): i) at room temperature; ii) at -70°C with a calcium-tungstate intensifying screen; iii) with a prefloashed film at -70°C with an intensifying screen.

[35S] labelled nucleic acids, in a fixed and dried gel, were detected by exposure to X-ray film (Cronex) at room temperature.
[³H] labelled nucleic acids, in a gel impregnated with Enlightning (DuPont) and then dried, were detected by exposure to preflashed Xray film (Hyperfilm MP) at −70°C.
2.3 PHAGE TECHNIQUES

2.3.1 PREPARATION OF PLATING CELLS AND \(\lambda\) TITRATIONS

For \(\lambda\) titrations a fresh 5ml overnight culture, grown from a single bacterial colony in LB at 37°C, was diluted twenty-fold into 20ml of prewarmed LB and grown at 37°C to a concentration of \(2-5 \times 10^8\) cells ml\(^{-1}\). Cells were then harvested by centrifugation in a MSE Minor 'S' bench-top centrifuge at 4100 rpm (2480g) for 5 minutes and resuspended in 10ml of 10mM MgSO\(_4\). These cells were then stored at 4°C.

\(\lambda\) phage were tenfold serially diluted in phage buffer. 0.1ml of these phage dilutions plus 0.2ml of cells were mixed and incubated at 37°C for 20 minutes to allow adsorption of the bacteriophage particles to the bacteria. 3ml of molten BBL top agar were added and the mixture poured onto a fresh BBL agar plate. The top agar was allowed to set at room temperature before the plate was inverted and incubated at 37°C overnight. Plaques were then scored.

2.3.2 \(\lambda\) PLATE LYSATES

Using a sterile toothpick, a single fresh plaque was picked into 1ml of phage buffer. Two drops of chloroform were added and mixed, before incubation at
37°C for 30 minutes. 0.1ml of phage suspension was added to 0.2ml of cells (prepared as described in section 2.3.1) and incubated at 37°C for 20 minutes. 3ml of heated BBL top agar was added, mixed and poured onto a fresh, moist LB agar plate. The plate was incubated at 37°C until confluent lysis was achieved (6 - 8 hours). The agar was then covered with 4ml of LB and the plate incubated at 4°C overnight. The next day, the broth was harvested, mixed with 2 - 3 drops of chloroform and clarified by centrifugation in a SS34 rotor at 8000 rpm (8000g) at 4°C for 10 minutes. The supernatant was carefully drawn off and titrated.

2.3.3 LIQUID LYSATES

A fresh overnight culture was diluted 40fold in 200ml prewarmed LB, supplemented with 10mM MgSO₄, and grown at 37°C to an A₆₅₀ of 0.45 - 0.6. Phage (prepared as described in section 2.3.2) were then added to give an MOI of 1. The A₆₅₀ of the culture was monitored until it reached a minimum (due to cell lysis). At this point, 0.5ml of chloroform was added and the lysate shaken for a further 10 minutes, before being clarified by centrifugation in 250ml plastic bottles in a GS-A rotor at 7000 rpm (8000g) at 4°C for 10 minutes. The supernatant was stored at 4°C with 20µl of chloroform per ml., and was titrated at this stage.
The phage were concentrated by sedimentation in a MSE ultracentrifuge at 21,000 rpm (40,000g) at 4°C for 3 hours and the pelleted phage resuspended in 0.05 volumes of phage buffer by shaking gently at 4°C. After further clarification by centrifugation in a SS34 rotor at 9000 rpm (10,000g) at 4°C for 15 minutes, the supernatant was treated with RNase A and DNase I (both at a final concentration of 10μgml⁻¹) at 20°C for 2 hours.

2.3.4 PHAGE PURIFICATION

In order to purify the phage, the nuclease-treated supernatant (see section 2.3.3) was separated on a CsCl step gradient. Three steps of CsCl in phage buffer were used, with densities of 1.3, 1.5 and 1.7gml⁻¹ (31%, 45% and 56% (w:w), respectively). The 1.7gml⁻¹ CsCl solution was loaded into a 14ml MSE polycarbonate tube first. The less dense solutions were then carefully overlaid using a syringe. The supernatant was loaded onto the surface of the gradient. The tubes were then placed in a MSE 6 x 14 Ti swing-out rotor and spun in a MSE ultracentrifuge at 33,000 rpm (140,000g) for 2 hours. After centrifugation, the visible phage band was collected through the side of the tube using a syringe fitted with a 19G needle. CsCl was removed from the collected phage band by dialysis against phage buffer at 4°C for one hour.
2.3.5 \( \lambda \) DNA PREPARATION

\( \lambda \) DNA was prepared from the dialysed phage (see section 2.3.4) by phenol extraction (three times). The final aqueous layer was then dialysed against several changes of TE buffer at 4°C over a 24 hour period. The phage DNA was precipitated with ethanol and the dried pellet resuspended in a desired volume of TE. DNA concentration and purity were estimated by UV spectrophotometry at 260nm and 280nm.

2.3.6 M13 PHAGE TITRATIONS

M13 phage were tenfold serially diluted in phage buffer. 10\( \mu l \) of these phage dilutions plus 0.2ml of a fresh overnight culture were added to 3ml of molten BBL top agar, containing 20\( \mu l \) of IPTG (24mgml\(^{-1}\)) and 30\( \mu l \) of Xgal (24mgml\(^{-1}\) in dimethylformamide). This mixture was then poured onto a fresh BBL agar plate. The top agar was allowed to set before the plate was inverted and incubated at 37°C overnight. This procedure was repeated if further plaque purification was required.

2.3.7 LARGE SCALE M13 PHAGE ISOLATION

5ml of LB, inoculated with a single fresh M13 plaque (prepared as described in section 2.3.6), and a separate 30ml host cell culture, were grown overnight at 37°C.
The following day the cell culture was diluted 25-fold in 400ml LB and grown at 37°C to an $A_{650}$ of 0.2. 200μl of the overnight phage culture were then added (MOI of 0.1 - 0.5) and the cells grown at 37°C for a further 5 hours. The cells were then collected by centrifugation in 500ml plastic bottles in a GS-3 rotor at 8000 rpm (10,000g) at 4°C for 10 minutes and the supernatant removed to a fresh plastic bottle. 8g of PEG 6000 and 11.7g of NaCl were added to the supernatant and thoroughly dissolved before the bottle was left on ice overnight.

The next day the sample was centrifuged in a GS-3 rotor at 8000 rpm (10,000g) and 4°C for 20 minutes, to harvest the phage. The resultant pellet was resuspended in 5ml of phage buffer. Any remaining cell debris was removed by centrifugation in a SS34 rotor at 8000 rpm (8000g) at 4°C for 10 minutes. This final supernatant was separated on a CsCl step gradient. Four steps of CsCl in phage buffer were used with densities of 1.25, 1.30, 1.35 and 1.40gml$^{-1}$ (27.0%, 31.2%, 35.2%, 38.8% (w:w) respectively). The 1.40gml$^{-1}$ CsCl solution was loaded first into a 14ml MSE polycarbonate tube and the others were carefully overlaid using a syringe. The phage-containing supernatant was gently loaded onto the surface of the gradient. Ultracentrifugation was then carried out exactly as described in section 2.3.4. The visible M13 phage band was then collected through the side of the tube using a syringe with a 19G needle. The
CsCl was removed from the sample by dialysis against several changes of phage buffer over a 12 hour period at 4°C.

2.3.8 LARGE SCALE M13 SINGLE-STRANDED DNA PREPARATION

DNA was prepared from the dialysed phage (see 2.3.7) by phenol extraction (three times) followed by ether extraction (twice) and finally precipitation of the phage DNA with ethanol. The dried pellet was resuspended in TE, and DNA concentration and purity were estimated by UV spectrophotometry at 260nm and 280nm.

2.3.9 PREPARATION OF SMALL QUANTITIES OF M13 SINGLE-STRANDED DNA

An overnight host bacterial culture was diluted 100-fold in LB and 1ml of this was inoculated with a fresh M13 plaque (prepared as described in section 2.3.6) and grown for 5 hours at 37°C. The culture was transferred to a microfuge tube and spun for 3 minutes in a microfuge (11,600g). The supernatant was carefully decanted into a fresh microfuge tube and 200μl of PEG/NaCl solution (20% (w:v) PEG 6000, 2.5M NaCl) added. The tube was vortexed and then transferred to ice for 20 minutes before the precipitated phage particles were recovered by centrifugation in a microfuge (11,6000g) for 5 minutes. The supernatant was drained off and discarded. The tube
was spun again for 1 minute (as above) and any remaining supernatant carefully removed. The pellet was then resuspended in 100μl TE and extracted once with an equal volume of phenol, before the DNA was ethanol precipitated. The dried DNA pellet was redissolved in 30μl TE and then stored at -20° C.
2.4 PLASMID TECHNIQUES

2.4.1 PREPARATION OF SMALL QUANTITIES OF PLASMID DNA

(modified from Birnboim and Doly 1979)

1.5 ml of an overnight culture (grown from an isolated colony in LB and under antibiotic selection) were transferred into a microfuge tube and spun for 2 minutes in a microfuge (11,600g). The supernatant was removed and discarded and the pellet resuspended by vortex-mixing in 100 μl of cold lysis solution (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose, 4 mg ml⁻¹ lysozyme) and left at room temperature for 5 minutes. 200 μl of freshly prepared alkaline – SDS solution (0.2 M NaOH, 1% (w:v) SDS) were then added, the samples mixed by inversion, and placed on ice for 5 minutes to allow completion of cell lysis and the selective denaturation of chromosomal DNA. 150 μl of cold potassium acetate pH 4.8 (3 M with respect to potassium and 5 M with respect to acetate) were added and the sample returned to ice for a further 5 minutes. During this time most of the chromosomal DNA aggregated into an insoluble network. It was removed, along with the co-precipitated high molecular weight RNA and proteins, by centrifugation in a microfuge (11,600g) for 5 minutes at 4°C. 400 μl of the supernatant were transferred to a fresh microfuge tube and extracted with an equal volume of phenol:chloroform. Nucleic acids were ethanol precipitated and the dried
pellet resuspended in 30µl of TE, containing RNase A at a final concentration of 20µgml⁻¹.

2.4.2 PREPARATION OF LARGE QUANTITIES OF PLASMID DNA

200ml of LB (plus appropriate antibiotic) were inoculated with a single bacterial colony and incubated at 37°C overnight. Cells were harvested by centrifugation in a GS-A rotor at 7000 rpm (8000g) at 4°C for 20 minutes, and the resultant pellet washed in 0.5 volume of cold TE buffer before being spun again, as before. This pellet was resuspended in 3ml of sucrose buffer (25% (w:v) sucrose, 50mM Tris-HCl pH8.0) and placed on ice. 1ml of lysozyme solution (10mgml⁻¹) was added and the tube left on ice for 5 minutes with frequent agitation. 1ml of 0.5M EDTA (pH8.5) and 0.4ml of RNase A (10mgml⁻¹) were added, mixed and left on ice for a further 5 minutes. 5ml of Triton solution were then added (0.1% (v:v) Triton X-100, 65mM EDTA pH 8.5, 50mM Tris-HCl pH8.0) and, after a further 10 minutes on ice, the debris from the lysed cells was pelleted by centrifugation in a SS34 rotor at 15,000 rpm (27,000g) at 4°C for 30 minutes.

Plasmid DNA was then isolated by equilibrium centrifugation in CsCl-EtBr. 9g of CsCl were added to the (9ml) supernatant and dissolved thoroughly before the further addition of 0.9ml EtBr (5mgml⁻¹). This solution
was transferred to a Beckman 12ml polyallomer tube which was sealed and centrifuged in a Beckman Ti50 rotor at 38,000 rpm (90,000g) for approximately 60 hours at 18°C. After centrifugation DNA bands were clearly visible when the tube was observed under long wave UV light. The lower (plasmid DNA) band was collected through the side of the tube, using a syringe and 19G needle. EtBr was removed from the sample by extracting three times with 0.5 volumes of isopropanol (equilibrated with saturated CsCl solution). The DNA solution was then dialysed against several changes of TE over a 24 hour period. The DNA was ethanol precipitated, and dried pellets resuspended in a desired volume of TE. DNA purity and concentration were estimated by measuring the UV absorbance at 260nm and 280nm.

2.4.3 PREPARATION OF SMALL QUANTITIES OF M13 DNA (RF)

An overnight culture was diluted 100-fold in LB. 2ml of this were inoculated with a fresh M13 plaque (prepared as described in section 2.3.6) and grown for 4 hours at 37°C. 1.5ml of the infected culture were transferred to a microfuge tube. The M13 DNA was isolated as described in section 2.4.1.
1ml of LB, inoculated with a fresh M13 plaque, as well as a 5ml cell culture, were grown overnight at 37°C. The cell culture was diluted 20-fold and grown to an $A_{650}$ of 0.5. 200ml of LB was then inoculated with 2ml of this culture and grown again to an $A_{650}$ of 0.5. 200μl of the overnight phage culture were then added and growth continued for a further 4 hours. The M13 DNA was isolated as described in section 2.4.2.
2.5 BACTERIAL TECHNIQUES

2.5.1 PREPARATION OF COMPETENT CELLS

An overnight culture, grown at 37°C in LB, was diluted 50-fold in prewarmed LB and grown to an A₆₅₀ of 0.5. The cells were cooled on ice for 20 minutes before being harvested by centrifugation in a MSE Minor 'S' bench-top centrifuge at 4100 rpm (2480g) for 10 minutes. The resultant pellet was washed in 0.5 volumes of 0.1M MgCl₂ and spun as before. The cells were then resuspended in 0.5 volumes of 0.1M CaCl₂ and left on ice for 30 minutes before being centrifuged as before, resuspended in 0.05 volumes of 0.1M CaCl₂ and returned to ice for a minimum of 1 hour before being used.

Competent cells were also prepared in advance and stored frozen for future use. In this case the final pellet, instead of being resuspended in 0.05 volumes of 0.1M CaCl₂, was resuspended in 0.05 volumes of MOPS (pH6.5), 50mM CaCl₂, 20% (v:v) glycerol, incubated on ice for 30 minutes, and stored in aliquots at -70°C. Before use these frozen aliquots were slowly thawed on ice.
2.5.2 TRANSFECTION AND TRANSFORMATION WITH DNA

Transfections were performed by adding up to 100ng of M13 DNA to 200μl of competent cells and incubating on ice for 30 minutes (to allow non-specific adsorption of DNA). The cells were then heat-shocked at 42°C for 2 minutes (to promote DNA uptake) before being returned to ice for a further 5 minutes. Samples were plated by adding 200μl of log-phase cells, 20μl IPTG (24mgml⁻¹) 30μl X-gal (24mgml⁻¹ in dimethylformamide) and 3ml of molten BBL top agar. This was mixed and poured as described in section 2.3.6.

For transformations, up to 50ng of plasmid DNA were added to 200μl of competent cells and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 2 minutes and chilled briefly on ice. 1ml of LB was then added and the samples incubated at 37°C for one hour, to allow expression of plasmid-encoded antibiotic resistance. 200μl aliquots were then spread on selective plates and incubated at 37°C overnight.
2.6 DNA TECHNIQUES

2.6.1 RESTRICTION ENDONUCLEASE DIGESTION

The restriction enzymes and buffers used during this work are shown in Table 2.4. Generally DNA was incubated with a two-fold excess of restriction endonuclease in the appropriate buffer as recommended by the enzyme's manufacturer. Incubations were generally of 60–90 minutes duration at the temperature recommended by the manufacturer. Reactions were stopped by heat-inactivation of the enzyme at 65°C for 10 minutes and/or phenol:chloroform extraction.
Table 2.4 **RESTRICTION ENZYMES AND BUFFERS**

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Manufacturer</th>
<th>Reaction Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccI</td>
<td>(Boehringer)</td>
<td>A</td>
</tr>
<tr>
<td>Clal</td>
<td>&quot;</td>
<td>H</td>
</tr>
<tr>
<td>EcoRI</td>
<td>&quot;</td>
<td>H</td>
</tr>
<tr>
<td>HindIII</td>
<td>&quot;</td>
<td>B</td>
</tr>
<tr>
<td>NarI</td>
<td>(New England Biolabs)</td>
<td>(1)</td>
</tr>
<tr>
<td>SalI</td>
<td>(Boehringer)</td>
<td>H</td>
</tr>
<tr>
<td>SmaI</td>
<td>&quot;</td>
<td>A</td>
</tr>
<tr>
<td>StuI</td>
<td>&quot;</td>
<td>B</td>
</tr>
<tr>
<td>XmaI</td>
<td>(New England Biolabs)</td>
<td>(2)</td>
</tr>
<tr>
<td>XmnI</td>
<td>&quot;</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Buffers A, B and H: supplied by Boehringer

Buffers (1), (2) and (3): made in the lab.

Buffer A (pH7.9) (33mM Tris acetate, 10mM Mg-acetate, 66mM K-acetate, 0.5M DTT)

Buffer B (pH8.0) (10mM Tris-HCl, 5mM MgCl₂, 100mM NaCl, 1mM β-mercaptoethanol).

Buffer H (pH7.5) (50mM Tris-HCl, 10mM MgCl₂, 100mM NaCl, 1mM β-mercaptoethanol)

(1) (6mM Tris-HCl pH7.4, 6mM MgCl₂, 100mM DTE, 0.01% (w:v) BSA).

(2) (10mM Tris-HCl pH7.5, 10mM MgCl₂, 25mM NaCl, 10mM β-mercaptoethanol, 0.01% (w:v) BSA).

(3) (10mM Tris-HCl pH8.0, 10mM MgCl₂, 10mM NaCl, 10mM β-mercaptoethanol 0.01% (w:v) BSA).
2.6.2 DNA LIGATION

Ligations were generally performed in a small volume (10 - 20μl total volume) containing digested DNA (generally 10 - 150μgml⁻¹ final concentration) and 0.1 volumes 10 x ligase cocktail (0.75M Tris-HCl pH7.8, 0.2M MgCl₂, 0.2M DTT, 10mM ATP, 0.01% (w:v) BSA). T4 DNA ligase (1 - 5 units) was added and the mixture incubated at 15°C overnight, or at room temperature for 4 hours. A portion of this reaction mix was then used directly for transformation of E. coli cells (see section 2.5.2).

2.6.3 DNA SEQUENCING (Sanger et al 1977, Biggin et al 1983)

DNA sequencing was carried out by the dideoxy nucleotide chain termination method on DNA cloned into M13mp10, M13mp18 or M13mp19 vectors (Messing et al 1977, Norrander et al 1983).

5μl of M13 single-stranded template DNA (prepared as described in section 2.3.9) were mixed with 5μl of primer mix [3μl universal sequencing primer 2.5μgml⁻¹, 1μl TM (100mM Tris-HCl pH8.5, 50mM MgCl₂), 1μl dH₂O] in a microfuge tube and annealed at 60°C for 1 hour. The samples were cooled by spinning briefly in a microfuge and were then split into 4 separate microfuge tubes (containing 2μl of primer : template mix per tube). 2μl
of the appropriate dideoxynucleotide mix (Tmix, Cmix, Gmix or Amix - see Table 2.5) were added to the appropriate tube followed by 2μl of Klenow mix (10mM Tris-HCl pH8.5, 10mM DTT, 4μCi [35S]-dATP, 1.5 units E. coli DNA polymerase I Klenow large fragment, dH2O to 8μl - sufficient for 4 reactions), and then the reaction started by spinning briefly to bring all solutions to the bottom of the microfuge tube. The reactions were left at room temperature for 20 minutes before 2μl of dNTP chase solution (0.25M each of dTTP, dCTP, dGTP and dATP) were added and the reactions left for a further 20 minutes. 2μl of formamide dye mix (100ml deionised formamide, 0.1g xylene cyanol FF, 0.1g bromophenol blue, 2ml 0.5M EDTA pH8.5) were added to each tube and the samples heated to 100°C for 3 minutes. Electrophoresis of the samples was then carried out on a buffer-gradient denaturing polyacrylamide gel as described in section 2.9.8.

