FUSIONS OF E. coli GENES lac, trp AND dea IN BACTERIOPHAGE LAMBDA

by

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A thesis presented for the Degree of Doctor of Philosophy at the University of Edinburgh

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November 1978
It is an impossible task to acknowledge everybody who has contributed to making my stay in Edinburgh so enjoyable. I would especially like to thank: the "in-mates" of 617A, Sue, Kathy, Graham and Alan, for providing a warm and friendly working environment; the ladies of the media and washing-up rooms, particularly Marion, Margaret and Ruby; Peter Southern for his invaluable advice; and Professor Ken Murray for his interest not to mention access to his wide range of restriction enzymes, etc.

Kathy Chalmers deserves a very special thankyou, a finer young lady I have yet to meet.

No words can possibly express my gratitude to my Supervisor, Dr. Noreen Murray. Especially for allowing me to make my own mistakes and always being available with good council. I have enjoyed working alongside her and have greatly benefited from our association.

I would especially like to acknowledge the ICL 14_75 and 2970 computers with whom I have spent many happy evenings. I would like to thank the staff of the Edinburgh Regional Computing Centre. Special thanks go to the members of the Computer Science Department (particularly Colin Adams) who bailed me out when the typewriter broke-down three days before this thesis was due to be typed.

Lastly, but definitely not least, I would like to thank my family. To Carol for being a typical younger sister, and to my parents - without whom none of this work could have been done.
Gene fusions have been constructed in bacteriophage lambda using in-vitro techniques in order to study two systems: convergent transcription and the regulation of polA expression.

Convergent transcription defines a situation in which two promoters are actively transcribing in opposition. λtrp transducing phages were used to investigate the effects of opposing transcription from the trp and APL promoters. It is shown that PL transcription completely blocks expression of the trp genes whilst transcription from the trp promoter both delays and attenuates leftward transcription from PL. The implications of these results are discussed.

Fusions between the polA and lacZ genes were constructed in which the transcription of lacZ is directed by the polA promoter. These fusions were characterized and used to investigate the regulation of polA expression. polA does not appear to regulate its own expression.
ABBREVIATIONS AND CONVENTIONS

kb       kilo-base-pairs
kd       kilo-dalton
pX       protein product of gene X
ACH      Casamino-acids - acid casein-hydrolysate: deficient in tryptophan
MMS      methyl methane-sulphonate
UV       Ultra-violet light
mM       milli-Molar
μM       micro-Molar
nm       nano-meters

1) Genetic nomenclature according to Bachmann et al (1976). Genotypes will be indicated by underlining the gene symbol.

2) In general bacterial strains will be referred to by their key genotype. In this case the gene symbol will not be underlined.

3) Restriction enzyme nomenclature according to Smith & Nathans (1973).

4) Lysogens will be designated by the bacterial host followed by the phage description enclosed in brackets. Where the genetic description of the phage is too long for convenience this will be replaced by the phage stock number.

5) Orientation of genes in a lambda transducing phage is defined according to their direction of transcription. Thus l-orientation denotes leftward transcription.
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CHAPTER 1

BACTERIOPHAGE LAMBDA
Although bacteriophage lambda is comparatively small, it has been the subject of much study. This interest culminated in 1971 in the production of a Cold Spring Harbor monograph, edited by Hershey, on "The Bacteriophage Lambda". Even today, seven years later, the reviews and articles still present an accurate overview of the development of phage lambda.

The mature lambda virion consists of protein and DNA in approximately equal proportions (Hershey & Dove, 1971). The DNA moiety is a single linear duplex, 49000 base-pairs in length (Philippsen & Davis, unpublished) with complementary 12 base-pairs long single-stranded projections at each 5'-end (Wu & Taylor, 1971). By means of these "cohesive" ends the linear molecule is able to circularize. Enclosing the DNA is a protein coat - the virion head - which is 50 nano-meters (nm) in diameter and attached to a protein tail 150nm long. The tail is flexible but non-contractile and is terminated by a single tail fibre which is responsible for the specific adsorption to the cell wall of the host bacterium Escherichia coli K12.

a) Life Cycle

The life cycle of lambda, like that of all bacteriophages, starts with the adsorption of the phage to the cell surface of the host. After this the DNA is injected into the cytoplasm and the phage "early" functions are expressed. These include the DNA replication functions which serve to direct the multiplication of the phage genome. The structural proteins of the virion are then synthesized and mature progeny start to appear. Some time later phage coded
products cause lysis of the host and the progeny are released.

The above cycle is often referred to as the "lytic-pathway" and is exhibited by almost all phages. Some phages, lambda included, are able to undergo a different course of development known as the "temperate-response" or "lysogenic-pathway". In this case, the phage DNA starts to replicate but the late structural genes are not expressed and no progeny appear. Instead, gene expression is turned-off and the phage becomes dormant. This is accompanied by the insertion of the phage genome into that of the host. The resultant lysogenic cells are now immune to further infection by lambda.

b) The Lytic Pathway

Immediately after the phage DNA enters the cytoplasm two promoters become active. These are designated $P_L$ and $P_R$ since their directions of transcription are respectively leftwards and rightwards on the phage lytic map (see Fig 1.2). The initial transcripts from these promoters are small and only two genes are expressed - $\mu$ and $\sigma$. The $\mu$ product, pN, acts to turn-on the expression of the delayed-early genes which include the replication functions and the $\sigma$ gene product. $\sigma$ acts to switch-off expression of these genes by repressing the two early promoters. This is apparently required not only to aid replication (see section IV) but also to facilitate the smooth transition to late gene expression. $\sigma$ controls the expression of the late functions which include the structural proteins and also the genes involved in host cell lysis. The yield of progeny in a typical infection is about 50-100. Regulation of the lytic pathway has been summarized by Echols (1971).
c) The Lysogenic Pathway

The lysogenic response is initially identical to that of the lytic pathway. pN switches on expression of the delayed early genes and phage replication ensues. However, two of the delayed-early products are those from genes cII and cIII. These are responsible for an activation of phage repressor synthesis. It is at this point that the decision has to be made as to whether the lytic or lysogenic pathway is followed. If expression of phage repressor dominates then transcription of the lytic functions will be blocked and the lysogenic pathway will ensue, otherwise expression of cII and cIII will be turned-off by cro and lytic development will occur.

Expression of the lysogenic pathway results in the production of the phage "integrase" system which catalyzes the site-specific recombination event that results in the integration of lambda into the host chromosome.

d) Immunity

The ability of the phage repressor to block expression of the lytic functions is central to the ability of lambda to maintain itself in the dormant lysogenic state. It has been shown that the repressor protein is able to specifically block early transcription (Chadwick et al., 1970; Steinberg & Ptashne, 1971). In the lysogenic state only two lambda genes are expressed and one of these is the repressor gene. The effect of this is that in such cells lambda repressor will be present. Should these lysogens be superinfected with lambda, the incoming phage genome will not be able to initiate expression of the lytic functions since their transcription is blocked by repressor. The cells survive and are said to be immune to superinfection by lambda. Other lambdoid phages are, however, still able to grow on
these lysogens and are said to be "hetero-immune" since they display a different immunity specificity. Kaiser & Jacob (1957) constructed a hybrid of lambda carrying the immunity specificity of phage 434, i.e. this hybrid phage will grow on a lambda lysogen but not on a lysogen of phage 434. The only difference between this hybrid and lambda was the replacement of a small region of lambda DNA with the non-homologous region from phage 434. This segment must be responsible for the specificity of immunity and is referred to as the "immunity-region".

e) Induction

In addition to the events leading to the establishment of the lysogenic state, lambda also has the ability to specifically reverse lysogeny and the integration of the phage into the chromosome. Experiments showed that induction of lambda, and the concomitant expression of the excision functions, was accompanied by a loss of immunity (Shinagawa & Itoh, 1973). Although induction occurs spontaneously at low frequency it can be made to occur at high frequency by treatments that alter the structure or synthesis of DNA. One response to such treatments, e.g. ultra-violet irradiation, is the activation of the SOS-repair pathway. Roberts, J. et al (1977) showed that this resulted in the proteolytic cleavage of the phage repressor which would explain the loss of immunity on induction. Since the phage repressor, either directly or indirectly, is responsible for repressing the lytic functions, its inactivation will result in the lytic development of lambda.
f) Transducing Phages

Lambda integrates into the E. coli chromosome at a single specific site located between gal and bio (Rothman, 1965). On induction, the integrated prophage excises exactly and undergoes lytic development. However, in some cases the excision event is aberrant and part of the lambda chromosome is lost. Its place is taken by the adjacent bacterial DNA (Campbell, 1962). Some of these phages carry the genes of the gal or bio operons and are said to be transducing phages since they are able to transduce these markers. The availability of such phages has contributed greatly both to our knowledge of lambda, by virtue of the genes lost, and also of the gal and bio operons.
Fig 1.1: Clustering of λ genes according to function
The lambda genes can be divided into four distinct functional categories:

1) Control genes regulating the lytic or lysogenic pathways.
2) Phage replication genes
3) The so-called "in-essential" genes concerned with phage recombination etc.
4) The late genes concerned with virion maturation and cellular lysis.

It is interesting to note that the genes of phage lambda are clustered on the genetic map according to their function (Fig 1.1).

The control genes are clustered at 70-80% on the vegetative map. (By convention, position in the lambda chromosome is defined as the distance from the left end of the vegetative map expressed as a percentage of the length of lambda wild-type). The non-homologous immunity regions for the related phages \( \lambda_{imm}^{434} \) and \( \lambda_{imm}^{21} \) are located here. Only the control gene \( Q \) is not closely linked to these regulation genes. In this case \( Q \) is located adjacent to the genes that it controls. These are the late genes which are concerned with phage morphogenesis. They are located in the left arm of the phage chromosome but are covalently linked to genes \( Q, \beta \) and \( R \) during lambda development. Such an arrangement lends itself to very easy control of the transition from early to late gene expression. The inessential genes are located in the centre of the genome and include the "silent" \( b2 \)-region and the \( \alpha \)-operon genes concerned with phage recombination.
Fig 1.2: Transcription in The λ Genome
Operationally the lambda genome can be considered to consist of five units (Fig 1.2): the N-operon; ope-operon; oI-operon; Q-operon; and the b2-region. Expression of these regions is regulated by the control genes. (During lytic development, lambda exists as a circular DNA molecule, and Fig 1.2 is drawn as a linear molecule only for clarity).

a) The Control Genes:

There are six lambda genes which control the development of lambda. These are N, ope, and Q which are involved in lytic regulation; and oII, oIII and oIV which are concerned with lysogenic development. The division of these control genes between the two pathways is, however, not clearly defined. Thus gene N is required for the synthesis of oII and oIV, and these in turn influence the lytic pathway via an inhibition of late gene expression (McMacken et al, 1970; and see Chapter 3).

The function of N will be described in more detail in section III. It primarily acts by preventing termination of the early mRNA transcripts initiated at Pl and Pr and thereby enables expression of the delayed-early genes in the N- and ope-operons. These include the genes involved in phage DNA replication (OP) and the late-gene regulator Q which are required for lytic development. In addition, the control functions oII and oIV are also switched-on and these promote the lysogenic pathway.

The role of N therefore seems to be that of switching-on lambda expression - which pathway is followed is decided at a later stage. Without this turn-on by N, lambda will remain dormant. Indeed, infections by lambda phage defective in N results in neither a lytic nor a lysogenic response and the inactive circular lambda DNA genome...
remains in the cytoplasm. Since the \( t_{R_1} \) terminator is leaky (see section IIIe), an appreciable amount of \( P_R \) initiated transcription does penetrate. This results in some expression of the replication genes with the consequence that the lambda DNA does replicate at a low rate. The net effect is that the lambda "phage" is maintained in the cell as a plasmid.

The function of \( Q \) is to allow the expression of the late genes. This is achieved by an interaction of \( pQ \) at a site located between \( Q \) and \( S \) (Herskowitz & Signer, 1970). It is possible that this site is the late-gene promoter \( P'_R \) but it is thought more likely that \( pQ \) acts in a similar manner to \( pN \), by permitting the extension of a short transcript originating from \( P'_R \). Unlike \( N \)-defective phage which totally fail to grow, \( Q \)-defective phage do form infective centres. This is not due to leakiness of the \( Q \) mutants since deletions of this gene also permit some progeny to be produced. Since this \( Q \)-independent expression of the late genes is dependent on the \( N \)-gene the explanation appears to be that \( P_R \) initiated transcription under the influence of \( pN \) is able to proceed through the \( Q \) gene and transcribe the late genes (Dambly & Couturier, 1971). In combination with a \( cro^- \) mutation, which fails to turn-off \( P_R \), expression of the late genes is sufficient to allow the phage to form a plaque.

The action of the \( cro \) gene is somewhat unclear (see section III). Its function is to repress the two early promoters \( P_L \) and \( P_R \) and thereby moderate expression of the delayed-early genes. The effects of a failure to achieve this are many fold. Replication is greatly impaired (Folkmanis et al., 1977) with the consequent reduction in the expression of the late genes. The result is that the phage fails to form a plaque. Indeed absolute defective \( cro \) mutations (as opposed to conditional-lethal temperature sensitive or amber mutations) are only...
Fig 1.3: The Lambda N-operon
isolated in strains carrying a temperature sensitive ci repressor gene. It is thought that weak repression by ci is able to compensate for the cag deficiency.

Although lysogeny is dependent on the ci repressor protein, lambda development is not dependent on lysogeny and the phage can be maintained in the lytic mode indefinitely.

The function of the ciI and ciIII control genes is to activate the expression of the ci repressor (Kaiser, 1957). Originally it was assumed that these two products activated a promoter for ci transcription. It is now becoming more likely that ciIII interacts with host proteins which antagonize the functioning of the ciII gene product which is responsible for activating ci transcription. In view of the action of pN as an anti-terminator and the probability that pQ also acts in the same way, it has been suggested that ciII might also function in this manner. The action of these genes will be described in more detail in section V.

b) The N-Operon:

The N-operon is transcribed leftwards from the early promoter PL (Fig 1.2). The genes of the N-operon are expressed from PL and include the genes: int, xis, red, gam, ciIII, kil, and ral together with a further three proteins whose functions are unknown.

The sizes of the proteins have been determined (Hendrix, 1971) and the genes have been mapped by deletion analysis (Szybalski & Szybalski, 1974). This in combination with the measurement of the ribosome binding sites (Calame & Ihler, 1977) for each gene has allowed the assignment of the genes as depicted in Fig 1.3. As can be seen, there is an oversaturation of genes in the promoter-proximal half of this operon. This suggests the possibility that genes in this
int & xis:

The int product is required for the integration of lambda into the host chromosome (Gottesman & Weisberg, 1971). It catalyzes the site-specific recombination between the phage att site and a corresponding site on the host chromosome. The regulation and function of int will be described in section V. In addition to its role in integration, int is also required for excisive recombination. Excision, the reversal of integration, also requires the function of the xis gene (Guarneros & Echols, 1970; Kaiser & Masuda, 1970). The involvement of both int and xis in excision was used for the isolation of several hundred mutants in these genes (Enquist & Weisberg, 1977). Mapping of these mutants indicated that the int gene starts within 50 base-pairs of att and extends about 1240 base-pairs rightwards. This is in agreement with the size of the polypeptide product of the gene. In contrast all xis mutants mapped within 110 base-pairs of each other. This would correspond to a protein of less than 4000 daltons which is in conflict with the size assigned by Hendrix (1971). However, it appears that the 32kd protein previously assigned may not be xis (Epp & Pearson - personal communication, cited in Nash, 1977). If this small size is correct then it would make its positive detection very difficult. The instability of xis (Weisberg & Gottesman, 1971) will also hamper its identification.

red and exo:

Infection by lambda results in the early appearance of an exonucleolytic activity. Purification of this lambda "exo" showed that the activity resided in two peaks after phosphocellulose
chromatography (Radding, 1966). Although both peaks were enzymatically identical, the second peak was shown (Radding & Shreffler, 1966) to be a complex of two subunits - alpha and beta. The sizes of the polypeptides were estimated at 24kd and 28kd, respectively (Carter & Radding, 1971).

Signer & Weil (1968) established that lambda coded for a function that was responsible for vegetative phage recombination. Shulman et al. (1970) isolated many recombination-defective (red-)

mutants and divided them into three complementation groups. Four temperature sensitive mutants in red were found to produce a temperature sensitive exo activity suggesting that exo is involved in recombination. Group B mutants were not found to affect exo activity but often affected the production of the beta subunit (Radding, 1970). Class A mutants were able to complement class C mutants and vice-versa. One pair of complementing redA and redC mutants gave a temperature-sensitive exo consistent with the hypothesis that these two complementation groups are part of the same gene. These results indicate that exo is a multimeric enzyme and that the phage recombination function is achieved by an exo-beta complex.

It appears that the red genes are also involved in lambda DNA replication. Phage deficient in red fail to form plaques on a lig-ts strain at 37°C where host ligase is limiting. Similarly, they fail to plate on hosts deficient in the polA gene (Zissler et al., 1971a).

gam:

In order to determine the role of recombination in the growth of lambda, Zissler et al. (1971a) isolated mutants unable to plate on recA hosts. Analysis of such mutants showed that they consisted of two mutations both of which were required for the inability to plate on
One of the mutations was located in \textit{red} whilst the second was located in a new gene which was called \textit{gamma} (\textit{gam}). Since amber and temperature sensitive mutants were obtained it was concluded that \textit{gam} was a protein. The native form of the protein is a dimer composed of two identical 16.5kd subunits (Karu \textit{et al}, 1975).

Partially purified \textit{gam} protein was shown to inhibit all the known catalytic activities of the \textit{recBC} enzyme (Sakaki \textit{et al}, 1973). Inhibition of the \textit{recBC} enzyme was only effective when the \textit{gam} product was added before the DNA suggesting the mode of action involved stoichiometric interaction. This action of \textit{gam} is required since the \textit{recBC} nuclease blocks the transition from the early theta replication mode to the more efficient late rolling-circle mode (Greenstein \& Skalka, 1975; and see section IV).

delta:

Wild-type lambda fails to plate on a P2-lysogenic strain (see Chapter 3). Mutants of lambda able to grow are found to be defective in both genes \textit{red} and \textit{gam} (Zissler \textit{et al}, 1971b). However, these phage produce only tiny plaques. The large plaque-forming phenotype requires a further mutation which was designated "delta". The use of \textit{cio} transducing phages suggested that deletion of \textit{del} was responsible for large plaque formation. This putative gene would therefore be located between \textit{int} and \textit{exo}.

That delta was a gene was cast into serious doubt by the discovery of chi mutations (Henderson \& Weil, 1975) which are "hot-spots" for recombination. Chi acts by promoting \textit{rec}-mediated recombination between circular lambda monomers. The resulting dimers, in contrast to monomers, may be packaged into phage heads and hence the burst size is increased resulting in a larger plaque.
Characterization of some delta mutants (Barta et al, 1974; Barta & Zissler, 1974) suggest delta may be distinct from chi.

cIII:

The role of cIII in regulation of lysogenic development will be described in section V. The mechanism of action of cIII is not clear as it does not appear to be as important as cII. There is evidence that cIII may act by antagonizing a host product which inhibits expression of phage repressor from the establishment promoter (Belfort & Wulff, 1971).

ral:

This product is responsible for relieving restriction by the host K or B systems (Zabeau et al, 1973). Very little is known about this function.

Ea9, Ea10 and Ea22:

These products have only been identified on polyacrylamide gels as proteins synthesized after lambda infection of ultra-violet irradiated hosts. Their function is unknown.

kil:

The nature of this gene is still of some controversy. Induction of a lambda prophage leads to phage replication and excision from the chromosome. Ultimately the cell dies and mature phage are released. It is also found that cell death occurs on induction of a prophage which is unable to either replicate or excise from the host chromosome. This was attributed to a function called "kil". Cell death was characterized by the formation of very long filaments and a
non-homology with $\text{imm}^\lambda$

*Fig 1.4: The Lambda cro-Operon*
decrease in the ability to reproduce a superinfecting heteroimmune phage (Greer, 1975). Deletion analysis with λbio phages showed that kil was located between the end-points of bio1 and bio252, i.e. between gam and gIII. The kil-function was inferred to be a protein since it was possible to isolate ochre mutants. (Pearson & Epp - personal communication cited in Calame & Ihler, 1977 - reported that the molecular weight of the protein is 16kd.)

kil does not seem to interfere with either RNA or protein synthesis. Pantoyl lactone, which stimulates cross plate formation, reverses the filamentation which occurs when kil is expressed but the cells still die. It therefore appears that killing is separate from the blockage of cell division.

c) The opo-operon:

The opo-operon is transcribed rightwards from the early promoter P₀ (Fig 1.2) and includes the genes oIII, O, P and Q in addition to oro itself (Fig 1.4).

The function of oro was introduced earlier and will be described in more detail in section III. The gene has been sequenced (Roberts,T.M. et al, 1977; Schwarz et al, 1978) and codes for a 66 residue protein.

oIII:

This gene, like oIII in the N-operon, is involved in the regulation of oI repressor expression (see section V). It apparently acts directly at the y region (defined by mutations defective in establishment of repression and mapping to the left end of oIII), though it is not known if y specifies the promoter site for establishment of repression.
Many amber mutants in \( \text{III} \) were isolated and characterized (Belfort \textit{et al.}, 1975). From the pattern and efficiency of suppression it was concluded that \( \text{III} \) plays a stoichiometric rather than a catalytic role and is not overproduced. The size of the gene product is 11kd (Schwarz \textit{et al.}, 1978).

\( \text{Q} \) and \( \text{P} \):

Lambda replication occurs in two stages (Carter \textit{et al.}, 1969). The early mode consists of the synthesis of monomeric circles and is followed by the late rolling-circle mode which produces long linear concatamers. The latter forms are the substrates for packaging into lambda virions. Two lambda genes are specifically required for the replication of the phage DNA (Brooks, 1965). These genes (\( \text{Q} \) and \( \text{P} \)) are expressed from \( \text{P}_\text{R} \) early in the infective cycle.

The action of genes \( \text{Q} \) and \( \text{P} \) is still not certain. The mechanism of action of \( \text{P} \) has been studied (Klinkert & Klein, 1978) by the use of temperature-sensitive mutants. It was shown that early replication was blocked after shifting to the non-permissive temperature whereas late replication continued for several rounds. This same effect was noted after inhibition of RNA or protein synthesis. In contrast to this, inactivation of \( \text{Q} \) resulted in an immediate cessation of replication at both early and late times. These results suggest that \( \text{P} \) is involved in the initiation of early theta replication whereas \( \text{Q} \) is required continuously. It was suggested that \( \text{Q} \) might be necessary for the structural integrity of the elongation complex.

There are indications that these two genes interact with each other. Revertants of \( \lambda \text{Qts81 Pam3} \) isolated by their ability to form plaques on a suppressor-free strain (Tomizawa, 1971) were found to be temperature insensitive. When the \( \text{Pam} \) and reversion mutations were
combined with another Qts mutation the phage were still temperature sensitive. The reversion mutation was shown to map at or very near to the Lam3 site. These results suggest that the Lam3 revertant is able to compensate for the Qts81 mutation to give a heat-stable complex, and this implies a direct interaction between these two proteins. This interaction has been confirmed by the use of phages carrying hybrid Q genes (Furth et al., 1978).

Using transient derepression of a λ857 lysogen showed that Q function was unstable, decaying with a half-life of only five minutes (Wyatt & Inokuchi, 1974). In contrast, P function was stable for periods in excess of an hour. The product of gene P has been identified (Oppenheim et al., 1977) as a 23kd protein.

The P product is found to interact with host components. Mutants known as groP (Georgopoulos & Herskowitz, 1971) have been isolated that fail to allow the replication of many of the lambdoid phages. Mutants of lambda have been isolated that are able to grow and these map in or near gene P. In addition to blocking phage replication these groP mutants also appear to be deficient in host replication since they are temperature-sensitive for growth. The groP mutations map in the dnaB gene (Skalka, 1977).

Similarly another class of mutant has been isolated (Sunshine et al., 1977) that affects lambda and P2 replication. These were originally called groPC but have now been renamed as dnaJ and dnaK (Yochem et al., 1978).

Another anomaly concerns the complementation behaviour of gene Q. Both Q and P mutants are effectively complemented by coinfection with Q\(^+\) P\(^+\) phage. However, when this is repeated in the absence of pN it was found (Kleckner, 1977) that P mutants were still complemented
whilst Q-amber mutants were not. This defect was alleviated by $LJ_{5b}$, which gives $N$-independent transcription of the Q gene region, and was not observed for a temperature sensitive mutant in Q. This suggested that in the absence of $N$-function, the Qam mutation exerts a cis-specific inhibition of replication. It was suggested that this might be due to an interference with transcriptional activation of replication.

d) The $qI$-Operon:

This operon consists of only two genes – $qI$ and rex. These are the only major genes that are expressed in the lysogenic state. The $qI$ operon is expressed from two promoters – $p_{rm}$ is used during the maintenance of repression whilst $p_{re}$ is used during the establishment phase. This will be described in more detail in section V.

The product of the rex gene is responsible for the exclusion of T4 rII mutants (Howard, 1967). Its function is not known and the closely related lambdoid phage 434 does not possess a rex gene.

The molecular weight of the $qI$ repressor is about 26kd (Ptashne, 1967). The active form may be a dimer or tetramer.

e) The Q-Operon and Late Genes:

The Q gene product is synthesized as part of the $pro$-operon. Its function is to enable the expression of the lambda late genes from the $P'_R$ promoter. The late genes can be sub-divided into two parts – firstly the genes concerned with cell lysis, and secondly, the genes concerned with the morphogenesis of the virion.
Endolysin and lysis functions:

Analysis of nine mutants defective in host cell lysis showed that seven were mutant in gene $R$ and produced either thermo-labile endolysin or none at all (Harris et al. 1967). The remaining two mutants produced high levels of apparently normal enzyme. It was therefore concluded that another gene must also be involved in lysis.

Endolysin is an endopeptidase which splits the cross-linking bond of $E. coli$ murein (Taylor, 1971).

This enzyme is produced in quantities that are in vast excess over requirements and its synthesis begins a long time before lysis occurs. This suggested that the second lysis gene, $S$, was probably involved in the control of the action of endolysin. However, the mechanism of action of $S$ has still to be satisfactorily explained.

Reader & Siminovitch (1971a) characterized five mutants belonging to the $S$-complementation group and found that they all shared two distinctive phenotypic properties. These were susceptibility of the host cells to chloroform induced lysis and overproduction of phage. Two and three factor crosses showed that the five mutations were closely linked and located between $Q$ and $R$.

It was shown that the $S$ gene product interferes, either directly or indirectly, with cellular respiration (Reader & Siminovitch, 1971b). The overproduction of phage on induction, or infection, with $S$ mutants would therefore be explained by the failure to block respiration. The normal yield of phage in $R^{-}$ infections, where lysis also fails to occur, indicates that blockage of cell lysis is not the reason why $S$-mutants overproduce phage.