As a method for rapidly screening several M13 plaques a slight modification of this protocol can be used. Only one dideoxynucleotide mix was used for each test template DNA (generally A or T-termed A-tracking or T-tracking respectively). Thereafter the protocol described above was followed exactly. In Fig. 2.1 a representative autoradiograph displays results of this tracking technique.
<table>
<thead>
<tr>
<th>Components</th>
<th>Tmix</th>
<th>Cmix</th>
<th>Gmix</th>
<th>Amix</th>
</tr>
</thead>
<tbody>
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<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0.5mM dCTP</td>
<td>500</td>
<td>25</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0.5mM dGTP</td>
<td>500</td>
<td>500</td>
<td>25</td>
<td>500</td>
</tr>
<tr>
<td>10mM ddTTP</td>
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<td>-</td>
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<tr>
<td>10mM ddCTP</td>
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<td>10mM ddGTP</td>
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<td>-</td>
</tr>
<tr>
<td>10mM ddATP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

TE 1000 1000 1000 500

Quantities given are in μl
Fig. 2.1

A representative autoradiograph displaying the results of A-track screening of candidate M13 mutants. Mut. is the deletion mutant M13mp19-11, produced by site-directed mutagenesis of M13mp19-5, and identified by this procedure. All other tracks are "parental" M13mp19-5.
2.7 RNA TECHNIQUES

2.7.1 PREPARATION OF RNA (Salser et al 1967, Shaw and Guest, 1982)

200 ml minimal cultures of the appropriate bacterial strains were grown to the desired A_{650} (generally 0.15 - 0.3). Where appropriate, cultures were treated with rifampicin (at 5μgml^{-1} final concentration) and grown for a further 15, 30 or 60 minutes. Cells were then harvested by centrifugation in a GS-A rotor at 7000 rpm (8000g) at 4°C for 10 minutes, washed in 20 ml TE and spun again, as above. After resuspension in 1 ml of cold 10 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl (pH7.3) the sample was transferred to a microfugetube. Lysozyme was added to a final concentration of 300 μgml^{-1} and the sample frozen at -70°C for 30 minutes. Upon thawing, 110 μl of 10% (w:v) SDS was added and the tube incubated at 64°C until the turbidity of the solution dropped (usually about 10 minutes). 40 μl of 3 M sodium acetate (pH5.2) were then added and the sample split between two microfuge tubes. Extraction with one volume of phenol was carried out by agitation at 64°C for 5 minutes and the phenol:aqueous phases separated by centrifugation in a microcentrifuge (11,600g) for 4 minutes. The aqueous layer was removed and phenol-extracted once more, as above, before ethanol precipitation. Pelleted RNA was washed 3 - 4 times in 70% (v:v) ethanol, 10 mM Tris-HCl pH7.5, 10 mM NaCl. RNA
concentration and purity were estimated by UV spectrophotometry at 260nm and 280nm.

2.7.2 **LABELLING AND ISOLATION OF RNA** (Dunn and Studier 1973)

Minimal cultures of the appropriate bacterial strains were grown to an A_{650} of 0.2. At this point small volumes (e.g. 2ml) were labelled with [{^5-^3}H] uridine at 20μCi/ml for 5 minutes. Rifampicin was then added to a final concentration of 100μg/ml. Immediately before, and 5 and 10 minutes after the addition of the drug, 0.5ml samples of the labelled culture were transferred to 1ml of 0.02M NaN₃ on ice. The cells were harvested by spinning in a microfuge (11,600g) for 2 minutes and resuspended in 20μl of 50mM Tris-HCl (pH6.8), 1% (w:v) SDS, 10% (v:v) glycerol, 0.14M β-mercaptoethanol. They were then placed in a boiling water bath for 2 minutes and transferred to ice. Labelled rRNA, extracted by this technique, is readily separated by electrophoresis on agarose/acrylamide composite gels (see section 2.9.5) and can be detected by autoradiography.
2.7.3 **LABELLING AND PREPARATION OF RNA** (Zengel *et al.* 1980)

Minimal cultures of the appropriate bacterial strains were grown to the desired $A_{650}$ (0.15 - 0.3). Where appropriate, cultures were treated with rifampicin (5μgml$^{-1}$ final concentration) and grown for a further 15, 30 or 60 minutes. 20ml samples were labelled for 60 seconds with [5-\textsuperscript{3}H] uridine at a concentration of 20μCi ml$^{-1}$. An equal volume of TSE1 (0.02M Tris-HCl pH7.4, 0.2M NaCl, 0.04M EDTA, 1% (w:v) SDS) preheated to 100°C, was added and the samples maintained at 100°C for a further 2 minutes. The samples were then allowed to cool to 20°C, extracted three times with phenol and precipitated twice with ethanol.
2.8 PROTEIN TECHNIQUES

2.8.1 PROTEIN LABELLING AND EXTRACTION

Cultures were grown exponentially in the appropriate minimal medium to the desired $A_{650}$ (0.15 - 0.3). Samples were pulse-labelled at various times thereafter, with or without addition of rifampicin at 5μgml⁻¹ final concentration. A 0.75ml aliquot was labelled for 2.5 minutes with L-[4,5⁻³H] leucine at a concentration of 20μCi/ml⁻¹. Unlabelled leucine was then added to 200μgml⁻¹ and growth continued for 3 minutes to allow completion of nascent labelled polypeptides. NaN₃ was then added to 20mM and the sample chilled on ice. The cells were collected by microcentrifugation (11,600g) for 2 minutes, washed by resuspension in Matzura buffer (plus leucine): 62.5mM Tris-HCl pH6.8, 0.1mM DTT leucine 200μgml⁻¹ : and spun as before. The cells were resuspended in 10μl of Matzura buffer (plus leucine) and lysed in 30μl SDS sample buffer (62.5mM Tris-HCl pH6.8, 3% (w:v) SDS, 10% (v:v) glycerol, 0.735M β-mercaptoethanol, 0.02% (w:v) bromophenol blue) at 100°C for 3 minutes, before being subjected to SDS-polyacrylamide gel electrophoresis (see section 2.9.9).

If required, cells were also labelled with L-[1⁻¹⁴C]-leucine at 0.5μCi/ml⁻¹ for approximately two generations prior to [³H]-leucine pulse-labelling.
Incorporation of $^{14}$C into protein was complete within about one generation.

Unlabelled cell extracts were prepared in essentially the same way as described above. 0.75ml aliquots were removed and NaN$_3$ added to 20mM. Samples were then chilled on ice and cells collected by microcentrifugation (11,600g) for 2 minutes. The cells were washed in Matzura buffer (without added leucine) and centrifuged as before. They were then resuspended in 10μl Matzura buffer and lysed and treated exactly as described above.
2.9 GEL ELECTROPHORESIS

2.9.1 HORIZONTAL AGAROSE GEL ELECTROPHORESIS

Horizontal 0.6 - 2.0% agarose slab gels were prepared by dissolving the correct concentration of agarose in 1 x TAE or 1 x TBE buffer in a microwave oven. The solution was allowed to cool to approximately 50°C before being poured into one of several possible gel formers with their combs in place (Bethesda Research Laboratories; 20 x 25cm (20 well); 11 x 14cm (14 well); 50 x 75mm (8 well)). The gel was allowed to set before being placed in the gel apparatus containing sufficient buffer (1 x TAE or 1 x TBE) to just cover the gel. 0.1 volume of loading buffer (50% (v:v) glycerol, 0.5%, (w:v) xylene cyanol FF, 0.5% (w:v) bromophenol blue, 10 x TAE or TBE) was added to the DNA sample prior to loading onto the submerged gel using a Gilson pipette. The gel was run at the desired voltage. After electrophoresis the gel was stained in EtBr (1μgml⁻¹) for 30 minutes, destained in water for 30 minutes and the DNA bands visualised using a short wavelength UV transilluminator. If required the gel was photographed using Polaroid 667 positive film and a suitable red filter.
2.9.2 **RECOVERY OF DNA FROM AGAROSE GELS**  
* (Ground Glass Method) 

DNA fragments were recovered from excised agarose gel slices using the "Geneclean" kit (BIO 101 Inc) precisely as stated in the manufacturer's instruction sheet.

2.9.3 **NONDENATURED POLYACRYLAMIDE GEL ELECTROPHORESIS**

DNA fragments of <500 bp were routinely separated on a 10% nondenaturing polyacrylamide gel. 30ml of a 10% acrylamide solution was prepared as follows. 10ml of 30% acrylamide solution (29% (w:v) acrylamide, 1% (w:v) bisacrylamide). 3ml 10 x TBE buffer, 250μl 10% (w:v) AMPS, dH2O to 30ml.

Two glass plates separated along their edges by plastic spacers were clamped together to create a mould of dimensions 25 x 14 x 0.1cm. Three edges were sealed with water agarose (0.5% (w:v) agarose in dH2O). 25μl of TEMED were then added to the 10% acrylamide solution and immediately poured into this glass sandwich. A 15 tooth comb was inserted and the gel left to polymerise for at least 60 minutes in an upright position. When polymerisation was complete the comb and bottom spacer were removed and the wells immediately rinsed out with 1 x TBE to remove any remaining acrylamide solution. The
gel was attached to an electrophoresis tank containing 1 x TBE in the upper and lower reservoirs. Care was taken to flush out the wells again, thoroughly and to remove any air bubbles trapped beneath the gel plates.

DNA samples for analysis were ethanol precipitated, dried and resuspended in 20μl TE. 10μl of loading buffer (0.5 x TBE, 0.125M EDTA, 0.1% (w:v) bromophenol blue, 50% (v:v) glycerol) were added to each sample before it was carefully loaded onto the gel using a Hamilton microsyringe. Electrophoresis was carried out at 150 - 200V until the bromophenol blue had migrated the appropriate distance. The gel was removed from the electrophoresis tank and stained in a solution of EtBr (1μg/ml) for 30 minutes, before being observed using a long wavelength UV transilluminator.

2.9.4 RECOVERY OF DNA FRAGMENTS FROM POLYACRYLAMIDE GELS

(Maniatis et al 1982)

Once located the DNA band of interest was excised from the polyacrylamide gel using a razor blade, and placed on a glass plate. The acrylamide slice was chopped into fine pieces with a razor blade and the fragments transferred to a microfuge tube where they were crushed against the wall of the tube using a disposable pipette tip. One volume of elution buffer (0.5M ammonium acetate, 1mM EDTA pH8.0) was added and the tube
incubated at 37°C overnight on a rotating wheel. The next day the sample was spun in a microfuge (11,600g) for 10 minutes. The supernatant was recovered and an additional 0.5 volume of elution buffer added to the pellet, which was then vortexed and recentrifuged as before. The two supernatants were combined, the DNA precipitated with ethanol and the pellet resuspended in 200μl TE. The DNA was precipitated once again, the pellet washed in 70% (v:v) ethanol and dried.

2.9.5 **COMPOSITE AGAROSE : POLYACRYLAMIDE GEL ELECTROPHORESIS** (Dunn and Studier, 1973).

To analyse high molecular weight RNA a 2% polyacrylamide gel plus 0.5% agarose was used. The agarose was added to strengthen the gel, making it easier to handle. A 50ml solution of this composite gel was prepared as follows: 2.5ml of 40% acrylamide solution (38% (w:v) acrylamide, 2% (w:v) bisacrylamide) 5ml of 10x sodium phosphate buffer (1M sodium phosphate pH6.8, 1% (w:v) SDS, 0.02M EDTA), dH₂O to 25ml. A solution of water agarose (1% (w:v) agarose in dH₂O) was made up separately. This water agarose was heated in a microwave oven and allowed to cool to 50°C before adding 25ml of it to the acrylamide solution and mixing both by swirling. 250μl of 10% (w:v) AMPS and 25μl of TEMED were added to the agarose/acrylamide solution, which was
then poured between two glass plates as described in section 2.9.3.

When polymerisation was complete the comb and bottom spacer were removed and the wells carefully rinsed out with 1 x sodium phosphate buffer. The gel was then attached to an electrophoresis tank containing 1 x sodium phosphate buffer in the upper and lower reservoirs. Any trapped air bubbles were removed from beneath the gel and the wells once more carefully flushed out with 1 x sodium phosphate buffer.

Labelled extracts (prepared as described in section 2.7.2) were loaded onto the gel using a Hamilton microsyringe. The gel was run at 70V for 3 hours. Afterwards it was impregnated with Enlightning and dried under vacuum. The radiolabelled rRNA bands were visualised after autoradiography as described in section 2.2.8.

2.9.6 **DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS**

Denaturing polyacrylamide gel electrophoresis was used to fractionate the products of S1 nuclease mapping and primer extension analyses. For these purposes 6% polyacrylamide/7.2M urea gels were used. To obtain gels of the desired acrylamide/urea concentrations, 9/10 volume stock solutions were prepared (e.g. 6.67% (w:v)
acrylamide:bisacrylamide (20:1), 8M urea for a final gel of 6% polyacrylamide/7.2M urea) deionised (see section 2.2.3), filtered and stored at 4°C. Gels were prepared by mixing 45ml of the stock solution with 5ml 10 x TBE buffer, 250μl 10% (w:v) AMPS and 25μl of TEMED and poured using a syringe into a 40cm x 20cm x 0.4mm mould formed by two glass plates separated by Plastikard strips (sealed at the edges with PVC tape). The flat side of a Sharkstooth comb (Bethesda Research Laboratories : either 24 sample, 6mm spacing or 48 sample, 3mm spacing) was inserted approximately 0.5cm into the top of the gel, which was then left to polymerise, for at least 60 minutes, laid at an angle a few degrees from the horizontal. After polymerisation was complete, the comb and PVC tape sealing the bottom edges of the plates were removed. The flat surface on top of the gel was washed with 1 x TBE and the Sharkstooth comb reinserted with its teeth just sticking into the gel surface. The gel was then placed in the electrophoresis tank filled with 1 x TBE buffer. After the gel was pre-electrophoresed for 40 minutes, the samples were denatured by heating to 100°C for 3 minutes in formamide dye mix (see section 2.6.3) or urea load buffer (10M urea, 1 x TBE, 0.2% (w:v) bromophenol blue, 0.2% (w:v) xylene cyanol; filter sterilised and stored at -20°C) and loaded using a drawn-out glass capillary. Electrophoresis was continued at 40W until the marker dyes had migrated the appropriate distance. At this point the gel plates were removed
from the electrophoresis tank and separated. The gel (attached to one glass plate) was covered with Saran Wrap and autoradiographed (see section 2.2.8).

2.9.7 **HIGH PERCENTAGE DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS**

20% polyacrylamide/8M urea gels were used to analyse radioactively labelled oligonucleotides (labelled as described in section 2.12.2). A 50ml solution of 20% polyacrylamide/8M urea was prepared as follows: 5ml 10 x TBE, 25ml 40% acrylamide solution (38% (w:v) acrylamide, 2% (w:v) bisacrylamide), 24g ultra-pure urea and dH2O to 50ml. 250µl 10% (w:v) AMPS and 25µl of TEMED were added and the gel poured, treated and pre-run exactly as described in section 2.9.6. Samples were denatured and loaded (as described in 2.9.6) and electrophoresis continued at 40W until the marker dyes had migrated the appropriate distance. After electrophoresis the gel was treated as described in section 2.9.6.

2.9.8 **BUFFER-GRADIENT DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS** (Biggin et al 1983)

Buffer-gradient denaturing polyacrylamide gels were used to fractionate the products of DNA sequencing reactions (see section 2.6.3). They are poured in such a way that there is a progressive increase in the
concentration of buffer towards the lower part of the gel. Because the voltage carried through the gel decreases as ionic strength increases, then the DNA moves more slowly as it approaches the bottom of the gel. This lessens the spacing between adjacent DNA bands in this region, allowing more sequence to be read from a single gel.

For each gel two solutions were prepared containing the following components: Solution A: 7ml 2.5 x TBE gel mix (150ml 40% acrylamide (38% (w:v) acrylamide, 2% (w:v) bisacrylamide) 200ml 10 x TBE, 460g urea, 50g sucrose, 50mg bromophenol blue, dH₂O to 1 litre), 14μl 25% (w:v) AMPS, 14μl TEMED.

Solution B: 35ml 0.5 x TBE gel mix (150ml 40% acrylamide (as above), 50ml 10 x TBE, 460g urea, dH₂O to 1 litre), 70μl 25% (w:v) AMPS, 70μl TEMED.

Using a 10ml pipette and "Pumpette", 4ml of Solution B was taken up, followed by 6ml of Solution A. A gradient was established by allowing 3 - 4 air bubbles up into the pipette, through the interface between Solutions A and B. This mixture was then poured into a glass gel sandwich (see section 2.9.6). The rest of Solution B was then taken up in a 20ml syringe and used to fill the remaining space. A Sharkstooth comb was inserted and the gel treated as described in section 2.9.6. DNA
sequencing reaction products (section 2.6.3) were loaded onto the gel using a drawn-out glass capillary and the gel run at 40W until the marker dyes had migrated the appropriate distance.

Following electrophoresis the plates were removed from the electrophoresis tank and separated. The gel, still attached to one plate, was fixed in a solution of 10% (v:v) methanol and 10% (v:v) glacial acetic acid for 15 minutes. It was then transferred to blotting paper, covered with Saran Wrap, dried using a heated vacuum gel drier and autoradiographed (see section 2.2.8).

2.9.9 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

(Laemmli 1970)

Proteins were separated on 5% linear or, more usually, 5 - 15% gradient SDS polyacrylamide gels. To prepare a 5% linear gel a 5% separating gel mix was prepared: 5ml 30% acrylamide solution (29.2% (w:v) acrylamide, 0.8% (w:v) bisacrylamide), 7.5ml 4 x lower gel buffer (1.5M Tris-HCl pH8.0, 0.4% (w:v) SDS), 25μl 25% (w:v) AMPS and dH2O to 30ml. 20μl TEMED was then added and the solution poured into a 25 x 14 x 0.1cm glass sandwich, pre-sealed with plastic spacers and 0.5% (w:v) water agarose, to within 5cm of the top of the notched plate. The gel solution was gently overlaid with H2O - saturated butan-2-ol and left to polymerise in
an upright position for at least 60 minutes. A 5% stacking gel mix was then prepared: 1.5 ml 30% acrylamide (as above) 2.25 ml 4 x upper gel buffer (0.5 M Tris-HCl pH 6.8, 0.4% (w:v) SDS) 10 μl 25% (w:v) AMPS and dH2O to 9 ml.

The H2O-saturated butan-2-ol was poured off the polymerised separating gel and the surface washed with 1 x upper gel buffer. 10 μl TEMED was then added to the stacking gel solution and this mix was poured onto the separating gel. A 15 tooth comb was inserted and the gel left to polymerise as before. Once polymerisation was complete the comb and bottom plastic spacer were removed. The wells were washed out with 1 x Tris-glycine running buffer plus 0.5% (w:v) SDS and the gel attached to the electrophoresis tank filled with this same 1 x Tris-glycine buffer. Any air bubbles trapped beneath the gel were removed before the protein samples (prepared as described in section 2.8.1) were loaded using a Hamilton microsyringe, and the gel run at 60 - 90 V overnight.

To prepare a 5 - 15% gradient SDS polyacrylamide gel, two 15 ml acrylamide solutions were prepared separately. 5% solution: 15 ml of this solution was prepared as described above. 15% solution: 7.5 ml 30% acrylamide solution (as above), 3.75 ml 4 x lower gel
buffer, 25μl 15% (w:v) AMPS, 20μl TEMED and dH₂O to 15ml.

The solutions were poured into a two chambered gradient maker which was used to mix a 5 - 15% gradient into a 25 x 14 x 0.1cm glass sandwich (as above). Thereafter, this gel was treated exactly as described above. Generally 5 - 15% gradient gels were run at 100V overnight.

Following electrophoresis, the protein gel was removed from the glass plates and fixed and stained in staining solution (50% (v:v) methanol, 10% (v:v) glacial acetic acid, 0.1% (w:v) Coomassie brilliant blue) at 37°C for 30 minutes. The staining solution was then removed and the gel washed in destaining solution (10% (v:v) methanol, 10% (v:v) glacial acetic acid) at 37°C for several hours.

If the protein samples were labelled with ³H-leucine (or double-labelled with ³H-leucine and ¹⁴C-leucine) then, at this stage, the gel was transferred to a clean glass plate, and the identified protein bands excised from the gel using a razor blade. Each piece of excised gel was then sliced into 1mm wide slices on a specially made razor blade stack. Each slice was transferred to 3ml of solubilising scintillation fluid (Petri Scintillant) (1 litre of Petri Scintillant comprises :
900ml toluene, 3.6g PPO, 101mg dimethyl POPOP, 100ml Soluene (Packard), 0.9ml 10% (w:v) SDS and dH₂O to 1 litre) and incubated, with shaking, at 37°C overnight. The next day samples were counted in a Beckman scintillation counter.

Once sufficiently destained, gels containing proteins not radioactively labelled were transferred to blotting paper, covered with Saran Wrap and dried using a heated vacuum gel dryer.
2.10 **β-GALACTOSIDASE ASSAY** (modified from Miller, 1972)

Assays of β-galactosidase activity were performed on AK14 strains harbouring pSKS106 (Shapira *et al.*, 1983) or its derivatives constructed during this work. AK14 carries lacIq, therefore lacZ expression must be induced by the addition of IPTG to the growth medium.

Assays were performed after 'long-term induction' (i.e. continuous growth of the culture in the presence of IPTG at a concentration of $10^{-5}$ M, for at least 18 hours) and 'short-term induction' (addition of IPTG at $10^{-3}$ M for 45 minutes).

Cells were grown in the appropriate medium to an $A_{600}$ of 0.3 and then assayed for β-galactosidase activity. Each assay contained 0.8 ml PM2 reducing buffer (0.1 M sodium phosphate pH 7.0, 1 mM MgSO$_4$, 0.2 mM MnSO$_4$, 100 mM β-mercaptoethanol), 0.2 ml 0.1% (w:v) CETAB, 0.01 ml 1% (w:v) sodium deoxycholate, 1 ml of a suitable dilution of cells (defined empirically) and 0.6 ml ONPG (13.5 mM in 1 x Spizizen salts). Assays were incubated at 28°C and the reaction stopped by the addition of 1.3 ml 1 M Na$_2$CO$_3$ once sufficient colour had developed (generally $A_{420}$ 0.3 – 0.8).
2.11 NUCLEIC ACID HYBRIDISATION

2.11.1 LABELLING OF DNA PROBES BY RANDOM PRIMING

(Feinberg and Vogelstein 1984)

This method permits the labelling of isolated DNA fragments to high specific activity and can even be used to label a DNA fragment in low melting-point agarose (LMP agarose) without the need for purification of the DNA.

Digested DNA was electrophoretically separated as described in section 2.9.1., but using 0.6% LMP agarose (Bethesda Research Laboratories). After staining with EtBr, and destaining, the gel was observed on a long wavelength UV transilluminator and the required band excised cleanly. The gel slice was weighed, dH₂O added (3ml per gram of gel) and the sample heated in a boiling water bath for 7 minutes to melt the LMP agarose and denature the DNA. This solution could either be used immediately for the labelling reaction or stored at -20°C (Before further use, such a stored sample was reboiled for 3 minutes).

To label the DNA the following reagents were added in the order stated to a microcentrifuge tube: dH₂O to a final volume of 50μl, 10μl oligonucleotide-labelling buffer (A : B : C mixed in the ratios 2 : 5 : 3 (v:v)) where A : 1.25M Tris-HCl pH8.0, 0.125M MgCl₂, 0.5mM each
of dATP, dTTP, dGTP and 0.25mM β-mercaptoethanol; B: 2M Hepes-NaOH pH6.6; C: 4.5mg/ml-1 random hexadeoxyxynucleotide pd(N)₆ mixture (in TE), 2μl BSA (10mg/ml-1), DNA in LMP agarose (up to 32.5μl containing 20 - 100ng DNA), 50μCi of α[³²P] - dCTP and 2 units of Klenow fragment. The mixture was incubated at room temperature for 2.5 - 18 hours to allow polymerisation to occur and the reaction then stopped by the addition of 200μl 'stop solution' (20mM NaCl, 20mM Tris-HCl pH7.5, 2mM EDTA, 0.25% (w:v) SDS, 1μM dCTP).

2.11.2 LABELLING OF DNA BY FILLING-IN OF RECESSED 3' TERMINI

DNA fragments which have been cut with restriction endonucleases to generate recessed 3' termini can subsequently be radiolabelled. If required, the cut DNA may be isolated and purified by a variety of techniques (as detailed in sections 2.9.1 - 2.9.4) although DNA can also be labelled directly after digestion (without any subsequent isolation/purification steps).

Which dNTPs are added to the labelling reaction depends on the sequence of the protruding 5' termini. During the course of this work all 4 dNTPs were required. Routinely, approximately 300ng of digested DNA were labelled by mixing 1μl 10 X TM (100mM Tris-HCl pH8.0, 100mM MgCl₂), 2μl of dGTP, dATP, dTTP mix (each at 1mM),
10μCi of α-[32P]-dCTP, 1 unit of Klenow fragment and dH2O to 10μl. This mixture was incubated at 37°C for 30 minutes, then extracted once with phenol, and ethanol precipitated.

2.11.3 LABELLING OF DNA OLIGONUCLEOTIDE PROBES

Oligonucleotide DNA was 5'-end labelled for use as a probe as follows: 30μCi γ[32P]-ATP was vacuum desiccated in a microfuge tube, to which were added 1.5μl (10pmolμl⁻¹) oligonucleotide DNA, 3μl 10X kinase buffer (500mM Tris-HCl pH8.0, 100mM MgCl₂), 1μl 100mM DTT and 24μl dH₂O. 2μl (20 units) of T4 polynucleotide kinase were added and the reaction incubated at 37°C for 30 minutes. After this, the probe was crudely purified by diluting with 3ml 6x SSC and passing the solution through a 0.45μm Acrodisc filter (Gelman Sciences). The filter was then washed with 1ml 6x SSC and the resulting 4ml of probe were stored frozen in a petri dish. This purification procedure is intended only to avoid the possibility of generating "hot-spots" on the hybridisation filters; it did not remove unincorporated label, but was nonetheless adequate for the purposes required.
2.11.4 PLAQUE BLOTTING

Transfected or duplicate stabbed plates were used as the source of plaques in this method. 2 - 3 day old BBL plates were used, and were well dried before use. The plates were incubated overnight in the usual way, before being chilled at 4°C for at least one hour to allow the top agar to harden. The nitrocellulose filters (Schleicher and Schuell, 82mm diameter, 0.45μm pore size) required no preparation prior to use, but were carefully marked to ensure easy orientation with the parent plate afterwards. A dry filter was carefully laid onto the surface of the plate for 90 seconds and then placed on a pad of blotting paper soaked in 0.5M NaOH (the surface that had been in contact with the plaques being placed uppermost). After 5 minutes the filter was transferred in sequence into the following solutions in a series of four petri dishes, allowing 20 seconds in each petri dish: a) 0.1M NaOH, 1.5M NaCl b) 0.5M Tris-HCl pH7.5, 1.5M NaCl c) repeat of b, d) 2 x SSC. Filters were dried at 37°C before being baked in a vacuum over at 80°C for 2 hours.

2.11.5 HYBRIDISATION OF PLAQUE BLOTS WITH LABELLED OLIGONUCLEOTIDE PROBES

This method was used to detect mutants produced by site-directed mutagenesis (using the labelled mutagenic
oligonucleotide as a probe). Filters were prepared as described in section 2.11.4 and then prehybridised in 10 x Denhardt's solution, 6 x SSC and 0.2% (w:v) SDS at 65°C for 1 hour. Each filter, treated individually, was then rinsed in 6 x SSC for 5 minutes and placed (plaque side down) into the petri dish containing the radiolabelled oligonucleotide probe (see section 2.11.3) and left for 60 minutes at room temperature. Occasionally, during this hour, the filter was lifted and replaced in the probe solution, in an attempt to ensure even hybridisation. The hybridised filter was washed for 3 x 5 minutes in 6 x SSC at room temperature, placed on blotting paper to remove excess liquid, sandwiched between two layers of Saran Wrap and exposed to X-ray film. If increased stringency washes were required then the washing procedure was carried out in 6 x SSC for 5 minutes at 5°C below the calculated dissociation temperature (Td) of the oligonucleotide. The Td was calculated using the Wallace rule (which only applies to oligonucleotides under 6 x SSC washing conditions).

\[ Td \ (°C) = 4 \ (G + C) + 2(A + T) \]

Under 6 x SSC washing conditions, increased stringency can be achieved by gradually increasing the wash temperature to the Td, with autoradiography at each step.
2.11.6 COLONY BLOTTING

The screening of bacterial colonies to identify recombinant clones is performed by the method of Grunstein and Hogness (1975) as detailed by Maniatis et al (1982). Transformed *E. coli* colonies were picked, using sterile toothpicks, and streaked (in a grid pattern) on duplicate LB agar (plus selective antibiotic) plates and grown at 37°C overnight. One of these duplicate plates was stored at 4°C as the master plate. Colonies which carried the desired recombinant DNA sequences were later recovered from this plate. The other plate was used for blotting.

A nitrocellulose filter (Schleicher and Schuell, 82 mm diameter, 0.45μm pore size) was laid on top of the agar, causing the bacterial colonies to stick to it. The membrane was then removed and laid, colony side uppermost, onto a pad of blotting paper soaked with 1.5M NaCl, 0.5M NaOH for 5 minutes. This step was repeated with blotting paper soaked in 1M Tris-HCl pH8.0, 1.5M NaCl for 5 - 10 minutes and blotting paper soaked in 2 x SSC for 5 minutes. The filter was then dried at 37°C and baked at 80°C under vacuum for 2 hours.
2.11.7 HYBRIDISATION OF COLONY BLOTS WITH LABELLED DNA PROBES

Filters (prepared as described in section 2.11.6) were pre-wetted in hybridisation solution (5 x Denhardt's solution, 2 x SSC, 0.5% (w:v) SDS, 0.1mgml⁻¹ salmon testes DNA (sheared by repeated passage through an 18G needle and boiled for 5 minutes prior to use), 50% (v:v) deionised formamide) and then transferred to a bag containing approximately 20ml of the same hybridisation solution. The bag was sealed, taped to a tray and placed, shaking, at 37°C for 2 hours to allow prehybridisation of the filters. After this time the solution was discarded. It was replaced by approximately 20ml of fresh hybridisation solution containing the radioactive probe (see section 2.11.1) (The probe had been heated to 100°C for 5 minutes prior to use). The bag was resealed and incubated, shaking, at 37°C overnight. The following day the filters were removed and washed at 37°C as follows: 2 x 20 minutes in wash 1 (2 x SSC, 0.5% (w:v) SDS, 50% (v:v) deionised formamide), 2 x 30 minutes in wash 2 (2 x SSC, 0.5% (w:v) SDS) and 2 x 30 minutes in wash 3 (2 x SSC). Excess liquid was removed on blotting paper, after which the filters were sandwiched between 2 layers of Saran Wrap and autoradiographed (see section 2.2.8).
Nitrocellulose filters (Schleicher and Schuell, 24 mm diameter, 0.45μm pore size) were prepared by numbering them with pencil and then incubating them in dH₂O at 80°C for 10 minutes. The filters were then stored in 10 x SSPE at room temperature until required. They were loaded (using a slight negative pressure) with either 2μg of SS M13 DNA or 4μg of denatured RF M13 DNA (in each case in a total volume of 500μl). The RF M13 DNA was first denatured in 4ml of alkaline SSPE (15 x SSPE, 0.5M NaOH) at 80°C for 10 minutes. The samples were neutralised by the addition of 600μl 0.5M HCl and 600μl of 1M Tris-HCl pH7.4, then placed on ice prior to loading. The loaded filters were dried at 37°C and baked at 80°C under vacuum for 2 hours.

The filters were prehybridised as described in section 2.11.7 and then placed in glass vials (which had been siliconised, DEP treated and baked prior to use). Hybridisation solution (see section 2.11.7) was added to the vials (0.75ml per vial) followed by the appropriate amount of labelled RNA (0.5, 0.25 and 0.125 volumes of the total RNA preparation - as described in section 2.7.3). The vials were incubated, with gentle shaking, at 42°C overnight.
Following hybridisation, each vial was aspirated using a water pump fitted with a Pasteur pipette. The liquid was replaced with 2.5ml wash 1 (2 x SSC, 0.1% (w:v) SDS). The vials were shaken at room temperature for 15 minutes and the above step repeated. The filters were then transferred to a plastic sandwich-box and washed as follows: 2 x 1 hour at 65°C in wash 2 (0.1 x SSC, 0.1% (w:v) SDS), 2 x 20 minutes at 37°C in wash 3 (2 x SSC), 1 x 2 hours at 37°C in wash 4 (2 x SSC, 25µg/ml-1 RNase A, 10 U/ml-1 RNase T1), 2 x 20 minutes at 65°C in wash 5 (2 x SSC, 0.5% (w:v) SDS) and finally 2 x 20 minutes at room temperature in wash 6 (2 x SSC). The filters were then placed on blotting paper and dried at 37°C, before the retained counts were determined by liquid scintillation counting in 3ml (per filter) of 0.4% (w:v) butyl-PBD in toluene.

A measure of the total labelled RNA was obtained by spotting a small volume onto a nitrocellulose filter. Once dried (and without any washing procedure) this can be counted as described above.

2.11.9 S1 NUCLEASE MAPPING OF RNA (Maniatis et al 1982)

20µg of RNA (prepared as described in section 2.7.1), approximately 10^4 cpm of labelled DNA probe (see
section 2.11.2) and 150μg of *E. coli* tRNA (Boehringer) as carrier were added to a microfuge tube and precipitated with ethanol. The dried pellet was resuspended in 30μl of hybridisation buffer (40mM PIPES pH6.4, 1mM EDTA pH8.0, 0.4M NaCl, 80% (v:v) deionised formamide). Optimal denaturation and hybridisation conditions must be determined for each separate DNA : RNA reaction. In the S1 nuclease mapping experiments carried out during this work I used the conditions set out in Railing and Linn (1987). Samples were immersed in a water bath at 80°C for 10 minutes to denature the DNA, followed by hybridisation at 53°C for 3 hours. With the body of the microfuge tube still submerged in the 53°C water bath, 0.27ml of ice-cold S1 nuclease buffer, containing S1 nuclease, was added (0.28M NaCl, 0.05M sodium acetate pH4.6, 4.5mM ZnSO₄, 20μgml⁻¹ carrier SS DNA, 400 Uml⁻¹ S1 nuclease). The samples were then transferred to 37°C for 30 minutes, placed on ice and the reaction stopped by the addition of 50μl 'STOP' mix (4M ammonium acetate, 0.1M EDTA). Each sample was then extracted once with phenol/chloroform. A further 20μg of carrier tRNA were added to the aqueous phase, then precipitated with an equal volume of isopropanol. The dried pellet was resuspended in formamide dye mix (see section 2.6.3) or urea load buffer (see section 2.9.6), boiled for 3 minutes, and subjected to denaturing polyacrylamide gel electrophoresis (see section 2.9.6).
2.11.10 PRIMER EXTENSION ANALYSIS OF RNA

This technique was used to map the 5' termini and/or processing intermediates of mRNA. The test RNA was hybridised with a synthetic oligonucleotide DNA primer radiolabelled at its 5' terminus by phosphorylation (see section 2.11.3). Reverse transcriptase was then used to extend this primer to produce a cDNA which was complementary to the RNA template. The cDNA was analysed by denaturing polyacrylamide gel electrophoresis (see section 2.9.6).

The oligonucleotide DNA primer was 5' end-labelled as follows: 20pmoles oligonucleotide, 20μCi \( \gamma \left[ ^{32}P \right] \) ATP, 3μl 10 x kinase buffer (500mM Tris-HCl pH8.0, 100mM MgCl₂), 3μl 100mM DTT, 1.5 U T4 polynucleotide kinase and dH₂O to 30μl were mixed in a microfuge tube and incubated at 37°C for 15 minutes. The enzyme was heat-inactivated at 70°C for 10 minutes and the sample then stored at -20°C until required. Varying amounts of RNA (30 - 150μg), prepared as described in section 2.7.1, were mixed with varying amounts (0.5 - 2μl) of the labelled oligonucleotide primer mix (see above) and hybridisation mix (50mM Tris-HCl pH7.5, 75mM KCl, 3mM MgCl₂) in a final volume of 30μl. This was incubated for 30 minutes at 5°C below the calculated Td of the oligonucleotide (see section 2.11.5). The sample was then cooled quickly on ice and the following reagents added: 1μl 1M Tris -
HCl pH7.5, 1.5μl 0.2M MgCl₂, 5μl 0.1M DTT, 2.5μl dNTP mix (each dNTP at 10mM), 1μl BSA 5mgml⁻¹, 7.5μl dH₂O and 1.5 U M-MLV RTase (Moloney Murine Leukemia Virus Reverse Transcriptase, Gibco BRL).

These components were mixed well and the sample incubated at 37°C for 1 hour. After this time, 12.5μl 0.5M NaOH were added and the sample placed in a boiling water bath for 3 minutes, then transferred to ice. 12.5μl 0.5M HCl and 12.5μl of 1M Tris-HCl (pH7.4), were then added and the sample precipitated with ethanol. After washing in 66% (v:v) ethanol, the dried pellet was resuspended in urea load buffer (see section 2.9.6), boiled for 3 minutes and subjected to denaturing polyacrylamide gel electrophoresis (see section 2.9.6).
2.12 OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS

2.12.1 THE ECKSTEIN METHOD (Taylor et al 1985a, 1985b)

All oligonucleotide site-directed mutagenesis reactions carried out during the course of this work used the 'Eckstein Method'. A schematic outline of this method is shown in Fig. 2.2. This procedure was performed using an oligonucleotide-directed in vitro mutagenesis kit supplied by Amersham International plc, and the protocol described in the booklet they provide was followed exactly.

Before use, the size and purity of each custom synthesised oligonucleotide were checked (see section 2.12.2). Also, prior to each mutagenesis reaction, the fidelity of oligonucleotide binding and optimal conditions for oligonucleotide : template DNA annealing were ascertained by using the oligonucleotide as a primer, at varied template-primer ratios and different temperatures, for dideoxy-sequencing (generally A- or T-tracking reactions, see section 2.6.3).

2.12.2 CHECKING THE SIZE AND PURITY OF AN OLIGONUCLEOTIDE

All of the oligonucleotides used in this work were custom synthesised by the OSWEL DNA service (Department
Fig. 2.2

The Amersham oligonucleotide-directed \textit{in vitro} mutagenesis system: The Eckstein Method.
Mutant oligonucleotide.

M13 recombinant ssDNA template.

15 hours 16°C
12 units Klenow
12 units Ligase

Extension + ligation with dCTPqS.

Remove unwanted remaining ssDNA template by filtration.

5 units Ncil
37°C 90 minutes.

Exonuclease III digestion

50 units ExoIII
37°C 30 minutes.

Repolymerization

3 units DNA PolI
2 units T4 DNA ligase
16°C 3 hours

Transformation
of Chemistry, Edinburgh). A small quantity of oligonucleotide was phosphorylated using $[^{32}\text{P}]\text{ATP}$, by mixing the following components: 10μCi $[^{32}\text{P}]\text{ATP}$, 10p moles oligonucleotide, 1μl 10 x kinase buffer (see section 2.11.10), 1μl 100mM DTT, dH2O to 10μl, and then adding 1 unit of T4 polynucleotide kinase. The reaction was incubated at 37°C for 15 minutes. 5μl of formamide dye mix (see section 2.6.3) were added and the sample boiled for 3 minutes before being loaded onto a high percentage denaturing polyacrylamide gel and subjected to electrophoresis (see section 2.9.7).

An example of the results of this method is shown in Fig. 2.3. These oligonucleotides are all essentially pure and are all of the correct size as judged by comparison with a previously determined oligonucleotide marker.
Check on the size and purity of oligonucleotides

**Tracks**

1. 202A (15 mer) oligonucleotide size marker  
2. 203A (17 mer)  
3. 211A (15 mer)  
4. 218A (16 mer) - not used in this work  
5. 219A (25 mer)  
6. 608A (21 mer)
CHAPTER 3
COMPARATIVE ANALYSIS OF rpoBC EXPRESSION
IN rnc+ AND rnc105 E. COLI STRAINS

3.1 INTRODUCTION

The rpoBC genes are transcribed in an operon with four 50S ribosomal protein genes, rplKALJ, included upstream (reviewed by Yura and Ishihama 1979). Approximately 80% of all transcripts which emanate from promoters PL11 and PL10 are terminated in the tL7 region. S1 nuclease analysis of this readthrough mRNA reveals that it is normally cleaved at sites between tL7 and the rpoB start, and that this processing does not occur in an rnc105 (RNase III deficient) strain (Barry et al 1980). These cleavage sites have been accurately mapped to a region of potential mRNA secondary structure which resembles other known RNase III target sites (Barry et al 1980, Fukuda and Nagasawa-Fujimori 1983, Downing and Dennis 1987, Ralling and Linn 1987). This and all other work to date (including Morgan and Hayward 1987) suggests that cleavage is slow or incomplete.

Dennis (1984) showed that the lack of any detectable RNase III processing, in the rnc105 strain N2077, has no observable effect on the rates of rplJL or rpoBC mRNA production or of ββ' protein synthesis under steady state
growth conditions, when compared with the isogenic $rnc^+$ control N2076. However, Portier et al (1987) presented evidence that $\beta\beta'$ proteins form a higher percentage of total protein in an $rnc105$ mutant (BL321) than in its isogenic wild type partner (BL322), although they did not measure the actual synthetic rates of $\beta\beta'$.

I decided to re-examine the rates of $\beta\beta'$ synthesis during exponential, steady state growth in N2076 and N2077, and also to determine these rates in BL322 and BL321.