It was suggested that by arresting energy production (respiration), $pS$ might initiate cellular lysis by preventing cell wall repair. Indeed metabolic poisons, such as cyanide, can cause premature lysis.
of induced lysogens. However, cyanide treatment fails to cause lysis of induced \( \Xi^- \) lysogens indicating that pS does not act simply as a direct inhibitor of energy metabolism. In contrast, the lipid solvent chloroform does cause lysis of induced \( \Xi^- \) lysogens suggesting that pS might promote lysis by an action at the cytoplasmic membrane. Electron microscopy on induced lysogens fail to show dissolution or extensive disruption of the cell membrane. However, lysogens of \( \Xi^- \)-mutants or non-lysogens showed a clear separation of the cell wall from the cytoplasmic membrane. This separation was not apparent in \( \mathcal{R} \) mutants. It was suggested that pS might cause the membrane to become more permeable. The hypertonic conditions of specimen preparation would then cause the non-permeabilized membrane in \( \Xi^- \) mutants to contract away from the cell wall, whereas this would not occur in \( \mathcal{R} \) mutants. Examination of the permeability of membranes confirmed that expression of \( \Xi^- \) did result in a more permeable membrane.

Morphogenetic Genes:

The genes concerned with morphogenesis are located in the left arm of the lambda genome. At least ten genes are involved in the formation of the phage head and 11 genes in tail formation. The biochemical steps in head and tail assembly are described in Hohn & Katsura, (1977). Of specific importance, however, is gene \( \mathcal{I} \) which specifies the tail fibre. Since this is the component that is involved in adsorption to the host cell, this gene determines the host range of the phage (Buchwald & Siminovitch, 1969). The proteins coded by many of these late genes have been identified (Murialdo & Siminovitch, 1972; Ray & Pearson, 1974) and the regulation of expression of these genes will be described in section III.
f) The b2-Region:

Phage lambda is extremely sensitive to chelating agents which cause a rapid loss in infectivity. However, it is found that lambda mutants with less DNA than wild-type are more resistant to such agents. This has enabled the isolation of deletions of lambda as phage resistant to the action of chelating agents. Parkinson & Huskey (1971) used this selection to isolate a series of deletions originating from the lambda att site (in the presence of the int protein it is found that the majority of deletions isolated have the phage att site as one of their end-points). It was found that most carried deletions extending leftwards. Since such deletion phages showed no defectiveness and no gene function was identified as being located in this region of the phage chromosome, the region was assumed to be "silent". The "prototype" deletion b2 has lent its name to this region.

Although proteins are produced from the b2-region, no function has yet been assigned to the genes in this region.

Rhoades & Meselson (1973), identified an endonucleolytic activity produced on lambda infection. Deletion mapping indicated a map position for the gene of between 52.2-53.8% (percent lambda wild-type from left end). Its expression was shown to be dependent on pN. There was no evidence that this endonuclease had any role in lambda development.

There was some suggestion (Roehrdanz & Dove, 1977) of a factor within the b2-region that affected site-specific recombination. Integration of lambda integration-defective phage is enhanced by a 434hy helper phage but not by 21hy. This defect was not observed when the b2-region of the helper was deleted. Three mutants that obviated this defect were shown to be small deletions located at about 2kb left of att. They had normal att sites.
Hendrix (1971) identified nine polypeptides which were expressed from the b2-region. The mapping of the genes for these proteins, by the use of b2-type deletions, suggested that not all of these proteins were the products of independent genes but that some were breakdown products. These proteins were produced at both early and late times after infection.

Experiments on escape synthesis of the host bio operon have identified in the b2-region at least two transcription termination sites and one replication termination site (Krell et al., 1972).

Escape synthesis in bacteria can be defined as the constitutive expression of a host gene as a consequence of phage development. There are three mechanisms by which it can occur. The first, or classical, mechanism is the result of phage DNA replication and escape synthesis is usually due to the depletion of repressor caused by replication of the operator. The second mechanism results from the extension of transcription from a phage promoter site into bacterial genes. This mechanism can only operate with either a prophage, whence the flanking bacterial genes may be expressed, or else with a lambda transducing phage. The third mechanism is the direct action of a lambda function on the expression of the gene. An example of this is the pN mediated stimulation of sigma synthesis (Nakamura & Yura, 1976).

Examination of escape synthesis of the bio operon showed that synthesis of bioA was low and dependent on prophage replication (Krell et al., 1972). In contrast, the escape synthesis of bioD showed extensive synthesis under the control of the lambda Q gene. The different result is presumably due to the fact that the bio operon is divergently transcribed with bioD being transcribed in the same direction as that from the pQ activated P'R promoter whilst bioA is
transcribed in the opposite direction. However, it was found that when the b2-region of the prophage was intact there was very little escape synthesis of $hLq$ even though escape synthesis of $gal$ occurred. This cannot be due to distance since $hLq$ is closer to lambda than is $gal$. Deletion of b2 resulted in a marked stimulation of $hLq$ synthesis. This expression was not due to the inability of the phage to excise from the host and occurred even in the absence of phage replication. In the lysogenic state the late gene promoter, $P'_R$, is not repressed but transcription of the late genes does not occur because of the lack of pQ. This product can be provided in-trans by a superinfecting phage and this results in the expression of the late genes carried by the prophage. This "trans-activation" also resulted in a stimulation of $hLq$ synthesis suggesting that its expression was directed from $P'_R$. Since the presence or absence of the b2-region on the helper phage was immaterial this excluded the possibility that b2+ might code for a diffusible inhibitor and suggested that the b2 deletion was required to remove a transcription termination site.

Deletion analysis suggested that there was more than one site. Similar experiments with $bioA$ escape synthesis showed that, in this case, escape was dependent on phage replication and that the b2-region contained a replication stop signal that prevented, or reduced, phage-induced replication of the $bio$ operon.

The presence of transcription termination sites in this region was further demonstrated by the expression of the $araC$ gene from a $\lambda araC$ transducing phage (Steffen & Schleif, 1977). Here the $araC$ gene is located just to the right of $att$ in such an orientation that it would be readable from the rightward promoters of lambda. Indeed the gene was found to be expressible from $P_R$ with the expression being switched-off at about 30 minutes post-infection. This was presumed to
be due to the action of orq since infection of a toh- bacteria (Inoko & Imai, 1974), in which orq action is defective, does not give this switch-off. Although P'R is oriented such that it could transcribe araC it does not do so unless the lambda b2-region is deleted. It was suggested that the b2-deletion removed transcription termination sites which pQ was unable to overcome.

It has also been reported (Adhya et al., 1976) that there is an N-insensitive transcription termination site present in this region which blocks leftward transcription from PL. It was proposed that this would prevent leftward transcription of the late structural genes. The significance of this is not clear since orq turns-off leftward transcription before rightward transcription reaches the structural genes.

Since many deletions are known that remove all of these sites they cannot be essential to lambda functioning. Therefore it appears that the b2-region serves no useful function and may be maintained only in order to retain sufficient DNA to package efficiently.
Heads Tails

\[ \text{immediate-early} \]

\[ \text{delayed-early} \]

\[ \text{late} \]

Fig 1.5: Transcription in the Lytic Cycle
The pattern of gene expression in the lytic development of lambda can be divided into three basic stages (Fig 1.5): immediate-early; delayed-early; and late.

The immediate-early genes, N and cro, are expressed immediately after lambda infection. Gene N is the positive regulator of lambda development. Its function is to turn-on the expression of the delayed-early genes. In contrast, the function of cro is to turn-off their transcription and allow the smooth transition to late-gene expression. The delayed-early genes flank N and cro and are transcribed from the same promoters as the immediate-early genes. The genes to the left of N include the phage recombination systems and are inessential for lytic development. Those to the right of cro include genes Q and P, which are vital for phage replication, and Q which is required for the expression of the late genes. The late genes are required for the production of the phage structural proteins and the consequent appearance of mature progeny. Also expressed late are the genes involved in cell lysis. In lytic growth the lambda genome is circular and all of the late genes are expressed from a common promoter. Lytic development can also ensue on induction of lambda from the prophage state. Here the pattern of expression is identical. In this case, however, expression of the delayed-early genes left of N is required to excise the prophage from the host chromosome.

a) The N Gene

The initial transcript from FL leftwards has a size of 12S (Roberts, 1969) and codes for the N gene product. In the absence of
the Ν gene product, leftward transcription of the Ν-operon, beyond Ν, does not occur and transcription of the rightward θN-operon is diminished (Schwartz, 1970). Gene Q is not transcribed and hence none of the late genes are expressed. By the use of transient de-repression of a prophage it was also shown that pN is unstable with a half-life of only five minutes.

The delayed-early genes map on the lambda chromosome on either side of the two immediate early genes. The simplest mechanism by which pN could cause expression of the delayed early genes would be if it activated new promoters. This proposal is, however, almost certainly incorrect.

Franklin (1971) isolated lambda transducing phages in which the E.coli trp operon replaced part of the lambda Ν-operon. In all cases where expression of the trp genes was from a phage promoter (i.e. the trp promoter was not present), expression was found to be completely blocked by the φX repressor. Since varying amounts of the Ν-operon had been deleted it was concluded that no immunity independent phage promoter existed in this region. Expression of the trp genes was dependent on pN. It was found that the related phage 434 could substitute for this dependence of pN indicating that the 434 Ν gene was similar to that of lambda. However, when ΛL transcription was blocked by repressor there was no expression of the trp genes even when pN was supplied in-trans by phage 434. This indicated that there is no Ν-activated promoter present in this region.

The alternative mechanism was proposed as a result of the work of Roberts (1969). Using a lambda DNA template in an in-vitro transcription system he was able to isolate a factor, rho, which decreased RNA synthesis. However, in the presence of rho the transcripts formed were of discrete sizes (12 and 7S), rather than the
heterogeneous sizes normally found. It is now known that rho is the factor responsible for termination of transcription at specific terminator sites. The 12S species was identified by hybridization as the N-gene transcript. The 7S species is that of the orf gene.

The conclusion was that there are terminator sites, to the left of PL and to the right of PR, which require rho factor to block transcription. This is supported by the finding that mutants deficient in rho function are permissive for lambda mutants defective in N function (Belfort & Oppenheim, 1976; Korn & Yanofsky, 1976).

By selecting bacterial mutants giving N-independent transcription of the N-operon, mutants were obtained called nitA (Inoko & Imai, 1976). These were mapped to between ilv and metE. The rho gene also maps here.

The suggestion that pN acted as an anti-terminator received further support by studies on polarity. Mutations are classed as being strongly polar when downstream genes are expressed at a much lower rate than normal. Such mutations, however, cause very little polarity when the genes are being transcribed as part of the N-operon (Franklin, 1974).

This was similarly demonstrated for the escape synthesis of the gal enzymes (Adhya et al, 1974). Here pN was found to overcome polar mutations which were rho-insensitive suggesting that pN does not act as an anti-rho factor.

b) Interaction of pN With the Host

The observed effect of pN as an anti-terminator suggests that it might act as an "anti-rho" factor, perhaps by binding at the terminator-sites and thereby preventing rho action.
This however, does not appear to be the case. There are two major lines of evidence against this: firstly, if pN acted as an anti-rho factor then it would result in a general suppression of termination and this is not observed. Secondly, pN is only effective (with a few exceptions) when transcripts are initiated at the lambda promoters \( P_L \) or \( P_R \). Furthermore pN is immunity-specific in that phage 21 N has no effect on transcription initiated at lambda promoters and vice versa (Franklin, 1974). This suggests that the site of pN action and the sites of pN effect are not identical.

It was therefore proposed that pN recognized the lambda promoters and that the transcription apparatus was so modified by interaction with pN that termination could no longer occur at termination sites subsequently encountered. This implies an interaction of pN with RNA polymerase. In support of this idea, Georgopoulos (1971) isolated bacterial mutants simultaneously resistant to infection by lambda and phage 434. These mutants are known as Gro- . One class, designated groN, was found to be defective in N function. Phi80 and phage 21 also failed to grow on this class of mutants but other lambdoid phages were able to plate. Infection of groN with \( \lambda trp46 \) (an in-vivo made trp-transducing phage carrying the \( trpDE \) genes expressible only from \( P_L \) in the absence of their own promoter) failed to give expression of the \( trp \) genes indicating a defect in \( P_L \) transcription. Since \( \lambda trp48 \) (similar to \( \lambda trp46 \) but carrying \( trpD \) fused to N deleting the termination site \( t_L \)) does give \( trp \) expression, this indicates that \( P_L \) is functioning normally but that termination at \( t_L \) is complete.

Similarly Friedman (1971) isolated mutants known as "nus" (N Utilizing Substance) which also interfere with lambda N gene function. The results showed that in nus mutants the expression of the N function is reduced or undersupplied and that nus interferes with all
stages of pN-action. Friedman & Baron (1974) mapped nus between argG and aspA (68' on the E. coli map). Transcription of N in a nus- host was found to be normal even though N-function was not expressed. A second class of nus mutants of similar phenotype, nusE, mapped at about 11' (Friedman et al., 1976).

Pironio & Ghysen (1970) used nitrosoguanidine mutagenesis of a strain carrying the supF amber suppressor to isolate mutants resistant to infection by λNam7. They obtained one mutant called ron which showed very close linkage to rif. Interestingly two out of 32 amber mutants of lambda also failed to grow on this strain as did their Sus+ revertants. This failure was attributed to a genetic alteration (mar) in these phages. mar apparently mapped in the right part of the N gene very close to the Nam53 marker. It was also found that the original 434hy phage exhibits this Mar characteristic but 21hy does not. Subsequent work (Ghysen & Pironio, 1972) identified a further four mar alterations. Three factor crosses indicated that three mapped very close to Nam7 and the original mar alteration (identified in λNam7 phage) could not be separated from Nam7.

Fine mapping of ron showed that it was located between rif and stl (streptolydigin resistance). Since both of these markers are known to be mutations of the RNA polymerase beta-subunit it was concluded that the ron mutation was also located in the beta-subunit.

This indicates an interaction between the N gene product of lambda (and phage 434) with the RNA polymerase beta-subunit. Since ron is recessive to wild-type this suggests that the mutant is defective in this interaction.

These results suggest that pN directly interacts with RNA polymerase probably via the beta-subunit. On this basis it should be possible to isolate a complex of pN and RNA polymerase. After
ultra-violet irradiation of the host prior to infection with lambda it was found that six phage coded proteins were immunoprecipitated with polymerase (Epp & Pearson, 1976). Four of these were encoded by the phage b2-region and the fifth appeared to be red-beta. The sixth was not identified. In addition small amounts of 11kd and 13kd proteins were found associated. Neither of these were present when N-mutants were used. After purification, the only lambda protein to co-sediment with RNA polymerase in glycerol high-salt gradients was the 11kd protein. Suppression of an Nام mutant by a tRNA^\text{tyr}^r amber suppressor showed that the tyr/met ratio for this protein increased from 1.1 for N^+ to 2.2 for Nام whereas the ratio did not change for other proteins. It was therefore concluded that the N product was an 11kd protein and did interact directly with RNA polymerase. Later work (Shaw et al., 1978) showed that this conclusion was incorrect and that pN has a size of 13.5kd.

c) Terminators

Immediately after infection the transcripts produced from the PL and PR promoters are short due to rho-mediated termination at two sites. The existence of the terminator of leftward transcription - t_L - was confirmed by the isolation of λtrp transducing phages (Franklin, 1971) in which the trp genes were not expressed from their own promoter but from PL. With most of these phages expression was dependent on pN. However, in some cases expression did not require pN. This independence was cis-dominant suggesting that a site had been deleted whose normal function was to prevent leftward transcription in the absence of pN.

Whilst the t_R terminator of rightward transcription, identified by Roberts (1969), was very efficient in-vitro, it is only about 50%
efficient in-vivo and transcription rightwards is found to pause here for about 60 seconds (Rosenberg et al., 1978; Adhya & Gottesman, 1978). Since expression of the late gene functions is absolutely dependent on pN this indicates that a second terminator site for rightward transcription must exist. This tR2 site was identified in two ways. Using a defective λbio phage in which the N gene was deleted, Court & Sato (1969) isolated plaque-forming phage. One of these mutants is the ninR5 deletion which removes the tR2 terminator thereby rendering transcription of Q independent of pN. By a similar selection for plaque forming derivatives of a · N- phage, mutants bypassing the normal requirement for N were isolated (Butler & Echols, 1970; Hopkins, 1970). These bvp mutants were deletion mapped to between genes P and Q. They also affect the tR2 terminator allowing pN independent expression of Q.

Recent results have established that the site originally called tL is not the only terminator within the N-operon. Two further sites were identified by deletion mapping (Salstrom & Szybalski, 1978b). The original terminator, tL1, was shown to be only about 80% efficient in the absence of pN whereas tL2 and tL3 are about 90% efficient.

The anti-termination effect of pN is usually only observed when the genes are being read from the lambda promoters P_L or P_R, implying that pN only acts at either of these promoters. However, Nakamura & Yura (1976) have shown that synthesis of the sigma (rpoD) gene product was stimulated by lambda infection. It was shown that pN was responsible for this stimulation and that this appeared to be a direct effect. It is possible to speculate that the mechanism of this stimulation of sigma expression might be that transcription of the structural gene is moderated by a terminator located between the promoter and the gene itself. This type of regulation would be analogous to the trp...
attenuator (see Chapter 2). \( pN \) could then be postulated to act at the \( rpoD \) promoter to overcome the normal termination event. Since infection with \( N \)-defective phages does not give rise to this stimulation of sigma synthesis, but nevertheless the phage are fully capable of growth when combined with the \( ninkR \) deletion, this effect cannot be vital to lambda growth and development. It is noteworthy that strains carrying lesions in the gene coding for the transcription termination factor rho also give a higher rate of synthesis of sigma than that in the isogenic wild-type strains. Richardson et al. (1977) have shown that \( N \)-defective mutants of lambda grow on strains defective in rho. In contrast to wild-type hosts, the lambda early exo function is expressed. This effect is not due to a general suppression of amber mutations since mutations in genes \( R \), \( P \) or \( Q \) are not suppressed. This inability to suppress \( Q \) is interesting in view of the suggestion that \( Q \) acts as an antiterminator like \( N \).

Recent results have indicated that \( pN \) does not interact with the \( P_L \) or \( P_R \) promoters but at two sites - respectively \( nutL \) and \( nutR \). This was originally proposed to explain the fact that the \( 43 \) \( N \) gene product is active with transcription from the lambda promoters, even though the promoters of these two phages are distinct. Mutants in the proposed \( nutL \) site have been isolated (Salstrom & Szybalski, 1978a). These are defective for leftward transcription of the \( N \)-operon even though expression of \( N \) is normal. This indicates that \( P_L \) transcription is unaffected and suggests that \( pN \) is no longer able to interact with RNA polymerase.

d) \( N \)-Operon RNA Processing

Transcripts from \( P_L \) appear to undergo a lot of processing. Kourilsky et al. (1968) identified three RNA species corresponding to
early gene transcription off the 1-strand - l₁, l₂ and l₃. The largest species, l₃, was not produced when using Ν-defective phage or when chloramphenicol was present to block the synthesis of pN. Thus the presence of pN is necessary for its production. Since all three species hybridize equally well to lambda and 434hy (a lambda hybrid phage carrying the immunity region of phage 434 - Kaiser & Jacob, 1957) they must all be transcribed from DNA left of immunity.

Portier et al (1972) used RNA-DNA hybridization-competition to show that neither species l₂ nor l₃ could compete with l₁. Sequence studies have shown that l₁ corresponds to the 5' proximal portion of nascent l₂. This result indicates that the l₁ species must be cleaved off from the l₂ transcript very rapidly. In contrast species l₃ is able to compete with l₂. About 20% of l₂ is not contained on l₃ but this is probably due to 5'-3' exonucleolytic digestion of l₃.

More recent work (Lozeron et al, 1976) has shown an even more complex degree of processing. Fragment l₁ was found to be rather stable and was coded for by the 89th to 190th base (relative to in-vitro transcript). The preceding 17 bases were missing as if excised endonucleolytically from the hairpin structure which exists in the in-vitro transcript at this point.

Exactly why such a complex series of events should occur is not clear. However, there are two important considerations. Firstly, it is apparent that the transcript from the Ν-operon genes and that of the Ν-gene is produced as a single polycistronic mRNA. The second consideration is that of the instability of pN. This coupled with the rapid destruction of the mRNA corresponding to the region of the Ν-gene would result in the very rapid disappearance of pN as soon as Pᵥ is turned-off. The consequence of this would be an abrupt cessation in the synthesis of the delayed-early genes. This blockage
of early expression may facilitate an efficient transition to the late mode.

e) The  

Pero (1970) showed that the "tof" function responsible for turning-off expression of lambda exo was located within the region of non-homology with 434hy, a phage identical to lambda except that it carries the immunity region of phage 434. The corresponding region of 434hy was also found to contain a tof function but this was ineffective with lambda. She concluded that the site of action of both tof gene-products was also located within this non-homologous immunity region. Deletion mapping (Pero, 1971) located tof (oro) between genes al and aII. The site of oro action for leftward expression was shown to be left of al and since it is immunity-specific it must lie between N and al.

Mutations in the oro gene have been isolated by a large variety of procedures. In many cases it was not until sometime after their isolation that they were realized to be mutations in the same gene. This state has lead to this gene having a number of names including: tof (turn-off); Ai (Anti-immunity); oro (control of repressor and other things); and fed.

N-defective phage are able to form plaques if they also contain the ninR deletion of terminator site t_R2. However, such phage do not form plaques on a recA host. This is the Fec phenotype and is the result of the inability of the phage to synthesize the red and gam gene products. By selecting for mutants able to plate on recA, Court & Campbell (1972) isolated mutants that mapped within the immunity region and appeared to be similar to oro. The ability of these fed" mutants to plate on recA hosts is presumably due to the
expression of some \textit{red} and \textit{gam} products. This was supported by the finding that the phage were excluded by P2 lysogens (Spi phenotype - see Chapter 3). The ability to plate on P2-lysogens depends on the inability to express both \textit{red} and \textit{gam}. However, exo activity (the product of \textit{red}) could not be detected. These \textit{fed}^− derivatives showed an increased rate of specific excision from lysogens whereas excision was immeasurably low in the parent. This indicated enhanced expression of \textit{xis}. The conclusion was that \textit{fed} resulted in elevated, but still low, expression of the genes of the \textit{N}-operon.

The \textit{cro} protein has been purified (Folkmanis \textit{et al}, 1976; Murotsu \textit{et al}, 1977; Takeda \textit{et al}, 1977) and appears to be a dimer of identical 8.6kd subunits. It binds specifically to \textit{O}_L and \textit{O}_R of lambda DNA. Using an in-vitro transcription system it was shown that \textit{cro} is an effective and specific repressor of RNA synthesis from the \textit{N}- and \textit{cro}-operons. In contrast to the complete turnoff of \textit{Ol} repression, \textit{cro} action gave a more gradual turnoff. Similarly, a protein presumed to be the phage 434 \textit{cro} gene product was isolated (Aono & Horiuchi, 1977). It was distinguished from the \textit{Ol} repressor by its requirement for magnesium for specific binding to phage 434 operators.

Phi80 has also been shown to have a \textit{cro} gene (Inoko \textit{et al}, 1974). It was also found (Inoko & Imai, 1974) that this gene failed to function in a derivative of HfrH. This Tob (Turn-Off in Bacteria) phenomenon was also shown to affect the lambda \textit{cro} function and suggests that \textit{cro} action might act in conjunction with a host function.
Whilst the mechanism of action of oro is clear, the ramifications of its role in lambda development are far from defined. It was originally thought that oro acted as an "anti-repressor" to prevent the synthesis of cI repressor. Mutants defective in this "anti-immunity" were isolated (Eisen et al., 1970) and shown to map within the oro gene. It was further shown (Echols et al., 1973) that lambda infection of a strain constitutively producing oro failed to permit cI expression because of an inability to synthesize the cII and cIII products. They proposed that the normal function of oro was to act as a repressor turning off synthesis of the delayed-early functions of replication, recombination and regulation, as lambda entered the late stage of lytic development.

oro- phage are unable to develop lytically under two circumstances. These are when the oro- mutation is combined with either a completely inactive or a fully active cI gene. In fact, absolute defective oro mutations are only viable when combined with a partially active cI gene such as would occur with a phage carrying a temperature sensitive mutation in the cI gene at 37°C. This suggests that a partially active cI gene can compensate for the defect in oro and further implies that weak repression of early functions is required for normal lytic development. It was originally thought that under oro- cI+ conditions the reason for the failure to form a plaque was due to very efficient lysogenization. However, Folkmanis et al. (1977) showed that under these circumstances there was no increase in the stable lysogenic response but rather the effect of oro- was to enhance the frequency of surviving non-lysogenic cells. This suggested that the lytic response had been blocked but that the lysogenic response had not been affected. oro- cI- phage are also non-viable. Deletion of most of the N-operon genes (including kil) does not prevent this
inviability. Mixed infection with the heteroimmune phage 434hy showed that the opr mutation exhibits "trans-lethality" in that the multiplication of 434hy is impaired. Deletion of the N-operon genes or inactivation of genes N, aL, P, or Q did not prevent this lethal effect on the superinfecting phage. It is therefore not clear what function is responsible for this effect. It was shown that DNA-replication was severely impaired and the late mode of lambda replication did not occur; even host DNA replication was reduced. It is therefore apparent that the effects of a loss of opr function are many fold.

f) Q and Late Gene Expression

Using amber mutants, Dove (1966) showed that the expression of the late genes was dependent on both phage replication and a general inducer of late functions identified by mutations in gene Q. It is known that super-infection of a lysogen by a hetero-immune phage can result in the activation of expression of the prophage's late genes. This "prophage-complementation" (Dambly et al, 1968; Thomas, 1970) is dependent on the super-infecting phage being able to express the Q-gene which supports the idea that late-gene expression is activated by pQ. This result also indicates that transcription of the late genes is not directly repressible by the immunity repressor which is responsible for preventing expression of the lambda early functions in the lysogenic state. It therefore implied that the late genes were expressed from a new promoter. Deletion-mapping identified a site, located between genes Q and S, which was essential for the expression of the late genes (Herskowitz & Signer, 1970).

Schleif et al (1971) studied a defective transducing phage in which the arabinose operon replaced the phage head and tail genes.
Expression of the *ara* genes started five minutes after that of endolysin and the rate of synthesis of both was linear with respect to time. This is interesting in view of the fact that phage replication increases the gene copy number and in most systems the rate of synthesis is proportional to the gene copy number. It therefore suggests that expression of the *ara* genes was being controlled similarly to the lambda late genes. The five minute lag in expression of *ara* is consistent with the distance between gene R (endolysin) and the *ara* genes. Heteroimmune infection of a lysogen of this phage showed a dependence on pQ for expression of the prophage *ara* genes. It was shown that pQ had no effect on the *ara* promoter. These experiments also showed that the lambda promoter for late gene expression was quite weak and explains why late gene expression is dependent on phage replication - to increase gene copy number. Endolysin synthesis was found to stop two minutes after the addition of rifampicin to induced lysogens whereas *ara* expression stopped after seven minutes. This again indicated expression from P'R.