The $rpoBC$ genes are also subject to translational feedback regulation. The drug rifampicin can interfere with this post-transcriptional control mechanism, as has been clearly demonstrated by studies in vitro (Bass et al 1979, Kajitani et al 1980, Lang-Yang and Zubay 1981, Fukuda and Nagasawa-Fujimori 1983) and more tentatively by in vivo results (Bass et al 1979). Rifampicin also causes increased transcriptional readthrough of the $t_{L7}$ terminator. This effect may be an artefact, since all tested terminators show increased readthrough in the presence of rifampicin (Howe et al 1982, Newman et al 1982, Cromie and Hayward 1984). I decided to determine the rates of $rpoBC$ mRNA synthesis and $\beta\beta'$ protein production in $rnc^+$ and $rnc105$ strains (N2076 and N2077 respectively) following rifampicin treatment.
3.2 INITIAL CHARACTERISATION OF *rnc* AND *rnc*105 STRAINS

It is known that RNase III deficiency leads to a reduction in the growth rate of *E. coli* (Apirion and Watson 1975, Studier 1975). Table 3.1 confirms that the *rnc*105 strains BL321 and N2077 grow markedly more slowly than their isogenic *rnc*+ partners BL322 and N2076, under all conditions tested.

Table 3.1 CELL DOUBLING TIMES

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Medium and Temperature</th>
<th>Min 30°C</th>
<th>Min 37°C</th>
<th>ENR 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL322 (rnc+)</td>
<td>MIN30°C</td>
<td>125 (±3)</td>
<td>106 (±3)</td>
<td>61 (±1)</td>
</tr>
<tr>
<td>BL321 (rnc105)</td>
<td>MIN 37°C</td>
<td>176 (±3)</td>
<td>133 (±2)</td>
<td>90 (±1)</td>
</tr>
<tr>
<td>N2076 (rnc+)</td>
<td>ENR 37°C</td>
<td>118 (±3)</td>
<td>70 (±2)</td>
<td>55 (±1)</td>
</tr>
<tr>
<td>N2077 (rnc105)</td>
<td>MIN30°C</td>
<td>177 (±3)</td>
<td>118 (±1)</td>
<td>73 (±4)</td>
</tr>
</tbody>
</table>

a) The time taken for doubling of cell number is shown in minutes. The figures in brackets are standard errors; each result is the mean of at least three observations.

b) MIN is Spizizen minimal medium: ENR is the same, enriched with all standard amino acids except leucine.
One major reason for this growth defect is likely to be the impaired processing of the 30S ribosomal precursor RNA to 16S, 23S and smaller RNAs, in the absence of RNase III (Dunn and Studier 1973, Nikolaev et al 1973, King et al 1984). This phenomenon is a good diagnostic characteristic of \textit{rnc105} strains, which can be readily checked. I verified it for our stock of strain N2077. Cultures of N2076 and N2077 were subjected to $[^{3}H]$ uridine pulse-labelling followed by rifampicin addition at 100\mu g ml$^{-1}$ and RNA extraction as described in section 2.7.2. Samples were fractionated by special, composite agarose : polyacrylamide gel electrophoresis (see section 2.9.5) and the \textit{rRNA} species detected by sensitive autoradiography, after fluorography (see section 2.2.8). This technique showed the presence of a 30S RNA in N2077 which was not detectable in N2076 (see Fig. 3.1). Upon 'chasing' with non-radioactive uridine the label in the 30S band disappeared, almost completely after 5 minutes and entirely after 10 minutes, whilst that in the 16S and 23S bands increased. This fits the expectation of slower processing in an RNase III deficient strain relative to its wild type partner and confirms that our stock of N2077 is indeed RNase III deficient. It is known that whilst maturation of 16S rRNA can proceed without prior RNase III cleavage, such processing is absolutely required to allow mature 23S rRNA production. RNase III deficient strains contain a variety of pre-23S \textit{rRNA} species with additional 5' and 3' sequences. The
quality of the resolution of the RNA bands in Fig. 3.1 is too poor to permit the detection of discrete pre-23S rRNA species: however, there is an indication of more diffuse 23S rRNA bands in tracks 4 and 6 (N2077) than in the corresponding "wild type" tracks 3 and 5 (N2076).
Fractionation of labelled RNA preparations from N2076 and N2077 by composite 0.5% agarose : 2% polyacrylamide gel electrophoresis.

Tracks

M  Markers are pNA60/ClaI, which should yield three fragments (approximately 6kb, 5kb and 3.5kb). These were denatured in 30% (w:v) DMSO, 1mM EDTA, 0.05% bromophenol blue at 90°C for 2 minutes, chilled on ice and loaded onto the gel. Sizes are given in nt.

1  N2076 0min., no rifampicin
2  N2077 0min., no rifampicin
3  N2076 +5min., rifampicin (100μgml⁻¹)
4  N2077 +5min., rifampicin (100μgml⁻¹)
5  N2076 +10min., rifampicin (100μgml⁻¹)
6  N2077 +10min., rifampicin (100μgml⁻¹)

30S, 23S and 16S mark the positions of 30SrRNA, 23SrRNA and 16SrRNA respectively.
3.3 **DETERMINATION OF ββ' SYNTHESIS RATES DURING EXPONENTIAL, STEADY STATE GROWTH**

Since \( rnc^+ \) and \( rnc105 \) strains display different growth rates (Apirion and Watson 1975, Studier 1975, see Table 3.1) and since it is known that ββ' production is growth-rate-dependent (Iwakura *et al* 1974) then it is important to study the expression of these proteins in \( rnc^+ \) and \( rnc105 \) strains under a variety of growth rates. In this way the relative contributions of growth rate and \( rnc \) phenotype to the level of ββ' expression can be elucidated. This was achieved by growing the \( rnc^+ \) and \( rnc105 \) strains in two different media (minimal and enriched minimal) and at two temperatures (30°C and 37°C). This provided a set of experimental data by which \( rnc^+ \) and \( rnc105 \) strains could be compared when growing at the same rate, as well as when growing in the same conditions. It is interesting to note that under these various growth conditions the \( rnc^+ \) strains N2076 and BL322 never grew exponentially above \( A_{650} \) of 0.5, and the \( rnc105 \) strains N2077 and BL321 never grew exponentially above \( A_{650} \) 0.35. Because of this, the experiments with all four strains were started at relatively low cell densities (e.g. \( A_{650} = 0.16 \)).

At this point the appropriate strains, grown under the correct conditions, were pulse-labelled for 2.5 minutes with \([^3H] \) leucine. After a 3 minutes chase with
unlabelled leucine, to allow completion of nascent labelled polypeptides, the cells were collected, washed and lysed (as described in section 2.8.1). To normalise for any variations in the recovery of $\beta$ and $\beta'$ from the different samples, the $[^{3}\text{H}]$ leucine labelled cell extracts were then mixed with an aliquot of a "standard" cell extract, prepared from a single culture which had been pre-labelled for two generations with $[^{14}\text{C}]$ leucine. A small volume of this 'mixed extract' was set aside to be used in determining the level of 'total protein' in each sample. This then allows one to determine accurately the rates of $\beta\beta'$ synthesis relative to total protein in the different cultures.

The labelled cell extracts were fractionated on SDS polyacrylamide gels (see section 2.9.9). $\beta$ and $\beta'$ proteins are sufficiently well purified from N2076, N2077, BL322 and BL321 cell extracts by this method to allow direct assay by scintillation counting of the excised protein bands. The $^{3}\text{H}:^{14}\text{C}$ ratios in $\beta\beta'$ and total protein were determined by direct scintillation counting of samples. The differential rates of $\beta$ plus $\beta'$ syntheses are summarised in Tables 3.2.1 and 3.2.2. The amount of incorporation of $[^{3}\text{H}]$ leucine into $\beta$ was virtually the same as that into $\beta'$ in all strains and in all conditions (data not shown). Therefore the sum of $\beta$ and $\beta'$ is given for greater clarity.
Two general conclusions can be drawn from the results presented in Tables 3.2.1 and 3.2.2. Firstly, the differential rate of $\beta\beta'$ synthesis is not significantly affected by the rnc phenotype when one makes a comparison between N2076 and N2077, or between BL322 and BL321, at similar growth rates. Secondly there is, as expected, a modest growth-rate-dependent effect on the differential rate of $\beta\beta'$ synthesis in strains N2076 and N2077. The data for BL322 and BL321 are compatible with a similar effect, but are insufficiently precise to allow any assertion.
Table 3.2.1
Rate of $\beta\beta'$ synthesis as a percentage of total protein $^b$

<table>
<thead>
<tr>
<th>Cell Doubling Time $^a$</th>
<th>N2076 ($rnc^+$)</th>
<th>N2077 ($rnc105$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 ($\pm 1$)</td>
<td>0.79 ($\pm 0.24$)</td>
<td></td>
</tr>
<tr>
<td>73 ($\pm 4$)</td>
<td>0.68 ($\pm 0.01$) 0.72 ($\pm 0.06$)</td>
<td></td>
</tr>
<tr>
<td>118 ($\pm 3$)</td>
<td>0.64 ($\pm 0.24$) 0.54 ($\pm 0.04$)</td>
<td></td>
</tr>
<tr>
<td>177 ($\pm 3$)</td>
<td>0.48 ($\pm 0.09$)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2.2
Rate of $\beta\beta'$ synthesis as a percentage of total protein $^b$

<table>
<thead>
<tr>
<th>Cell Doubling Time $^a$</th>
<th>BL322 ($rnc^+$)</th>
<th>BL321 ($rnc105$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61 ($\pm 1$)</td>
<td>0.65 ($\pm 0.10$)</td>
<td></td>
</tr>
<tr>
<td>99 ($\pm 10)^1$</td>
<td>0.83 ($\pm 0.27$) 0.62 ($\pm 0.18$)</td>
<td></td>
</tr>
<tr>
<td>128.5 ($\pm 6.5)^2$</td>
<td>0.58 ($\pm 0.07$) 0.65 ($\pm 0.10$)</td>
<td></td>
</tr>
<tr>
<td>176 ($\pm 3$)</td>
<td>0.52 ($\pm 0.09$)</td>
<td></td>
</tr>
</tbody>
</table>
1) BL322 and BL321, respectively, 106 (± 3) and 90 (± 1): see Table 3.1.

2) BL322 and BL321, respectively, 125 (± 3) and 133 (± 2): see Table 3.1.

a) Cell doubling time is shown in minutes. Except as noted (1,2) the figures in brackets are standard errors, and each result is the mean of at least three observations. Growth conditions are those shown in Table 3.1.

b) ββ' protein levels are expressed as a percentage of the total protein level i.e. \( \frac{(\text{H}^3/\text{C}^{14}) \text{ in } \beta\beta'}{(\text{H}^3/\text{C}^{14}) \text{ in total protein}} \times 100 \). The figures in brackets are ranges; each result is the mean of two independent experiments.
3.4 **DETERMINATION OF ββ' SYNTHESIS RATES FOLLOWING RIFAMPICIN ADDITION**

As described in Chapter 1, addition of sub-bacteriostatic quantities of the drug rifampicin, which binds to the β subunit of RNA polymerase and aborts initiation, leads to an increase in synthesis of β, β' and other RNA polymerase subunits in exponential cultures of sensitive *E. coli*. I compared the kinetics of this ββ' response in the strains N2076 and N2077 (rnc* and rnc105 respectively).

These experiments involved exponential growth of N2076 and N2077 with addition of rifampicin at $A_{650} = 0.16$. The low drug concentration used (5μg/ml) had no detectable effect on growth rate for at least 90 minutes after addition, and similarly no measurable effect on [³H] leucine incorporation into total protein for at least 60 minutes after addition (data not shown). Samples were pulse-labelled with [³H] leucine immediately before, and at various times after drug addition (as well as in parallel drug-free controls). Results were normalised using [¹⁴C] leucine which had been added to the culture two generations prior to [³H] leucine pulse-labelling, to serve as an internal control.
Incorporation of $^{14}$C leucine into protein had been complete within about one generation.

The differential rates of $\beta$ and $\beta'$ protein syntheses prior to, and following, rifampicin addition are shown for N2076 (Table 3.3) and N2077 (Table 3.4). Since $\beta$ and $\beta'$ subunits showed essentially parallel responses to rifampicin in both strains and all growth conditions, the results for $\beta$ plus $\beta'$ are again taken together and expressed graphically (as $\beta\beta'$) in Fig. 3.2.

The drug-free control data confirm that the cultures were in steady state exponential growth throughout the experiments (and that the $^{14}$C labelled control $\beta\beta'$ proteins were stable). The $rnc^+$ (N2076) strain shows the expected rapid increase in $\beta\beta'$ synthesis following drug addition, the kinetics being somewhat slower at the lowest growth rate. The extent of the effect (a 2 - 3 fold increase in the rate of $\beta\beta'$ synthesis) is very similar to that seen previously for example by Nakamura and Yura (1976) using low rifampicin concentrations in the drug-sensitive strain KY1400, or by Hayward and Fyfe (1978a) using the rif$s$/rif$r$ merodiploid RSH74. The $rnc105$ strain (N2077) also shows the effect, but the kinetics are quite different. This is particularly striking if one compares the two strains growing at the same rate [Fig. 3.2 a) and e) and especially Fig. 3.2 b) and f)], but is also true if one compares the two strains
growing under the same conditions (marginally so in Fig. 3.2 a) and d), but more clearly in Fig. 3.2 b) and e), and, particularly at early time points, in Fig. 3.2 c) and f)]. It is clear that the rifampicin effect is expressed more slowly in the rnc105 mutant, N2077, than in its isogenic rnc+ partner, N2076.
Table 3.3

β and β' synthesis rates in N2076 (rnc*). Synthesis rates are given relative to the rate at zero minutes (designated 1). -Rif. (no rifampicin added); +Rif. (rifampicin added at 5μgml⁻¹). Growth conditions are: MIN 30°C (minimal medium at 30°C), MIN 37°C (minimal medium at 37°C), ENR 37°C (enriched medium at 37°C). The drug-free control experiments were carried out only once (except for the zero time points). The results for N2076 ENR 37°C +Rif. are the means of duplicate experiments. All other results are the means of three independent experiments. Ranges or standard errors are not shown (but see Fig. 3.2).
| Time | MIN 30 C | | | MIN 37 C | | | ENR 37 C | | |
|------|----------|-------|-------|----------|-------|---------|-------|-------|
|      | -Rif     | +Rif  |       | -Rif     | +Rif  |       | -Rif  | +Rif  |
|      | β         | β'    | β     | β'       | β     | β'     | β     | β'     |
| 0    | 1         | 1     | 1     | 1        | 1     | 1      | 1     | 1      |
| 5    | 0.97      | 0.99  | 1.07  | 1.02     | 0.92  | 0.87   | 1.16  | 1.18   |
|      | 0.90      | 0.87  | 1.47  | 1.64     | 0.94  | 0.95   | 1.40  | 1.43   |
| 15   | 0.92      | 0.90  | 1.61  | 1.83     | 0.88  | 0.93   | 1.64  | 1.55   |
| 20   | 0.95      | 0.92  | 1.87  | 2.05     | 0.93  | 0.94   | 1.63  | 2.06   |
| 30   | 1.02      | 1.08  | 1.5   | 1.6      | 0.88  | 0.86   | 2.40  | 2.33   |
|      | 1.01      | 1.01  | 1.74  | 1.86     | 0.96  | 0.96   | 1.98  | 1.97   |
| 45   | 0.93      | 0.91  | 1.93  | 1.74     | 0.85  | 0.93   | 2.43  | 2.57   |
|      | 1.02      | 0.93  | 2.90  | 2.96     | 0.98  | 1.00   | 1.95  | 2.21   |
Table 3.4

β and β' synthesis rates in N2077 (rnc105). Synthesis rates are given relative to the rate at zero minutes (designated 1). -Rif. (no rifampicin added); +Rif (rifampicin added at 5μgml⁻¹). Growth conditions are exactly as for Table 3.3. The drug-free control experiments were carried out only once (except for zero time points). The results of N2077 MIN 30°C +Rif. are the means of duplicate experiments. All other results are the means of three independent experiments. Ranges or standard errors are not shown (but see Fig. 3.2).
### Table 3.4

$\beta$ and $\beta'$ synthesis rates in N2077 (rncl05)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>MIN 30°C</th>
<th>MIN 37°C</th>
<th>ENR 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Rif</td>
<td>+Rif</td>
<td>-Rif</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.90</td>
<td>1.02</td>
<td>1.07</td>
</tr>
<tr>
<td>10</td>
<td>0.91</td>
<td>1.01</td>
<td>1.15</td>
</tr>
<tr>
<td>15</td>
<td>0.85</td>
<td>0.98</td>
<td>1.14</td>
</tr>
<tr>
<td>20</td>
<td>0.95</td>
<td>1.05</td>
<td>1.21</td>
</tr>
<tr>
<td>30</td>
<td>0.95</td>
<td>1.08</td>
<td>1.33</td>
</tr>
<tr>
<td>45</td>
<td>0.96</td>
<td>1.09</td>
<td>1.39</td>
</tr>
<tr>
<td>60</td>
<td>0.96</td>
<td>1.06</td>
<td>1.83</td>
</tr>
</tbody>
</table>
The differential rate of ββ' synthesis in N2076 (rnc+: a,b,c) and N2077 (rnc105: d,e,f) following the addition of rifampicin at 5μg/ml. Cultures were grown in minimal medium at 30°C (a,d) or 37°C (b,e), or in enriched medium at 37°C (c,f). Open circles: no rifampicin added; closed circles: 5μg rifampicin ml⁻¹ added at zero minutes. The heights of the vertical bars on the latter points represent twice the standard errors of the mean results, all of which were derived from three independent experiments (except for c and d, which show means and ranges derived from duplicate experiments). Where no bars are shown, the errors were less than ±0.04. The drug-free control experiments were carried out only once (except for the zero-time points). The vertical arrows link the rnc+ (upper panels) and rnc105 strains for comparison when growing under the same conditions. The diagonal arrows link them when growing at the same rates.
Fig 3.2
3.5 DETERMINATION OF \( rpoB \) AND \( rpoC \) mRNA SYNTHSESES

RATES FOLLOWING RIFAMPICIN ADDITION

Obviously one important, initial question is whether this delayed response of \( \beta\beta' \) synthesis in the \( rnc105 \) strain reflects a transcriptional or post-transcriptional effect. To determine this, I monitored the rates of synthesis of \( rpoB \) and \( rpoC \) mRNA, as well as control \( rps \) and \( rpl \) mRNAs, in both strains (N2076 and N2077) using the DNA : RNA filter hybridisation method described in section 2.11.8. Both strains were pulse-labelled with \([^{3}H]\) uridine before and after rifampicin addition. I studied N2076 in Spizizen minimal medium at 30°C and 37°C, and N2077 in minimal and enriched media at 37°C, to allow comparisons at two different doubling times (see Table 3.1), as well as in the same growth condition (minimal medium at 37°C). The culture absorbance at the time of rifampicin addition, and the drug concentration were exactly as in the protein labelling experiments. The low drug concentration used (5\( \mu \)g/ml\(^{-1} \)) had no detectable effect on \([^{3}H]\) uridine uptake into total RNA for at least 60 minutes after addition (data not shown).

The labelled RNA was extracted as described in section 2.7.3. Several DNA probes were used in these
filter hybridisation experiments. These are listed in Table 3.5.

Table 3.5

**DNA probes for filter hybridisation experiments**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene</th>
<th>DNA type</th>
<th>Hybridisation to mRNA</th>
<th>Probe size (nt)</th>
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<tbody>
<tr>
<td>M13mp8</td>
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<td>SS</td>
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<tr>
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<td>rpsMKD</td>
<td>SS</td>
<td>+</td>
<td>1320</td>
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<td>rplJL</td>
<td>SS</td>
<td>+</td>
<td>916</td>
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<tr>
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<td>rplJL</td>
<td>SS</td>
<td>-</td>
<td>916</td>
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<tr>
<td>M13mp18-11</td>
<td>rpoB</td>
<td>SS</td>
<td>+</td>
<td>1090</td>
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<td>M13mp18-12</td>
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<td>M13mp11-23</td>
<td>rpoC</td>
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<td>-</td>
<td>2542</td>
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</tbody>
</table>

a) (SS) is single stranded M13 DNA

b) (+) means the M13 SS DNA strand which is complementary to the gene's mRNA, and will hybridise. (-) means the M13 SS DNA strand which has the same sequence as the gene's mRNA, and should not hybridise.

c) Probe size refers to the length of bacterial DNA inserted into the M13 vector and is given in nucleotides (nt).
As can be seen from Table 3.5, the probes were all single-stranded M13 DNA, prepared from clones in M13mp vectors. M13mp8 SS DNA acts as a control for non-specific hybridisation; it contains no bacterial DNA insert and should have no sequence complementarity to bacterial messenger RNA. Filters without any DNA were included as further negative controls. M13mp11-5 was constructed as described in Meek and Hayward (1986), and serves as a positive control responding to mRNA sequences outwith the rpoBC operon. It binds to mRNA from the α operon: rpsMKD. I prepared the rplJL positive-hybridisation probe (M13mp18-9) by cloning a 916bp EcoRI-StuI DNA fragment (derived from \lambda DWM321) into M13mp18 between the EcoRI and SmaI sites. I made the complementary, negative-hybridisation probe (M13mp19-4) by cloning the same fragment in M13mp19. I constructed the rpoB positive- and negative-hybridisation probes by cloning the 1090bp SalI DNA fragment from \lambda DWM321 into the SalI site of M13mp18, in both orientations. In JM101, M13mp18-11 was unstable and readily generated deletions, whilst M13mp18-12 was stable. This phenomenon has been observed before with rpoB DNA sequences (Meek and Hayward 1986), where a 2873bp EcoRI rpoB insert was stable in one orientation in M13mp11, but highly unstable in the opposite orientation in the same vector. Interestingly the same 2873bp EcoRI DNA fragment is stable in both orientations when cloned into M13mp8 (Ralling and Linn 1987).
In the recA strain NM570, both M13mp18-11 and M13mp18-12 are stable. All my M13 SS DNA probe preparations were therefore made in this strain.

The DNA probes made during this work: M13mp18-9, M13mp19-4, M13mp18-11 and M13mp18-12 were all thoroughly checked by restriction endonuclease analysis (of their replicative form DNAs) to verify that, in each case, a single fragment of the correct size had been inserted; and by DNA sequencing, to ensure that the orientation of the insert was as expected. These results are presented in Figs. 3.3, 3.4 and 3.5.

The presence of excess probe DNA (immobilised on nitrocellulose filters) during these hybridisation experiments was verified by the linear relationship between input and hybridised counts, using increasing amounts of RNA, in every experiment. This is shown in Fig. 3.6.

The results of hybridisation of the labelled RNA to the various SS DNA probes are shown in Table 3.6 and graphically in Fig. 3.7.

From these data one can conclude that the rnc phenotype has no significant effect on the rates of synthesis of the rplJL, rpoB or rpoC mRNAs of the rpoBC
operon. Also, it does not significantly affect the efficiency of the partial terminator t_{L7} (lying between \textit{rplL} and \textit{rpoB}), nor the degree to which rifampicin increases readthrough of this terminator. Accordingly, the effect of \textit{rnc105} on the response of \beta\beta' synthesis to drug addition must be post-transcriptional.
Fig. 3.3

Restriction analysis of M13mp18-9 and M13mp19-4. 1% agarose gel electrophoresis. The upper part shows partial maps of M13mp18-9 and M13mp19-4 (not to scale). Open boxes represent the EcoRI - StuI fragment from the rpoBC operon cloned into M13mp18 and M13mp19. The lower part shows the agarose gel photograph.

Tracks

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The sizes of all DNA fragments are shown in kb.
Fig 3.3

**M13mp18-9**

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**M13mp19-4**

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**Gel**

- Lane M: 2.03, 1.90, 1.58, 1.33, 0.98, 0.83, 0.56, 0.6
Restriction analysis of M13mp18-11 and M13mp18-12. 1% agarose gel electrophoresis. The upper part shows partial maps of M13mp18-11 and M13mp18-12 (not to scale). Open boxes represent the SalI fragment from the rpoBC operon cloned into M13mp18 in both orientations. The lower part shows the agarose gel photograph.

Tracks

M  Markers are $\lambda$ / HindIII + EcoRI
1  M13mp18-11 - uncut
2  "  - SalI
3  "  - EcoRI
4  M13mp18-12 - uncut
5  "  - SalI
6  "  - EcoRI

The sizes of all DNA fragments are shown in kb.
Fig 3.4

M13mp18-12

\[ \text{EcoRI SalI} \quad \text{EcoRI} \quad \text{SalI} \]

\[ 0.59 \quad 1.08 \]

M13mp18-11

\[ \text{EcoRI SalI} \quad \text{EcoRI} \quad \text{SalI} \]

\[ 0.50 \quad 1.08 \]

M  1  2  3  4  5  6

2.03  1.90  1.58  1.33  0.98  0.83

0.56

-1.08

-0.59

-0.50
Fig. 3.5

DNA sequencing analysis of M13mp18-9, mp19-4, mp18-11 and mp18-12. Sequences are read 5' - 3', upwards from left to right. In each case a representative portion of the sequence (ten nucleotides) at the junction of M13 and the cloned insert is highlighted to indicate the orientation of the inserted fragment.
Hybridisation of pulse-labelled RNA to specific DNA probes. In every experiment the presence of excess probe DNA (immobilised on nitrocellulose filters) was verified by the linear relationship between input and hybridised counts, using increasing amounts of RNA. In each case, the RNA preparation was divided into three fractions (0.5, 0.25 and 0.125) and hybridised, in separate assays, to an excess of SS DNA probe. (1) is N2076 MIN 30°C (0, 15, 30 and 60 minutes after rifampicin addition at 5μg ml⁻¹) (2) is N2077 MIN 37°C (as above) (3) is N2076 MIN 37°C (as above) and (4) is N2077 ENR 37°C (as above). Growth conditions are as described in Table 3.6.

Symbols:-

- is rplJL mRNA
× is rpsMKD mRNA
○ is rpoB mRNA
● is rpoC mRNA
Fig 3.6

![Graphs showing cpm vs. time (0', 15', 30', 60') with increasing [RNA].](image-url)
<table>
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<tr>
<th>Genotype and Conditions(a)</th>
<th>Min after rif (b)</th>
<th>Input RNA (c)</th>
<th>RNA hybridised, normalised for probe length(d)</th>
<th>Efficiency of termination (%) (e)</th>
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<td>2.19</td>
<td>82</td>
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<td>1.56</td>
<td>84</td>
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<td>60</td>
<td>4.85</td>
<td>2.11</td>
<td>61</td>
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</table>

(a) MIN is minimal medium; ENR is the same, enriched with all standard amino acids except leucine. Growth rates for rnc<sup>+</sup>, MIN, 30° and rnc105, MIN, 37° were identical; as were those for rnc<sup>+</sup>, MIN, 37° and rnc105, ENR, 37°. "Zero time" samples were taken 1 min before drug addition.

(b) Minutes after addition of rifampicin, to 5 µg ml<sup>-1</sup>.

(c) The figure given is cpm[<sup>3</sup>H]x 10<sup>-6</sup> for the highest input; in all cases parallel hybridisations used 50% and 25% as much RNA.

(d) Shows the % of input counts in hybrids per nucleotide length of probe x 10<sup>-6</sup>. Background counts and non-specific hybridisation (generally 5-20cpm) have been subtracted.

(e) The efficiency of termination at t<sub>L7</sub> was estimated by comparing the rpoB and rplJL mRNA levels.
Graphic illustration of the rates of mRNA syntheses in *E. coli* rnc*+* and rnc105 strains during rifampicin treatment. The data are taken from Table 3.6.

Symbols:
- ○ is N2076 MIN 30°C
- ● is N2076 MIN 37°C
- □ is N2077 MIN 37°C
- ■ is N2077 ENR 37°C
Fig 3.7

Time (mins) after rif addition
3.6 S1 NUCLEASE ANALYSIS OF THE rplL - rpoB INTERGENIC REGION, FOLLOWING RIFAMPICIN ADDITION

As described in Chapter 1, transcription and processing in the rplL - rpoB intergenic region of the rpoBC operon have been extensively studied in rnc⁺ and rnc105 strains during steady state exponential growth (Barry et al 1980, Downing and Dennis 1987, Morgan and Hayward 1987, Railing and Linn 1987). Furthermore Morgan and Hayward (1987) have directly demonstrated, using S1 nuclease analysis, that following rifampicin addition at 10μg/ml⁻¹ transcriptional readthrough of tL7 is increased approximately twofold in the rnc105 strain AB301-105. This level of increased transcriptional readthrough is equivalent to that which I observed in DNA : RNA filter hybridisation studies of N2076 and N2077, using rifampicin at 5μg/ml⁻¹ (see section 3.5). Whilst the results presented in section 3.5 give a detailed account of the rates of rpoB and rpoC mRNA syntheses, and the efficiency of termination at tL7 in N2076 and N2077 following rifampicin addition, they do not provide information as regards RNase III processing in this region under such conditions. I therefore attempted to extend my analysis of rpoBC expression by performing S1 nuclease mapping of mRNA in the rplL - rpoB intercistronic region in N2076 and N2077 following addition of rifampicin at 5μg/ml⁻¹. Previous reports have indicated that the pattern of hybrids formed in this
region during S1 analysis may be too complex to allow quantitative assessment of the effect of rifampicin, in the \textit{rnc}\textsuperscript{*} strain (see Morgan and Hayward 1987). Nonetheless I decided to make an attempt using both N2076 and N2077.

I grew both strains in Spizizen minimal medium at 37°C to an \(A_{650}\) of 0.16. RNA was then prepared from cultures grown in the absence, or presence, of rifampicin (5\(\mu\)gml\(^{-1}\) final concentration) as described in section 2.7.1.

As a probe I used a 1533bp \textit{HindIII} DNA fragment (which spans the \textit{rplL} - \textit{rpoB} intergenic space) from M13mp10-21. (M13mp10-21 has a 1886bp \textit{PstI} \textit{rplL'} - \textit{rpoB'} DNA fragment cloned into the \textit{PstI} site of M13mp10, and was constructed by Brian Morgan, in this lab.) The fragment was labelled at the 3' ends by the method described in section 2.11.2. This 1533bp \textit{HindIII} DNA fragment contains 1524bp of DNA sequence from the \textit{rpoBC} operon, plus 9bp of M13mp10 polylinker sequence DNA. The DNA strand which is complementary to \textit{rpoBC} mRNA has the 9 nucleotides of M13mp10 polylinker DNA at its 5' end. After S1 nuclease treatment any 'full-length' DNA : RNA hybrids should be 1524bp long (since the unpaired extra 9nt of probe DNA would be degraded by S1 nuclease). On the other hand, re-annealing of the probe DNA strands
(i.e. a DNA : DNA hybrid) should produce a S1 nuclease resistant hybrid which is 1533bp long.

I performed the S1 nuclease analysis as described in section 2.11.9. Each DNA : RNA hybridisation reaction contained 20µg RNA, 150µg tRNA and approximately $10^4$ cpm of 3' end-labelled probe DNA in 30µl of hybridisation buffer. The denaturation step was carried out at 80°C for 10 minutes, followed by hybridisation at 53°C for 3 hours (Railing and Linn 1987). S1 nuclease digestion was then performed as described in section 2.11.9. Transcripts were sized by resolving the single-stranded DNA probe fragments, which had survived S1 nuclease treatment on a denaturing polyacrylamide gel (see section 2.9.6). Predicted transcript sizes, and the experimentally observed bands, are shown in Fig. 3.8.

Despite the very poor quality of the data presented in Fig. 3.8, this S1 nuclease analysis indicates that the rnc$^+$ strain N2076 does process rpoBC mRNA in the rplL - rpoB intergenic region, as predicted (see fragment B). Processing occurs in the absence, and presence, of rifampicin at 5µgml$^{-1}$. Also the rnc105 strain, N2077, shows no indication of mRNA processing in this region, again as expected.

Due to the poor quality of the S1 nuclease mapping, any quantitative interpretation of the data must be limited. A summary of the densitometric analysis of Fig. 3.8 is given in Table 3.7.
Fig. 3.8

S1 nuclease mapping of transcript ends derived from the *rplL - rpoB* intergenic space. The structural organisation of this region is illustrated (top). *rplL* is the carboxy-terminal portion of the gene which encodes r-protein L7/L12. *rpoB'* is the amino-terminal portion of the gene encoding RNA polymerase β subunit. *tL7* is a terminator, *rns* is the RNase III target site. Predicted transcript sizes (A,B,C) are shown in nt. Closed circles represent the labelled 3' ends of the protected single-stranded DNA fragments after S1 nuclease treatment. The experimentally observed fragments are marked (A,B,C).

Tracks

M Markers (in both cases) are pBR322/*MspI* and pBR322/*AvaI + NheI* mixed
1 N2076 0min., no rifampicin
2 " +15min., rifampicin 5μg/ml⁻¹
3 " +30min., rifampicin 5μg/ml⁻¹
4 " +60min., rifampicin 5μg/ml⁻¹
5 N2077 0min., no rifampicin
6 " +15min., rifampicin 5μg/ml⁻¹
7 " +30min., rifampicin 5μg/ml⁻¹
8 " +60min., rifampicin 5μg/ml⁻¹

The sizes of all DNA fragments are shown in nt.
Table 3.7

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<th>Track</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<td>15.8</td>
<td>15.8</td>
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<td>13.6</td>
<td>12.1</td>
<td>9.1</td>
<td>-</td>
<td>-</td>
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<td>tL7 terminated transcript C (566 nt)</td>
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<td>42.1</td>
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a) Numbers 1 - 8 refer to tracks 1 - 8 respectively in Fig. 3.8.

b) Density per band is expressed as a percentage of the total in each (vertical) track.
The results presented in section 3.5 and previously published data (discussed in Chapter 1, e.g. Blumenthal and Dennis 1978, Bass et al 1979) give a good indication of the synthesis rates of the various transcripts of the rpoBC operon, and the efficiency of terminator tL7, before and after rifampicin treatment. However the quantitative analysis of Fig. 3.8 presented in Table 3.7 does not agree with these results. In particular the data indicate an accumulation of fragment C (tL7 terminated transcript) in the rnc+ strain during rifampicin treatment. Other published S1 nuclease mapping studies of this operon have also shown a large excess of tL7 terminated transcripts relative to readthrough transcripts (Downing and Dennis 1987, Ralling and Linn 1987, Morgan and Hayward 1987). That the quantitative relationship between tL7 terminated and full-length transcripts seen in the S1 nuclease mapping experiments does not match the ratio observed in the DNA : RNA filter hybridisation tests (i.e. synthetic rates), could, of course, be due to differential stabilities of the various transcripts.

There is little published information as regards the stability of rpoBC transcripts. One indirect study of the functional half-life for E. coli mRNAs (Pedersen et al 1978) estimated that rplJL message has a half-life of 85 - 107 seconds, while that of rpoB mRNA is 58 - 70 seconds. However, this same study also estimated L11
mRNA half-life to be 60 seconds, whereas a more recent, direct analysis by Cole and Nomura (1986) concluded that (in normal, translationally regulated strains) L11 mRNA chemical half-life is 22 seconds.

One plausible explanation for the apparent accumulation of rplJL (i.e. tL7 terminated, 566nt) transcripts in N2076, after rifampicin addition, is that it actually represents full-length message which has been processed at the RNase III site and then rapidly degraded by 3' - 5' exonucleases to the potentially stable secondary structure at tL7. This argument has also been used by Raling and Linn (1987) to explain apparent discrepancies in the results of their S1 nuclease mapping and filter hybridisation experiments on rpoBC message.

In summary, my attempts to quantitatively assess the effect of rifampicin on transcription of the rpoBC operon in the rnc* strain, N2076, appear to vindicate the assertion of Morgan and Hayward (1987) that the pattern of hybrids formed during S1 nuclease analysis may be too complex to interpret. This, allied to the poor quality of my own data, limits my conclusion to the qualitative judgement that N2076 does process rpoBC mRNA in the rplL - rpoB region whilst N2077 does not.
3.7 DISCUSSION

The results presented in this chapter on the differential rates of ββ' synthesis in steady state, exponential cultures of N2076 and N2077 confirm and extend those of Dennis (1984). His work (also using N2076 and N2077) produced clear evidence that the differential rates of β and β' protein syntheses were essentially identical in the rnc+ and rnc105 strains, when grown exponentially at 37°C in M9 glucose minimal medium, supplemented with sixteen amino acids and necessary vitamins.

In my own work, when one compares the two strains growing at identical rates (either in two different media at 37°C, or in identical media at two different temperatures) there is no significant difference between them as regards the differential rates of ββ' syntheses (see Table 3.2.1). There are slight differences between the two strains when grown in identical conditions: the rate of ββ' synthesis appears to be marginally higher in N2076 than in N2077 in each case. However, these differences appear to be due solely to the growth-rate-dependent regulation of ββ' synthesis, rather than to the rnc phenotype. Iwakura et al (1974) derived an equation (to which the data of Matzura et al (1973) also fit very well) to express the relationship between growth rate and the differential synthesis rate of ββ' in E. coli K12: α
\[ 0.7\mu + 0.45, \] where \( \alpha \) is the differential rate of synthesis and \( \mu \) represents growth rate as generations per hour. The degree to which \( \beta \beta' \) synthesis varies with doubling time for both strains in Table 3.2.1 is similar to that predicted by the equation.

This observed growth-rate-dependent regulation of \( \beta \beta' \) synthesis in both N2076 and N2077 serves to highlight the importance of making comparisons between \( rnc^+ \) and \( rnc105 \) strains at similar growth rates when analysing the effect of the \( rnc \) phenotype on expression of \( rpoBC \).

In a paper primarily concerned with the effect of RNase III processing on the expression of the \( pnp \) operon, Portier et al (1987) also reported a preliminary finding that \( \beta \) and \( \beta' \) proteins were approximately twofold more abundant in extracts of the \( rnc105 \) mutant BL321 than in the isogenic \( rnc^+ \) control BL322. (In fact this result has proved difficult to reproduce - Claude Portier, personal communication). Their figure refers to total amounts of protein, not synthetic rates. When I measured the differential rates of \( \beta \beta' \) synthesis in BL322 and BL321, growing with the same doubling time, there was no significant difference between them. Again there were slight differences between the strains when they were grown in identical conditions (as for N2076 and N2077), but these were compatible with the differences in growth rate.
The major finding presented in this chapter is that when N2076 and N2077 are subjected to a constraint (namely the addition of sub-bacteriostatic levels of the drug rifampicin) which causes ββ' oversynthesis, the rnc105 strain shows a markedly delayed response as compared with the isogenic wild type control.

The rate of rpoBC mRNA synthesis increases to the same extent in both strains, following rifampicin addition. Therefore the delayed response of ββ' synthesis in the rnc105 strain is a post-transcriptional effect. Since I have demonstrated that rpoBC mRNA is cleaved at the RNase III site between rplL and rpoB in N2076 (rnc) but not in N2077 (rnc105), both in the absence and presence of rifampicin, then I conclude that (contrary to the view of Dennis, 1984) processing does play an important role in the post-transcriptional regulation of rpoB gene expression. Since this is only apparent in constrained cells (i.e. after the addition of rifampicin at 5μgml⁻¹) I suggest that during steady state, exponential growth, the significance of RNase III processing is concealed.

Consider for instance, the possibility that cleavage of the rpoBC mRNA at the RNase III target site is
required to achieve the optimal mRNA conformation for efficient initiation of \textit{rpoB} translation. Such efficient translation would then be countered by an appropriately strong degree of operation of the known feedback regulation mechanism. In the \textit{rnc105} strain, however, failure to process the \textit{rpoBC} mRNA would, according to this model, produce a message which was inefficient for translation initiation. This would be compensated for by little or no autogenous translational repression. Hence the outcome, during unconstrained growth, would be the production of similar levels of \(\beta\) protein synthesis in \textit{rnc}\textsuperscript{+} and \textit{rnc105} strains, as observed in Tables 3.2.1 and 3.2.2, and by Dennis (1984).

Addition of rifampicin not only increases the transcriptional readthrough of \textit{tL7} to the same extent in \textit{rnc}\textsuperscript{+} and \textit{rnc105} strains (see Table 3.6 and Fig. 3.7), it also interferes with feedback repression, as mentioned in Chapter 1. A combination of these two effects could lead to the prompt stimulation of \(\beta\) synthesis observed in the \textit{rnc}\textsuperscript{+} strain after the addition of rifampicin since, according to this hypothesis, the extra, newly-synthesised and processed mRNA would be intrinsically translationally efficient and, due to the presence of the drug, subject to less autogenous regulation than normal.
According to the above argument, feedback regulation would be operating weakly, or not at all, in the rnc105 strain. The addition of rifampicin should therefore have a less significant effect here. Thus the slow response to rifampicin in the mutant strain may reflect only the increased synthesis (due to increased tL7 readthrough) of unprocessed, translationally inefficient mRNA. If this view is correct, then by applying an artificial constraint (rifampicin) upon the supply of active RNA polymerase, I may be observing the disadvantage that would apply to an E. coli strain which had lost the rpoB mRNA RNase III target site: an inability to respond as rapidly as the wild-type strain to a sudden demand for increased RNA polymerase output.