Addition of rifampicin to induced lysogens results in a rapid drop in the rate of mRNA synthesis. In contrast it has only a very slow effect on late-gene expression. This was used (Gariglio & Green, 1973) to selectively label late gene mRNA. It was found that such labelled RNA sedimented heterogeneously at rates up to 60S which corresponds to 15-27kb. Such a transcript is large enough to code for the entire late gene region. Hybridization analysis confirmed that this RNA did correspond to the late-gene region of the chromosome. It was also shown (Ray & Pearson, 1974) that all the late genes were transcribed equally and transcripts were equally stable. Thus transcriptional control cannot account for the vast disparity in molar ratio of late proteins synthesized.
Although the late genes are transcribed as a single polycistronic messenger, expression of the gene products is not equal. Measurements of the amounts of late proteins synthesized after infection of ultra-violet irradiated cells showed that there was an 870-fold difference on the rate of synthesis of these gene products (Murialdo & Siminovitch, 1972).

By the use of lambda phages carrying duplications, the rate of synthesis was found to be directly dependent on the gene copy number (Ray & Pearson, 1976) suggesting that the different amounts of protein synthesized is due to differential efficiency of translation.

The mechanism of turn-on of late gene expression by pQ is thought to be analogous to the action of pN. In the lysogenic state the $P_R^I$ promoter is active and results in the production of a 6S transcript. This has been sequenced (Lebowitz et al, 1971) and has two interesting features. Firstly, an extensive region of duplex-formation adjacent to a U6A sequence which is characteristic of transcription termination sites. Secondly, the RNA potentially encodes a 41 residue polypeptide which is very analogous to the $\text{irp}$ attenuator (see Chapter 2). It has been suggested that pQ acts by preventing termination of this transcript thereby enabling transcription of the late genes. This suggestion gains some weight from the finding that in spite of the many mutations isolated in the late genes there is very little evidence of strong polarity. Weak polarity does exist (Murialdo & Siminovitch, 1972) but this seems to be clustered into polarity groups. This low polarity suggests that pQ might act as an anti-terminator agent like pN. However, transcripts from $P_R^I$ are terminated at signals that fail to impede those from $P_R$. 

1-38
When lambda infects a sensitive bacterium the first event is the circularization of the genome (Young & Sinsheimer, 1964) via the cohesive ends. This is then followed by the replication of the circular DNA molecule involving "theta" shaped structures (Ogawa et al, 1968). By the use of isotopic pulse-chase experiments it was shown (Carter et al, 1969) that lambda replication occurs in two stages. The first stage was characterized by the production of covalently closed circles which were not packaged into mature virions. The second phase started after about 15 minutes and resulted in the formation of linear DNA structures as found in mature phage particles. Analysis by ultracentrifugation and electron microscopy showed that the first mode consisted of a circular form of lambda containing two forks whilst the second mode contained DNA of greater than unit length. Since it has been shown (Enquist & Skalka, 1973; Freifelder et al, 1974) that circular lambda monomers are not efficiently matured, the burst size of a phage is a reflection of the amount of oligomeric DNA produced during the infective cycle. Oligomers can be produced by recombination of two monomers which would then be a substrate for the maturation functions. However, in normal infections the oligomeric DNA is produced via the rolling-circle mode.

Enquist & Skalka (1973) studied the roles of the host and phage recombination systems. They showed that the rate of phage DNA replication is abnormally low in the absence of red but that this replication was normal and the DNA was packaged at good efficiency. They concluded that red is involved in replication and its function cannot be assumed by the host rec system. gam mutants were also found to be defective in replication but in this case abnormal forms.
were produced. Infection of recA reverted the observed structures to normal. This suggested that recA blocks the production of the normal late-replicative structures and that gam counteracts this. By the use of a temperature sensitive mutation in gam it was shown (Greenstein & Skalka, 1975) that the recBC enzyme blocks the transition from early to late modes but does not prevent rolling-circle replication once this has started.

Carter & Smith (1970) studied the pools of the various forms of lambda DNA produced during infection. The size of the pool of closed-circular DNA rose until about 15 minutes post-infection then remained constant at about 20 phage DNA equivalents/cell. Similarly the pool of oligomeric DNA increased to a constant level of 90 equivalents at 27 minutes at which time phage-sized DNA began to accumulate at a constant rate. The pool of phage-sized DNA exceeded that contained in mature phage by about 30-40 equivalents.

a) Early Mode

The early mode of replication in lambda consists of "circle-to-circle" replication. Both open and closed circular forms are the primary components synthesized (Young & Sinsheimer, 1968). Although the early mode stops after 15 minutes (Carter & Smith, 1970) it appears that covalently-closed circular forms may still be synthesized in small amounts very late in the infective cycle. Since the amount of this form does not decrease it is probable that it is not a major precursor of phage DNA. Pulse chase experiments qualitatively support this.

Schnos & Inman (1970) examined the replicating structures of Ogawa et al (1968) which had been shown to consist of wild-type length lambda DNA plus an extra length of DNA extending between two branch points. They showed by partial denaturation mapping that replication
starts at $14.3 \pm 0.5$ micron from the left end of lambda DNA (lambda is about 17.5 micron long) corresponding to $81.7 \pm 2.9\%$ from the left end. Gene $P$ is located at about $81.4\%$. They pointed out that there is a region of high GC content at $14.5$ micron from the left end which is very close to ori, the replication origin. Their results also showed that replication is bidirectional but the rate of migration of the two branch points was not always identical. Stevens et al (1971) used deletion mapping to locate the ori site. Their results indicated a position of $79.8-80.5\%$ and also confirmed that replication is bidirectional.

Previous experiments had established that lambda genes $O$ and $P$ (Brooks, 1965) and at least one host gene (Georgopoulos & Herskowitz, 1971) were required. However, Dove et al (1971) showed that a repressed lambda phage fails to replicate even when all necessary gene functions are provided in-trans. Experiments with mutants such as $\Delta 11$, which are defective in rightward transcription, lead to the suggestion that rightward transcription is required for replication - the failure of a repressed phage to replicate would therefore be a consequence of repression of $P_R$ rather than a direct effect of $O^R$. This is supported by the observation that mutants, such as $O17$ or $X_1X_3$, which are insensitive to repressor are able to replicate even in the presence of $O^R$-repression. They also observed that multiple initiations from ori do not occur and that a complete round of replication seems to be performed before a new initiation occurs. Whether this reflects some control mechanism or a low frequency of initiation is not clear.

Although transcription is required for replication, transcription of the ori site itself is not required (Furth, 1978). $ri^O$ mutations create a new, repressor-insensitive, promoter which is able to activate replication. These mutations were found to be located to the
right of and separated from ori mutations by an R.EcoRI target. Therefore transcription will not pass ori and transcription of ori cannot be required. This suggests that the role of rightward transcription is not to act as a primer for DNA replication.

Sogo et al (1976) studied the forms of the early mode of replication by the use of SpI phage (see Chapter 3). These phage are defective in both red and gam functions (see section II, this chapter) and as a consequence are unable to undergo the transition from early to late replication modes. Consequently all replication throughout the lytic cycle is via the early mode. Infections by SpI phage produce an approximately equal number of relaxed and covalently closed monomeric circles. However, even though about 30-40 phage DNA equivalents are made, very few phage are produced since monomeric circles are not a substrate for packaging (Enquist & Skalka, 1973; Freifelder et al, 1974).

Although initiation of replication occurs at a specific site, there does not appear to be a specific termination site. Termination probably occurs simply as a result of the collision of the growing forks (Valenzuela et al, 1976). Although the b2-region has been shown to be a termination site (Krell et al, 1972), this cannot be efficient nor can it be essential to phage viability. How important this site is in the normal lambda life cycle is not known.

Inman & Schnos (1971) used electron microscopy to examine the structure of the early replicative forms. Not only did they observe the double-branched "theta" structures but they also frequently found single strand connections at the branch points. It was suggested that these represented the daughter DNA strand being replicated discontinuously. They were unable to size the length of these single-stranded regions but indicated that they were probably of the
order of 200-700 bases long. Since not all branch points showed these single-stranded regions it was proposed that when their size reached a certain value, DNA synthesis in the 5'-3' direction would be initiated and would proceed at a high rate completing the replication of the daughter strands.

b) Late Mode

Early observations (Young & Sinsheimer, 1968) on the nature of the late replication showed that the DNA produced late in infection sedimented heterogeneously faster than mature DNA and that the kinetics of its synthesis suggested turnover during the period of viral DNA accumulation.

Skalka et al (1972) used electron microscopy to show that the late DNA was present as long linear concatemers. Molecules were not whole multiples of wild-type length nor did they appear to have specific start/end points. These forms comprised about 20% of the late DNA. About 40-60% of the molecules were of lambda wild-type length and consisted of circular permutations of mature sequence as though derived by random breakage of circular DNA molecules. It was proposed that both of these molecular forms were generated from rolling-circles which had fallen apart at the growing point.

Takahashi (1975) later confirmed that 87% of the structures isolated at 30-50 minutes after infection were single branched circles. Denaturation mapping showed that many of these forms were partially replicated bi-directionally from ori suggesting that during DNA replication, one of the forks may have been scissored leaving the other fork to continue. The free ends were found at many positions on the lambda chromosome but many shared a few common sites (7/20 had their free suggesting this scission may be produced by some specific
mechanism. DNA isolated at about eight minutes post-infection has about three nicks/genome and it was suggested that these might be responsible for the early-theta to late-rolling-circle transition. It was further shown that rolling circles were formed independently of rec, red, int, gam and phage maturation (Skalka et al., 1972; Takahashi, 1975).

In the majority of molecules the direction of movement of the intact replication fork was shown to be right to left with only a minority progressing in the opposite direction (Bastia et al., 1975). The direction of DNA packaging into the virion heads is left-to-right (Emmons, 1974) which implies that packaging occurs from the linear concatemer and not from the end of the replicating molecule.

It was also shown that under conditions of limited replication, by suppression of QamPam, rolling circle forms were still found but the predominant direction of replication was left to right.

Interestingly it was shown that if initiation delayed 30 minutes by the use of a temperature sensitive mutation in Q then nearly all replicative structures were single branched rolling circles even for the first round of replication (Takahashi, 1977). Thus the early theta-mode of replication is not an essential prerequisite for rolling circle replication.

One interesting facet of lambda replication is its ability to cause the replication of the host when a prophage is induced. This was shown (Imae & Fukasawa, 1970) by studying escape synthesis (uncontrolled expression of a gene as a result of phage development) of the host gal operon. Experiments showed that most of the gal escape synthesis was dependent on the lambda Q and P function, i.e. lambda replication. It was also shown that newly synthesized DNA was not pure lambda DNA but contained a significant amount of gal operon
DNA. This suggests that DNA replication initiated at the phage ori site is able to extend outwards into the host chromosome adjacent to the prophage. The consequence of this is that it implies that on induction of a prophage, replication precedes excision.

c) Host Genes

The involvement of host genes in lambda replication has been reviewed by Skalka (1977). At least four genes are involved - dnaE, dnaR, dnaG, and dnaZ. The role of dnaE (polymerase III) is self-explanatory since lambda does not produce its own DNA polymerase. The involvement of dnaB was originally shown by the isolation of groP mutants (Georgopoulos & Herskowitz, 1971) also showed that this product interacted with the lambda P gene (see section II). It was shown that the C-terminal segment of P was responsible for this interaction (Furth et al, 1978).

The lack of involvement of dnaA and dnaC (Skalka, 1977) is explainable by the production of the P gene product. This probably substitutes for dnaC. It was further shown that P seems to act by reducing the initiation potential of dnaA (Kellenberger-Gujer & Podhajska, 1978).

Recent work has identified a further two genes, dnaJ and dnaK, which were originally isolated as groPC mutants (Sunshine et al, 1977; Yochem et al, 1978)
After infection of a sensitive bacterium by a virulent phage, such as T4, there is only one outcome - the phage develops lytically and the host cell is killed. Temperate phages, such as lambda, have another option. This is the "temperate" or "lysogenic" response. Its major characteristic is that the phage lies dormant within the host chromosome sometimes for many hundreds of cell generations. At some later point in time the phage can escape from this state and develop lytically.

Early studies on the lysogenic state indicated that the delayed early genes cII and cIII, and hence by inference all the delayed-early genes, were not expressed (Bode & Kaiser, 1965). This suggested that in the lysogenic state, lambda expression was blocked. Lambda mutants are known which are temperature sensitive and form lysogens at low temperatures but not at high temperature. Such lysogens, when transferred to high temperature, are killed since the lysogenic state can no longer be maintained and the phage develops lytically. However, superinfection with wild-type lambda shows that some cells become heat resistant within three minutes (Lieb, 1966). These cells segregate heat-stable progeny even though genetically they are still temperature sensitive. This persistence suggests that the product responsible for maintaining the lysogenic state is a stable substance which is able to maintain the heat-resistance phenotype for several generations.

There are two requirements for the lysogenic state. Firstly, some method is required to prevent expression of the genes involved in lytic development and secondly, some mechanism is required to insert the phage genome into that of the host. For lambda, the first of
these requirements is met by the production of a repressor protein which acts at the early leftward and rightward operators thereby preventing expression from the two early promoters (Steinberg & Ptashne, 1971). As indicated earlier, transcription from these promoters is required for the synthesis of pN which in turn is required for expression of all delayed-early and late functions. In this manner the repressor is effectively able to block the expression of all functions involved in the lytic response.

The second requirement, that of integration of lambda into the \textit{E. coli} chromosome, is met by the "integrase" system. This is a phage-coded protein which results in a site-specific recombination event between a specific site on the host chromosome and a specific site on the phage DNA. These are the attachment sites. This will be dealt with more fully later.

In contrast to some similar systems, lambda also codes for a system that specifically reverses the integration step. This is the excision reaction by which the prophage is accurately excised from the host chromosome. This reaction also requires integrase but in addition a further gene product is required - xis. After excision, the phage develops lytically.

a) Regulation of Repressor Expression

The normal morphology of a lambda plaque is turbid. This is because lambda does not kill all of the bacteria that it infects. Early experiments (Kaiser, 1957) on lambda showed that mutations to a "clear-plaque" morphology could be obtained and these mutants were defective in the formation of lysogens. Mapping of these mutants showed that all could be assigned to three chromosomal regions according to their phenotype. Complementation was observed between a
mutant in any of these classes and either of the other two classes. Mutants of the same class failed to complement each other. These groups are now referred to as genes cI, cII, and cIII. A fourth class of mutant was found that appeared to be cII on the basis of its phenotype and map location but which was nevertheless able to complement mutants in all of the other three genes. This mutant was called cv and because of its cis-dominance was presumed to be a site rather than a gene.

Mutants of class cI give completely clear plaques on a sensitive host. The few colonies that do appear are found to be resistant to lambda indicating a total inability to form lysogens. This suggests that the cI mutations define the gene required for the maintenance of the lysogenic state. In contrast to this, mutants in cII, cIII or the cv site could form lysogens, albeit at a very low frequency. Lysogens once formed were found to be stable and indistinguishable from normal lambda lysogens. This implied that the latter three mutations are not required for maintenance of the lysogenic state but in some manner control the establishment of lysogeny. Once this state has been achieved they are no longer involved. This is supported by the finding that genes cII and cIII are not expressed in the lysogenic state (Bode & Kaiser, 1965).

These results imply that there are two modes of expression of the cI repressor gene - firstly, the establishment mode involving genes cII and cIII together with the site cv, and secondly, the maintenance mode which is independent of these genes.

The kinetics of expression of the cI gene were studied by Reichardt & Kaiser (1971). Their results showed that the rate of cI synthesis was very low during the first five minutes after infection. Between five and 16 minutes the rate became extremely rapid reaching about.
80-90 times the rate of synthesis in a mono-lysogen. After 16 minutes the expression of the cI gene was dramatically curtailed. This pattern of expression is not due to phage replication since λam29, which is unable to replicate, shows the same pattern with the rate of cI synthesis being about 70% that of wild-type. The involvement of cII, cIII and cy in cI expression was amply demonstrated since the rate of cI synthesis in cy and cII mutants was only 1-4% wild-type levels. With cIII mutants the rate was 18% which is in keeping with the observation that cIII mutants are able to form lysogens at a higher frequency than either cy or cII mutants. In spite of the altered rate of repressor synthesis the pattern of expression remained the same in all of these mutants. These results were confirmed by Echols & Green (1971) who also investigated the nature of the cy mutation. Complementation studies showed that cI+ cy− does not complement cI− cy+ for repressor synthesis. This cis-dominance of cy suggests it defines a site for cI expression. Since cy mutants are able to form lysogens this suggests that cy might define the promoter for the expression of cI during the establishment mode. This suggestion has neither been confirmed nor refuted.

b) The Maintenance Mode

Experiments (Heinemann & Spiegelman, 1970) with lysogens containing the temperature sensitive repressor mutation cI857 showed that raising the temperature to 42°C, to denature the repressor, resulted in a rapid drop in the rate of cI mRNA synthesis. Lowering the temperature to 30°C, to renature the repressor, resulted in the restoration of cI transcription. It was therefore suggested that active repressor was required to enable its own synthesis in the lysogenic state. This "auto-control" is independent of any other lambda gene since NN− X−
mutants (which are blocked for expression of all lambda genes with the exception of $\alpha I$ and rex) gave the same result.

It is now believed that the $\alpha I$ protein acts by activating a promoter ($\alpha II$) which lies immediately to the right of the $\alpha I$ gene. Indeed, Yen & Gussin (1973) described the isolation of a mutant which is phenotypically $\text{rex}^- \alpha I^-$ and unable to form lysogens. Since it was able to complement a $\delta y$ mutant, it was concluded that this mutant was defective in its ability to express $\alpha I$ from the maintenance promoter but which was proficient in expression from the establishment promoter. This mutant has been called $\text{pr}m116$ and has been deletion mapped to within $Q_R$ (Smith et al., 1976).

c) Autoregulation

In vivo work suggests that $\alpha I$ not only positively controls its own synthesis but also exerts a negative control (Ptashne et al., 1976).

Meyer et al. (1975) used a 790 base-pairs HaeIII restriction fragment of lambda, containing the rightward operator-promoter, as primer for transcription in-vitro. This fragment not only carries the $P_R$ promoter but also the $\alpha II$ promoter. They were therefore able to study the effect of repressor concentration on transcription leftwards for $\alpha I$ expression and rightwards for $\alpha \text{cro}$ expression. Addition of increasing concentrations of repressor showed that synthesis of $\alpha I$ mRNA was markedly stimulated at first but at higher concentrations of repressor was inhibited. In contrast, synthesis of $\alpha \text{cro}$ mRNA was progressively inhibited and very little transcription occurred at levels that stimulated $\alpha I$ expression. Using a template containing the $\text{pr}m116$ mutation gave no $\alpha I$ mRNA. Transcription of the $\alpha \text{cro}$ gene from a template containing the rightward operator virulent mutations $\nu_1\nu_3$ was not inhibited by high concentrations of repressor.
Experiments with an in-vitro protein synthesis system (Dottin et al., 1975) showed that repressor synthesis was stimulated about four fold at a concentration of repressor equivalent to 35 dimers/operator. Above this level there was no further stimulation.

d) Control by cli and cIII

The involvement of two genes, cli and cIII, in the regulation of the establishment mode of cl expression could indicate two points at which positive control of transcription is performed. In view of the involvement of the site og, a more likely hypothesis would be that the cli and cIII gene products interact with each other at the site og and thereby switch-on the transcription of the cl gene.

Support for this latter view comes from "clear-plaque" mutants of phage $\lambda_{imm434}$ (Strack & Ziegler, 1969). Some of these mutants were found to complement cli mutations but neither cli nor cIII. Similar mutants were also obtained from lambda but at a lower frequency. Hybrid phages with the immunity region of phage 21 did not give rise to this type of mutation. One of the mutants mapped very close to the cli CoI mutation and was found to lie outside of the non-homology region between phage 434 and lambda. The inability of this class of mutant to complement either cli and cIII suggested that these two gene products interact with each other and the complex then activates pre. These clia mutants were proposed to be defective in this interaction of cli with cIII. However, it was later demonstrated that clia is partially dominant to wild-type (Wulff, 1974) so that these mutations cannot be taken as evidence for an oligomeric complex of cli and cIII.

Reichardt (1975a) studied the involvement of N, cli and cIII in the regulation of cl synthesis. He concluded that the role of N in the establishment of lysogeny was to enable the synthesis of cli and cIII.
At low multiplicity of infection, repressor synthesis was found to be limited by the copy number of genes \(aI\) and \(aIII\) but not that of \(N\) or \(cII\). Phage replication was also shown to promote \(aI\) expression at low multiplicities but not at high multiplicities of infection suggesting that its major role was to increase the gene dosage. Similarly the action of \(croc\) was thought to be mainly via repression of \(O_L\) and \(O_R\) thereby reducing \(N\), \(cII\) and \(aIII\) synthesis. Since these gene products are unstable they would decay quickly.

It is interesting to note that phage 434 and lambda share the same \(cv\) site and \(cII\) gene but that phage 21 does not. This suggests that \(cII\) is specific for its homologous \(cv\) site. In contrast to this the \(aIII\) gene is common to all three phages and suggests that the specificity for \(aIII\) is lower. It could also be taken to indicate that \(cIII\) does not interact directly with either \(cv\) or \(cII\). This point will be taken up in the next section.

The involvement of \(cII\) and \(cIII\) in the establishment of repression took on a new light with the finding that these products also acted to inhibit late gene expression (McMacken et al., 1970). This action was independent of \(aI\). Expression of the \(cII\) and \(cIII\) functions resulted in a five minute delay in transcription of the late genes. In contrast, early gene expression, as monitored by exo synthesis, was unaffected. It was suggested that the \(cII + cIII\) action could be produced by an inhibition of \(cII-O-P-Q\) transcription (but see Chapter 3). \(cv\) mutants which are not only defective in \(aI\) transcription (in the establishment mode) were also found to be defective for this \(cII + cIII\) inhibition of late gene expression (Court et al., 1975).
e) Involvement of Host Genes

The host genes *cya* and *orpe* have been implicated in the control of establishment of repression (Grodzicker *et al*, 1972). Lambda forms clear plaques on *cya*⁻ or *orpe*⁻ bacteria and the frequency of lysogenization is reduced about 4-7 fold. (In such hosts the catabolite-sensitive maltose regulon is not expressed, hence the cells are resistant to phage lambda infection. The use of a hybrid of lambda and phi80 with the host-range of phi80 and the immunity of lambda overcomes this problem.) Lysogens once formed are, however, quite normal indicating that these host genes are only involved in the establishment phase. Lambda phage defective in the *orpe* gene and carrying a temperature-sensitive *cI* gene fail to plate at 33°C, a fact attributed to overproduction of active *cI* repressor. Such phages will, however, plate on *cya*⁻ or *orpe*⁻ bacteria at this temperature which again suggests that these hosts are defective in the expression of the *cI* gene. Mutagenesis of *λh*80 allowed the isolation of phage able to form turbid plaques on *cya orpe* hosts. These mutants lysogenize wild-type hosts at >70% efficiency and are very unstable - reverting at frequencies of about 0.5%. The mutants were found to be of two classes - one mapping outside the phage 434 immunity-region and the other within it. The nature of these mutations is not known.

Belfort & Wulff (1974) have also studied the involvement of the host catabolite gene activation system. Lambda *cII* mutants lysogenize wild-type hosts poorly. With a *cya orpe* host the lysogenization frequency is depressed about ten-fold. In the case of a *cIII* mutant the depression was greater than 500 fold - lysogeny was undetectable in the mutant hosts indicating that either *cIII* or the host catabolite gene regulator system is required for lysogeny. Host mutants, called *hfl* for High Frequency of Lysogenization, were isolated (Belfort &
Wulff, 1971) which act as though they have lost a function which antagonizes the establishment of lysogeny. When a cIII mutant infects a hCI- host, lysogeny results with 100% efficiency suggesting the possibility that cIII may act by inhibiting hfl function. Lysogenization of a cya cpr host by lambda cIII mutants was also restored to normal when the host was hCl-.

A major criticism of the preceding results was that the catabolite gene activation system has a multitude of side-effects. It is therefore possible that the effects observed in cI expression could be due to a secondary action rather than a direct effect. To circumvent this problem the effect of varying the cyclic-AMP level after infection was examined (Jordon et al, 1973). These results showed no effect on either repressor or endolysin synthesis. It was therefore suggested that cyclic-AMP is not a major regulatory influence between lysis and lysogeny and that the previous results were due to secondary effects.

This finding was confirmed (Pastrana & Brammar, 1976) by the use of gene fusions in which the trpED genes were expressed from the cI promoters. Infection in the presence of λcI helper resulted in the same enzyme level irrespective of whether the infected cells were grown on glucose or glycerol. This demonstrated that the host catabolite system does not have any effect on pre expression.

Infection of a lysogen by this λtrp fusion phage gave an elevated rate of trp enzyme synthesis as expected by the stimulation of cI expression from pre. Co-infection with helper phage allowed the effect of cII and cIII on pre expression to be studied. Helper phages defective in both of these functions gave no stimulation of enzyme synthesis whereas there was a ten-fold stimulation when both functions were supplied in-trans. Using a helper defective in cIII still gave a
five-fold enhancement demonstrating that cI has only a slight effect on pre expression. In contrast, when the helper was defective in cII, the enhancement was only 2.5-fold indicating that the cII product is much more important. The lack of stimulation when both cII and cIII were mutant shows that PN supplied in-trans has no effect on pre.

The involvement of host functions was further demonstrated by the finding that infection of ultra-violet irradiated hosts resulted in an elevated production of a 14kd protein when the host was mutant in lyo (Lathe & Lecocq, 1977). lyo is probably equivalent to hfl. This protein was tentatively identified as the cII gene product. These results suggested that the lyo protein normally acted by suppressing the appearance of cII, possibly by increasing the rate of degradation. It can therefore be speculated that the function of cIII is to inhibit this lyo function.

f) Turn-off by cro

As mentioned earlier, at about 16 minutes after infection the rate of synthesis of cI is curtailed. Since cro mutants do not show this switch-off, Reichardt & Kaiser (1971) concluded that this turn-off was due to the action of the cro gene.

Heat induction of an N- cI857 Q- prophage results in a loss of immunity but the host is not killed. This is because the N-defect prevents expression of the excision and late gene functions and the Q-defect prevent phage replication. On returning the cells to a low temperature, immunity is not restored because active repressor is required for cI expression from pre and the mutation in gene N blocks the synthesis of cII and cIII required for pre activation. Mutagenesis of such lysogens with ethyl methane-sulphonate allowed Eisen et al (1970) to isolate mutants that were able to regain
immunity. One of these was studied and found to map in the cro gene. This suggested that the cro-product antagonized the synthesis of repressor from prr. It was shown that cro represses prr about five-fold (Reichardt, 1975b).

It was originally thought that cro function was the critical component in the decision between the lysogenic and lytic pathways. The results of Folkmanis et al (1977) indicate that cro does not have a role in the control of lysogeny since the frequency of lysogen formation is not enhanced by a cro mutation. However, cro, is necessary for lytic development.

g) Integration

After establishment of repression by the cI gene product the formation of a stable lysogen requires a further event, the insertion of the lambda genome into that of the host chromosome.

Early studies on integration showed that the prophage genetic map was a circular permutation of the vegetative map (Calef & Licciardello, 1960) and led to the proposal of the "Campbell" model (Campbell, 1962) whereby lambda was assumed to insert into a specific part of the E.coli chromosome. Transduction analysis with P1 (Rothman, 1965) confirmed that lambda integrated at a specific site on the host chromosome (between gal and his) and that a specific site on lambda was also involved. The lambda gene int was found to be involved (Gottesman & Weisberg, 1971) and this product was identified (Weil & Signer, 1968) as a site-specific recombination system. It was therefore proposed that integration involved a reciprocal cross-over event between two specific sites - attB on the chromosome and attP on the phage - mediated by the int protein. Such an event would result in the insertion of the circular lambda chromosome into the host.
Freifelder et al. (1975) followed insertion of lambda into an F' plasmid carrying the bacterial att site and showed that such integration was primarily dependent on the int function and the correct combination of att sites, e.g. a phage carrying attB would not integrate into the attB site on the plasmid. It was also shown that a large pool of lambda DNA was required, though replication itself was not essential. Interestingly, some expression of the xis function was required. It was further found that cl repression was not required for integration provided replication and expression of excision functions were blocked by mutation. In these experiments integration did not commence until about 10-15 minutes after infection.

Partial purification of int showed that its ability to bind to DNA was specific for the correct att sites suggesting int protein directs a sequence-specific recognition (Kotewicz et al., 1977).

Because of the location of the int gene in the N-operon it was assumed that int function would be expressed from a PL transcript. Shimada & Campbell (1974) used an abnormal lambda lysogen in which lambda had integrated into trpC in such an orientation that tryptophan synthetase (the product of trpA and trpB) was expressed from PL. In the lysogenic state (PL repressed) it was found that expression of tryptophan synthetase was dependent on a site within the prophage. By the use of deletion mutants this site was shown to be located close to the left prophage end. By selecting for growth on low concentrations of indole (a procedure selective for high expression of tryptophan synthetase) they were able to isolate intO mutants in which the only phage gene derepressed was int. These mutants were found to be defective in xis function even when the phage was derepressed.

The expression of the int product has been studied by determining the pattern of expression of lambda-specific products after infection.
of ultra-violet irradiated hosts. It was shown that both \( c_{II} \) and \( c_{III} \) together activate synthesis of \( \text{int} \) (Katzir et al., 1976). This stimulation was not found for \( \text{int}^c \) mutations. In \( \sigma_\rho^* \) infections, all the proteins subject to \( \sigma_\rho \) repression were turned-off except \( \text{int} \). It is interesting to note that the synthesis of the \( c_I \) gene product, also required for lysogeny, is also stimulated by \( c_{II} \) and \( c_{III} \). The site of action of these proteins is, however, not the same for the two genes (Chung & Echols, 1977). The implications of these findings are that they suggest that the \( \text{int} \) protein is synthesized from a transcript initiated at the \( \text{int} \) promoter and not from the \( \text{P}_L \) initiated transcript.

Using an in-vitro integration system, Nash et al. (1977) showed that only closed circular DNA was an efficient substrate for the integration reaction. This form seems to be necessary since it is the only form that can be converted into a supertwisted form by the action of DNA gyrase. Gyrase is not required if negatively supertwisted closed circular DNA is used (Mizuuchi et al., 1978).

The integration reaction has also been shown to involve host factors (Miller & Friedman, 1977; Williams et al., 1977). These are under investigation.
CHAPTER 2

trp, polA and lac-FUSIONS
There are six genes in E. coli that are specifically concerned with the biosynthesis of tryptophan. Five of these are genetically linked and comprise the structural genes for the biosynthetic enzymes whilst the sixth, the regulatory gene, is unlinked. The five structural genes are expressed co-ordinately as an operon.

The first step in the biosynthesis of tryptophan from chorismate is catalyzed by anthranilate synthetase, the first enzyme in the trp operon - trpE. However, it was observed that some trpD nonsense mutants were also defective in anthranilate synthetase (Ito & Yanofsky, 1966). Since these two genes are genetically distinct this suggested that the two products might interact at the protein level. This was confirmed. The normal assay, by the conversion of chorismate to anthranilate, is therefore a measure of the trpE and trpD products.

In a similar manner, tryptophan synthetase is a complex of the trpE and trpA gene products.

a) Regulation

The five genes of the trp operon are co-ordinately transcribed as a single polycistronic mRNA initiated at the promoter which is located adjacent to the first structural gene trpE. Expression of the operon is repressed by the product of the trpR gene in concert with tryptophan. That tryptophan itself is the co-repressor was verified using an in-vitro transcription system (Rose et al, 1973). It was also shown that binding of repressor prevents the binding of RNA polymerase to the operon (Squires et al, 1975).

Using indole-3-propionic acid to synchronously derepress transcription of the trp operon, followed by high concentrations of
tryptophan to repress, Baker & Yanofsky (1968) showed that it took about 6.5 minutes to transcribe the operon. It was also shown that initiations occurred with a periodicity of about 2.5 minutes. This indicated that at most three RNA polymerase molecules can be transcribing the operon simultaneously. This periodicity of mRNA synthesis was also reflected in the enzyme synthesis (Morse et al., 1968). Later experiments (Rose et al., 1970) using trpR hosts and rifampicin to block transcription initiation indicated that only three minutes were required to transcribe the whole operon. This discrepancy probably reflects the different experimental conditions used and the original failure to realize the lability of the mRNA. It is possible that the periodicity observed could have been due to the use of indole-3-propionic acid as the derepressing agent.

Baker & Yanofsky (1972), using exhaustive hybridization to determine the cellular level of trp mRNA, showed that in trpR cells the rate of transcription was equivalent to 2.6 initiations/trp operon/minute.

Using cells deficient in control by the trpR product it was shown (Rose & Yanofsky, 1972) that the rate of synthesis of trp mRNA and enzymes varied directly with the cell growth rate. This effect was shown to be due to an alteration in the frequency of transcription initiation.

b) Attenuator

The action of trpR at the operator seemed to explain the regulation of the trp operon. This situation was, however, shattered by the discovery (Jackson & Yanofsky, 1973) that certain internal deletions of the trp operon resulted in a significant elevation in the expression of the remaining genes. In all cases the promoter-proximal
end-point of the deletions preceded the first structural gene. Since repression of the operon was normal it was concluded that the operator was intact. It was therefore proposed that a second site, located between the operator-promoter and structural genes, reduced expression of the \textit{trp} operon. Deletions of this site would therefore result in an increased rate of expression. This site defines the \textit{trp} attenuator.

Examination of the messenger RNA (Bronson \textit{et al}, 1973) showed that the translation initiation codon for the first structural gene was preceded by a leader sequence of at least 150 nucleotides.

Using an in-vitro transcription system it was shown that transcription was frequently arrested at a site early in the operon (Pannekoek \textit{et al}, 1975). This short transcript is referred to as the \textit{trp}-leader RNA.

Bertrand \textit{et al} (1976) used deletions of the attenuator to show that only one out of 8-10 RNA polymerase molecules actually transcribed beyond the attenuator. It was further shown (Bertrand & Yanofsky, 1976) that tryptophan starvation (in \textit{trpR} bacteria to eliminate repressor-control) relieved termination at the attenuator up to five-fold. Isoleucine starvation did not have any effect suggesting that termination at the attenuator was regulated by either tryptophan, or a derivative. None of the gene products of the operon was found to be involved.

Squires \textit{et al} (1976) sequenced the initial part of the \textit{trp} mRNA transcript and found there were about 160 nucleotides preceding the start of \textit{trpE}. Lee \textit{et al} (1976) showed that the leader transcript terminated in a run of uridine-residues which were preceded by a GC-rich region. Such sequences are characteristic of transcription termination sites. Termination was found to occur about 20 base pairs.
upstream of the start of the trpE gene.

Bertrand et al (1977) compared the in-vivo trp leader RNA with that produced in vitro and showed that the two were essentially identical. The exception was that whilst the in-vitro transcript terminated in U7 or U8 the in-vivo transcript was more heterogeneous and terminated in U4-U8. Deletions which extended into this polyU tract reduced the frequency of termination.

When the sequence of the attenuator was determined (Squires et al, 1976) it was found that the leader RNA potentially encoded a 11-residue polypeptide. Although attempts to identify such a polypeptide in vivo have not succeeded there are indications that it might be produced. Ribosomes bind at two sites within the first 190 nucleotides of trp mRNA (Platt et al, 1976). One site is the initiator for the trpE gene whilst the second, located near the 5' end, also contains an AUG codon which suggests that ribosomes may be able to initiate translation of the leader sequence, in vivo.

Analysis of the secondary structure of the leader RNA's of E.coli and S.typhimurium reveals a well conserved pattern of RNA base pairing (Lee & Yanofsky, 1977). Interestingly, the UGA translation terminator for the proposed polypeptide is only three nucleotides away from a region of base-pairing which includes the actual point of transcription termination. This suggests that ribosome movement might be involved in transcription termination, possibly by disrupting the secondary structure of the RNA. A further interesting point in that two codons for the amino-acid tryptophan occur in phase and adjacent to each other only three codons upstream of the translation termination site. Since tryptophan codons are comparatively rare the occurrence of two adjacent to each other seems significant. It has been shown (Toulme & Helene, 1977) that a lys-trp-lys tripeptide binds
selectively to unpaired regions of DNA and is therefore able to distinguish the conformation of DNA. It is very tempting to speculate that translation of the leader could be involved in controlling termination at the attenuator.

Although there is no direct evidence for translation of the leader RNA, it is considered quite probable that translation does occur. In two deletion mutants, which fuse the initial region of the trp leader to the distal segment of trpE, novel fusion polypeptides were synthesized at high efficiency both in-vivo and in-vitro (Miozzari & Yanofsky, 1978). Despite the different sizes of polypeptides the N-terminal amino-acid sequence was common to both. This suggests that both polypeptides had been initiated at the AUG-codon in the leader region.

One controversy about the attenuator is the role of tRNA\textsuperscript{trp}. With the proposal that translation of the leader region may control the frequency of attenuation and the occurrence of tandem tryptophan codons near to the site of termination, it might be expected that tRNA\textsuperscript{trp} would play an important role in regulation. Early experiments by Ito (1972) suggested that trps (tryptophanyl-tRNA synthetase) interacted with the trp\textsuperscript{R} repressor. However, in-vitro transcription studies (Squires et al, 1973) showed that neither trps nor tRNA\textsuperscript{trp} was involved in attenuation which argues against a direct role in regulation. Later experiments (Morse & Morse, 1976) showed an inverse correlation between the degree of amino-acylation of tRNA\textsuperscript{trp} and the expression of the operon. This regulation was shown to act after initiation of transcription and presumably acted at the attenuator. Using a mutant in tRNA\textsuperscript{trp} it was found (Yanofsky & Soll, 1977) that there was a seven-fold elevation in the rate of trp enzyme and mRNA synthesis. When conditions permitting increased charging were
imposed, the normal rate of expression of the trp operon was restored. A second mutant which alters tRNA\textsuperscript{trp}, but which is not actually in the structural gene, also resulted in increased expression but in this case increased charging had no effect.

It is therefore not clear how tRNA\textsuperscript{trp} is involved. It was suggested (Bertrand et al, 1975) that the attenuator functions by permitting fine regulation of the trp operon so that cells could respond to conditions of mild tryptophan limitation as well as to extremes. The correlation between degree of charging of tRNA\textsuperscript{trp} and the expression of the operon might therefore be two consequences of the same situation - namely tryptophan limitation.

c) Atbp Transducing Phages

Phage phi80 integrates into the E.coli chromosome near to trp. Occasionally when this phage excises from the host, the excision event is aberrant and part of the phage genome is replaced by E.coli DNA. This is similar to the generation of Abio transducing phages. In the case of phi80 some of these illegitimate events result in the formation of transducing phages carrying parts of the trp operon. Such phages provide a convenient source of trp operon DNA and much use has been made of this, particularly in transcription studies. The ability of the trpR repressor to control transcription of the trp operon was described earlier. This observation was used as an assay to permit the purification of the trpR protein by its ability to repress in-vitro transcription from the trp promoter (Zubay et al, 1972).

The structure of these phages is such that the trp DNA replaces that of the N-operon. Naomi Franklin (1971) isolated a series of these transducing phages and many of them were found to express the
trp operon from P_L. In this manner she was able to monitor expression from P_L by assaying the enzymes of the trp operon. These experiments contributed to our understanding of the mechanism of action of pN (see Chapter 1).

The cloning of the trp operon by in-vitro techniques using the restriction endonuclease R.HindIII (Hopkins et al, 1976) provided an extension to the above in-vivo phages. Not only were they integration-proficient but the genes could be inserted into lambda in both orientations. Three classes of transducing phage were obtained - containing respectively trpE, trpC and trpA. It was shown that the trp operon has only two restriction sites for R.HindIII located in genes D and B. The restriction fragment carrying the trpE gene was also found to carry the promoter. The trp genes were inserted into a lambda vector at the HindIII target left of the attachment region. Insertion of both the trpE and trpC fragments in the appropriate orientation reconstituted the trpD gene.
The proposal of the double helix structure of DNA suggested a mechanism by which an organism's genetic material could be duplicated. A search for suitable enzymes resulted in the discovery of DNA polymerase in *E. coli* (Kornberg *et al.*, 1956). It has become apparent that this enzyme, now called DNA polymerase I (polI), is only one member of a group of enzymes involved in the copying of DNA templates.

a) Properties:

The DNA polymerases catalyse the addition of deoxy-ribonucleotides to the 3'-hydroxy terminus of a primer DNA chain. The direction of synthesis of a DNA chain is therefore 5'-3'. Although most polymerases have an associated 3'-5' exonucleolytic activity, *E. coli* polymerase I has the additional ability to degrade DNA progressively from either end of the duplex.

The isolation of purified polI (Jovin *et al.*, 1969) has enabled much of the mechanism of action of this enzyme to be studied. It consists of a single polypeptide chain of 109kd containing one disulphide bond and a single reactive sulphydryl group which is not essential for activity.

A summary of the properties of polI is given in Kornberg (1969) and will only briefly be described here. The enzyme has a single binding site for the DNA template and will not bind to covalently closed circular DNA unless the DNA has been nicked. Binding seems to occur only at single-stranded regions and nicks. The enzyme has a single binding site for the deoxyribonucleotide triphosphates and only the triphosphate moiety is of primary importance.
Limited proteolysis of polII yields two polypeptides. The larger fragment of size 76kd contains the polymerase activity and 3'-5' exonucleolytic activity. It also contains the binding sites for the nucleotides and the primer (Setlow et al., 1972). The smaller 36kd-fragment contains the 5'-3' exonuclease activity (Setlow & Kornberg, 1972). These two polypeptides are able to interact with each other and are then indistinguishable from the intact protein.

The increased understanding of the mechanism of action of polII cast serious doubt as to whether this enzyme could be responsible for DNA replication in-vivo. The requirement for a primer was absolute and hence there was no way for polII to initiate replication de novo. In addition, the activity of the enzyme in-vitro was two orders of magnitude too low to account for the observed in-vivo rate of DNA synthesis. More seriously, it would only polymerize in a 5'-3' direction. Since DNA must also be synthesized in the opposite direction this indicated that other enzymes should also be involved. This latter deficiency was somewhat ameliorated by the demonstration that synthesis in the 3'-5' direction could occur by the synthesis of small DNA fragments in the 5'-3' direction which were then joined by host DNA ligase (Okazaki et al., 1968).

The role of polII in DNA replication received a bitter blow when DeLucia & Cairns (1969) isolated a mutant defective in polII activity but which was still completely viable. This mutant contained only about 1% the normal level of enzyme activity. The effect of this defect was a marked increase in sensitivity to ultra-violet irradiation and alkylating agents such as methyl methane-sulphonate. As a consequence of the characteristics of this polA1 mutant and the inadequacies of polII as a replication enzyme it was suggested that the role of this enzyme was not in replication but in the repair of
damaged DNA.

The residual polymerase activity in polA1 cells was found to be due to a small amount of fully active enzyme. Despite the low level of polymerase activity, that of the 5'-3' exonuclease remained almost normal. In the wild-type enzyme this exo activity co-sediments with the polymerase activity, however, in the mutant strain the exo activity sediments more slowly than the polymerase. Other non-suppressible polA mutants retain exo activity that co-sediments with the polymerase activity. The size of the fragment containing the 5'-3' exo in polA1 cells is the same as that of the small fragment generated by proteolysis of polI. It is possible that the truncated polypeptide produced from the polA1 amber mutation is coincidentally the same size as the small fragment. However, a more likely explanation would be that the truncated polypeptide is even more susceptible to proteases than the wild-type enzyme and is rapidly cleaved to generate the small fragment.

b) DNA Replication:

The availability of the polA1 mutant, with its greatly decreased polymerase activity, allowed the search for other DNA polymerases which were present in smaller amounts. A second enzyme was identified and subsequently purified (Kornberg & Gefter, 1970 and 1971). A third enzyme present in even smaller amounts was also identified. The function of the second enzyme, polII, is not known but the third enzyme, polIII, was later identified as the product of the dnaE gene. The isolation of the dnaE mutants as defective in DNA replication suggested that polIII was the primary enzyme responsible for in-vivo replication. The properties of this enzyme (see Kornberg, 1974) are consistent with this role.
Although polI has been displaced from the primary role in replication it may still play an important role. It is found that conversion of newly synthesized low molecular weight DNA to high molecular weight is impaired in polA1 mutants (Kuempel & Vecomett, 1970; Okazaki et al, 1971) suggesting a role for polI in gap-filling between the nascent short segments synthesized by the replication complex. A consideration of the properties of the available polA mutants, particularly conditional lethal mutants, (Lehman & Uyemura, 1976) led to the conclusion that polI is essential for viability in E.coli and that it was essential for discontinuous replication. No absolute defective mutants have been isolated (yet).

b) Mutants:

After the initial isolation of polA1 several other mutants have been isolated. polA107 has been shown to be defective in the 5'-3' exonuclease but is apparently normal with respect to its polymerase activity (Heijneker et al, 1973). This suggests that both the exo and polymerase activities are essential to the role of polI in replication. A comparison of the properties of polA1 and polA107 is given in Glickman et al (1973) The only apparent difference is that polA107 is slightly less sensitive to irradiation (both ultraviolet and X-ray). Another mutant, polA6, is similar to polA1 (Kelley & Whitfield, 1971). Mapping of these three mutants (Kelley & Grindley, 1976) established the direction of transcription of the gene as being clockwise on the E.coli genetic map (Bachmann et al, 1976). Fine mapping of polA mutants is made very difficult by the high reversion frequency of these mutants.
The repair of DNA damaged by treatment with such agents as ultra-violet (UV) light consists of a complex series of reactions which have still not been totally resolved. Damage by UV appears primarily to be caused by the production of pyrimidine dimers - predominantly between adjacent thymine residues (Setlow & Carrier, 1964). There appear to be three mechanisms for repair. The first consists of photoreactivation which seems to act directly by reversing the dimerization. The integrity of the DNA is retained and no DNA synthesis occurs. The second mechanism has been classed as an error-free dark-repair system, whilst the third is an error-prone or "SOS" pathway involving the host rec system and in particular recA.

The original isolation of UV sensitive mutations (Howard-Flanders et al., 1966) led to the genetic identification of three loci concerned with repair of UV damaged DNA. These were designated uvrA, B and C. Since double mutants were only slightly more sensitive that the single mutants it suggested that all three are defective in the same reaction pathway. uvrA and uvrB are now known to define an endonuclease activity (Braun & Grossman, 1974) which specifically binds to UV-irradiated DNA. uvrC may act by allowing turn-over of this endonuclease thereby increasing the rate of incisions (Seeberg et al., 1976).

The accepted mechanism of DNA dark repair commences with the binding of the uvrA + uvrB endonuclease to the region of DNA containing the thymine dimer. The dimer can then be removed by one of several reactions. Excision by polA requires both the polymerase and 5'-3' exonuclease activities (Deutsch et al., 1976) and is consequently slow in polA1 cells. However, since repair synthesis continues for longer, full repair does eventually occur. Repair by
this system results in very little DNA synthesis indicating that the DNA "patches" used to replace the excised thymine dimers are small (Cooper & Hanawalt, 1972b).

It was shown that some of this repair was achieved by patches greater than 1500 nucleotides long (Cooper & Hanawalt, 1972a). Since the average size is only about 30 nucleotides, these results indicated that >99% of dimer excision must be repaired with short patches characteristic of repair by the polI enzyme. It was shown that in polA− cells repair synthesis was enhanced. This was presumably because repair was now predominantly by the "long-patch" repair system. This alternative system was shown to require polIII (Youngs & Smith, 1973). Later results showed that any of the three polymerases could effect repair but that polII repair was limited and incomplete (Tait et al., 1974).

**e) Incompatibility With Other Genes**

Enzymatic repair of DNA damaged by ultra-violet irradiation occurs predominantly via two pathways. One of these is mediated by the host rec system whilst the second involves repair by polymerase I.

When both pathways are inactivated by mutations the bacteria are unable to grow (Gross et al., 1971). Presumably this is due to a total inability to repair even naturally arising damage during DNA replication.

polA1 is also found to be incompatible with several other genes involved in DNA repair (Morimyo & Shimazu, 1976).

**f) In-Vitro Cloning of polA**

Lambda phage defective in either the red or gam genes are unable to plate on hosts defective in the polA gene (Zissler et al., 1971a).
This observation was used as the basis for the isolation of polA transducing phages from lysates made by in-vitro cloning of E.coli DNA using the restriction endonuclease R.HindIII (Kelley et al, 1977). The vector used was deleted for the lambda red gene and recombinants were selected by their ability to form plaques on polA1. Unfortunately, it is found that many in-vitro recombinants not carrying the polA gene are also able to plate on polA1. Consequently this selection was only used as an initial enrichment and a second screen was performed for those phage able to transduce polA1 to resistance to methyl methane-sulphonate, hence PolA+. The resultant phage were confirmed to contain the polA gene. Apart from the genetic evidence, the presence of the polA gene on this phage has been conclusively demonstrated by the elevated levels of polII protein produced after infection. Levels up to 100 times normal were produced in this manner. The presence of polII was confirmed not only by its activity but also by visualization of the protein on sodium dodecyl sulphate polyacrylamide gel electrophoresis. Under the conditions used expression of the polA gene could not be from a lambda promoter which suggests that the promoter is also present on this HindIII fragment. This is supported by the finding that lysogens of this phage are PolA+. The HindIII restriction fragment containing the polA gene is about 5kb long. Since the polA gene will be about 3kb long this leaves very little room for other genes.
Gene fusions, and in particular fusions to the *lac* operon, have been of immense assistance to studies on the mechanism of regulation. The literature on gene-fusions is too extensive to be covered exhaustively. This introduction will be confined to three aspects.

The first of these is the fusion of the *trp* operon to *lacZ*. Such fusions were constructed by transposing the *lac* genes into close proximity to the *trp* operon by the integration of phi80*lac* at its attachment site near *trp*. The *tonB* gene is now located between the *lac* and *trp* genes. Mutants in *tonB* can readily be selected and many are found to be deletions. In some of these deletions, the *trp* and *lac* operons were found to be fused such that *lacZ* was expressed from the *trp* promoter (Miller *et al.*, 1970). It was also shown that mRNA corresponding to *lacZ* was covalently linked to that of the *trp* operon (Eron *et al.*, 1971).

Another approach to the construction of *lac*-gene fusions has been developed by Casadaban (1976a). This method is generally applicable and easy to perform - factors attested to by the many gene fusions which have now been constructed by this procedure. The technique involves the integration of phage Mu into the gene of interest thereby providing homology to direct the integration of a lambda phage carrying a portion of Mu in addition to the *lacZ* gene. The lambda phage is integration defective and the alternative integration site on the host at *lacZ* is removed by deletion. This also provides a Lac phenotype. The strain of Mu used carries a temperature-sensitive repressor and hence by looking for heat-resistant survivors, deletion derivatives can be isolated. Strains able to express the *lacZ* gene can easily be identified by the use of a suitable indicator plate.
These strains should now have the lacZ gene fused to the promoter of interest. Using this technique, fusions to araBAD, araC and leu were constructed (see also Casadaban, 1976b). Similarly fusions to ilv (Smith & Umbarger, 1977) and argA (Eckhardt, 1977) were constructed. thr-lacZ fusions (Saint-Girons, 1978) were used to isolate thr regulatory mutants by selecting for elevated expression of beta-galactosidase.

The above examples are cases of gene-fusions. The technique is also readily applicable to the construction of protein fusions. This allowed the isolation (Silhavy et al., 1977) of fusions containing the N-terminal region of lamB (the lambda receptor) and the major C-terminal portion of beta-galactosidase. Since these fusions have beta-galactosidase activity it is possible to use this to determine the cellular location of the enzyme. One strain with very little lamB-specified polypeptide had the beta-galactosidase activity in the cytoplasm whereas another fusion with more of the lamB product was found to be located in the outer membrane. These results suggested that the N-terminus of the lamB polypeptide contains the information necessary to direct the protein to the outer membrane.

A different approach to constructing gene-fusions was the assembly of a hybrid operon on a lambda transducing phage using restriction enzymes (Ptashne et al., 1976). In this manner lacZ expression was coupled to the lambda prM promoter.
CHAPTER 3

EXPRESSION INTERFERENCE
The original proposals of the "operon-theory" (Jacob & Monod, 1961) have resulted in the accumulation of much information on the regulation of expression. One unfortunate connotation of operon-theory is that it has been taken to imply that regulatory events occur only at the operator-promoter region. Furthermore that once transcription has been initiated no further regulation occurs and all genes of the operon are expressed equally. It has now become apparent that this simplified view of regulation is far from correct. Course regulation at the operator-promoter is indeed important but fine regulation mechanisms are beginning to be seen as being of increasing importance.

The discovery of the termination factor rho (Roberts, 1969) and its involvement in regulation in lambda, introduced a new concept. The regulation of the lambda N-operon by pN and rho has already been described (Chapter 1). In this case it is known that the N gene and genes to its left are transcribed as a single mRNA unit initiated at PL. However, early in infection the transcript is small due to the action of rho at the tL1 terminator. When pN is available it antagonizes the action of rho and the N-operon genes are expressed. This second site of control of lambda development is of equal importance to that occurring at the operator.