An interesting feature of Tables 3.3 and 3.4 is the parallel behaviour between β and β' synthesis which was observed in both strains. Rifampicin certainly stimulates both rpoB and rpoC mRNA syntheses (Blumenthal and Dennis 1978, also Table 3.6 and Fig. 3.7), and opposes translational repression of β' as well as β in vitro (Fukuda and Nagasawa-Fujimori 1983). However it is also clear that the two genes can be independently regulated post-transcriptionally (Meek and Hayward 1986). My results suggest that processing at the RNase III site between rplL and rpoB may affect rpoC translation. Presumably this is as a consequence of the (proposed)
direct affect processing has on rpoB translation. But how is this mediated? It may be an indication of "translational coupling" of rpoB and rpoC. For instance, translation of rpoB may be required to "open up" a translation initiation site in rpoC. Another possible explanation is that this reflects a shared component in translational regulation i.e. β subunit may be part of a repressor which regulates the translation of both genes. This does not exclude the possibility that the genes are regulated independently by separate repressor assemblies (e.g. α2β regulates rpoB, and holoenzyme regulates rpoC (and rpoB) ?) If this were the case then rifampicin interaction with β subunit could interfere with the translational regulation of both genes (rpoB and rpoC). Work is currently underway in our laboratory to study the post-transcriptional regulation of rpoC.

One interesting "spin off" from these results is that they may provide a basis for research into the mechanism of co-ordination of the synthesis of the major RNA polymerase subunits. As well as stimulating ββ' synthesis, rifampicin also induces α and σ70 syntheses. (Nakamura and Yura 1976, Hayward and Fyfe 1978b). The drug induces ββ' synthesis directly through transcriptional anti-termination and by lifting translational repression. The observed increases in α
and $\sigma^{70}$ syntheses may be an indirect result of higher $\beta\beta'$ levels. Rifampicin mediated readthrough of terminator t1 in the rpoD operon (see Fig. 1.3) is a possible alternative explanation for increased $\sigma^{70}$ production. However, $\beta\beta'$ oversynthesis in a rpoBC merodiploid also leads to increased $\sigma^{70}$ production (Hayward and Fyfe 1978a). Moreover, there is no known transcriptional terminator which could mediate the effect of rifampicin on $\alpha$ synthesis. The idea of co-ordinated RNA polymerase subunit synthesis is therefore favoured.

If the rifampicin-induced stimulation of $\alpha$ and/or $\sigma^{70}$ syntheses is dependent upon $\beta\beta'$ induction, then the markedly delayed response of $\beta\beta'$ synthesis in N2077 should consequently delay $\alpha$ and $\sigma^{70}$ induction in this strain. This possibility is currently under investigation in our laboratory.

The major interest generated by the results presented in this chapter was in the possibility that RNase III processing could play a direct role in rpoB gene expression. Therefore a system was devised to permit the detailed analysis of the role of RNase III processing in rpoB expression. This is described in Chapter 4.
CHAPTER 4

CONSTRUCTION AND CHARACTERISATION OF A \textit{rpoB-lacZ} TRANSLATIONAL FUSION PLASMID AND DELETION MUTANT DERIVATIVES

4.1 INTRODUCTION

Dennis (1984) concluded that processing \textit{per se} is unimportant in \textit{rpoB} expression and that sequences around the RNase III processing site, in the \textit{rplL - rpoB} intergenic region, dictate the translational efficiency of \textit{rpoB} mRNA. I have found that processing \textit{does} play a role in \textit{rpoB} expression and that translational regulation probably masks this by balancing the level of $\beta$ production in \textit{rnc}$^+$ and \textit{rnc105} strains. Dennis (1984) also observed that autogenous translational regulation plays a major part in controlling the expression of \textit{rpoB}. In his work four different, extensive deletions of the \textit{rplL - rpoB} region were made in plasmids carrying a large EcoRI fragment of the \textit{rpoBC} operon ('\textit{rplKAJL rpoBC}'). An important feature of these plasmids is that they all contain the entire \textit{rpoB} structural gene and thus can all direct the production of $\beta$ protein. Dennis found that the introduction of the multicopy "parent" plasmid (carrying an intact \textit{rplL - rpoB} intergenic region) into N2076 caused the relative translational efficiency of
*rpoB* mRNA to drop by 58%. This demonstrates the compensatory effect of autogenous translational repression. Furthermore, deletion of the attenuator tL7 not only caused the expected increase in the levels of plasmid-derived *rpoB* messenger RNA, but also a compensatory decrease in the translational efficiency of *rpoB* mRNA by a further 13%. Thus translational repression alone can account for (at least) a 71% drop in the translational efficiency of *rpoB* mRNA. Against this background the reported changes in translational efficiency caused by deletion of sequences around the RNase III site (roughly a further 15% drop) are less striking (although still interesting). A better system to study these deletions would have been one in which variations in protein output from the plasmid-borne gene could not lead to confusing effects of autogenous translational repression.

With this in mind I devised a strategy for the construction of a *rpoB* - *lacZ* translational fusion. This construct contains most of the *rplL* - *rpoB* intergenic region plus only the first 15bp of the *rpoB* structural gene and therefore does not produce β protein.

Hence the plasmid protein product should not have any feedback effects. It now appears likely that this approach has also destroyed the feedback target on the mRNA, part of which resides well within the *rpoB* gene.
(Passador and Linn 1989). This was unplanned, but is a welcome advantage in that variations in mRNA output from the plasmid are therefore unlikely to alter the cellular level of \textit{rpoB} feedback by competing for the repressor protein complex. The \textit{rpoB} and \textit{lacZ} sequences are in the same translational phase. The level of translation of \textit{lacZ} (which can readily be determined by $\beta$ galactosidase assay, see section 2.10) is dependent upon the frequency of translation initiation at the \textit{rpoB} start. This provides an easy way of accurately determining the efficiency of translation initiation of \textit{rpoB}, since this system should not be subject to autogenous feedback repression.

Precise, defined deletions around the RNase III target site can then be introduced and their effect on \textit{rpoB} translational efficiency monitored by $\beta$ galactosidase assay. Choosing exactly which deletions to make is a difficult task. There is no satisfactory "all encompassing", consensus RNase III target site: therefore defining precise sequences necessary for processing is, as yet, impossible. One proposed RNase III target site "consensus" is shown in Fig. 4.1. This is the result of a survey by Daniels et al (1988) of 17 known RNase III target sequences. Twelve have the boxed sequence (Fig. 4.1) at exactly this location relative to the mapped cut sites. However, no bacterial mRNA was used in this survey and certainly this "consensus"
sequence is not present in the *rpoBC* RNase III target (nor in the RNase III processing site in the *pnp* operon). Therefore with no clear indication of which small mutations to make at the RNase III target, I decided to create several large RNase III target site deletions. I have also noted that the triplet UUU is commonly found near the cut-site in RNase III target sequences. Therefore I decided to delete the two copies of this UUU triplet, present in the 5' arm of the *rpoBC* RNase III target site, which are located near the known, mapped cleavage sites.

The construction and characterisation of the *rpoB* - *lacZ* translational fusion and its RNase III target site deletion derivatives are detailed in this chapter.
Fig. 4.1

"Consensus" RNase III target site, from Daniels et al (1988). The boxed sequence is the conserved feature. It lies at exactly this location, relative to the mapped RNase III cut sites (shown by the arrows) in 12 out of 17 cases surveyed.
Fig 4.1
4.2 **CONSTRUCTION OF THE rpoB - lacZ TRANSLATIONAL FUSION PLASMID**

4.2.1 **INTRODUCTION OF A SmaI/XmaI SITE INTO rpoB**

This laboratory already possessed various M13 derivatives carrying rpoB fragments. One such derivative, designated M13mp10-21 was chosen. (The construction of M13mp10-21 has already been explained, see section 3.6). The synthesised oligonucleotide (608A) has the sequence 5' CGTTTTTTCCGGATAGGAG 3' and is complementary to a sequence within the rpoB gene (see Fig. 4.2). Oligonucleotide 608A allows two bases to be changed in the rpoB sequence, thereby creating a SmaI/XmaI site, as shown in Fig. 4.2. The size and purity of oligonucleotide 608A were checked as described in section 2.12.2 (see Fig. 2.3) and it was then used initially as a sequencing primer to check that it was binding to the correct (and unique) location on M13mp10-21. This also allows one to optimise the conditions for hybridisation between oligonucleotide 608A and M13mp10-21. An example of the results of such a test is shown in Fig. 4.3.

Oligonucleotide site-directed mutagenesis was performed as described in section 2.12.1. The oligonucleotide 608A and M13mp10-21 SS DNA were incubated at 70°C for 5 minutes and hybridised at 37°C for 30
Fig. 4.2

Complementarity between oligonucleotide 608A and *rpoB* sequence. Upper line shows a portion of *rpoB* DNA sequence. Lower line shows the oligonucleotide 608A DNA sequence. X indicates positions of mutations which 608A introduces during the mutagenesis reaction. The initiation codon of *rpoB* is boxed and the target for the restriction enzymes *SmaI/XmaI* is underlined.
Fig 4.2

5' rpoB start 3'
- C C C T A T G G T T T A C T C C T A T A C C G A G A A A A A A A C G T A T T -
  G A G G A T A G G G C C C T T T T T T G C
3' 5'

SmaI / XmaI

608A
Fig. 4.3

Optimisation of the conditions for hybridisation between oligonucleotide 608A and M13mp10-21 SS DNA. The autoradiograph depicts the results of T-tracking after using the annealing conditions detailed below. The ratios shown below are the molar ratios of oligonucleotide 608A: M13mp10-21 SS DNA.

Tracks

1 2:1 - 60°C, 5 minutes and 37°C, 30 minutes
2 10:1 - "  
3 20:1 - "

*4 2:1 - 70°C, 5 minutes and 37°C, 30 minutes
5 10:1 - "  
6 20:1 - "

7 2:1 - 80°C, 5 minutes and 37°C, 30 minutes
8 10:1 - "  
9 20:1 - "

* marks the optimal conditions for annealing oligonucleotide 608A and M13mp10-21 SS DNA.
minutes, at a 2 : 1 molar ratio (608A : M13mp10-21). Routinely the restriction endonuclease NciI is used to nick the non-mutant DNA strand during the Eckstein mutagenesis procedure. However in this case there is a NciI site within the oligonucleotide 608A sequence. Therefore PvuI was used as an alternative nicking enzyme.

One hundred candidate mutant plaques were screened by hybridisation (as described in section 2.11.5) using labelled oligonucleotide 608A as a probe. Three putative positive plaques were identified by this procedure (designated A, B and C in Fig. 4.4). These plaques were purified, and large scale RF M13 DNA preparations were made from each one (as described in section 2.4.4). The successful introduction of a SmaI/XmaI site into two of these three M13 phage (B and C) was verified by restriction endonuclease analysis of the M13 RF DNA (see Fig. 4.5). The mutant was designated M13mp10-22.

4.2.2 CREATION OF pRAMI

A 256bp NarI - XmaI fragment from M13mp10-22 was then cloned between the AccI and XmaI sites of M13mp19 to produce M13mp19-5. This was checked by restriction endonuclease analysis (see Fig. 4.6) and by DNA sequencing of the entire 256bp insert to ensure that no
Fig. 4.4

M13 plaque hybridisation using labelled oligonucleotide 608A. The filter was washed in 6 x SSC at 59°C for 5 minutes, prior to exposure to Xray film. The calculated Td of oligonucleotide 608A is 64°C. Three putative positive plaques were identified, designated A, B and C.
Fig 4.4
Restriction analysis of M13mp10-22 candidates. 1% agarose gel electrophoresis. The upper part shows a partial map of M13mp10-22 (not to scale). X Smal shows the position of the newly created Smal site (introduced by site-directed mutagenesis). The open box represents the PstI fragment of rpoBC operon DNA cloned into M13mp10 (to produce the parental M13mp10-21). The lower part shows the agarose gel photograph.

Tracks

M  Markers are $\lambda / HindIII + EcoRI$
1  Candidate A - uncut
2    "    A - Smal
3    "    B - uncut
4    "    B - Smal
5    "    C - uncut
6    "    C - Smal

The sizes of all DNA fragments are given in kb.
Fig 4.5

M13 mp10-22

SmaI

\text{rplL-rpoB}

SmaI

SmaI

10

M 1 2 3 4 5 6

2.03

1.90

1.58

1.33

0.98

-10
other, unplanned, mutations had been introduced during the mutagenesis reaction (see Fig. 4.7).

M13mp19-5 contains a 256bp fragment of the *rpoBC* operon (co-ordinates 2732 - 2987 inclusive, from Post et al 1979, as corrected by Morgan 1986). However it should be noted that the mutagenesis reaction, which introduced a *SmaI/XmaI* site into the *rpoB* gene, changed the nucleotide at position 2985 from A to C. This alters codon 6 of the *rpoB* structural gene from ACC to CCC (resulting in the substitution of a proline for a threonine at this position in the *rpoB* - *lacZ* fusion protein). It is also worth noting that (by design) M13mp19-5 does not contain the attenuator tL7 from the *rpoBC* operon. The important features carried on the 256bp insert in M13mp19-5 are outlined in Fig. 4.8.

M13mp19-5 SS DNA was then used as the template for further oligonucleotide site-directed mutagenesis reactions (see section 4.3.1).

To construct the plasmid-borne *rpoB* - *lacZ* translational fusion a 277bp *HindIII* - *XmaI* fragment from M13mp19-5 was cloned between the same sites in pSKS106. The resultant plasmid was checked by restriction endonuclease mapping in comparison with pSKS106 and then designated pRAMI (see Fig. 4.9).
Competent AK14 cells were transformed with each plasmid. AK14 harbouring pSKS106 produces deep blue colonies on LB plates (plus ampicillin, Xgal and IPTG) due to the high level of β galactosidase activity from pSKS106. AK14 harbouring pRAMI, on the other hand, produces pale blue colonies when grown on similar plates.
Fig. 4.6

Restriction analysis of M13mp19-5. 1% agarose gel electrophoresis. The upper part shows a simple map of M13mp19-15 (not to scale). The open box represents the 256bp *NarI* - *XmaI* fragment from M13mp10-22 which was cloned between the *AccI* - *XmaI* sites of M13mp19 to produce M13mp19-5. The lower part shows the agarose gel photograph.

**Tracks**

M  Markers are $\lambda$ / *HindIII*
1  M13mp19-5 - uncut
2  M13mp19-5 - *XmnI*

The sizes of all DNA fragments are shown in kb.
Complete DNA sequence of the 256bp NarI-XmaI insert in M13mp19-5

*NarI
GT[C GCCAGTAGCGTTTTCACACCTGTTTGACTACTGCTGTGCCTTTCJTGC
TTGTTTCTATCGACGACTTTAAATATACTGCGACAGGACGTCCGTCTCTGTGTGAATCC
AATGAAATGGTTTAAAGCTGCTAGCAACACGGCATTCGCGAAAAGTGTGCCATTTTCCGG
TCAACAAAAATAGTTGCTGCAAAAAACTGTGCCGCTCAATGGACAGATGGGTGCGACTTGCTCA
* GCGAGCTGAGGAACCCTATGTTTACTCTATCCC]GGG

Notes

Brackets [ ] denote the boundaries of the 256 bp insert sequence

* shows the A - C point mutation made by site-directed mutagenesis.

'NarI denotes the destroyed NarI site

Sequencing gel was run at 40W for:   A 1 hour 45 minutes
                                           B 3 hours 30 minutes
                                           and  C 5 hours 30 minutes
Fig. 4.8

Features in the 256bp NarI - XmaI insert in M13mp19-5. Co-ordinates are from Post et al (1979) as corrected by Morgan (1986). ATG is the initiation codon of rpoB. The A --> C point mutation was introduced by site-directed mutagenesis, this work.
Fig 4.8

- RNaseIII target region

A>C point mutation (2985)
Fig. 4.9A

Simple map of pSKS106 (not to scale). The polylinker sequence in the 5' end of lacZ contains restriction targets for HindIII, PstI, SalI, BamHI, SmaI/XmaI, EcoRI and HaeIII. The open boxes represent the lacZ, lacY and lacA structural genes. Apr denotes ampicillin resistance.
Fig 4.9 A

polylinker

Plac^+
lacZ

Ap^r

pSKS106

lacY

lacA
Restriction analysis of pSKS106 and pRAM1. 0.6% agarose gel electrophoresis. Upper part shows a simple map of pRAM1. The open box represents the 277bp HindIII - XmaI DNA fragment from M13mp19-5 cloned into pSKS106 (between the same sites) to produce pRAM1. This is inserted into the polylinker region at the 5' end of lacZ (see Fig. 4.9A) - to produce a translational fusion. Digestion of pSKS106 with the following enzymes should yield these fragments: HindIII (10.1kb); EcoRI (9.9 + 0.2kb); XmnI (8.2 + 1.9kb). Digestion of pRAM1 with the following enzymes should produce these fragments: XmnI(7.7 + 1.9 + 0.7kb); HindIII, XmaI(10.1 + 0.25kb) EcoRI (9.9 + 0.45kb). Lower part shows the agarose gels.

Tracks

M Markers (in both cases) are $\lambda /$ HindIII + EcoRI
1 pSKS106 - uncut 5 pRAM1 - uncut
2 "  - HindIII 6 "  - XmnI
3 "  - EcoRI 7 "  - HindIII + XmaI
4 "  - XmnI 8 "  - EcoRI

The sizes of all DNA fragments are given in kb. The 2.6kb fragment (Track 6) represents partial cutting of pRAM1 DNA with XmnI (1.9 + 0.7).
4.3 CONSTRUCTION OF DELETED \textit{rpoB} - \textit{lacZ} \\
TRANSLATIONAL FUSION PLASMIDS \\

4.3.1 OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS OF \\
M13mp19-5 \\

As described in section 4.2.2 and Fig. 4.8, M13mp19-5 carries a short fragment of the \textit{rpoBC} operon containing the RNase III target site, the weak promoter \textit{P}_\text{B}, the translation initiation site of \textit{rpoB} and the first five codons of the \textit{rpoB} structural gene. I decided to create a series of RNase III target site deletion mutants. These were made using M13mp19-5 SS DNA and various custom-synthesised DNA oligonucleotides. The creation of each mutant is detailed below.

\textbf{M13mp19-6, mp19-7, mp19-8 and mp19-9} \\

The synthesised oligonucleotide 211A has the sequence 5' GGAACACGGCACAGC 3' and is complementary to sequences surrounding the RNase III target site. Oligonucleotide 211A allows a deletion of \textit{rpoBC} operon sequence to be made from position 2771 - 2878 inclusive. This deletes the entire putative stem-loop structure of the RNase III target site. The size and purity of oligonucleotide 211A were checked as described in section 2.12.2 (see Fig. 2.3). Optimal conditions for hybridisation of oligonucleotide 211A and M13mp19-5 SS
DNA were determined. They were mixed and incubated at 70°C for 5 minutes and hybridised at 37°C for 30 minutes, at a 2 : 1 molar ratio (211A : M13mp19-5). The mutagenesis reaction was carried out exactly as described in section 2.12.1.

Twenty plaques were screened by T-tracking. Of these, 30% were M13mp19-5, 35% appeared to have the planned deletion (2771 - 2878) and a further 35% consisted of various unplanned deletions. Representative plaques from each class of deletion mutant were purified and sequenced fully (see Fig. 4.10). Due to aberrant binding of oligonucleotide 211A to sequences in M13mp19-5, three extra mutants had been created (in addition to the planned deletion). These were designated M13mp19-6 (deletion is 2827 - 2929 inclusive) M13mp19-7 (deletion is 2823 - 2925 inclusive) and M13mp19-8 (deletion is 2771 - 2905 inclusive, plus a C to G point mutation at 2911). The planned deletion mutant (2771 - 2878 inclusive) was designated M13mp19-9. A schematic outline of these deletions is given in Fig. 4.11. In this work only M13mp19-6 and M13mp19-9 were used for further investigation (M13mp19-7 and M13mp19-8 were stored for use at a later date).
DNA sequencing analysis of M13mp19-6, mp19-7, mp19-8, mp19-9. Sequences are read 5' - 3', upwards from left to right. In each case a portion of the sequence (twelve nucleotides), spanning the deletion, is highlighted. The exact position of the deletion is indicated, = in each case.
Fig. 4.11

A schematic outline of the RNase III target site deletion mutants created during this work (not to scale). Co-ordinates are from Post et al (1979), as corrected by Morgan (1986). The deleted sequences are presented as gaps. Stem-loop structure represents the putative secondary structure of the RNase III target site. The UUU triplets are discussed in the text.
This deletion mutant was created by a summer visitor to the lab, Dr. Margarita Martin, using the oligonucleotide 219A which has the sequence 5' CGTCGATAGCAAGCATTGGGCACAG 3'. This oligonucleotide allows the deletion of two copies of the triplet sequence UUU (positions 2771 - 2773 inclusive and 2783 - 2785 inclusive) from the 5' arm of the putative stem-loop RNase III target structure. The size and purity of oligonucleotide 219A were checked as described in section 2.12.2 (see Fig. 2.3). After determining the optimal conditions for oligonucleotide 219A : M13mp19-5 SS DNA hybridisation (incubation at 70°C for 5 minutes and 37°C for 30 minutes at a molar ratio of 2 : 1, 219A : M13mp19-5) the oligonucleotide site-directed mutagenesis reaction was carried out as described in section 2.12.1.