Another important example of second-site control was the discovery of the trp operon attenuator (Jackson & Yanofsky, 1973). Here again the dual regulation allows the operon to be regulated over a much wider range than would be possible with only a single control site. Attenuators have been found in other systems (see Goldberger, 1974) and there is suggestive evidence that the expression of the RNA polymerase sigma factor may also involve an attenuator (Nakamura & Yura, 1976).
These are examples of regulation of transcription. Whilst all the genes of an operon are usually required to be regulated co-ordinately it is often found that the amounts of each protein synthesized are not the same. Thus the lambda late genes differ in their molar rate of synthesis by a factor of nearly a thousand-fold (Murialdo & Siminovitch, 1972). This is thought to be due to a differential efficiency of translation of the mRNA. Similarly the lambda N-operon genes are not produced equally (Hendrix, 1971).

This chapter is primarily concerned with phenomena that can best be described as "interference with expression". Very little is known about such events and their mechanisms are unknown. Examples include the lambda Hin function and transcriptional interference.

a) HOST INHIBITION

It is normally propounded that virulent phages, such as T\textsubscript{4}, completely subvert the host whilst the temperate phages, such as lambda, have no specific effect on the host cell synthesis other than by a simple competition for resources. This, however, is not correct, lambda does interfere with host gene expression. The function involved is known as "Hin" - Host Inhibition - and has been mapped within the N-operon. Even so, the responsible agent is not known nor is the mode of action. Cohen & Chang (1970) showed that net RNA synthesis was affected within a few minutes of infection. This effect was strongly dependent on the multiplicity of infection but at multiplicities of ten only a four-fold inhibition was observed. Addition of chloramphenicol ten minutes before infection prevented this inhibition. N-defective phage did not express the Hin-function suggesting that either pN or an N-controlled function was responsible.
Using the expression of beta-galactosidase as a monitor of host protein synthesis gave similar results. \( \lambda \) blO phages with different sizes of deletion of the \( \Lambda \)-operon confirmed that the function responsible was located within the \( \Lambda \)-operon and indicated that there were probably two components involved.

Brown & Cohen (1974) identified a lambda function which was responsible for an alteration in the specificity of the host RNA polymerase resulting in a nine-fold increased activity for lambda DNA. This alteration was prevented by chloramphenicol treatment. Since pN synthesis is required for transcription of the lambda genes this does not necessarily imply that the function is a protein. Again deletion mapping using \( \Lambda \) blO phages, showed this function was located within the \( \Lambda \)-operon. Electrophoresis of partially purified RNA polymerase showed no alteration in its subunit composition. However, a 72kd protein was especially prominent. None of the \( \Lambda \)-operon genes codes for a protein of this size suggesting that it is probably a host protein stimulated by lambda. Induction of a non-replicating \( Q^- \) prophage resulted in a five-fold inhibition of beta-galactosidase synthesis. This inhibition was found to be completely reversed by the addition of 2mM cyclic AMP (Wu et al, 1971). The extent of catabolite repression by glucose on beta-galactosidase synthesis is also about five-fold and is known to be mediated by a reduction in the cellular level of cyclic AMP. This suggests that the Hin effect might be mediated via a reduction in the concentration of cyclic AMP.

The involvement of cyclic AMP was further demonstrated by results obtained with the escape synthesis of lacZ resulting from transcription from the lambda P\(_L\) promoter (Mercereau-Puijalon & Kourilsky, 1976). \( \lambda \) pla5 (Ippen et al, 1971) is an in-vivo generated lambda transducing phage carrying the lacZ gene and promoter in the
phage b2-region oriented such that it is potentially transcribable from \( P_L \). Expression, apparently from a phage promoter, was observed and found to be controlled by the same factors that regulate expression of the \( N \)-operon. Such synthesis from \( P_L \) has two characteristics: firstly, an absolute requirement for cyclic AMP and secondly, a low efficiency. The requirement for cyclic AMP was found to be the result of the expression of at least one lambda gene located in the \( N \)-operon. This position co-incides with the location of the \( His \)-function. In deletions removing this region, cyclic AMP was no longer required for the \( P_L \) promoted synthesis of beta-galactosidase. This would suggest that \( His \)-function grossly lowers the cellular cyclic AMP level.

In addition to cyclic AMP it was also found that expression of \( lacZ \) required \( crp \) (catabolite gene activator protein) which suggested that the \( lac \) promoter might be involved in this synthesis. Addition of rifampicin resulted in a blockage of enzyme synthesis within three minutes indicating that transcription of \( lacZ \) was initiated in close proximity to the \( lac \) promoter. Mutants in the \( lac \) promoter failed to show this synthesis. Measurements of the amount of transcription of the \( lacZ \) gene showed that transcription was in vast excess over the rate of beta-galactosidase synthesis. This transcription was independent of cyclic AMP. It was proposed that expression from \( P_L \) resulted in transcription of \( lacZ \) at a high rate but that this mRNA was not translatable. At low efficiency, initiation of transcription at the \( lac \) promoter could occur and, because of the high rate of transcription from \( P_L \), this was not blocked by the \( lac \) repressor. This mRNA is translatable. The failure of the \( lac \) repressor to block transcription from the \( lac \) promoter might be explained by its displacement from the operator as a result of "through-transcription"
from \( P_L \).

Since host DNA synthesis requires the production of host specified RNA and protein synthesis it might be expected that Hin might also affect host DNA synthesis. There is a suggestion that this might occur (Waites & Fry, 1964).

It therefore seems that two components participate in concert to partially repress the host's expression. These map in the vicinity of genes red and \( \text{gam} \). Blockage of protein synthesis prevents their action which might indicate they were protein in nature were it not for the fact that the \( H \)-operon is not transcribed in the absence of \( pN \) which would require protein synthesis for its production. It is therefore conceivable that the function could be RNA. Their mechanism of action is not clear but at least one effect seems to involve an alteration in the cellular level of cyclic AMP. Whether or not this is a primary effect or a consequence is not known.

b) \( \text{Spi} \) PHENOTYPE

This phenotype seems to be closely related to the Hin function. Wild-type lambda is unable to form plaques on a strain lysogenic for phage P2 because of its "Sensitivity to P2 Interference" - the Spi phenotype. However, there is no interference with injection of the phage DNA and it does not appear to be damaged. Nevertheless phage replication is severely impaired. Lindahl et al (1970) were able to isolate P2 mutants (old) which no longer gave this interference. Since the mutants were recessive to wild-type it was suggested that the P2 prophage codes for a diffusible product which interferes with lambda development. This same P2 function was also responsible for cell-death on injection of recombination-deficient bacteria.
Similarly lambda mutants resistant to this interference were also found to be recessive to wild-type implying that lambda actively participates in the interference. These mutants are unable to synthesize at least two non-essential proteins and are unable to grow on recA hosts.

There are two aspects to the Spi phenotype. The first is concerned with lambda replication whilst the second is associated with an inhibition of host functions (Hin function).

Whilst investigating the role of recombination in the growth of lambda, Zissler et al. (1971) found that lambda mutants defective in both red and gam functions were able to plate on a P2 lysogenic strain (Spi- phenotype) but the plaque size was very small. However, some of the λbio phages formed large plaques. This was originally explained as the deletion of a gene, delta (see Chapter 1, section II) which prevented the large-plaque phenotype. The reason for the small plaque phenotype can be explained by considering the mechanism of replication of lambda (see Chapter 1, section IV). The effect of a mutation in gam is that the infected host will remain RecBC+ since the function of gam is to inhibit this exonuclease. Since the recBC enzyme prevents the transition to the late rolling-circle mode this means that replication will be entirely in the early theta form. The products will therefore be circular monomers of lambda DNA which are not substrates for packaging into mature virions. In order to be packaged they must first be converted to dimeric circles by recombination. The most efficient mechanism of achieving this is the phage-coded red system. However, Spi- mutants are also deficient in the red function. Whilst the host rec system is active it is not found to be very efficient at dimerization. Consequently very few dimeric circles are produced and the yield of mature progeny is correspondingly low. The
plaque size will therefore be small. Under such circumstances the selection pressure for large-plaque formation is high and secondary mutations can occur. These occur at high frequency and are known as the \textit{chi} mutations (Henderson & Weil, 1975). They provide "hot-spots" for recombination by the host \textit{rec} system (Stahl \textit{et al}, 1975). This aspect of the Spi phenotype is not restricted to growth on a P2 lysogen but applies to growth on all strains (except those mutant in \textit{recBC}).

The second aspect of Spi seems to be analogous to the Hin function described in the previous section. This must, however, be treated with caution since the Hin-effect is small whereas the result of interference between P2 and lambda is a total turn-off of cell expression.

After lambda infection of either a P2-lysogen or a non-lysogenic strain, the synthesis of beta-galactosidase was induced in order to study the effects of interference (Cohen & Chang, 1971). Induction was unaffected with lambda mutants which were deleted for the N-operon. Shorter deletions gave a partial inhibition (30\%) in a non-lysogen but in the P2-lysogen there was still a complete blockage of enzyme synthesis. Wild type lambda gave a four-fold inhibition in the non-lysogen and again the inhibition was complete in the P2-lysogen. These results suggest that the P2 prophage is able to supply a component that is lacking in the shorter lambda deletions. This P2 function together with the lambda function combine to result in the total inhibition of host cell synthesis. There is a very good correlation amongst the lambdoid phages between the ability to express the Hin function and the Spi phenotype. Thus phi80 which gives no host inhibition is not sensitive to P2 interference.
Using pulse labelling to study RNA synthesis gives an essentially similar result to that for beta-galactosidase induction. It appears however, that one effect of the P2 prophage is to block transcription of the lambda late functions and this is supported by the absence of lambda endolysin synthesis. Infection of a P2-lysogen with a $\lambda_{trp}$ transducing phage shows that lambda early expression is turned off after about ten minutes (this was not due to cro mediated turn-off as this gene was mutant in these phages). Identical results were obtained with lambda exo.

The "mechanics" of P2-\lambda interference have been studied by Bregegere (1974, 1976 and 1978). Although interference was marked by a shut-off in protein, RNA and DNA synthesis, the kinetics showed that the first to be turned-off was amino-acid incorporation. This was not the consequence of a blockage of transcription. It was found that the acylation potential of tRNA's were markedly lower than normal as was their efficiency at promoting translation in-vitro. This interference was found to be only partially dependent on the lambda gam function and red was not involved. Although Q and P were involved, DNA replication does not occur. It was further shown that mutations in dnaB (croP) or the lambda ori site suppress the inhibition of amino-acid incorporation. Partial suppression was observed with recA mutants provided that the red function was expressed. It was suggested that P2-\lambda interference was due to the action of the P2 old-function at an early step in the initiation of lambda replication. Why this should affect cellular protein synthesis is not clear.
Under normal circumstances, transcription initiates at a promoter and continues through the gene, or operon, finally terminating at a specific site (e.g. the *trp* operon, Guarente *et al.*, 1977). However, in some cases transcription ends prematurely and this may be termed "transcription interference". An example is provided by polarity in operons.

A different kind of interference has been found in phiX174 in-vitro. Three promoters and four termination sites have been located on phiX174 DNA. Studies on in-vitro transcription (Axelrod, 1976) showed that at low ratios of RNA polymerase:DNA transcripts terminated at the terminator sites. However, when a large excess of polymerase was used, the transcripts were found to terminate at the sites of downstream promoters. This suggests that the binding of polymerase at downstream promoters is able to block the progress of polymerase.

In contrast to this, Naomi Franklin isolated *Atrp* transducing phages (see Chapter 2) in which the genes were expressed from both the *trp* promoter and P_L (Franklin, 1974). In this case repression of the *trp* promoter did not prevent P_L expression from transcribing the operon. This suggests that regulator proteins bound to the DNA in-vivo are not a hindrance to transcription. However, the two situations are not analogous. The *trp* repressor dissociates from the DNA with a half-life of less than two minutes (Rose & Yanofsky, 1974). Also pN-modified RNA polymerase is noted for its ability to prevent termination. Furthermore the levels of RNA polymerase used in the phiX174 experiment were very high and hence it is not clear whether this situation would occur in vivo.
Convergent transcription defines a situation where two promoters are actively transcribing in opposing directions. Just such a situation was observed (Bovre & Szybalski, 1969) to occur in the lambda b2 region. However, although both strands of DNA are transcribed during the infective cycle, they were quick to point out that transcription probably does not occur simultaneously in both directions since early leftward transcription will be turned-off by cro long before late rightward transcription occurs.

However, lambda mutants defective in cro do not turn-off leftward transcription. Under these circumstances it is possible that leftward transcription might occur simultaneously with rightward transcription. Indeed, λ cro mutants are inviable because of two effects. The first was called Tro (Eisen et al., 1975) and is probably analogous to the Hin function whilst the second effect is cis-specific. It is possible that this latter effect might be due to leftward transcription interfering with rightward transcription of the phage structural genes.

It has been reported that there may be a transcription termination site left of b2 which is resistant to the action of pN (Adhya et al., 1976). This should prevent leftward transcription from reaching the late structural genes. However, many of the in-vitro generated transducing phages will have deleted this site. It is found that many of these phages when combined with a cro– mutation grow very poorly (Noreen Murray, personal communication, and personal observations).

Fusions have been isolated in which the lac and trp operons were oriented in opposition to each other (Miller et al., 1970). One such fusion in which all termination sites between the two operons had been deleted was studied. It was shown that derepression of the trp operon had very little effect on either the basal or the induced level of
beta-galactosidase synthesis. The reverse was also true. These results implied that productive expression, both transcription and translation, was unaffected by a polymerase transcribing in the opposite direction.

This conclusion is at variance with an analogous fusion between the his and roughP operons in Salmonella typhimurium (Levinthal & Nikaido, 1969). Derepression of the his operon resulted in a five-fold depression in the expression of the roughP operon, and vice versa.

The in-vitro cloning of the trp operon using R.HindIII (Hopkins et al., 1976; and see Chapter 2) resulted in the availability of λtrp transducing phages in which the trp genes were carried in the 'r'-orientation, i.e. transcribed from the 'r'-strand of lambda. The consequence of this is that the trp genes are now oriented in opposition to the lambda P_L promoter. It could therefore be asked whether or not P_L expression had any effect on expression of the trp genes. The results showed that not only did P_L interfere but the interference was almost total.

The discrepancies between these results could be explained by the nature of the gene fusions. In the case of the lac-trp fusions it is possible that transcription proceeding in both directions does not pass very far beyond the fusion point before reaching a natural termination site. This would mean that, although the genes were oriented in opposition, neither the trp nor the lac genes assayed were capable of being transcribed in both directions.

In the case of the lambda-trp fusion this criticism would not hold. Transcription from the lambda P_L promoter is modified by the action of the N gene product such that termination does not occur. Therefore leftward transcription from this promoter will be able to proceed into the trp genes with the consequence that the trp operon will be
transcribed in both directions. However, in view of the lambda Hin function a serious criticism of this work must be that the interference was not proved to be due to transcriptional effects and could have been due to a general effect of lambda on non-lambda promoters. This is unlikely but not impossible.

The possibility that the inhibition observed for the lambda-trp situation might not be due to convergent transcription gains some weight from work on the expression of bacterial genes from lambda promoter. Steffen & Schleif (1977) used a λaraC phage in which the bacterial DNA was located to the right of att. The orientation of this gene was such that it could potentially be transcribed from one of the rightward promoters of lambda. Indeed, expression from PR was observed. This causes no conflict since by the time PR transcription had progressed round the chromosome and was reading araC, PL transcription would have been shut-off by the action of opr. Therefore the two main lambda promoters would not be opposing each other. However, they also used tetR bacteria, which blocks opr-action (Inoko & Imai, 1974), in order to prevent the normal opr-mediated turn-off of PR and thereby prolong expression of araC. This was exactly the result they obtained. However, the lack of opr-effect in this strain will mean that PL will not be switched-off and will therefore be transcribing araC in the anti-sense direction whilst PR is transcribing it in the sense-direction. Since the araC protein was synthesized at a high rate this would indicate that PL was not interfering with the opposing transcription from PR. It is possible, however, that PL transcription was prevented from opposing PR transcription by the presence of an N-insensitive termination site. It is found in some λtrp transducing phages (Franklin, 1974; Hopkins et al, 1976) that there is a site upstream of the trp promoter that
Fig 3.1: Opposing Transcription in Phage Lambda
severely restricts transcription from \( P_L \). It is possible that such a site might exist near the \( araC \) gene.

In all of the above cases the instances of opposing transcription were artificially constructed. One case that occurs naturally and is of particular relevance concerns the early transcription in lambda. Early expression of lambda results in the appearance of the \( cII \) and \( cIII \) gene products. These are known to activate \( pre \) transcription of \( cI \). Although it is not known where the \( pre \) promoter is located it is most certainly located to the right of the \( oro \) gene. Indeed RNA transcribed from the anti-sense strand of \( oro \) was shown to be present after lambda infection (Spiegelman et al., 1972). Therefore \( pre \) transcription leftwards will be directly opposing \( P_R \) transcription rightwards (Fig 3.1) and the \( oro \) gene will be transcribed in both directions simultaneously.

McMacken et al. (1970) showed that after infection by lambda phage mutant in genes \( cII \) or \( cIII \), synthesis of the tail antigen began at least five minutes earlier than in infections with wild-type phages. The same result was obtained for the synthesis of endolysin. The conclusion was that \( cII \) and \( cIII \) in concert acted to inhibit or delay late protein synthesis. These gene products had no effect on exo synthesis. The inhibition was also apparent in the synthesis of late mRNA. The \( cI \) gene was found to have no effect and therefore the inhibition was not due to repression of \( P_R \) transcription. It was suggested that the \( cII/cIII \) effect was the result of an inhibition of rightward transcription of the \( cII, Q, P \) and \( Q \) genes. Whilst it is possible that \( cII + cIII \) might be directly responsible it is possible that the inhibition could be due to transcriptional interference resulting from the opposing transcription. This is supported by the finding that \( cy \) mutants, which are defective in leftward transcription
of cII from pre fail to show the cII + cIII mediated inhibition of late-gene expression (Court et al, 1975).
CHAPTER 4

MATERIALS AND METHODS
All media were sterilized by autoclaving at 15 lb/sq in for 15 minutes before use.

**Phage Buffer**

- Potassium dihydrogen phosphate: 3g
- di-Sodium hydrogen phosphate (anhydrous): 7g
- Sodium Chloride: 5g
- Magnesium sulphate: 10ml of 0.1M
- Calcium chloride: 10ml of 0.01M
- Gelatin: 1ml of 1% w/v
- Distilled water: to 1 litre

**L-broth**

- Difco Bacto Tryptone: 10g
- Difco Bacto Yeast Extract: 5g
- Sodium chloride: 10g
- Distilled water: to 1 litre

Adjusted to pH 7.2 with sodium hydroxide before autoclaving. For the growth of bacterial strains that carry the thyA mutation, the L-broth was supplemented with thymine at 40μg/ml.
**5X Spizizen Salts**

- Ammonium sulphate: 10g
- di-Potassium hydrogen phosphate: 70g
- Potassium dihydrogen phosphate: 30g
- tri-Sodium citrate, 2H₂O: 5g
- Magnesium sulphate, 7H₂O: 1g
- Distilled water: to 1 litre

**20X Vogel & Bonner Salts**

- Magnesium sulphate, 7H₂O: 4g
- Citric acid, 1H₂O: 40g
- di-Potassium hydrogen phosphate: 200g
- Sodium, ammonium, hydrogen phosphate, 4H₂O: 70g
- Distilled water: to 1 litre

**Vogel & Bonner Nutrient Medium:**

- 20X Vogel & Bonner Salts: 5ml
- 20% Glucose: 1ml
- 20% Difco Bacto Casamino acids: 0.25ml
- Distilled water: to 100 ml
- L-tryptophan: 200μg/ml
- Uridine: 20μg/ml
- 5-fluorouracil: 20μg/ml

The 5-fluorouracil solution was made up fresh for each experiment.
**BBL Agar**

- **Tryptase** (Baltimore Biological Labs.) 10g
- **Sodium chloride** 5g
- **Distilled water** to 1 litre

Bottom layer agar was prepared by adding Difco Bacto Agar to 1%. Top layer agar was prepared with the same agar but at 0.65%. The top layer agar was brought to 1mM magnesium sulphate before use.

**L-Agar**

- **Difco Bacto Tryptone** 10g
- **Difco Bacto Yeast Extract** 5g
- **Sodium chloride** 10g
- **Difco Bacto Agar** 15g
- **Distilled water** to 1 litre

The suspension was adjusted to pH7.2 with sodium hydroxide before autoclaving. For the growth of phage P1, 2mM calcium chloride was added to the agar immediately before pouring.

**Minimal Agar**

- **Davis New Zealand Agar** 16g
- **Distilled water** to 800ml

After autoclaving, 200ml of 5X Spizizen salts were added. Supplements were added when required in the following concentrations:

- Carbon sources 0.2%
- Amino-acids 20μg/ml
- Difco Bacto Casamino Acids 0.05%
- Thymine 40μg/ml
- Indole 10μg/ml
- Thiamine 2μg/ml
Top Layer For Minimal Agar Plates

New Zealand Agar 6.5g
Distilled water to 1 litre
Magnesium sulphate 1ml of 1M

Lactose-MacConkey Agar

40g Oxoid MacConkey Agar No. 3 made up to 1 litre with distilled water.

Xg-Indicator Plates

40mg/litre of 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (Xg) in BB1 Bottom layer agar. Xg was purchased from Bachem Inc.
### SECTION II - PHAGE STRAINS

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**R.HindIII in-vitro DERIVATIVE PHAGES (Note 2)**

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**lacZ Fusion Derivatives (Note 3)**

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4-6
Notes:

1) Strain numbers prefixed by: "NEM" were from Noreen Murray; those with the "D"-prefix were constructed during this thesis.

2) All phages in this section are derivatives of phages constructed in-vitro using R.HindIII. In all cases the bacterial genes are inserted into lambda at R.HindIII site 3 and carry a deletion of the DNA between R.EcoRI sites 1 and 2. shn 60 indicates that lambda site 6 for R.HindIII is not present.

3) These phages all have the left arm of JDW36 generated by restriction with R.HindIII. They carry the lacO gene fused to trpA via the W205 fusion (Mitchell et al, 1975).
### SECTION III - BACTERIAL STRAINS

All strains are derivatives of *Escherichia coli* K12.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
</table>
| C600        | supE,
|             | tonA,
|             | thr,
|             | leu,
|             | thi,
|             | lac               | NEM    | Appleyard (1954)   |
| C600(P2)    | "               | "      | NEM                |
| C600(λimm^21) | "             | (λimm^21) | NEM |
| C600(λimm^34) | "             | (λimm^34) | NEM |
| 594(λ)      | gal strR (λ)     | NEM    |                    |
| QR47        | supE             | NEM    | Signer & Weil (1968) |
| QR48        | supE recA        | NEM    | "                  |
| W3110       | prototroph       | NEM    | Campbell (1961)    |
| W3350       | gal              | NEM    | Murray & Brammar (1973) |
| trpE        | trpOPR1A supE    | NEM    | Yanofsky et al (1971) |
| trpD        | W3110 trpD9778 supE | NEM |
| trpC/C      | W3110 trpCam10243 trpCam9870 | NEM |

4-8
trpA88  W3110  trpA88  supE  WJB  Drapeau et al (1958)
trpA9761  W3110  trpF9777  trpA9761  lacZocU188  trpR  ilv  aziR  WJB
trpA23  trpA23  tonA  supE  WJB
trpED24  W3110  trpED24A  tna  Korn & Yanofsky (1976)
ED8614  trpBE9A  trpR  thyA  Yanofsky Jackson & Yanofsky (1972o)
trpLD102  W3110  trpLD102A  cysB  Hopkins et al (1976)
C24  trpBE9A  trpR  thyA (λoInd−)  WJB
trpR+  trpBE9A  thyA  trpR+  Yanofsky (1976)
AA125  gal-uvrA  his  strR  trpLD102A  taxR  lacI3  lacZm15  tonA  This Thesis page 5.3
polA1  polA1  endA  WJB  Algis Anilionis (1977)
polA107  C53  polA107  thyA  lacY14  strR  Delucia & Cairns (1969)
lig-ts  lig-ts7  supE  NEM  Kelley via NEM  Kelley & Grindley (1976)
polA6  polA107  thyA  lacY14  strR  Kelley via NEM
feb10  polA6  C571  thyA  lacY14  strR  ""
feb10  polA10  supE  tonA  NEM
N259  803  supE  supF  met−  his−  hadM+  Zissler et al (1971a)
N261  lacZam  tonA  Borck et al (1976)

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NEM  NEM  NEM

Drapeau et al (1958)
Korn & Yanofsky (1976)
Yanofsky Jackson & Yanofsky (1972o)
Hopkins et al (1976)
This Thesis page 5.3
Algis Anilionis (1977)
Delucia & Cairns (1969)
This Thesis page 6.20
Kelley via NEM  Kelley & Grindley (1976)
Kelley via NEM  ""
Zissler et al (1971a)
Borck et al (1976)

NEM  NEM  NEM
N301 Ymel supF $\lambda^R$ ($\lambda_{imm}^{21}$) NEM

C38 C600 lacZ$_{415}$ supF tonA hadN$^-$ ($\lambda_{imm}^{21}$) DFW

C48 C600 lacZ$_{415}$ hadN$^-$ ($\lambda_{imm}^{21}$) (D48) This Thesis page 6.11

C49

C50

C51

C52

803 ($\lambda_{clind}^-$) 803 hadN$^-$ met$^-$ ($\lambda_{clind}^-$) WJB

N14-4 uvrD3 trp56 galT23 strA178 Bachmann Ogawa et al (1968)

AB2545 HfrH thi1 metE46 relA1 Bachmann Eggertsson & Adelberg (1965)

Notes:

1) Origin: NEM= Noreen Murray; WJB= Bill Brammar
a) PREPARATION OF PLATING CELLS:

An overnight bacterial culture was diluted 50-fold into fresh L-broth and grown to mid-logarithmic phase, i.e. \(3-5 \times 10^8\) cells/ml. They were then harvested and resuspended in an equal volume of \(1\text{mM MgSO}_4\) and stored at \(4^\circ\text{C}\). The cells retained their viability for at least a month in this form but were not generally used when more than ten days old.

b) PHAGE TITRATIONS:

Phage stocks were serially diluted in phage buffer. 0.1ml of a suitable dilution was adsorbed to 0.2ml of plating cells (usually C600) for 10-15 minutes at room temperature. 3ml of molten BBL top layer agar was then added and the mixture poured onto a BBL agar plate. Plates were incubated for 12 hours or overnight at the required temperature, usually \(37^\circ\text{C}\).

Thymine (40\(\mu\text{g/ml}\)) was added to the agar if required. For the titration of P1 stocks, calcium chloride was added to the agar at \(1\text{mM}\).

c) PHAGE STOCK PREPARATION:

An isolated single plaque was picked into 1ml of phage buffer containing 3-4 drops of chloroform and briefly whirlimixed. 0.1-0.2ml of this suspension was adsorbed for ten minutes at room temperature to 0.1ml of a fresh overnight culture of bacteria, usually C600 or QR47, containing \(5\text{mM}\) magnesium. 3ml of BBL top layer agar was added and
then poured onto a fresh L-agar plate. Confluent lysis was observed after 6-10 hours incubation at 37°C. The top layer was then harvested in 2.5ml of L-broth. Chloroform was added and whirlimixed for 5-10 seconds to release phage from the agar. Agar, chloroform and cell debris were removed by centrifugation. The supernant was decanted, titred and stored at 4°C. Titres varied from $1 \times 10^9$ to $5 \times 10^{11}$ depending on the phage and bacterial host but were typically greater than $2 \times 10^{10}$ pfu/ml.

Where a single plaque did not contain sufficient phage to give a high titre stock a second stock was prepared using about $5 \times 10^6$ pfu from the first stock.

Lysates of P1.kc were propagated on the required strains using an inoculum of $5 \times 10^6$ pfu/plate. The L-agar plates were supplemented with 2mM calcium chloride.

d) PHAGE CROSSES:

Freshly prepared plating cells, usually QR47, were coinfected at a multiplicity of infection of five of each of the two parent phages. After 10-15 minutes adsorption at room temperature the infected cells were diluted twenty-fold into pre-warmed L-broth and grown at 37°C for two hours. Chloroform was then added and the supernatant titred on a permissive host for total progeny and on a selective host for the required recombinant. Recombinants were picked and tested as appropriate. After purification by single-plaque isolation, phage stocks were prepared.
CONSTRUCTION OF LYSOGENS:

The bacteria to be lysogenized was grown in L-broth to mid-logarithmic phase, about 3-4 X 10^8 cells/ml, pelleted and resuspended in 3mM MgSO_4. After 30 minutes starvation at room temperature the cells were infected at a multiplicity of about six and the phage allowed to adsorb for 15 minutes at room temperature. The cells were then diluted 20-fold into fresh L-broth and grown for 3-16 hours at 37°C. The resultant culture was then streaked onto an L-plate. When Rec-mediated integration was desired it was found better to use an L-plate that had been spread with 10^9 pfu each of two homoinmune phages of differing host-ranges and carrying a clear mutation in the repressor gene. Survivors were picked into broth and tested for lysogeny. Typically >80% of surviving colonies are lysogens. Lysogens were purified by at least two single colony isolations without phage selection.

f) INDUCTION OF A LYSOGEN BY ULTRAVIOLET IRRADIATION:

The culture to be induced was grown in L-broth at 37°C to early-logarithmic phase, about 2X10^8 cells/ml, harvested and resuspended in a quarter volume of 1mM MgSO_4. The cells were then irradiated with ultraviolet light to give a total dose of 400 ergs/mm^2 (40 joules/m^2) followed by a four-fold dilution into fresh warm L-broth. They were then grown at 37°C in the dark for two hours. Chloroform was added and cell debris removed by centrifugation.
g) **TEST FOR red, gam OR polA GENES:**

Serial dilutions of the phage were spotted (ca. 10μl) onto a lawn of either lig-ts at 37°C or polA1 cells on BBL-agar plates. Wild-type phage, red+ gam+, give normal size plaques whereas phage that are mutant in either of red or gam do not grow or give very tiny plaques. polA transducing phages which are red- do grow on PolA- hosts but not on lig-ts.

h) **TEST FOR THE Spi PHENOTYPE:**

Phages which have lost the activity of both of the red and the gam genes are able to plate on a strain which is lysogenic for phage P2 (Spi phenotype). The phages were tested by spotting serial dilutions onto a P2-lysogenic host, typically C600(P2) was used.

i) **COMPLEMENTATION BY trp-TRANSUDUCING PHAGES:**

A glucose minimal agar plate supplemented with ACH (casamino-acids) was overlaid with 0.1-0.2ml of fresh plating cells of the Trp- bacteria in 3ml minimal top layer agar. Approximately 10μl spots at the appropriate dilution of the transducing phages were spotted onto the plate. Complementation of the bacterial lesion by the transducing phage allows tryptophan to be synthesized and bacterial growth results. This was scored after 24-72 hours incubation at 37°C.

With some of the Trp- bacteria, notably supE trpOE1, the complementation was enhanced by the addition of a drop of L-broth to the top layer agar before pouring onto the plate.
Alternatively, phage were titred for "complementation plaques". The procedure was similar to the titration of phage as in (b) above except that glucose ACH minimal plates were used.

j) GENERALISED TRANSDUCTION BY PHAGE P1.kc:

1 ml of a fresh overnight culture of the bacteria, supplemented with 2.5 mM calcium chloride, was mixed with about $10^8$ pfu of a lysate of P1.kc previously grown on the required donor strain. The mixture was incubated at 37°C for 20 minutes, the cells pelleted, washed with 0.1 M tri-sodium citrate pH 7 and finally resuspended in 1 ml of citrate. 0.01 ml and 0.03 ml portions were spread onto selective plates.

A control of untransduced bacteria was always run in parallel. A 0.1 ml portion of this mixture was spread onto the same selective plates.

When time for phenotypic expression was required the cells were diluted 50-fold into L-broth and grown at 37°C for 2-5 hours prior to spreading on selective plates.

k) SPECIALIZED TRANSDUCTIONS BY PHAGE LAMBDA:

A fresh culture of the bacterial strain to be transduced was prepared as described in (a) above. After at least 30 minutes starvation at room temperature the cells (0.5-1 ml) were infected at a multiplicity of five with the transducing phage. 15 minutes at room temperature were allowed for adsorption after which the infected cells were pelleted and washed with 1 ml of 0.1 M tri-sodium citrate pH 7 and resuspended in the original volume of citrate. Dilutions of this were then spread onto selective plates.
When time for phenotypic expression was required, the washed infected cells were resuspended in 1ml L-broth and grown for 30 minutes before being given a final wash and plating out as above.

When the transducing phages carried the $\text{gi857}$ mutation (temperature sensitive repressor) the selective plates were incubated at 32°C, otherwise 37°C was used.

Selective plates for $\text{trpA}$ transductions were glucose, ACH, indole minimal plates. For $\text{polA}$ transductions, glucose, thiamine, ACH (+thyamine if required) minimal plates containing 0.04% methyl methane-sulphonate. These plates were prepared fresh.

1) HOMOGENETIZATION:

The homogenetization frequency between a $\text{polA-lacZ}$ fusion phage and the chromosomal $\text{polA}$ mutation was measured as follows:

A single colony of the lysogen was inoculated into 5ml L-broth and grown up over-night. This was then checked for sensitivity to methyl methane-sulphonate (MMS). Logarithmic cells were prepared from this overnight culture as in (a) above. These were then titred for total bacteria on the appropriate minimal plates (usually containing glucose, ACH, and thiamine with thyamine if necessary) and for PolA$^+$ cells on the same plates containing 0.04% MMS. Plates were incubated at 37°C and scored after 1-2 days.

Homogenetization frequency is expressed as MMS resistant cells divided by the total number of cells.
m) PHAGE SENSITIVITY TO PYROPHOSPHATE:

Wild-type lambda is extremely sensitive to chelating agents such as pyrophosphate, whereas deletion derivatives are not. The sensitivity of a phage was tested by titering on plates containing varying concentrations of pyrophosphate, typically 4-10mM sufficed. The higher the concentration the greater the cut-back in titre.

Pyrophosphate can also be used for the selection of deletions. A single plaque was picked into 1ml phage buffer. 0.1ml was absorbed to 0.2ml of plating cells and then plated out onto plates containing pyrophosphate. The concentration was determined by measuring the sensitivity of the phage.

n) ORIENTATION OF λtrp PHAGES:

Phi80trp and λtrp phages have no phage homology between the host-range and immunity determinants. However, should the trp DNA be in the same orientation then this provides homology for recombination between the two phages. The phages are crossed as in (d) above and the frequency of hλ imm80 recombinants measured using C600 lysogenic for a lambda phage of the same immunity as the trp phage. In general phi80pt190 (Deeb et al, 1967) was used. This carries the entire trp operon.

o) TEST FOR Lac PHENOTYPE:

Phage were tested for this phenotype by spotting a suitable dilution onto a lawn of a Lac- bacteria (e.g. N261) on a Lac-indicator plate. Either Lac-MacConkey agar or BBL agar
supplemented with Xg was used.

p) PREPARATION OF PHAGE BY LIQUID LYSATE:

The host strain, typically C600, was diluted about 20-fold from a fresh overnight culture into L-broth containing 1mM MgSO$_4$ and grown to mid-logarithmic phase, about 2-3 x $10^8$/ml. The cells were then infected with the phage at a multiplicity of one. The turbidity of the culture was followed at 650nm and when lysis occurred (usually after 2-4 hours) chloroform was added. The lysate was then clarified by centrifugation for ten minutes at 15,000g in an MSE HS18 centrifuge. The supernant was decanted and titred.

q) CONCENTRATION OF PHAGE BY POLYETHYLENE GLYCOL PRECIPITATION:

This was performed essentially as per Yamamoto et al (1970).

To the un-clarified liquid lysate prepared as in (p) above was added per litre: 40g Sodium chloride, 2ml chloroform, and 1mg each of DNase and RNase. After at least a one hour digestion at room temperature the lysate was clarified by centrifugation at 15,000g for ten minutes in an MSE HS18 centrifuge. Polyethylene glycol 6000 (MW range 6000-7500, British Drug Houses) was added to the decanted supernatant to give a final concentration of 10% w/v and dissolved by gentle shaking.

The lysate was left at 4°C for at least an hour and usually overnight. A flocculent precipitate can be seen which settles out with time leaving a clear supernatant. The phage was pelleted by centrifugation at 15,000g for 10 minutes in an MSE HS18 and resuspended gently in about 1/25th the original volume of phage.
buffer.

Yields of viable phage were typically 60-100%. Phage to be used for the preparation of DNA were further purified by CsCl step-gradient.
a) PREPARATION OF PHAGE:

200ml to 1 litre of phage was prepared as described in the previous section. After concentration by polyethylene glycol precipitation and purification by CsCl step-gradient the phage were phenol extracted. DNA prepared in this way was used satisfactorily for restriction and ligation experiments.

b) CsCl STEP-GRADIENTS:

These were performed using the 6X14ml MSE Titanium swing-out rotor. Three steps of 1-1.5ml of each of the following densities of CsCl were used: 1.3, 1.45, and 1.65 gm/ml made up in phage buffer and clarified by filtration before use. The lightest CsCl solution was put into the centrifuge tube first and the denser solutions underlayed using a syringe and hypodermic needle. Finally the phage sample (up to a maximum of about 10ml per tube) was overlayed onto the gradient with a pipette.

Centrifugation was for 1-2 hours at 35krpm at 20°C in an MSE preparative ultracentrifuge. Examination of the resultant typically showed three bands. The lower band contained the phage and was collected by side-puncture of the tube with a 21 guage needle.

c) PHENOL EXTRACTION OF PHAGE DNA:

CsCl banded phage were dialysed against 10mM tris pH7.5 + 1mM EDTA (TE buffer) for at least one hour to remove the cesium.
Phenol (redistilled under nitrogen and stored frozen at -20°C) was equilibrated with an equal volume of 0.5M tris.HCl pH7.5. Phage and phenol, in equal proportions, were mixed by gentle shaking for several seconds. The tube was briefly centrifuged to separate out the two layers and the lower phenol phase removed. Fresh phenol was added and the extraction repeated a further three times. Finally, dissolved phenol was extracted from the DNA solution by chloroform/amyl alcohol (24:1) and the DNA precipitated with 2-3 volumes of ethanol at -20°C. The DNA was redissolved in a smaller volume of TE buffer and stored at 4°C.

d) RESTRICTION OF DNA:

DNA was digested with restriction endonucleases in a total reaction volume of 40μl containing 10mM tris.HCl pH7.5, 10 mM MgCl₂, and 50 mM KCl. The amount of enzyme and the time of incubation required was determined by trial digestions. Reactions were performed at 37°C.

Digests with R.AvaI were performed in the absence of KCl. In some cases, digests with R.EcoRI and R.HindIII were performed in the presence of 0.005% Triton X100.

Restriction reactions were stopped by heating at 70°C for ten minutes followed by storage at -8°C until required.

The following are gratefully acknowledged for their donations of enzymes: Ken Murray for R.HindIII, R.EcoRI, R.HindII, R.HpaII, and R.Sali; John Boothroyd for R.Avi; Steve Hughes for R.XhoI; Ed Southern for R.HindIII; Bela Sain for R.BglII and R.KpnI; Peter Southern for R.HpaI and Douglas Ward for R.BamHI.
LIGATION OF DNA:

Restricted DNA was diluted to between 5-30μg/ml with 10mM tris.HCl pH7.5 + 0.1M NaCl and one tenth volume of 10X (freshly made-up) ligase cocktail added. T4 polynucleotide ligase was then added (the amount of enzyme required was determined in trial experiments) and the reaction mix incubated at 10°C for 3-6 hours and then kept on ice. At various times thereafter the ligation reaction was sampled by transfection.

10X cocktail was: 660mM tris.HCl pH7.5, 10mM EDTA, 0.1M MgCl₂, 0.1M dithiothreitol, and 1mM ATP. T4 ligase was a gift of Noreen and Ken Murray.

TRANSFECTION:

DNA from ligation reactions was recovered as plaque forming units by transfection. The host strains usually used were N259 or AA125 (sup+ and suppressor-free, respectively).

Competent cells were made by diluting a fresh overnight L-broth culture approximately 40-fold into L-broth and growing for 90 minutes at 37°C (cell density approx 3 X 10⁸/ml). The cells were then harvested by centrifugation, washed with half volume of ice-cold 0.1M MgCl₂, resuspended in 1/20th volume of ice-cold 0.1M CaCl₂ and kept on ice. Competence was not found to vary much between one and two hours after this point but cells were usually used at 90 minutes after resuspension in calcium.

DNA was added to 0.2ml of competent cells and left on ice for 30 minutes. The cells were then given a heat-shock (about two minutes at about 55°C - not critical) and left on ice for a further 30 minutes.
when they were then plated for infective centres on BBL agar.

Transfection efficiency varied but was usually within the range $0.2-1 \times 10^6$ pfu/µg intact DNA. Recoveries from ligation reactions were usually of the order of $4-10 \times 10^3$ pfu/µg.

**g) AGAROSE GEL ELECTROPHORESIS:**

Restriction endonuclease digests in 5% poly-ethylene glycol (6000) were analyzed by electrophoresis in 1% agarose. The buffer used contained: 40mM tris.HCl pH8.2; 20mM Sodium acetate; 1mM EDTA.

Electrophoresis was at 1volt/cm for 16 hours. The DNA bands were visualized by staining in a solution of 2 mg/litre ethidium bromide for 30 minutes followed by de-staining for 30 minutes in water. The gels were then photographed (using an X4 red filter) under ultra-violet light using Ilford FP4 film.
a) THE INFECTION EXPERIMENT:

The host bacteria were grown aerobically overnight in L-broth supplemented with 40μg/ml thymine for thyA hosts. The cultures were diluted 20-fold into fresh medium and grown for about 90 minutes to mid-logarithmic phase (3 X 10^8 cells/ml). The generation time for all cultures was always checked and slow growing cultures were discarded. Doubling times were typically 30-40 minutes depending on the bacterial strain.

The cells were then pelleted by centrifugation at 15,000g for five minutes at 4°C, washed with an equal volume of ice-cold 1mM MgSO_4 + 20μg/ml 5-fluorouracil (5FU) and finally resuspended in 1/10th volume of the same Mg/5FU medium.

The bacteria were infected with phage at a multiplicity of two (with the exception of infections in which tryptophan synthetase was to be assayed in which case the multiplicity was five). After 15 minutes at 32°C for adsorption the cells were pelleted and resuspended in pre-warmed Vogel and Bonner nutrient medium at the original cell density. Point of resuspension was taken as zero time.

Infected cells were grown at 37°C with vigorous aeration. At suitable times after infection, samples were removed from the culture flasks, brought to 10mM sodium azide and kept on ice until all of the samples could be processed. Sample size was 10ml where anthranilate synthetase was to be measured and 50ml where tryptophan synthetase was being assayed.

The samples were pelleted at 4°C and washed in an equal volume of ice-cold 1X Vogel & Bonner salts and resuspended in 0.8ml of
sonication buffer (50mM tris-HCl pH7.8, 1mM EDTA, and 1mM reduced glutathione). The cells were then sonicated for two bursts of ten seconds using an MSE 100-watt ultrasonicator and cell debris removed by centrifugation at 15,000g for ten minutes.

The cell extracts were assayed for enzyme activity and protein content. Activity is expressed in Enzyme Units/mg protein. In some of the experiments the samples, before resuspending in sonication buffer, were quick frozen in liquid nitrogen and stored at -70°C for up to a week before processing. This caused no loss of activity – see Chapter 5.

The infection hosts used were: ED8614 (trpBE9 thyA trpR) and its lysogenic derivative C24.

b) ANTHRANILATE SYNTHETASE ASSAY:

Anthranilate synthetase was assayed by measuring the rate of conversion of chorismate to anthranilate. The anthranilate was estimated by its fluorescence at 386nm with an excitation wavelength of 313nm.

The enzyme sample (50μl) was added to 1.5ml assay mix (containing 0.034mg chorismate in 10mM potassium phosphate pH7.6, 1mM beta-mercaptoethanol, 10mM glutamine and 4mM magnesium sulphate) which had been prewarmed to 37°C. After mixing by inversion the rate of increase of fluorescence was measured using a Locarte spectrofluorimeter with a water-heated cuvette holder equilibriated to 37°C.

1 unit of enzyme activity is defined as that required to produce 0.1 μmoles anthranilate / 20 minutes at 37°C

Enzyme specific activity is expressed as units/mg cell protein
c) TRYPOTOPIAN SYNTHETASE ASSAY:

Enzyme activity was determined by the rate of conversion of indole to tryptophan.

The reaction mix consisted of 6.3mg DL-serine; 10μg pyridoxal phosphate; 0.1M tris.HCl, pH7.8; 0.03ml saturated Sodium Chloride; 0.4μmole indole in 0.9ml. A 50μl enzyme sample was added and incubated at 37°C for up to 2 hours. The reaction was stopped by the addition of 0.1ml 0.1M Sodium hydroxide.

The indole remaining in the sample was then estimated by extraction with 2ml of toluene. 0.5ml of the toluene-extract was then mixed with 2ml ethanol and 1ml indole-reagent (containing 9gms p-dimethyl-amino-benzaldehyde; 45ml concentrated HCl made up to 250ml with ethanol). After 20 minutes the optical density at 540nm was read against a no-indole blank.

1 Unit of enzyme activity is defined as that required to remove 0.1μmole indole/20 minutes at 37°C.

d) PROTEIN DETERMINATION:

50μl samples from the sonicates, prepared as in (a) above, were diluted to 1ml with water and mixed with 5 ml of Folin's reagent (1 part 1% copper sulphate; 1 part 2% sodium, potassium tartrate; and 100 parts 2% sodium carbonate + 0.1M sodium hydroxide). After ten minutes at room temperature, 0.5ml of Folin & Ciocalteu's phenol reagent (diluted 1:1 with water) was added with vigorous mixing. The absorbance at 650nm was read after 20-30 minutes against a "no-protein" blank. A calibration curve was always determined using Bovine Serum Albumin.
e) DETERMINATION OF STEADY-STATE LEVEL OF _lacZ_ EXPRESSION:

An overnight culture of the bacteria was prepared using minimal growth medium. This was then diluted as appropriate into the same medium and grown at 37°C in a shaking water bath. When the turbidity at 600nm reached 0.1-0.2, two 1ml samples were withdrawn. The turbidity (at 600nm) was measured for one. To the second was added one drop of 0.1% sodium dodecylsulphate and three drops of chloroform followed by vigorous whirlimixing for ten seconds and storage on ice. Typically, a further five samples were withdrawn at intervals until the turbidity reached about 0.8. The enzyme samples were assayed as described below.

After plotting enzyme activity against turbidity, the differential rate of enzyme synthesis was determined from the gradient, and expressed as enzyme units/mg protein by using the conversion factor - 1 O.D. unit at 600nm = 220μg protein/ml.

f) beta-GALACTOSIDASE ASSAY:

The sample to be assayed was diluted to 1ml with 1X Vogel & Bonner salts. After five minutes at 37°C, 0.2ml of pre-warmed o-nitrophenyl-β-D-galactoside (4mg/ml in 0.1M phosphate buffer pH7) was added to start the reaction. When sufficient yellow colour had developed the reaction was terminated by the addition of 0.5ml of 1M Na₂CO₃. Usually the samples were clarified by centrifugation and the absorbance (A) was then read at 420nm. The turbidity of the sample at 550nm was usually checked.

1 Enzyme Unit = 241 X (A₄₂₀ - 1.75xA₅₅₀) / (Assay time in minutes) / nanomoles o-nitrophenol/min/ml.
CHAPTER 5

CONVERGENT TRANSCRIPTION
The objective of the series of experiments to be described was to examine the consequences of the $P_L$ promoter of lambda actively transcribing in opposition to the trp promoter. As described in Chapter 3 the pilot experiment (Hopkins et al., 1976) had shown that under such circumstances, expression of the trp genes from their promoter was turned off as a result of expression from the lambda promoter. The main criticism of this experiment was that it was not conclusively shown that this was due to transcriptional interference and it is therefore possible that the inhibition could have been the result of translational interference or a general interference with host gene expression such as the Hin function (see Chapter 3).

The following experiments were designed to prove, or disprove, that the inhibition was due to interference by RNA polymerases initiating at $P_L$ in opposition to the trp promoter, and to try to establish what factors influence this inhibition.

a) The experimental system:

The in-vitro generated $\lambda$trp transducing phages (Hopkins et al., 1976) provide a system in which the orientation of the trp promoter can be altered. In this way a pair of otherwise isogenic phage strains can be constructed in one of which the trp promoter will be opposing $P_L$ transcription. An additional reason for the use of this system is that a great deal is known about the regulation of the trp operon; furthermore, the enzyme assays for anthranilate synthetase and tryptophan synthetase are comparatively easy and sensitive.

The structure of these phages is illustrated in fig 5.1. In both cases the trp DNA was inserted into lambda using R.HindIII. The site
Fig 5.1: Directions of transcription in λtrp phages
of insertion into the phage is at site 3 which is located slightly to the left of the phage attachment site. The E.coli DNA is about 9kb long.

In the 'r'-orientation the trp genes are inserted into lambda such that transcription from the trp promoter is from the phage 'r'-strand and is directed rightwards in opposition to leftward transcription from P_L. It was in this situation that trp expression was found to be turned-off by P_L transcription.

In the 'l'-orientation, the trp genes are inserted such that transcription from the trp promoter is in the same orientation as leftward transcription from P_L. Therefore transcription from the two promoters is no longer in opposition.

Under normal circumstances, the cro product turns-off P_L transcription within about five minutes after infection. This turn-off was prevented by the use of a mutation in the cro gene.

The hosts used for these phages were ED8614 (trpER9 thyA trpR) and a λcInd* lysogenic derivative, strain C24. The deletion trpER9 removes all of the trp operon except the promoter and trpA. There is therefore no expression of anthranilate synthetase from the host chromosome. Consequently the level of this enzyme after infection reflects expression from the phage. The thyA mutation is required so that replication of the phage can be blocked by 5-fluorouracil in the absence of thymine. This block is necessary to eliminate any trivial effects due to phage replication. The trpR mutation is required experimentally for reproducibility. Its function is to genetically derepress expression from the Lrp promoter. The alternative of using a trpR* host in the presence of low concentrations of tryptophan proves to be very unsatisfactory in practice. The reason for this is that the efficiency of termination at the trp attenuator is very
variable in the presence of low levels of tryptophan. This results in the power of the trp promoter varying from experiment to experiment. By using high concentrations of tryptophan, termination at the attenuator is maximal and constant thereby enabling reproducibility to be achieved. High tryptophan concentrations necessitate the use of a trpR+ host in order to permit expression of the operon.

The lysogenic derivative of ED8614 was made by transduction using P1.kc grown on strain 803(λpLind-). Selection was for transductants immune to lambda as described for the construction of lysogens (Methods & Materials). The reason for using a pLind- lysogen was that thymine starvation results in the induction of lambda prophages. However this does not occur when the prophage carries the pLind- allele.

The protocol of the Infection-experiment is described in the Methods. Essentially it consisted of infecting the host with the trp phage and then diluting into glucose-casamino acids minimal medium. Samples were removed over the next 30 minutes and after sonicating were assayed for either anthranilate synthetase or tryptophan synthetase.

b) Liquid Nitrogen Freezing

In most of the later infection experiments the samples for enzyme assay were not assayed on the same day as the infection was performed. After washing each sample, and before resuspending in sonication buffer, the cell pellet was quick-frozen in liquid nitrogen and stored at -70°C for as long as a week. The following experiment showed that such treatment had no effect on the enzyme activity subsequently found when the samples were thawed, sonicated and assayed.
Fig 5.2: Effect of Freezing

- *x* = Samples Frozen
- *o* = Samples Not Frozen
Host ED8614 (trpB trpR thyA) was infected with λtrpEDC+ oro (NEM69) as described in the Methods. Two samples were removed at each time-point up to 30 minutes post infection. One set of samples was processed immediately, i.e. was sonicated and assayed. The second set was washed and pelleted but was not resuspended in sonication buffer. Instead the centrifuge tubes containing the cell pellets were plunged into liquid nitrogen. The samples were then stored at -70°C. Five days later the samples were thawed-out on ice and sonication buffer added. After sonication and clarification the samples were assayed for anthranilate synthetase. Fig 5.2 shows the resultant activities obtained for the two sets of samples. This graph shows no detrimental effect of freezing on the enzyme activity.
Fig 5.3: Convergent Transcription with cro- phage
SECTION II - EFFECT OF PL ON trp EXPRESSION

a) Effect of Orientation on Inhibition

Three hypotheses can be proposed to explain the inhibition of trp gene expression. The most obvious is a transcriptional mechanism which requires that the inhibition will only be observed when the trp genes are being read in a direction directly opposing transcription initiated at the lambda PL promoter. A variant of this would be a translational interference in which the inhibition might be due to the production of double-stranded RNA which would be non-translatable. The third mechanism would be the production by lambda of an inhibitor-function. In this case the inhibition will be observed irrespective of the orientation of the trp genes. To distinguish between these mechanisms the effect of orientation on the inhibition of anthranilate synthetase synthesis was determined.