Twenty plaques were screened by A-tracking, of which 25% appeared to have the planned deletions. DNA sequencing confirmed that the double deletion mutant had been created as planned (see Fig. 4.12). This was designated M13mp19-10 and is outlined schematically in Fig. 4.11.
DNA sequencing analysis of M13mp19-10 and M13mp19-11. Sequences are read 5' - 3', upwards from left to right. Note that Dr. M. Martin sequenced M13mp19-10 (and that the tracks in this case are ordered CGTA). A portion of the sequence spanning the deletions is highlighted in each case, and the exact positions of the deletions are indicated (=).
M13mp19-11

The synthesised oligonucleotide 203A has the sequence 5' CGCAGTATAGGCACAGC 3'. This oligonucleotide allows a deletion from positions 2771 - 2800 inclusive (which deletes the 5' arm of the proposed stem-loop secondary structure of the RNase III target site). The size and purity of oligonucleotide 203A were checked (see section 2.12.2 and Fig. 2.3). The optimal conditions for hybridisation of oligonucleotide 203A and M13mp19-5 SS DNA were determined as incubation at 70°C for 5 minutes and 37°C for 30 minutes at a molar ratio of 10 : 1 (203A : M13mp19-5). Twenty plaques were screened by A-tracking, of which 5% (i.e. one plaque) appeared to be the planned deletion. This was purified and checked by DNA sequencing to confirm that the deletion mutant had been created as planned (see Fig. 4.12). This was designated M13mp19-11 and is outlined in Fig. 4.11.

4.3.2 CREATION OF pRAM2, pRAM3, pRAM4 and pMRM1

The cloning strategy used to create pRAM1 was also used to construct four deleted rpoB - lacZ translational fusion plasmids. The creation of each plasmid is detailed below.
pRAM2

This plasmid was constructed by cloning the 169bp HindIII - XmaI fragment from M13mp19-9 between the same sites in pSKS106. Transformation of competent AK14 cells produced pale blue colonies on LB (plus ampicillin, Xgal and IPTG) plates. These were screened by the hybridisation technique detailed in section 2.11.7, using a labelled 1.53kb HindIII - PstI ('rplJL - rpoB') DNA fragment from M13mpl0-21 as a probe. An identified putative positive clone (see Fig. 4.13) was purified and plasmid DNA prepared and analysed by restriction endonuclease mapping (see Fig. 4.14). This plasmid was designated pRAM2.

pRAM3

This plasmid was made by cloning the 174bp HindIII - XmaI fragment from M13mp19-6 between the same sites in pSKS106. Upon transformation of competent AK14 cells pale blue colonies were produced, which were screened as described above. An identified putative positive clone (see Fig. 4.13) was purified and plasmid DNA prepared and analysed by restriction endonuclease mapping (see Fig. 4.15). This plasmid was designated pRAM3.
Fig. 4.13

Detection of clones containing pRAM2 and pRAM3 by colony hybridisation. The DNA probe was the 1.53kb HindIII - PstI ('rplJL - rpoB') DNA fragment from M13mp10-21. Filter was washed as described in section 2.11.7 and exposed to Xray film at room temperature for 60 minutes. From the top of the filter (proceeding left to right), the first 5 colonies were candidate AK14 (pRAM2) clones - one of which was picked as a positive putative clone. The next 43 colonies were candidate AK14 (pRAM3) clones - one of which was picked as a putative positive clone. The last two colonies were controls.
Fig 4.13

AK14(pRAM2) +ve clone

AK14(pRAM3) +ve clone

AK14(pRAM1) "+ve control"

AK14(pSKS106) "-ve control"
Fig. 4.14

Restriction analysis of pRAM2. 2% agarose gel electrophoresis. The upper part shows a partial map of pRAM2 (not to scale). The open box represents the (approximately) 0.15kb HindIII - Xmal DNA fragment from M13mp19-9, inserted between the same sites in pSKS106. The lower part shows the agarose gel photograph.

Tracks
M  Markers are λ / HindIII + EcoRI
1  pRAM2 - uncut
2  " - HindIII + XmaI
3  " - EcoRI

The sizes of all DNA fragments are shown in kb.

Fig. 4.15

Restriction analysis of pRAM3. 1% agarose gel electrophoresis. The upper part shows a partial map of pRAM3 (not to scale). The open box represents the (approximately) 0.15kb HindIII - XmaI DNA fragment from M13mp19-6 inserted between the same sites in pSKS106. The lower part shows the agarose gel photograph.

Tracks
M  Markers are λ / Hind III + EcoRI
1  pRAM3 - uncut
2  " - HindIII + XmaI
3  " - EcoRI

The sizes of all DNA fragments are shown in kb.
Fig 4.14

pRAM2

EcoRI  HindIII  XmnI  EcoRI

0.35

0.98  0.83  0.56

Fig 4.15

pRAM3

EcoRI  HindIII  XmnI  EcoRI

0.35

2.03  1.98  1.93  1.83  0.98  0.93  0.83  0.56  0.35  0.15  0.12
pRAM4

This plasmid was constructed by cloning the 247bp HindIII-XmaI fragment from M13mp19-11 between the same sites in pSKS106. Pale blue colonies (see above) were picked and small scale plasmid DNA preparations were made from them. These were then screened by restriction endonuclease analysis. An identified positive clone was further purified and reanalysed as before (see Fig. 4.16). This plasmid was designated pRAM4.

pMRM1

Dr. M. Martin made this plasmid by cloning the 271bp HindIII - XmaI fragment from M13mp19-10 between the same sites in pSKS106. Pale blue colonies were picked and screened by restriction endonuclease analysis (as for pRAM4). One correct clone was identified, purified and analysed again (see Fig. 4.17). This plasmid was designated pMRM1.
Fig. 4.16

Restriction analysis of pRAM4. 0.6% agarose gel electrophoresis. The upper part shows a simple map of pRAM4 (not to scale). The open box represents the (approximately) 0.22kb HindIII - XmaI fragment from M13mp19-11, cloned between the same sites in pSKS106. Digestion of pRAM4 with the following enzymes should produce these fragments (sizes are approximate): HindIII + XmaI (10.1 + 0.22kb), EcoRI (9.9 + 0.42kb), XmnI (7.7 + 1.9 + 0.7kb). The lower part shows the agarose gel photograph.

Tracks
M  Markers are λ / HindIII + EcoRI
1  pRAM4 - uncut
2   "  - HindIII + XmaI
3   "  - EcoRI
4   "  - XmnI

The sizes of all DNA fragments are shown in kb.

Fig. 4.17

Restriction analysis of pMRM1. 0.6% agarose gel electrophoresis. The upper part shows a simple map of pMRM1 (not to scale). The open box represents the (approximately) 0.25kb HindIII - XmaI fragment from M13mp19-10 cloned between the same sites in pSKS106. Digestion of pMRM1 with the following enzymes should produce these fragments (sizes are approximate) HindIII + XmaI (10.1 + 0.25kb), EcoRI (9.9 + 0.45kb), XmnI (7.7 + 1.9 + 0.7kb). The lower part shows the agarose gel photograph.

Tracks
M  Markers are λ / HindIII + EcoRI
1  pMRM1 - uncut
2   "  - HindIII + XmaI
3   "  - EcoRI
4   "  - XmnI

The sizes of all DNA fragments are shown in kb.
Fig 4.16

Fig 4.17

pRAM4

pMRM1
4.4 CHARACTERISATION OF A rpoB – lacZ TRANSLATIONAL FUSION PLASMID AND DELETION-MUTANT DERIVATIVES

4.4.1 PRIMER EXTENSION ANALYSIS

Having made pRAM1, containing an intact RNase III target site upstream of the rpoB start, one important initial question was whether mRNA derived from the plasmid was processed faithfully at this site. Also, since all four mutant rpoB – lacZ fusion plasmids contain partial or entire deletions of the RNase III target site, it was important to ascertain whether mRNA derived from these plasmids was cleaved in this region. Primer extension analysis of RNA extracted from cells harbouring each plasmid was performed. Cultures of AK14 (transformed with the appropriate rpoB – lacZ fusion plasmid) in Spizizen-glycerol minimal medium (plus ampicillin) were grown to an A600 of 0.3. At this point RNA was extracted as described in section 2.7.1.

The synthesised oligonucleotide 083C (sequence 5' CTGCAAGGCGATTAAGTTGG 3') kindly provided by John Morran and Dr. Jean Beggs, was labelled at its 5' end as described in section 2.11.3. This oligonucleotide is complementary to a sequence within the lacZ structural gene (spanning codons 21-29) and is ideal for analysing mRNA derived from the rpoB – lacZ fusion plasmids exclusively, since the host strain AK14 is Δ(lac – pro)
and carries F' lacZ M15 (a deletion extending from codons 11-41 inclusive). Thus only lacZ mRNA derived from the fusion plasmids will contain sequences with which this oligonucleotide can hybridise. Universal sequencing primer (sequence 5' TCCCAGTCAGACGT 3') has essentially the same features as 083C: it is complementary to a sequence within the lacZ structural gene (spanning codons 12-17 inclusive) which is missing in (lac-pro) and lacZ M15. Thus it too can be used in these primer extension experiments.

The results of primer extension analysis of RNA derived from pRAM1, pRAM2, pRAM3 and pMRM1, using oligonucleotide 083C as the primer, are shown in Fig. 4.18, and those of RNA from pRAM4 (using the same oligonucleotide primer) are shown in Fig. 4.20. pRAM1 derived RNA was also analysed using the universal sequencing primer, results of which are shown in Fig. 4.19.

Analysis of pRAM1 derived mRNA, using oligonucleotide 083C, produced four fragments (175, 185, 270 and 278nt in length) which map to co-ordinates 2888, 2878, 2793 and 2785 respectively (on the scale of Post et al 1979) in the rpoBC operon sequence. 2785 and 2793 are at previously mapped cleavage sites in the RNase III target region (see Fig. 4.21.1), thus possibly indicating RNase III processing of pRAM1 derived mRNA at these sites. However position 2888 lies outwith the proposed RNase III target site and 2878 has not been previously mapped as a cleavage site. These fragments may be due to processing of pRAM1 by an unidentified enzyme; may indicate that the transcript has an abnormal secondary structure thereby causing altered processing; or may represent previously undetected RNase III cleavage sites in this region. Analysis of pRAM1-derived mRNA from an rnc-strain might help to clarify this matter (see also
Similarly, four major fragments were observed when using universal sequencing primer to analyse this RNA (the identical RNA preparation). These fragments (152, 158, 170 and 172nt long) map to co-ordinates 2876, 2870, 2858 and 2856 respectively. All four align to the 3' arm of the proposed RNase III target site. Cleavage sites in this arm have been reported previously (see Fig. 4.21.1), although not accurately defined. Processing in this arm may well be specific to plasmid-derived rpoBC transcripts. It is surprising that the analysis of a single RNA preparation (using two different primers) should yield the variety of end-points observed (see Fig. 4.21.1). A diagram showing the position of all these processing sites (relative to the known mapped cut sites) in the context of putative stem-loop RNase III target site secondary structure is presented in Fig. 4.21.1.

Primer extension analysis of RNA from the four deletion mutant plasmids showed that none were processed in the vicinity of the RNase III target site. However it must be noted that the quality of the data presented in Fig. 4.20 (pRAM4) is very poor and hence is not entirely conclusive. In each case the predominant fragment corresponded to an uninterrupted transcript initiated at (or near) Plac. A less abundant, secondary band was observed which mapped to a position upstream of the cloned rpoBC operon fragment in pRAM3, pMRM1 and pRAM4. It was designated S3 in pRAM3 (Fig. 4.18), S4 in pMRM1 (Fig. 4.18) and S1 in pRAM4 (Fig. 4.20) and detailed in Fig. 4.21.2. Its origin will be discussed later.
Primer extension analysis of pRAM1, pRAM2, pRAM3 and pMRM1-derived transcripts, using oligonucleotide 083C. The upper part shows simple maps of the relevant region for each plasmid (not to scale). The position of the promoter Plac* is shown by (Pr) in each case. Open boxes represent the HindIII - XmaI fragments cloned from the appropriate M13s into pSKS106 to generate each plasmid. The position of the oligonucleotide 083C binding site, relative to this insert sequence, is shown for each plasmid. 1,2,3,4 P2,P3,S3,P4 and S3 all show the approximate positions of the DNA fragments produced by primer extension analysis. The lower part shows an autoradiograph of the acrylamide gel.

Tracks

M Markers are pBR322/MspI and pBR322/AvaI, NheI, mixed
1 70μg pRAM1 RNA : 0.3 pmoles 083C - fragments 1,2,3,4
2 70μg pRAM2 RNA : 0.3 pmoles 083C - fragment P2
3 70μg pRAM3 RNA : 0.3 pmoles 083C - fragments P3,S3
4 100μg pMRM1 RNA : 1.3 pmoles 083C - fragments P4,S4.

The sizes of all DNA fragments are given in nt.
Fig. 4.19
Primer extension analysis of pRAM1-derived transcripts using universal sequencing primer. The upper part shows a simple map of the relevant region of pRAM1 (not to scale). The position of Plac+ is shown by (Pr). The open box represents the 277bp HindIII - XmaI fragment from M13mp19-5 cloned into pSKS106 to generate pRAM1. The position of the universal sequencing primer binding site (U.P.) relative to this insert sequence is shown. 1,2,3,4 show the approximate positions of the DNA fragments produced by primer extension analysis. The lower part shows an autoradiograph of the acrylamide gel.

Tracks
M Markers are pBR322/MspI and pBR322/AvaI + NheI mixed
1 140μg pRAM1 RNA : 1.3pmoles UP - fragments 1,2,3,4
2 70μg pRAM1 RNA : 0.3pmoles UP - " "
The sizes of all DNA fragments are shown in nt.

Fig. 4.20
Primer extension analysis of pRAM4-derived transcripts using oligonucleotide 083C. The upper part shows a simple map of the relevant region of pRAM4 (not to scale). The position of Plac+ is shown by (Pr). The open box represents the 247bp HindIII - XmaI fragment cloned from M13mp19-11 into pSKS106 to generate pRAM4. The position of the oligonucleotide 083C binding site relative to this insert sequence is shown. P1 and S1 show the approximate positions of the DNA fragments produced by primer extension analysis. The lower part shows an autoradiograph of the acrylamide gel.

Tracks
M Markers are pBR322/MspI and pBR322/AvaI + NheI, mixed
1 140μg pRAM4 RNA : 1.3pmoles 083C - Note
2 70μg pRAM4 RNA : 0.3pmoles 083C - P1,S1.
The sizes of all DNA fragments are shown in nt.

Note This RNA preparation contained sufficient salt (especially in Track 1) to affect the mobilities of the final product bands.
Fig. 4.21.1

Analysis of primer extension data. Positions of RNase III cut sites.

Open boxes (a)-(e) show the previously reported RNase III cut sites in this region (mapped using S1 nuclease mapping).

(a) 2770 (±3) (Ralling and Linn 1987)
(b) 2776 (±1) (Downing and Dennis 1987)
(c) 2785 (±3) (Fukuda and Nagasawa-Fujimori 1983, Downing and Dennis 1987, Ralling and Linn 1987)
(d) 2864 (Fukuda and Nagasawa-Fujimori 1983)
(e) 2860 (Barry et al 1980)

083C 1-4, show the positions of the fragment termini (see Fig. 4.18, Track 1) in the context of the RNase III target stem-loop. UP 1-4, show the positions of the fragment ends (see Fig. 4.19, Tracks 1 and 2) in the context of the RNase III target stem-loop.

Fig. 4.21.2

Analysis of primer extension data. Position of the secondary band terminus (S3, S4, S1). The sequences at the junction of lacZ and the M13mp19 polylinker region are shown in each case. The arrow marks the position of the end of each fragment (see Fig. 4.18 and Fig. 4.20) within this region.
Fig 4.21.1

083C3 (2793±2)
083C4 (2785±2)

UP4 (2856±2)
UP3 (2858±2)

UP2 (2870±2)
UP1 (2876±2)

083C2 (2878±2)
083C1 (2888±2)

Fig 4.21.2

pRAM3
lacZ
mp19
CCAAGCTTTG

S3

pMRM1
lacZ
mp19
ATTACGCCAAGCTTTG

S4

pRAM4
lacZ
mp19
CCAAGCTTTGCATGCCTGCA

S1
4.4.2 \( \beta \) GALACTOSIDASE ASSAYS

Since pRAM1 is processed faithfully at its RNase III target site (see Fig. 4.18, Fig. 4.19 and Fig. 4.21.1), whilst the messenger RNAs of all four deletion mutant derivatives are not cleaved at this site then the effect of processing on the expression of the \( rpoB - lacZ \) fusion proteins can be judged. This was done by measuring the level of \( \beta \) galactosidase activity produced from each plasmid in the host strain AK14. This strain carries \( lacI^q \); therefore transcription from \( Plac^+ \) must be induced with IPTG. Spizizen-glycerol minimal cultures of AK14 (alone or transformed with the appropriate plasmid) were grown in the absence or presence of IPTG (10\(^{-5}\)M). The next day these were diluted and grown in the same medium (plus or minus IPTG) to an \( A_{600} \) of 0.3. Assays were then performed as described in section 2.10. Results were calculated using the following equation:

\[
\text{Miller Units} = 1000 \times \frac{A_{420} - 1.75 \times A_{550}}{t \times v \times A_{600}}
\]

(per ml of culture at \( A_{600} \) of 1.0)

where \( t \) is the duration of incubation of the sample at 28°C (in minutes)

\( v \) is the volume of cell culture in the sample (in ml)

\( A_{600} \) is the culture absorbance at time of sampling (i.e. approximately 0.3).
(ONPG does not absorb at 550nm. Thus the $A_{550}$ reading only measures light-scattering by cell debris. For *E. coli* light-scattering at 420nm is 1.75 times that at 550nm).

The levels of β galactosidase activity for pSKS106, pRAM1 and the deletion mutant derivatives are shown in Table 4.1.

Table 4.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>β Galactosidase Levels $-\text{IPTG}^1$</th>
<th>$+\text{IPTG}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot; pSKS106</td>
<td>3</td>
<td>5506 (±283)</td>
</tr>
<tr>
<td>&quot; pRAM1</td>
<td>0</td>
<td>53 (±7)</td>
</tr>
<tr>
<td>&quot; pRAM2</td>
<td>2</td>
<td>510 (±19)</td>
</tr>
<tr>
<td>&quot; pRAM3</td>
<td>1</td>
<td>341 (±16)</td>
</tr>
<tr>
<td>&quot; pRAM4</td>
<td>1</td>
<td>378 (±6)</td>
</tr>
<tr>
<td>&quot; pMRM1</td>
<td>0</td>
<td>15 (±0.3)</td>
</tr>
</tbody>
</table>

β galactosidase levels are expressed in Miller Units.

1) Figures are the result of a single observation.

2) Each result is the mean of at least three observations; figures in brackets are standard errors.
The results presented in Table 4.1 show that, in the absence of IPTG, \(lacIq\) (carried on \(F'\)) strongly represses transcription from \(P_{lac}\), leading to little or no \(\beta\) galactosidase activity. Under IPTG induction, the results show that the introduction of the 277bp \(HindIII - XmaI\) fragment of \(rpoBC\) operon sequence (from M13mp19-5) between the same sites in pSKS106 has a profound effect on \(\beta\) galactosidase expression: pRAM1 \(\beta\) galactosidase levels are approximately 100 fold lower than those of the "parent", pSKS106. The reason for this is not clear. Perhaps the alteration of codon 6 of the \(rpoB\) structural gene (see p.183) has affected the translational efficiency of this message (e.g. due to altered codon usage?). An investigation of this region using site-directed mutagenesis to change specific nucleotides may yield some useful information. Large deletions in the \(rplL - rpoB\) intergenic region of this plasmid result in increased \(\beta\) galactosidase levels of approximately 6 - 10 fold (pRAM2, pRAM3 and pRAM4). Intriguingly the small deletions in pMRM1 caused the level of \(\beta\) galactosidase activity to drop below that of pRAM1 (by about threefold). In order to relate the effects of all these deletions on \(\beta\) galactosidase expression to the role of RNase III processing of \(rpoBC\) mRNA one could transfer this series of plasmids into a suitable host strain: an isogenic \(rnc105\) derivative of AK14, generated by P1 transduction. In this way the effects of RNase III processing and of the deletions could be independently monitored (by \(\beta\) galactosidase assays, as above), thereby clarifying their contributions to the levels of \(rpoB-lacZ\) expression.