Anthranilate synthetase synthesis was measured following infection with λtrpEDC" oro⁻ (NEM669) which carries the trp genes in the 'r'-orientation (see Fig 5.1). This was repeated with λtrpEDC¹ oro⁻ (NEM692), which has the trp genes in the 'l'-orientation.

The hosts were ED8614 and its lysogenic derivative C24. Infection of the non-lysogenic host by λtrp" showed (Fig 5.3) that anthranilate synthetase was synthesized for about five minutes and then no further enzyme was produced. However, infection of the lysogenic host, when transcription from PL is repressed, resulted in an approximately constant rate of enzyme synthesis. This is a measure of the power of the trp promoter.

With λtrp¹, anthranilate synthetase was synthesized at the same constant rate in both the lysogenic and non-lysogenic hosts. Thus no inhibition was observed in the expression of the trp genes when they
were inserted into lambda in the 'l'-orientation.

This result is, in fact, surprising since in this orientation it would be expected that \( P_L \) transcription would be augmenting transcription initiated at the \( trp \) promoter. Consequently it would be expected that in the non-lysogenic host, where \( P_L \) is active, the rate of anthranilate synthetase synthesis would be higher than in the lysogenic host where \( P_L \) is not active. This clearly does not happen and the explanation appears to be the presence of a transcriptional stop-signal upstream of the \( trp \) promoter. The presence of such a site has been shown previously (Franklin, 1974; Hopkins et al, 1976).

This experiment was repeated (data not presented) with identical results. In this case the two phages showed the same rate of synthesis of anthranilate synthetase in the lysogenic host.

As no inhibition of \( trp \) expression was found with \( \lambda trp^1 \), it was concluded that expression from \( P_L \) (or \( P_R \)) does not lead to the production of an inhibitor which blocks expression of the \( trp \) operon.

Since inhibition was only observed when the \( trp \) promoter and \( P_L \) were actively transcribing in opposing directions, the most probable explanation of the inhibition would be a transcriptional interference resulting from 'convergent-transcription'.

Transcriptional interference is presumably due to the cessation of transcription on collision of two RNA polymerases moving in opposing directions. If no mechanism exists to remove one, or both, of the polymerases from the DNA then the complex will remain frozen. This might also occur if RNA polymerase cannot be removed from the DNA before the next polymerase hits the complex. In these cases the inhibition of \( trp \) expression would be irreversible once established. However, if the release of polymerase is rapid then the inhibition should be reversible when \( P_L \) expression is turned-off.

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Fig 5.4: Convergent Transcription with cro+ phage
The preceding results, however, do not exclude a translational mechanism of interference. This might be mediated by the production of double stranded RNA. There is no precedent for this.

b) Reversibility of Inhibition

In order to examine the mechanisms suggested in the preceding section, the reversibility of the inhibition was determined by turning-off $P_L$ transcription and then measuring how rapidly $trp$ expression was restored. The previous experiment was repeated using $\lambda_{trp}$ phages carrying the $cro^{+}$ allele. (NEM691 = 1-orientation; NEM870 = r-orientation) Under these circumstances, when expression from $P_L$ and $P_R$ is allowed, synthesis of the $cro$-product will result in the turn-off of $P_L$ expression after 5-8 minutes (Franklin, 1971).

The results (Fig 5.4) confirm that with the $trp$ genes in the '1'-orientation there is no inhibition of the expression of anthranilate synthetase. When the $trp$ genes are inserted in the phage in the 'r-orientation', the rate of synthesis of anthranilate synthetase in the lysogenic state is linear with respect to time. However, infection of the non-lysogenic host with the $\lambda_{trp}$ phage showed a rise in enzyme activity up to five minutes post-infection, a plateau to ten minutes followed by an increase in anthranilate synthetase activity at a rate comparable to that in the lysogenic host.

This result demonstrates that the inhibition of $trp$ expression is reversed extremely rapidly. It further implies that continuous transcription in opposition is required in order to maintain the inhibition.
Fig 5.5: Fine Time course with cro⁺ Phage
c) Kinetics

In the preceding experiment, samples for enzyme assay were only taken at five minute intervals. Consequently it is not possible to deduce at what time the reversal of inhibition occurs. Therefore the infection with the \( \lambda_{trp} \) phage (NEM870) was repeated but in this case samples for enzyme assay were taken at one minute intervals over the first 15 minutes after infection.

As can be seen (Fig 5.5), infection of the non-lysogenic host resulted in the synthesis of anthranilate synthetase during the first three minutes of infection. After this time the rate of synthesis was drastically reduced. 5-6 minutes later the inhibition was rapidly alleviated and the rate of synthesis of anthranilate synthetase returned to that characteristic of the un-hindered \( trp \) promoter as depicted by infection of the lysogenic host. Since the \( cro \) gene product turns \( P_L \) off at about 5-8 minutes after infection this indicates that enzyme synthesis recommences almost as soon as opposing transcription ceases.

The preceding results have shown that anthranilate synthetase synthesis is only inhibited when the \( trp \) operon is being transcribed in opposition to \( P_L \). This suggests that the interference is the result of transcription from \( P_L \) and is due to collision between RNA polymerase molecules travelling in opposing directions. The rapidity of the reversal of this inhibition indicates that continued transcription from \( P_L \) is required to maintain this inhibition of enzyme synthesis and that whatever mechanism exists to release the inhibitory state must act very quickly.

For these proposals to be valid the times of onset and relief of the inhibition must be compatible with the calculable transcription times for the system. In other words, the time of onset of inhibition
should be compatible with the time taken for RNA polymerases initiating at \( P_L \) to reach the \( trp \) operon. Similarly, relief of inhibition should not occur until the \( cpo \) product has turned-off \( P_L \) and the last RNA polymerase has proceeded past the \( trp \) operon.

From the physical structure of the \( \lambda trp \) phages used, the distance between the end of the \( trpD \) gene and \( P_L \) is about 9.3 kb. Estimates of the rate of nucleotide polymerization by RNA polymerase vary quite significantly but a figure of 45 nucleotides/sec (Manor et al, 1969; Rose et al, 1970) seems reasonable. It should be stressed that it is not known if \( pN \)-modified RNA polymerase transcribes at the same rate and hence this figure may be in considerable error. Nevertheless it does provide a working figure. With this assumption, transcription from \( P_L \) will take 3.5 minutes to reach the end of the \( trpD \) gene and the proposed inhibition of \( trp \) mRNA synthesis should then occur. The time taken for RNA polymerase initiating at the \( trp \) promoter to transcribe the \( trpED \) genes will be about 70 seconds and enzyme should start to appear slightly after this. Therefore no enzyme synthesis should occur before about one and a half minutes after infection. In general, enzyme synthesis does appear to occur before this point suggesting that the true zero time of infection occurs at about one minute before resuspension in growth medium.

Fig 5.5 indicates that the inhibition starts at about 3 minutes and is complete by about 4 minutes after resuspension. Therefore the inhibition of anthranilate synthetase synthesis must occur at about 4-5 minutes after infection.

Transcription from \( P_L \) will reach the \( trp \) genes after 3-4 minutes. At this point it is proposed that synthesis of anthranilate synthetase mRNA will cease. From this time mRNA molecules that have already transcribed the \( trpED \) genes will continue to direct the synthesis of
anthranilate synthetase until they are degraded. Consequently the
turn-off of enzyme synthesis would be expected to lag behind the
turn-off of mRNA synthesis. Therefore transcription from \( P_L \) will
reach the \( trp \) genes after 3-4 minutes at a time that is compatible
with the onset of the inhibition of anthranilate synthetase synthesis
which occurs after 4-5 minutes.

It is proposed that when the last RNA polymerase initiated at \( P_L \)
has passed through the \( trp \) operon, inhibition of anthranilate
synthetase expression will cease and enzyme synthesis will resume.
Calculation of the time at which this would be expected to occur is
difficult since nobody has measured the time at which \( cro \) turns-off \( P_L \)
transcription. In all cases, enzyme synthesis from a gene in the
\( \lambda \)-operon has been used as a monitor of \( P_L \) transcription thereby making
it necessary to allow for the distance between this gene and \( P_L \). Much
more serious is the observation (Franklin, 1971) that the time of \( cro \)
turn-off is extremely dependent on the multiplicity of infection. At
a multiplicity of two, turn-off of anthranilate synthetase synthesis
from \( \lambda trp \delta \) occurred at about 8-10 minutes post-infection. Since it
will take about 2-3 minutes to transcribe from \( P_L \) to the \( trp \) genes
this suggests \( cro \) turn-off occurs before about 5-7 minutes of
infection. In view of the enhanced stability of \( P_L \) initiated
transcripts it is possible that \( cro \) turn-off of transcription could be
a couple of minutes before this.

Thus \( P_L \) transcription will stop within about five minutes after
infection. The distance from \( P_L \) through the \( trp \) operon is about
12.6kb which corresponds to a transcription time of slightly less than
5 minutes. Therefore the last \( P_L \) initiated RNA polymerase should have
completed the transcription of the \( trp \) operon by ten minutes after
infection. Anthranilate synthetase should then appear about one
minute later. Fig 5.5 shows that the restoration of anthranilate synthetase synthesis is more gradual than the onset of inhibition. However, by ten minutes after resuspension, corresponding to 11 minutes post infection, significant enzyme synthesis has begun. This is compatible with the calculated time of relief of the inhibition. Although this crude analysis makes many assumptions it does show that the observed effect of P_L transcription on trp expression can be explained by a simple model of transcriptional interference.

d) **Variation of the Powers of the Promoters**

The preceding results showed that the inhibition of expression of the trp genes was only observed when both the trp promoter and P_L were actively transcribing in opposing directions. These two promoters are functionally quite distinct. There are two important features about the lambda P_L promoter. Firstly, it is a very powerful promoter and secondly, transcription initiated here is modified by the N gene product. Although the trp promoter is also quite powerful, the presence of the trp attenuator (see Chapter 2) reduces its power by approximately 5-8 fold (Bertrand et al, 1975). Thus P_L is effectively ten times more powerful than the trp promoter system (Davison et al, 1974). It is therefore plausible that the inhibition of trp expression could be the simple consequence of the overwhelming power of P_L. If this is so, then alteration of the relative powers of the two promoters should affect the inhibition.

An alternative explanation would be that this inhibition is not due simply to convergent transcription but that it is a specific effect of RNA polymerase initiating at P_L under the influence of the N-gene product. Although the mechanism of action of pN (see Chapter 1) is still not fully resolved it is clearly apparent that pN affects the
host cell machinery such that termination of transcription does not occur. There is also evidence that pN binds to RNA polymerase. Transcription termination seems to be characterized by a kinetic pause in transcription (e.g. Rosenberg et al., 1978). Presumably collision of two polymerases would result in a pause in transcription. This situation might then be resolved by the termination of transcription, in one or both directions, possibly mediated by the rho factor. With pN-modified RNA polymerase such termination might not occur and hence the trp promoter transcript would preferentially be terminated and released leaving the P_L polymerase to continue transcribing leftwards. If this is the case then even if P_L is made much weaker than the trp promoter the inhibition of anthranilate synthetase synthesis would still be observed.

To try to answer these questions the relative powers of the two promoters were altered:

Alteration of the power of the trp promoter:

As indicated above, the power of the trp promoter is significantly reduced by the action of the attenuator. From the different rates of anthranilate synthetase synthesis from λtrp phages in which the trpE and trpD genes are either transcribed from the trp or P_L promoters, the difference in powers of these two promoters is almost exactly 10-fold in favour of P_L (Davison et al., 1974). Deletions, however, have been isolated which remove the attenuator whilst not affecting the trp promoter itself. The result of this is to effectively increase the power of the trp promoter by a factor of about 8-fold thereby making its strength more comparable to that of P_L. One such deletion is trpL102 (Jackson & Yanofsky, 1973) which deletes from within the trp leader region, through the trpE gene and removes most
Fig 5.6: Structure of trp-deletion phages
of the trpD gene (Jackson & Yanofsky, 1972b). In the absence of the genes coding for anthranilate synthetase it is necessary to assay tryptophan synthetase (the product of the trpA and trpE genes).

In order to eliminate any trivial effects, the trpED24 deletion (Jackson & Yanofsky, 1972b) was also used in parallel. This deletion is very similar to trpLD102 in that it deletes the trpE gene and much of trpD, but it leaves the trp attenuator intact. Comparison of enzyme production from these two phages when PL transcription is active therefore serves to establish whether raising the power of the trp promoter has any effect on the observed inhibition.

Phage carrying these trp deletions were constructed using strain NEM773 which is an integration-defective lambda transducing phage carrying the trpBE9 deletion (or to be more accurate, carrying the DNA on either side of this deletion) in the 'r'-orientation. As in the previous cases the trp operon was inserted at R.HindIII site 3, just to the left of attL. The trpBE9 deletion was replaced with the shorter deletions by lysogenizing either strain trpED21 or trpLD102. Since phage NEM773 is integration-defective, it will insert into the host chromosome using the homology at trp via Rec-mediated recombination. Ultraviolet-induction of these lysogens should give two possibilities - either the original phage carrying the trpBE9 deletion or alternatively a phage carrying the shorter trp deletions (trpED24 or trpLD102, respectively). These should occur at approximately equal frequency and can be distinguished by virtue of the ED24 and LD102 derivative phages being TrpC+. The LD102 derivative is phage D34.

Phage able to complement a trpC/C were isolated and after purification were also shown to complement trpA88 but neither the trpD nor trpE strains. These phage, which have the immunity of phage 21, were converted to λ pco+ by crossing to NEM897 (λ80 cIAt2 pco1) and

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Fig 5.7: Effect of varying power of trp promoter
selecting for $\lambda^+$ $\lambda^-$ recombinants on C600($\lambda$imm$^{21}$). The resultant strains are D36 and D37, carrying the ED24 and LD102 deletions respectively. The structure of the trp genes carried on these phages are shown in Fig 5.6.

As before, cro- derivatives were used to maintain the opposing transcription and to prevent reversal of the inhibition.

These phage include a deletion extending rightwards from att into xis. This deletion was necessary to reduce the size of the genome so that it could be packaged within the phage head and should have no effect on transcription from $P_L$ with the exception that RNA polymerase will now reach the trp genes slightly earlier than before. The use of the phage carrying the trpED24 deletion controlled against any effects this might have.

Because of the greatly reduced sensitivity of the tryptophan synthetase assay the infection procedure was modified. The multiplicity of infection was increased from two to five and larger samples were taken. It was found that samples removed at zero time of infection appeared to have enzymatic activity. Since the host (ED8614) is deleted for trpE, this activity cannot be genuine. This was confirmed by the finding that uninfected cells have an apparent level of enzyme activity equal to that of the zero time points (data not shown). It is thought likely that this activity is due to other enzymes such as tryptophanase.

Despite the lower sensitivity, hence lower accuracy, of the tryptophan synthetase assay the results of Fig 5.7 clearly show that the increased power of the trp promoter, without attenuator, does not prevent the inhibition of enzyme expression when $P_L$ is active.

Comparison of the two phages in the lysogenic host where only the trp promoter was active indicated that with the trpLD102 deletion the
rate of expression of tryptophan synthetase was about five times greater than in the case of \textit{trpED24}. This is compatible with the accepted value for the effect of the \textit{trp} attenuator (Bertrand \textit{et al}, 1976).

Alteration of the power of the \(P_L\) promoter:

Since raising the power of the \textit{trp} promoter did not abolish the inhibition due to convergent transcription, it was decided to reduce the power of \(P_L\). It was initially thought that it might be possible to use the down promoter mutation \textit{sex}". (This mutation was isolated by Max Gottesman by its inability to express the excision functions. Roberts (1969) showed it to be defective in leftward transcription in-vitro). However, the results of Franklin (1971) indicated that combination with a \textit{orc}" mutation restored \(P_L\) expression to normal. This was confirmed (personal observations, data not presented). The reason for the conflict between the in-vitro results, which show a ten-fold lower transcription from \(P_L\), and those in-vivo, which show normal expression from \(P_L\) in the absence of \textit{orc}, is not known.

An alternative approach to reducing transcription from \(P_L\) was tried. In the absence of \textit{pN}, transcription from \(P_L\) cannot proceed past the \(t_L\) terminator sites. However, if limited amounts of \textit{pN} are supplied then only a portion of RNA polymerases initiating at \(P_L\) will become \textit{pN}-modified. Only the fraction that are so modified will be able to pass through the leftward terminators and proceed towards the \textit{trp} operon. Thus by restricting the availability of \textit{pN} the power of the \(P_L\) promoter can be attenuated. However, all polymerases that do proceed past \(t_L\) will be \textit{pN}-modified. Therefore if the inhibition of enzyme expression is due to the action of \textit{pN}-modified RNA transcription, a turn-off in the expression of \textit{trp} will still be
observed.

As judged by the low level of N-operon transcription, strains carrying an N<sup>ts</sup> mutation are grossly deficient in N function at the non-permissive temperature (Franklin, 1974). Accordingly it was decided to use such a mutation at the restrictive temperature in combination with cro<sup>-</sup>. The reason for the use of cro<sup>-</sup> was, as before, to prolong leftward transcription.

The N<sup>ts</sup><sub>8</sub> cro<sup>-</sup> recombinant was constructed in two steps. Firstly, N<sup>ts</sup><sub>8</sub> was crossed with h<sup>80</sup> NN<sup>-</sup> imm<sup>434</sup> (NEM744) selecting h<sup>+</sup> imm<sup>434</sup> recombinants on a suppressor-free lambda-lysogenic strain (C24) at 32°C. These were then tested and verified to be N<sup>ts</sup> imm<sup>434</sup>. This phage was then crossed with NN<sup>-</sup> fed<sup>+</sup> (NF62) selecting against N<sup>-</sup> phage on W3350 (suppressor-free) at 37°C. After screening for phage with lambda immunity, and hence able to form plaques on C600(λimm<sup>434</sup>), one plaque was obtained with the characteristics expected of an N<sup>ts</sup> cro<sup>-</sup> recombinant. This is strain D63.

The presence of the N<sup>ts</sup> mutation was confirmed by back-crossing to NEM744 (NN<sup>-</sup> imm<sup>434</sup>) selecting on 594(λ) - suppressor-free - at 32°C. All recombinants were found to be temperature sensitive for growth confirming that the original strain still contained the N<sup>ts</sup> allele.

Strain D63 (N<sup>ts</sup> cro<sup>-</sup>) was crossed with h<sup>-80</sup> trp<sub>46</sub> imm<sup>21</sup> (D47) selecting on strain N301, λ<sup>R</sup> (λimm<sup>21</sup>), for h<sup>-80</sup> imm<sup>+</sup> recombinants. In the resultant strain, h<sup>-80</sup> trp<sub>46</sub> N<sup>ts</sup> cro<sup>-</sup>, the trp genes are expressed only from P<sub>L</sub> and hence serve to monitor the strength of leftward transcription. Infection of host ED8614 at 37°C showed that leftward transcription was not turned off, confirming the presence of the cro<sup>-</sup> mutation. However, the rate of expression was identical to that observed for h<sup>-80</sup> trp<sub>46</sub> N<sup>+</sup> cro<sup>-</sup> (strain NEM777). At 30°C, N<sup>ts</sup><sub>8</sub> cro<sup>+</sup> was shown to give only 10% the normal level of leftward
transcription (Franklin, 1974). At higher temperatures, P\textsubscript{L} transcription was reduced even further. This finding had been taken to infer that pN was not overproduced and hence limited supply of pN would result in limited leftward transcription. The observation that N\textsuperscript{ts} or\textsuperscript{o} gives the same level of leftward transcription as N\textsuperscript{+} or\textsuperscript{o} would suggest that pN is overproduced but that this only becomes apparent under conditions where or\textsuperscript{o} turn-off does not occur.
The results of the preceding sections have established that when the trp promoter is transcribing in opposition to PL, the result is the turn-off of trp expression. Raising the power of the trp promoter did not affect this inhibition. However, these results do not answer the reverse question, namely, "does trp transcription interfere with PL transcription?" One method of answering this question would be to construct a phage with trp transcription opposing PL but in addition carrying a second set of genes oriented and positioned in such a manner that their sole means of transcription is from the PL promoter located beyond the opposing trp operon. With such a phage, assaying this second gene product, in addition to anthranilate synthetase, allows the simultaneous monitoring of leftward and rightward transcription and would allow the effect of the trp promoter on PL transcription to be determined.

The obvious candidate for this second gene is lacZ. There are two major reasons for this choice. Firstly, the enzyme assay is simple and sensitive. It can also be performed with enzyme samples prepared as for anthranilate synthetase thereby simplifying the experimental procedure. The second reason is that lambda transducing phages are available with the lacZ gene positioned as required. These are based on λlacS (Ippen et al, 1971) which carries the lacZ gene within the phage b2 region, i.e. to the left of the trp genes inserted at R.HindIII site 3, and in the 1-orientation. The lac promoter is present on this phage and was removed by the use of a derivative which contains a trp-lac fusion (W205 - Mitchell et al, 1975) in which the lac promoter was deleted during the fusion event between lacZ and trpA.
Fig 5.8: Construction of lacZ-trp Fusion Phages
a) Construction of lac-trp Fusions

The construction of trp-lac fusions in which the lacZ gene was expressed from the trp promoter has been described (Chapter 2, section III). In this present study, however, it is necessary to construct a lac-trp? hybrid phage in which the trp genes are in the reverse orientation, i.e. transcription from the trp promoter is oriented away from lacZ.

trp-lac fusion W205 is deleted for all transcription termination sites between the lac and trp genes. This fusion had been transferred to a lambda phage (Barnes et al, 1974) in an orientation such that both the lac and trp genes were expressible from P_L. The location of the lac gene is within the b2-region just to the right of the tail gene f. There is a R.HindIII target within the trpB gene (Hopkins et al, 1976) and hence restriction of such a phage with R.HindIII yields a fragment containing the lambda head and tail genes, lacZ fused onto the operator-distal end of the trp operon, the whole of trpA and most of trpB. This fragment therefore provides the lacZ gene suitably positioned for monitoring leftward transcription from P_L. The trp operon, to provide rightward opposing transcription and the trpE and trpD genes for monitoring this transcription, were added by the addition of a HindIII fragment containing these genes. Finally, a right arm, also generated by R.HindIII, would complete the construction of a plaque-forming phage and provide the P_L promoter.

The construction of this recombinant is outlined in Fig 5.8. The two donor phages used were D314 and D144. The construction of D144 will be described in Chapter 6 (see Fig 6.1). This phage supplies the phage left arm carrying the lacZ gene and also the right arm of the phage. The construction of D314 was described earlier (see section IId) and was used since it carries the trpL102 deletion. The reason
Fig 5.9: Transcription Directions in lacZ-trp^ fusion
for this was so that a single HindIII fragment would carry the promoter and trpC gene. The use of a trpEDC phage would have precluded this by virtue of the presence of a R.HindIII target in trpl. This would inactivate anthranilate synthetase activity and thereby prevent the normal assay for rightward transcription.

Essentially the rationale of the construction of this lacZ-trp fusion strain (D57) was to use the lac-polA hybrid phage D44 as a replacement vector - replacing the polA fragment with the trpC fragment from phage D34.

Consideration of the sizes of the derivatives that would be made from this lac-trp phage necessitated that it must also carry the att-red deletion. This deletion is present in D44.

After restriction and ligation, the recombinants were recovered by transfection. The required phage has the following characteristics: Lac+ TrpC+ Red-. Out of 100 recombinants tested, 95% were Lac+. Only 8 were also TrpC+ and of these half were Red-. The orientation of the trp genes in four of these phages was tested, but in this case phi80pt6A (trpEDC) was used because of the trpA gene present in the trp-lac fusion left-arm. All three Red- phage tested had the trp genes in the 'r'-orientation. The only Red+ phage tested carried the trp fragment on the 'l'-orientation.

One of the isolated lacZ trpC red- phage was called D57. It was also found to weakly complement trpA88.

The structure of this page is indicated in more detail in Fig 5.9. The HindIII fragment carrying the trpC gene is oriented such that the trn promoter is transcribing in opposition to P_L. The presence of the att-red deletion should not affect the interference of trp expression by P_L except that the inhibition should start slightly earlier than normal (the size of this deletion is about 4kb corresponding to a.
transcription time of about 90 seconds). If \( P_L \) transcription is not totally cut-off by \( trp \) expression then transcription should continue leftwards through the \( trp \) operon, to the \( lacZ \) gene.

A Trp\(^+\) version of the \( trpLD102 \) deletion phage D57 was made so that anthranilate synthetase assays could be used to monitor transcription from the \( trp \) promoter. This was achieved by lysogenizing QR47, and after induction by ultra-violet irradiation, TrpD\(^+\) plaques were selected. Since the phage \( int \) gene is deleted, formation of a lysogen will occur by Rec-mediated recombination. Insertion will therefore occur either in the vicinity of \( trp \) or within \( lacZ \). Ultra-violet induction of lysogens containing the prophage at \( trp \) should give phage that have reconstituted the \( trp \) operon. This phage is stock number D58. The \( trp\)EDC genes were confirmed to be in the 'r'-orientation.

Both the \( lacZ-trpLD102" \) (D57) and \( lacZ-trpEDC' \) (D58) \( \lambda imm^21 \) phages were converted to \( i^+ cro^- \) by crossing to \( h^60 cro^- \) (NEM897). Selection was for \( h^6 i^+ \) recombinants on C600(\( \lambda imm^21 \)). The resultant phages are stock numbers D65 and D66.

b) Effect on \( lacZ \) Expression

The \( lac-trp" \) fusion strains constructed above provide a method of examining the effect of \( trp \) transcription on \( P_L \) expression. Under infection conditions where both the \( trp \) and \( P_L \) promoters are active, assaying anthranilate synthetase provides a monitor of productive \( trp \) transcription whereas beta-galactosidase provides a monitor of leftward transcription from \( P_L \) that has passed through the \( trp \) operon in the anti-sense direction.

The experimental conditions were as before. Hosts ED8614 and C24 (\( trpBE9 \) \( trpR \) thyA and its lysogenic derivative) were infected with D66 (\( \lambda lacZ \) \( trp\)EDC" \( cro1 \)) at a multiplicity of two. Samples were removed
Fig 5.10: beta-Galactosidase Expression in lac-trp Phage

- Ant hranilate Syhtase

Time After Infection (Minutes)

- o Δ non-lysogenic host
- x v lysogenic host
at up to 30 minutes after infection and after sonicating were assayed for anthranilate synthetase and beta-galactosidase as described in the Methods.

The results (Fig 5.10) for trp expression confirm the previous findings. In the lysogenic host, anthranilate synthetase synthesis is linear whereas in the non-lysogenic host, enzyme is synthesized for about five minutes and then no further synthesis occurs. In contrast, beta-galactosidase levels in the lysogenic host were very low demonstrating that there is no effective promoter located in the interval between lacZ and the trp promoter. In the non-lysogenic host, however, no expression occurred for 5-10 minutes after which synthesis was switched-on at a high rate. Since samples were only taken at five minute intervals it is not possible to ascertain at what time synthesis of beta-galactosidase commences but it is nevertheless apparent that its synthesis does not start until after anthranilate synthetase synthesis has been turned-off.