4.4.3 CRUDE ESTIMATION OF \(lacZ\) AND FUSION PROTEIN LEVELS

In order to ensure that the differences in \(\beta\) galactosidase enzyme activity shown in Table 4.1 reflected differential gene expression rather than low enzymatic activity of the hybrid \(\beta\) galactosidase, the level of \(lacZ\) or fusion protein produced from each plasmid was crudely determined. Thus a comparison could be made between \(lacZ/fusion\) protein levels and \(\beta\) galactosidase enzyme activities in the series of plasmids.
AK14 (alone or transformed with the appropriate plasmid) was grown in the absence or presence of IPTG (10^{-5}M) exactly as described in section 4.4.2. At an A_{600} of 0.3 unlabelled cell extracts were made as described in section 2.8.1, and fractionated on SDS polyacrylamide gels as described in section 2.9.9. The gels were stained, destained and dried as described in section 2.9.9, and then photographed. Fig. 4.22.1 shows an "uninduced gel" (i.e. no IPTG added) and Fig. 4.22.2 shows an "induced gel" (IPTG at 10^{-5}M). The rpoB - lacZ fusion proteins are virtually identical in size to the lacZ protein encoded by pSKS106 (just four amino acids shorter) and are approximately 116,000 Daltons in size. The uninduced protein gel (Fig. 4.22.1) shows no protein of this size, which is in agreement with the β galactosidase enzyme assays showing very little or no activity in the absence of IPTG. In the presence of the inducer (Fig. 4.22.2) the levels of fusion protein observed correlate well with the levels of β galactosidase enzyme activity shown in Table 4.1. Thus it appears that the differences in β galactosidase activities do reflect differences in rpoB - lacZ expression by the various plasmids.
Fig. 4.22.1

Crude estimation of $\text{lacZ}$ and fusion protein levels by SDS polyacrylamide gel electrophoresis of cell extracts. Extracts were prepared from cultures grown in the absence of IPTG.

**Tracks**

M Markers are from the MW-SDS-200 kit (Sigma) which contains six proteins of known molecular weights. Two of these [phosphorylase B, rabbit muscle - 97,400 Daltons and $\beta$-galactosidase, $E. coli$ - 116,000 Daltons] are shown.

1 AK14 pRAM4 - IPTG (1)
2 " pSKS106 " (3)
3 " pRAM2 " (2)
4 " pRAM3 " (1)
5 " pRAM1 " (0)
6 " pMRM1 " (0)
7 AK14 " (0)

Figures in brackets are the observed levels of $\beta$-galactosidase activity (in Miller Units) - taken from Table 4.1.

Fig. 4.22.2

Crude estimation of $\text{lacZ}$ and fusion protein levels by SDS polyacrylamide gel electrophoresis of cell extracts. Extracts were prepared from cultures grown in the presence of IPTG ($10^{-5}$M). The location of the fusion proteins (and $\text{lacZ}$ from pSKS106) is marked by F.P.

**Tracks**

M Markers are the same as in Fig. 4.22.1

1 AK14 pRAM4 + IPTG (378±6)
2 " pSKS106 " (5506±283)
3 " pRAM2 " (510±19)
4 " pRAM3 " (341±16)
5 " pRAM1 " (53±7)
6 " pMRM1 " (15±0.3)
7 AK14 " (0)

Figures in brackets are the levels of $\beta$-galactosidase activity (in Miller Units) taken from Table 4.1.

All sizes are shown in Daltons.
4.4.4 DETERMINATION OF mRNA SYNTHESIS RATES DURING IPTG INDUCTION

The differential expression of the various rpoB - lacZ fusions may be due to transcriptional or post-transcriptional effects. Obviously since each plasmid has the same promoter (Plac+) one would expect that the level of transcription of the lacZ or rpoB - lacZ fusion genes should be similar in each case, and would therefore conclude that post-transcriptional events are responsible for determining the level of expression from the various plasmids. However, the possibility that the deletion mutants are responsible for specific modulations in the rate of transcription of the rpoB - lacZ fusion genes cannot be entirely excluded. Therefore I determined the mRNA synthesis rates of lacZ and the rpoB - lacZ fusion genes in pSKS106, pRAM1 and the four deletion-mutant plasmids.

AK14 (alone, or transformed with the appropriate plasmid) was grown as described in section 4.4.2 in the presence of IPTG (10^{-5}M) to an A_{600} of 0.3. At this point the cultures were pulse-labelled with [^{3}H] uridine for one minute and the RNA extracted as described in section 2.7.3.

M13mp8 was used as the source of both the positive and negative hybridisation probes. It contains no
bacterial DNA insert in the polylinker region, but does have the $lacZ$ $\alpha$ complementation region. M13mp8 SS DNA was used as the "negative-hybridisation" probe since it has no sequence complementarity to any bacterial messenger RNA. Filters without any DNA were included as further negative controls. Denatured M13mp8 RF DNA was used as the source of the "positive-hybridisation" probe (obviously it includes both negative and positive hybridisation probe DNA). It contains a sequence (altered only at the polylinker region) which is complementary to 372nt of $lacZ$ mRNA extending from Plac through the $\alpha$ complementation region of $lacZ$. This probe will hybridise with 372nt (again with some deviations at the polylinker) of $lacZ$ mRNA from plasmids pSKS106, pRAM1, pRAM2, pRAM3, pRAM4 and pMRM1. It will also hybridise with 279nt of $lacZ$ mRNA derived from the $lacZ$ $\Delta$M15 fragment carried on F', which is present in strain AK14. The level of hybridisation determined in untransformed AK14 must be subtracted from the levels determined in transformed AK14 strains to achieve an accurate measure of the transcription rate for each plasmid.

DNA : RNA filter hybridisation experiments were performed as described in section 2.11.8. The presence of excess probe DNA (immobilised on nitrocellulose filters) during these experiments was verified by the linear relationship between input and hybridised counts,
Hybridisation of pulse-labelled RNA to specific DNA probe. In every experiment, the presence of excess probe DNA (immobilised on nitrocellulose filters) was verified by the linear relationship between input and hybridised counts, using increasing amounts of RNA. In each case the RNA preparation was divided into three fractions (0.5, 0.25 and 0.125) and hybridised in separate assays to excess probe DNA.
Fig 4.23

cpm

AK14  pRAM1  pSKS106  pRAM2

pRAM3  pMRM1  pRAM4

increasing [RNA] →
using increasing levels of RNA, in every experiment (see Fig. 4.23). The results of hybridisation of the labelled RNAs to the M13mp8 "positive-hybridisation" probe are shown in Table 4.2.

Table 4.2

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of input RNA per nt. (x 10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK14</td>
<td>(0.32)(^a)</td>
</tr>
<tr>
<td>&quot; pSKS106</td>
<td>1.57</td>
</tr>
<tr>
<td>&quot; pRAM1</td>
<td>2.69</td>
</tr>
<tr>
<td>&quot; pRAM2</td>
<td>3.88</td>
</tr>
<tr>
<td>&quot; pRAM3</td>
<td>3.43</td>
</tr>
<tr>
<td>&quot; pRAM4</td>
<td>4.50</td>
</tr>
<tr>
<td>&quot; pMRM1</td>
<td>3.43</td>
</tr>
</tbody>
</table>

\(^a\) This value has been subtracted from all the other results. It is the level of hybridisation due to F' derived lacZ mRNA. Background counts and non-specific hybridisation (generally 5-20cpm) have been subtracted.
The results presented in Table 4.2 show that the rates of mRNA synthesis from the various plasmids used in this work are, broadly speaking, similar. There is an approximately three-fold difference between the lowest and highest rates of synthesis. This compares with an approximately three hundred fold difference between the lowest and highest levels of β galactosidase activity amongst the plasmids. More importantly, there is no correlation between these small variations in rate of mRNA synthesis and the big variations in level of β galactosidase activity. In particular pSKS106 has the lowest mRNA synthesis rate but yields by far the highest level of β galactosidase activity. Clearly the differences in β galactosidase activity between the various plasmids shown in Table 4.1 are not due to differential rates of mRNA synthesis.

4.4.5 ARE THE *rpoB - lacZ* FUSIONS SUBJECT TO TRANSLATIONAL CONTROL?

The *rpoB - lacZ* translational fusion proteins only contain the first five amino acids of the β subunit. Thus it is almost certain that they are incapable of acting as autogenous translational repressor molecules (or components thereof). However, although the results of Passador and Linn (1989) suggest that the target for feedback repression is not fully intact on the *rpoB - lacZ* fusion mRNA, the site may be partially retained.
The repressor complex might well be present in high enough molarity to affect it, even when being transcribed from the multiple copies of the Plac promoter.

Using the knowledge that rifampicin can alleviate translational repression of rpoB in vitro (Lang-Yang and Zubay 1981, Fukuda and Nagasawa-Fujimori 1983) I decided to test whether any of the rpoB - lacZ fusion genes were subject to translational repression in vivo, by comparing the kinetics of β galactosidase induction (with IPTG) in the absence and presence of rifampicin. If translational repression were in operation then rifampicin might interfere with this, and consequently increase the level of β galactosidase expression during IPTG induction. If not, then rifampicin should not alter the kinetics of induction with IPTG.

Rifampicin was used at a concentration of 3μgml⁻¹. At this level the drug caused a 2 - 3 fold increase in the differential rate of ββ' synthesis after 45 minutes in AK14, AK14 (pSKS106) and AK14 (pRAM1) (data not shown), as expected from previous observations. For β galactosidase induction experiments, AK14 (transformed with the appropriate plasmid) was grown to an A₆₀₀ of 0.3 in Spizizen-glycerol minimal medium (plus ampicillin). At this point IPTG (10⁻³M) and/or rifampicin (3μgml⁻¹) were added and growth continued. Samples were taken 15, 30 and 45 minutes later and β galactosidase activity
measured as described in section 2.10. The results of these tests are shown graphically in Fig. 4.24. In every case it is clear that there is no increase in the level of β galactosidase induction (with IPTG $10^{-3}$M) in the presence of rifampicin compared to that without the drug. If anything, the addition of rifampicin (3μgml$^{-1}$) decreases the rate of β galactosidase induction with IPTG. Thus, from this series of tests, it would appear that the $rpoB$ - $lacZ$ fusion genes are not subject to translational repression.
Induction of β-galactosidase expression from the various plasmids, in the absence and presence of rifampicin. Time (after induction) is shown in minutes on the X-axis, in each case. The level of β galactosidase activity from each plasmid is shown in Miller Units. IPTG induction, in the absence of rifampicin, is shown by open circles, and induction in the presence of the drug, by closed circles.
Fig 4.24
4.5 DISCUSSION

The combination of sequence and structural elements which make up a RNase III target site is, as yet, not fully understood. Therefore it was interesting that all four RNase III site deletions, created during this work, completely abolished processing. This was expected for the deletion in pRAM2 which has lost the RNase III site entirely. The total abolition of processing in pRAM3 and pRAM4 (both of which contain partial RNase III site deletions) demonstrates the absolute requirement for secondary structure in this cleavage event, as previously observed by e.g. Srivastava and Schlessinger (1989b) in the RNase III processing of 16S rRNA. The relatively "subtle" deletion of two copies of the triplet UUU (the deletion in pMRM1) from the RNase III target site results in the entire loss of processing. This may be due to disruption of the secondary structure caused by the deletions, an explanation which has been used previously to explain the effects of point mutations in the sib region, downstream of the int gene in bacteriophage λ (Guarneros et al 1982). However, the locations of the UUU triplets are such that their loss need not necessarily cause the collapse of the RNase III target site secondary structure (see Fig. 4.11). Accordingly it may be that failure to process rpoBC mRNA after the deletion of the UUU triplets reflects a loss of some primary sequence information required for cleavage. The
elucidation of pMRM1 derived mRNA secondary structure in this region would be interesting.

The secondary fragments observed during primer extension analysis, designated S3 in pRAM3, S4 in pMRM1 and S1 in pRAM4, all map to a region upstream of the rpoBC insert fragment, either in M13mp19 polylinker sequence or in the start of the lacZ sequence. Cannistraro and Kennell (1985b) observed "ragged" 5' ends of lacZ mRNA, which they assumed were the products of degradation to the beginning of the ribosome-protected region of lacZ message. The secondary bands seen in the primer extension analysis may be due to a similar phenomenon; or could represent the formation of a novel cleavage site; or, of course, could reflect premature termination during the reverse transcription reaction.

The set of rpoB - lacZ translational fusion plasmids constructed during this work has provided an extremely useful, and relatively easy, way of monitoring the effects of the RNase III target site deletions on the expression of rpoB. The deletions were all made in a short rpoBC operon fragment (which lacks the tL7 attenuator) and then cloned downstream of Plac+. This arrangement ensured that the rate of mRNA synthesis was similar from plasmid to plasmid. These rates were determined using an amino-terminal DNA probe (M13mp8 RF DNA) which allows the detection of lacZ mRNA proximal to
the known site(s) of rho-mediated transcription termination. This site(s) is certainly distal to nucleotide position 200 of the coding region (Stanssens et al 1986), and could be near the middle of the lacZ gene (de Crombrugghe et al 1973). As described in Chapter 1, termination here is related to the efficiency of translation initiation. Therefore it was important to avoid this complication when attempting to monitor the rate of mRNA synthesis from the various plasmids, since they had already shown a wide range of β galactosidase enzyme activities (i.e. varied translation initiation efficiencies). The deliberate omission of the attenuator tL7 from these plasmid constructs also eliminated a possible source of variation at the transcriptional level.

One of the major difficulties in interpreting previous studies of rpoB expression has been assessing the contribution of autogenous translational repression. The "in frame" fusion of a very short amino-terminal portion of rpoB to the lacZ reporter gene appears to have overcome this problem. It seems certain that the fusion will not be susceptible to "full-blown" translational feedback regulation, because the target for this control has been, at least partially, deleted. Passador and Linn (1989) presented evidence which suggests that part of the translational regulation site may lie between 126 and 403bp inside the rpoB gene. It should be stressed
that this is probably just part of the regulatory site, since weakened translational control was still observed in a construct containing *rplK* and only the first 126bp of the *rpoB* structural gene (Passador and Linn 1989). Regulation was also noted by Dennis (1984) in a very similar construct (containing the same length of *rpoB*) (Hui et al 1982). In the fusions used in the present work, however, only 15bp of *rpoB* remain; thus the feedback target structure might well have been totally lost. The fact that the presence of rifampicin did not enhance β galactosidase expression from the fusion plasmids, during IPTG induction, serves to reinforce the view that these *rpoB − lacZ* fusion plasmids are not subject to translational regulation.

This allows a clearer interpretation of the results of the β galactosidase assays presented in Table 4.1. One conclusion which can be drawn is that any effect RNase III processing might have on *rpoB − lacZ* mRNA stability is neither the sole nor predominant means of controlling *rpoB − lacZ* expression. Similar quantities of non-processed transcript produce higher (pRAM2, pRAM3, pRAM4) and lower (pMRM1) levels of β galactosidase than processed mRNA (pRAM1). Thus there is no systematic way in which RNase III processing (or the lack of it) could, solely by affecting messenger stability, account for these β galactosidase assay results.
Instead, it is likely that the variations in β galactosidase activities are due to differences in the efficiency of translation initiation at rpoB in the various plasmids. Echoing the argument proposed in section 3.7, I suggest that the efficient initiation of translation at rpoB is dependent upon attaining an optimal mRNA conformation, and that RNA processing is required to reach this state. An outline of this model is presented in Fig. 4.25. The secondary structure shown between positions 2879 and 2977 is the optimal one for this sequence using the "RNA fold" programme of Zuker (1981). It is possible that by "helix-stacking", one large extended secondary structure is formed from position 2770 - 2977. It is proposed that RNA processing (presumably by RNase III, although this has yet to be rigorously tested - see p. 192) is required to destroy the "closed" structure in Fig. 4.25 (1) and convert this to the "open" structure Fig. 4.25 (3). In particular it should be noted that the novel cleavage site mapped at position 2888 (this work) which lies outwith the proposed RNase III target stem-loop structure could destabilise the lower stem-loop structure. "Closed" and "open" refer to the accessibility of the rpoB Shine and Dalgarno sequence and translation initiation site for interaction with 16SrRNA of the small ribosomal subunit, and the initiator tRNA respectively. Thus processing would be required to achieve the optimal mRNA conformation for efficient translation initiation. According to this model the deletions in pRAM2, pRAM3 and pRAM4 all destabilise the "closed" structure and thus achieve an "open" conformation without the need for RNase III processing. The mRNA derived from these plasmids is therefore intrinsically translationally efficient, and thus each one produces a high level of β galactosidase activity relative to wild-type mRNA, which must first be processed
Fig. 4.25

A model for the role of processing in rpoB expression.

(1) Shows the proposed secondary structure of rplL - rpoB mRNA. 2770-2879 is the putative stem-loop of the RNase III target. Nucleotides 2879-2977 are shown in the predicted optimal conformation (Zuker 1981). The closed box shows the Shine and Dalgarno sequence. The open box is the rpoB structural gene. ATG shows the rpoB initiation codon. This is the "closed" conformation.

(2) Arrows show the positions of mapped RNase III cut sites (this work, and previously published data). Other symbols as in (1).

(3) Symbols as in (1). This is the "open" conformation, achieved by RNase III processing (stage (2)).
Fig 4.25

(1) 2770

2879
2977

ATG
S-D

(2) ATG
S-D

(3) S-D ATG
by RNase III. This requirement for cleavage may partly explain the low levels of β galactosidase activity in pRAM1 relative to pSKS106. The deletions in pMRM1 also abolish processing but, it is argued, allow the maintenance of the "closed" conformation, resulting in translationally inefficient mRNA and explaining the low levels of β galactosidase activity in pMRM1.

Such a role of RNase III processing in determining translational efficiency is not without precedent. Processing greatly stimulates translation of gene 0.3 mRNA of phage T7, \textit{in vivo} and \textit{in vitro} (Dunn and Studier 1975). It can also play a role in the regulation of genes 1.1 and 1.2 of the same phage. Incomplete processing at a RNase III site downstream of gene 1.2 in a particular T7 mutant produces an aberrant transcript which is 29nt longer than in wild-type T7. Apparently this extra sequence can hybridise to the gene 1.1 ribosome binding site and exclude ribosomes. Since the translation of genes 1.1 and 1.2 is coupled, both are regulated by this mechanism (Saito and Richardson 1981). More recently it has been shown that RNase III stimulates the translation of the cIII gene of bacteriophage \(\lambda\). Intriguingly it appears that RNase III binding, not necessarily processing, at a site upstream of the cIII gene is responsible for the translational enhancement (Altuvia \textit{et al} 1987), possibly by favouring an mRNA structure which increases the translational efficiency of
cIII mRNA (Kornitzer et al 1989). These models, in which RNase III or RNase III processing shift the equilibrium between two mRNA conformations to increase cIII mRNA translational efficiency are very similar to the one which I have outlined in Fig. 4.25 to explain the role of RNase III processing in the expression of rpoB. Further mutational analysis is currently underway in this laboratory in order stringently to test the model.

A possible alternative explanation of the data is that RNase III binding (as with the c III gene) is responsible for altering the secondary structure and translatability of the rpoB - lacZ transcript. This could be tested by producing transcripts from each of the deletion-plasmids in vitro and elucidating their mRNA secondary structures in the presence and absence of purified RNase III.
An important factor in \textit{rpoB} gene expression is the contribution made by autogenous translational repression. This mechanism is probably responsible for balancing the expression of \textit{rpoB} (and \textit{rpoC}) between \textit{rnc} and \textit{rnc105} strains. Only when translational control is lifted, for example by rifampicin treatment (as detailed in Chapter 3) or by the creation of a plasmid-borne \textit{rpoB} - \textit{lacZ} fusion system (as detailed in Chapter 4), is the effect of RNase III processing revealed. However an important question remains: if translational control ultimately dictates the level of 1313' synthesis, what is the physiological significance of RNase III processing at this site? It may be that any sudden demand for increased RNA polymerase output can only be met by the increased translational efficiency of \textit{rpoB} mRNA which RNase III cleavage can provide. Alternatively RNase III processing and translational control may themselves be intimately related: processing at the RNase III site, upstream of \textit{rpoB}, may be required for translational regulation (perhaps to create a target structure for translational repression?)

The \textit{rpoB} - \textit{lacZ} fusion which I have constructed seems well suited to a study of the sequence requirements
for RNase III processing at the rpoB site, and of the effect of such processing on the translatability of rpoB mRNA. Further studies along these lines are intended. The fusion is probably not susceptible to feedback regulation of translation. This could be more rigorously tested by in vitro methods. Alternatively the fusion could be transferred from a multicopy to a single copy system, in case the observed loss of regulation is due (at least in part) to an overproduction of target sites relative to repressor complexes. If, as expected, the fusion cannot be translationally regulated (due to the loss of part of all of the target site), then the fusion of longer stretches of rpoB to the lacZ reporter gene seems the best way ahead in attempting to locate the target site for translational repression.
BIBLIOGRAPHY


American Society for Microbiology, Washington D.C.