The interval between the lacZ gene and the trp promoter is about 5kb and it was originally thought possible that promoters for other genes might be present and result in the expression of beta-galactosidase. Previous experiments have shown that the closest major gene, cysB, is not carried on the trpE HindIII fragment (Hopkins et al, 1976) but of course this would not preclude the promoter being present. The gene opp (oligopeptide transport- Bachmann et al, 1976) seems to be located close to trp and might be present. Either transcription of this gene does not allow expression of lacZ (because of a termination site or the wrong orientation) or else its rate of expression is low.

At a transcription rate of 45 nucleotides/second (see earlier), it would take about three minutes to transcribe the distance from the
Fig 5.11: Effect of trp Promoter on $P_l$ Expression

![Graph showing the effect of trp Promoter on $P_l$ Expression. The x-axis represents time after infection in minutes, ranging from 5 to 30. The y-axis represents beta-Galactosidase (Units/ml), ranging from 0 to 600. The graph includes data points for different phages and hosts.

- Phage: trpEDC
- Host: (-)
- Phage: trpEDC
- Host: (λ)
- Phage: trpLD102
- Host: (-)
- Phage: trpLD102
- Host: (λ)
trpEDC genes to the end of the lacZ gene. Thus at least a three minute gap would be expected between the switch-off of trp RNA synthesis and the appearance of beta-galactosidase. The results are compatible with this.

In parallel to the above experiment phage D65 (λlacZ trpL102r cro) was also used. This phage is similar to the trpEDC phage but carries the trpL102 attenuator deletion to boost the power of the trp promoter. Since the genes for anthranilate synthetase are deleted, and those for tryptophan synthetase are not present, it was not possible to perform trp enzyme assays to measure the inhibition of trp expression. However, it was possible to compare the rates of beta-galactosidase synthesis to determine whether or not a higher power promoter in opposition to P_L has any effect on read-through transcription of lacZ.

The results (Fig 5.11) clearly demonstrate two features. Firstly, the rate of expression of beta-galactosidase when P_L is opposed by the high power trp promoter, with attenuator deletion, is considerably lower than when the attenuated trp promoter is in opposition. Secondly, the time of onset of beta-galactosidase expression is delayed. Because of the low temporal resolution of this experiment it is not possible to estimate how long this delay is. The delay is made even more apparent when it is considered that the trpL102 deletion is about 3.6kb long (Anilionis, 1977) and therefore in this phage the lacZ gene is 3.6kb closer to P_L. This corresponds to a transcription time of about a minute and hence beta-galactosidase synthesis should have started one minute earlier in this phage than in the non-deleted phage.

These results give rise to two conclusions. Firstly, whilst P_L expression blocks trp operon expression totally, the reverse does not
Fig 5.12: Inhibition of $P_l$ expression

\[ \text{Time After Infection (Minutes)} \]

\[ \text{beta-galactosidase (Units/ml)} \]

- $o = trpEDC$ - attenuator present
- $x = trpLD102$ - attenuator deletion
hold. $P_L$ transcription leftwards is able to proceed through an operon whose promoter is oriented in opposition. Secondly, whilst leftward transcription will pass through the opposing operon, when the power of the opposing promoter is increased, leftward transcription takes longer to "penetrate" the operon. Even when transcription has penetrated, the rate of expression of the $lacZ$ gene is reduced. Thus opposing transcription both reduces and delays leftward transcription from $P_L$.

These results clearly demonstrate that expression from the $trp$ promoter is interfering with $P_L$ transcription.

c) Fine Time Point

In order to establish at what time beta-galactosidase synthesis commences in these two phages (D66 and D65), a modification of the preceding experiment was performed. Only beta-galactosidase assays were required and this simplified the procedure allowing 1ml samples to be taken for assay. These could be permeabilized by chloroform/sodium dodecyl sulphate rather than sonication. Since infection of the lysogenic host gave no enzyme expression, only the non-lysogenic host was used. It was therefore possible to take samples at one minute intervals for infection by both phage.

The results (Fig 5.12) again show the disparity in the rates of synthesis of beta-galactosidase. The start time of synthesis for the $\lambda trpEDC\gamma$ phage appears to be at about 10-12 minutes after infection. The distance from $P_L$ to the end of $lacZ$ is about 16kb which would require a transcription time of 6 minutes. Therefore the opposition of the $trp$ promoter is responsible for a delay of about 4-6 minutes. This delay could be explained by two mechanisms. Firstly, transcription from $P_L$ during the first 4-6 minutes after infection
fails to penetrate the trp operon. This would imply that a head-on collision between RNA polymerases initiating at the trp and P_L promoters results in both polymerases being cleared from the DNA. The alternative would be that, in the face of oncoming transcription, the rate of movement of pN-modified RNA polymerase from P_L is considerably slowed but that transcription does not actually stop. To account for the above delay the transcription rate would have to slow down from 45 nucleotides/second to about 10 nucleotides/second. The initiation frequency of P_L can be estimated from the observed initiation frequency of the trp promoter (2.6 initiations/minute, Baker & Yanofsky, 1972). It is found that P_L is almost 11-times more powerful than the trp promoter (Davison et al, 1974). This would give about 30 initiations/minute for P_L which is equivalent to an initiation occurring every 2 seconds and would correspond to a polymerase spacing on the DNA template of only about 20 base-pairs.

The radius of gyration of RNA polymerase was estimated as 6nm (Pilz et al, 1972) which corresponds to about 18 base pairs. Thus a polymerase spacing of only 20 base-pairs would imply that RNA polymerases moving leftwards are in physical contact with each other. This is highly improbable.

With the higher power trp promoter (attenuator deletion LD102) expression of beta-galactosidase did not commence until about 14-16 minutes after infection. Since unimpeded transcription from P_L should express beta-galactosidase after about 5 minutes (the LD102 deletion brings lacZ closer to P_L), this indicates a delay of about 9-11 minutes. In this case, however, the region of DNA over which convergent transcription will operate is reduced considerably by the LD102 deletion and the transcription rate would have to slow down to 2 nucleotides/second to account for this. This is totally unrealistic.
and it must therefore be suggested that leftward transcription does terminate and $P_L$ initiated RNA polymerases are removed from the DNA template.

It therefore seems much more probable that the mechanism of interference is that both leftward and rightward moving RNA polymerases are cleared from the DNA. Under these circumstances the collision point will move in the direction specified by the more powerful promoter. $P_L$ transcription will therefore penetrate the $\text{trp}$ operon only when the collision point has moved to the $\text{trp}$ promoter.

However, if the $\text{trp}$ promoter remains active then subsequent initiations, albeit infrequent compared to $P_L$, will collide with leftward transcription thereby reducing expression of $\text{lacZ}$. The time delay before leftward transcription can penetrate the $\text{trp}$ operon will depend on the number of collisions that occur before leftward moving RNA polymerases reach the $\text{trp}$ promoter. This will depend on two factors. Firstly, the initiation frequency of the opposing promoter which specifies the polymerase spacing and hence the distance between collisions with leftward moving polymerases. Secondly, the distance over which convergent transcription is occurring. This determines the distance over which $P_L$ transcription must "battle" against oncoming polymerases. Whilst the initiation frequency of the $\text{trp}$ promoter is well known, the second point is problematical. It has clearly been established that $P_L$ transcription can transcribe through the $\text{trp}$ operon, however, it is not known how far $\text{trp}$ transcription will proceed into the lambda genome. The DNA sequence of the region to the left of the lambda $\text{att}$ site shows several interesting features (Landy & Ross, 1977). Translation termination codons occur in all reading frames for rightward transcription thereby making it very unlikely that translation could be occurring. In addition to this there are two
occurrences of a T₆A sequence which is characteristic of some rho-independent transcription termination sites. It can therefore tentatively be suggested that trp initiated transcription does not proceed past the lambda att site.

If so then in the case of the trpEDC phage there is about 5kb of DNA, between the trp promoter and the lambda att site, over which opposing transcription should act. In the case of the attenuator deletion LD102 the distance over which opposing transcription will occur will be only about 1.4kb.

d) Repression of trp Transcription

The preceding results showed that the trp promoter with attenuator caused a five minute delay in the expression of beta-galactosidase from P₁. In the absence of the attenuator, there was a ten minute delay, despite the two promoters being 3.6kb closer together. On the basis of the proposal that this delay is the result of transcription initiated at the trp promoter, then repressing this promoter should abolish this delay. This effect can be achieved by the use of a trpR⁺ host in the presence of high concentrations of tryptophan. Under these conditions the trp promoter will be shut off.

The experiment was performed using the two phages above (D65 and D66, with and without attenuator) and infecting a trpR⁻ host where trp expression will be derepressed, and a trpR⁺ host where very little trp expression will occur. The results (Fig 5.13) for the trpR⁻ host confirm the previous results. The absence of the attenuator results in about an extra four minutes delay and the level of beta-galactosidase expression is reduced. However, the time of onset of enzyme synthesis for both phages occurred two minutes later than in the previous experiments.
Fig 5.13: Effect of trp Promoter Power on Inhibition

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>λtrpEDC</td>
<td>trpR⁺</td>
</tr>
<tr>
<td>λtrpEDC</td>
<td>trpR⁻</td>
</tr>
<tr>
<td>λtrpLD102</td>
<td>trpR⁺</td>
</tr>
<tr>
<td>λtrpLD102</td>
<td>trpR⁻</td>
</tr>
</tbody>
</table>
Infection of the trpR+ host by the trpEDC+ phage (D66) resulted in the commencement of beta-galactosidase synthesis at about 6 minutes after infection. This is exactly the time at which P_L initiated transcription would reach laQZ if it is unimpeded and supports the suggestion that blockage of the opposing trp transcription should prevent the inhibition and delay of leftward transcription. The level of expression was also about 60% higher than in the trpR- host. This suggests that the trp promoter maintains its interference of leftward transcription even after P_L transcription has broken through the trp operon. This might suggest that trp transcription initiation still occurs.

In the case of infection of the trpR+ host with the attenuator-deletion phage (D65) there is clearly a delay of about three minutes before beta-galactosidase starts to appear. There is also, possibly, a slightly lower rate of enzyme expression. At first sight this result looks surprising since the objective of using the trpR+ host was to shut-off trp transcription. However, repression by tryptophan is not complete. In the presence of high tryptophan concentrations the trp promoter is repressed about 70-fold (Jackson & Yanofsky, 1972a). However, in the absence of the trp attenuator, transcription of the trp operon is 8-fold higher than normal. Thus the power of a repressed promoter when combined with an attenuator deletion will be only about 10-fold lower than a derepressed promoter with attenuator (the normal situation). This is still a significant rate of rightward transcription and explains the delay in leftward transcription and also the slight decrease in the rate of beta-galactosidase synthesis. These results will be discussed in the following section.
Several situations in which two promoters were actively transcribing in opposing directions were described in the introduction (Chapter 3). In some cases the consequence of this opposition was an interference with expression. However, in the remaining cases no inhibition was observed which might suggest that simultaneous transcription of both DNA strands could occur. It is important to realize, however, that in the cases where no inhibition occurred, there was no evidence that transcription was actually opposing even though the promoters were directed in opposition. Indeed one of the main problems with gene fusions is that the new junction can result in polarity, caused by translation termination, with the consequent elimination of transcription past the fusion point. It is therefore quite possible that with those fusions that failed to show an inhibitory effect, there was no opposing transcription. In the case of the lambda-trp fusions, transcription from the lambda P_L promoter is modified by the N gene product with the consequence that transcription from this promoter will almost certainly be able to transcribe into the trp operon. In this case, therefore, transcription from P_L will be occurring off the anti-sense strand of the trp DNA that is simultaneously being transcribed from the sense-strand by the trp promoter, i.e. transcription will be occurring in opposition. Under these circumstances an inhibition of trp enzyme expression was observed (Hopkins et al., 1976). However, it is important to realize that there are many mechanisms by which gene expression can be inhibited (see Chapter 3). It was therefore possible that the inhibition observed could have been due to other causes.
The experiments described in this chapter were designed to examine the inhibition observed when the trp and P_L promoters are transcribing in opposition; to establish what factors were responsible and to determine the effect on transcription from P_L.

The experiments described in section II investigated the effect of transcription from P_L on the expression of the trp operon. It was shown (Fig 5.3) that inhibition of anthranilate synthetase (trpE + trpD) expression only occurred when the trp genes were oriented in opposition to P_L. This eliminated the possibility that the interference was due to the production of a diffusible product since this would have caused an inhibition irrespective of the orientation of the trp genes.

Since the interference is only observed when the two promoters are transcribing in opposition, the simplest mechanism to account for the inhibition would be an interference with transcription of the trp operon. However, it is possible that a blockage of translation could be involved. One possible mechanism might be the formation of double-stranded RNA due to the vast excess of anti-sense transcription from P_L. Although feasible it is hard to imagine how this could occur in view of the tight coupling between transcription and translation. This together with the rapid decay of trp mRNA makes it very unlikely that significant amounts of trp-specific mRNA would be available in a form able to hybridize to the anti-sense strand transcript.

The rapidity with which the inhibition of anthranilate synthetase synthesis was reversed (Fig 5.4) indicates that continuous transcription from P_L, in opposition, is required to maintain the interference. The simplest explanation of this would be that RNA polymerases initiating at P_L prevent transcription from the trp promoter - once leftward transcription ceases, as a result of turn-off
by ovo, then the block is lifted and expression of trp recommences. The analysis of the times of onset and relief of inhibition (section IIc) indicate that the kinetics are compatible with such a transcriptional interference model. It should be stressed, however, that several assumptions were made which may not be valid. The transcription rate of pN-modified RNA polymerase may be much higher than the figure assumed. Also the time of ovo turn-off is not known accurately and therefore may be in considerable error. More seriously, the inhibition was monitored via the synthesis of anthranilate synthetase and not by measuring transcription itself. This means that a finite time will elapse after transcription of the genes before enzyme activity appears, and also, enzyme synthesis will continue after transcription has stopped. In this latter case it is not clear how quickly the trp mRNA will decay. Under normal circumstances this would be comparatively fast. However, it could be proposed that, in the face of opposition from P_L, trp transcription continues but is unable to completely transcribe the trpE and trpD genes. The continued production of uncompleted trp mRNA could delay the decay of completed mRNA molecules.

It might be thought that measurement of mRNA synthesis would be a better monitor of the inhibition resulting from convergent transcription. This approach is, however, technically relatively complicated. Although trp-specific mRNA synthesis, can be measured comparatively easily by using hybridization of pulse-labelled RNA to phi80trp DNA, it is insufficient to do so. The reason is the vast excess of anti-sense strand transcription from P_L which would also hybridize. It would therefore be necessary to specifically measure trp sense-strand transcription and this would necessitate the separation of the DNA strands of the phi80trp phage. Although this
was claimed to be possible using the polyUG method (Pannekoek & Pouwels, 1973) the resolution, i.e. separation of one strand from the other, was not good. The sense strand would therefore be heavily contaminated with anti-sense strand DNA which would inevitably lead to a high background and would probably obscure the low level of transcription of the trp operon. However, under conditions in which the DNA strands of lambda separated cleanly, no separation was observed for phi80pt190 (personal observations). A further complication would be that in order to attain sufficient resolution the pulse-labelling would have to be of short duration. This would result in low incorporation and hence large errors in estimating trp mRNA. For these reasons this approach was not pursued.

Nevertheless, the preceding results clearly demonstrated that the inhibition of trp expression was the consequence of the opposing transcription from P_L. However, these results did not show what facet of P_L expression was responsible. The lambda P_L promoter is very powerful (11-fold more than that of the trp promoter - Davison et al, 1974) and therefore might block trp expression by sheer brute force. Alternatively, the anti-termination action of pN might be responsible. By altering the relative powers of the two promoters it was hoped to distinguish between these two possibilities.

The result of raising the power of the trp promoter (Fig 5.7) showed that the inhibition of trp expression was as strong as before suggesting that the relatively high power of P_L may not be responsible. However, it was not possible to increase the power of the trp promoter so that it became more powerful than P_L.

Two approaches to reducing the power of the P_L promoter met with failure. In-vitro experiments had shown that the sex^- mutation resulted in a 10-fold lower rate of leftward transcription than normal.
(Roberts, 1969) and it was therefore presumed to be a promoter mutation. It was also defective in-vivo as this was the method by which the mutation was isolated. The normal level of expression of sex− in combination with a cro− mutation is inexplicable. It can only be suggested that the OPL region may be functionally more complex than was imagined and that, in-vivo, the involvement of cro and host functions (e.g. tob - Inoko & Imai, 1974) modifies the efficiency of initiation at PL. Similarly the results with the Nt88 cro1 phage were unexpected. It is normally proposed that pN is not overproduced (Franklin, 1974; Adhya & Gottesman, 1978) since the level of leftward transcription is found to be directly proportional to the amount of pN present. However, in these experiments, cro+ phages were used. It is possible that full leftward transcription from PL might be maintained by a low but continuous rate of synthesis of pN. This is feasible in view of the rapidity of pN decay.

It is quite possible that this Nt88 cro− phage might give reduced leftward transcription at 42°C but this has not been tried.

From the foregoing results it can be concluded that PL completely blocks trp expression. However, these data provided no insight as to what was happening to PL expression as a result of the opposition from the trp promoter.

In section III, the construction of a phage was described (Fig 5.8) which could be used to answer this problem. The "transcription-diagram" (Fig 5.9) shows that in addition to the trp and PL promoters being oriented in opposition, the lacZ gene is positioned such that it might be expressed by leftward transcription from PL but only if it is able to pass through the opposing trp operon.
In this phage there is a considerable distance (5kb) between the 
lacZ gene and the trp promoter and it was originally thought that 
another promoter, capable of expressing lacZ, might be present in this 
interval. This proved not to be of concern since the rate of lacZ 
expression in the absence of PL transcription was very low.

The results (Fig 5.10) show that the pattern of expression of 
anthranilate synthetase in this λlacZ-trp" phage is exactly as normal 
with a total shut-off of trp enzyme synthesis when PL is in 
opposition. The key part of this experiment, however, was the 
beta-galactosidase assays. These show very little expression when PL 
is repressed. In the non-lysogenic host, beta-galactosidase is not 
produced until after anthranilate synthetase synthesis has been 
turned-off. Since expression of lacZ occurred at a high rate this 
clearly demonstrates that whilst PL completely turns off trp 
expression, the reverse does not apply and PL transcription can 
proceed through the opposing operon.

The result (Fig 5.11) with the higher powered trp promoter, i.e. 
with the LD102 attenuator deletion, in opposition shows that leftward 
transcription is still able to pass through the trp operon.

However, two features are clearly apparent. Firstly, the rate of 
beta-galactosidase synthesis is markedly reduced. This indicates that 
the trp promoter does impede leftward transcription even when this is 
proceeding through the trp promoter region. Secondly, the time at 
which beta-galactosidase synthesis starts appears to be delayed, 
possibly by as much as 5 minutes.

These results are interesting for another reason. It has been 
shown that close to the trp promoter, and outside the operon, there is 
a transcription termination signal (Franklin, 1974; Hopkins et al, 
1976). When the trp genes are inserted into lambda in the
1-orientation this site severely reduces transcription of the \textit{trp} genes from P$_L$. The above result showed that expression of \textit{lacZ} from P$_L$ did occur at a high rate indicating that this site is not effective as a terminator when transcription passes through it in the opposite direction. This implies that the site is asymmetric.

The temporal resolution of the previous experiment was too low to permit any conclusion as to the time at which beta-galactosidase synthesis commences. By taking samples for enzyme assay at one minute intervals (Fig 5.12) it was possible to establish that \textit{lacZ} expression started about 12 minutes post-infection when P$_L$ transcription was opposed by the normal \textit{trp} promoter plus attenuator combination. With the phage carrying the attenuator deletion, beta-galactosidase was not detected until after 15 minutes. It should only take about 6 minutes to transcribe the distance between P$_L$ and \textit{lacZ} which indicates a considerable delay in both cases.

The analysis given in section IIIc showed that these figures were incompatible with the proposal that P$_L$ transcription continues in spite of head-on collision from RNA polymerases initiating at the \textit{trp} promoter; therefore, leftward transcription must stop. Since rightward transcription from the \textit{trp} promoter stops, as deduced from the lack of anthranilate synthetase, this suggests that collision between two polymerases results in a blockage of transcription in both directions. It is probable that this is mediated by the release of the polymerases from the DNA.

By the use of a \textit{trpR}$^+$ host it was possible to establish whether or not the physical presence of the \textit{trp} operon was responsible for the delay in beta-galactosidase synthesis. This allowed the power of the \textit{trp} promoter to be varied by a factor of nearly a thousand-fold. The results (Fig 5.13) showed no inhibition or delay in beta-galactosidase synthesis.
### TABLE 5.1 - SUMMARY

<table>
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<th>att</th>
<th>trpR</th>
<th>( P_{trp} )</th>
<th>( P_{L-trp} )</th>
<th>Distance of Opposition</th>
<th>Time Delay</th>
<th>Delay-factor Minutes/kb</th>
<th>Enzyme Synthesis</th>
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<td>9</td>
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</tr>
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Notes:
1) att = \( trp \) attenuator
2) trpR = Genotype of infection host
3) \( P_{trp} \) = Power of \( trp \) promoter relative to \( P_L \)
4) kb = kilo-base-pairs
5) Enzyme synthesis Units = nano.moles o-nitrophenol/min/ml
synthesis when the $trp$ promoter was fully repressed suggesting that it is rightward opposing transcription that is responsible for the interference with leftward transcription from $P_L$. As the power of the $trp$ promoter is raised not only does the delay in beta-galactosidase expression increase but also the rate of synthesis decreases.

These results are summarized in Table 5.1. The power of the $trp$ promoter is presented relative to that of $P_L$ and varies from virtually no transcription (1 initiation every 30 minutes) up to about 80% that of $P_L$. On the basis of the proposal that the inhibition resulting from opposing transcription is due to transcripitional interference and termination of transcription, then the time delay before beta-galactosidase synthesis occurs should be dependent on two factors - the relative power of the two promoters and the distance over which opposing transcription is occurring. This latter distance will depend on exactly where rightward $trp$ transcription terminates and is therefore difficult to determine. These figures can be combined into a "delay-factor" expressed as the time delay per "unit distance of opposition". In the case of the highest power $trp$ promoter (without attenuator in $trpR^-$ host) this amounts to about 8-9 minutes/kb whereas with the attenuator present this is reduced 5-fold to about 1.6 minutes/kb. The effect of the attenuator is to reduce rightward transcription by about 9-fold. However, it is more realistic to consider the excess power of $P_L$ over that of the $trp$ promoter since this is the factor that determines $lacZ$ expression. In this case the highest power $trp$ promoter reduces leftward transcription by a factor of five which is exactly the same degree by which the delay-factor is increased.

In the two cases where the $trpR$ repressor is present the situation is less clear. With the attenuator present the $trp$ operon will only

5-36
be transcribed rightwards approximately once in 30 minutes, which is effectively zero. Therefore no interference with leftward transcription would be expected. However, when the attenuator is deleted there is a substantial delay in expression of beta-galactosidase. Indeed the delay-factor (2.5 minutes/kb) is higher than in the case where the promoter is 9-fold more powerful. It can only be suggested that perhaps leftward transcription from \( P_L \) displaces the trp repressor from the operator and thereby artificially simulates a trp\(^R\) condition where the power of the trp promoter will be very high.

It therefore seems that the inhibition caused by convergent transcription is due solely to the action of opposing promoters.

The conclusion that opposing transcription results in the dominance of the more powerful promoter can be applied to the situation in lambda whereby the \( P_R \) and pre promoters are in opposition (see Fig 3.1). In this case, if pre is more powerful than \( P_R \) then it will "win". The lysogenic functions will be expressed and transcription of the lytic functions will be blocked. However, if \( P_R \) is more powerful than pre then \( P_R \) transcription will dominate and lead to expression of the lytic functions. Therefore by modulating the power of the pre promoter it would be possible for lambda to respond by completely committing itself either to lysogenic development or to lytic development.

There are two lines of experimental evidence that support this proposal. Firstly, the mechanism of establishment of repression (see Chapter 1, section V) involves an activation of pre transcription by \( cII \). Host factors appear to control this activation by either varying the amount or activity of \( cII \). Both \( cII \) and the host catabolite gene activation system seem to exert their effect on lysogeny by acting via
these host factors which suggests that the physiological state of the cell might be "communicated" to lambda via the control of cII activity mediated by these host factors. Since the requirement for cII in the activation of repressor synthesis appears to be stoichiometric it would therefore be reasonable to suggest that the power of leftward transcription from pre would be dependent on the activity of cII. Conditions favouring lysogeny would be those favouring expression of cIII and therefore in which pre transcription is greater than that from F_R.

Secondly, it was found that when either cII or cIII was mutant, and hence leftward transcription from pre opposing F_R was absent, expression of the late gene functions occurred five minutes earlier than normal (McMacken et al, 1970). This could be interpreted as pre transcription normally being responsible for a five minute delay in transcription from F_R. This is very analogous to the effect of the trp promoter on transcription from P_L.

It therefore appears that the effects of convergent transcription can be applied to this situation and at least supply one possible explanation as to how lambda decides whether or not to enter the lysogenic pathway.
CHAPTER 6

CONSTRUCTION AND CHARACTERIZATION OF polA-lacZ GENE FUSIONS
The recent cloning of the *E. coli* polA gene (Kelley et al., 1977) presented the possibility of using this lambda transducing phage to examine the regulation of the polA gene. As was discussed in the introduction (Chapter 2), polymerase I (the product of the polA gene) plays an essential role in DNA replication and is involved in DNA repair. It has the special significance of being the first DNA polymerase to be discovered and studied in depth.

Infection with this λpolA transducing phage showed that the rate of polI synthesis was higher in a polA1 host than in the isogenic polA+ host. It was therefore suggested that polI might participate in the regulation of polA. However, in this experiment replication of the phage was allowed and since polI is involved in DNA replication it is possible that other factors could be affecting polA expression. Nevertheless there is some indication that T4 DNA polymerase regulates its own expression at the level of transcription whereas T4 gene 32 is regulated at the translational level (Krisch et al., 1977).

Whilst it is possible to assay polI protein synthesis after infection with λpolA it is neither sensitive nor is it easy. In addition, the infections would have to be performed under conditions in which phage replication did not occur and this would greatly reduce the level of enzyme synthesis. Furthermore, on the proposal that polI regulates its own synthesis, expression of this protein from the phage would affect the intracellular level of polI and thereby disturb the rate of expression of the polA gene. It was for these reasons that the polA gene was fused to lacZ so that beta-galactosidase synthesis could be used as a monitor of polA transcription. Three methods of constructing gene fusions were discussed in the introduction.
a) Construction of polA-lacZ Hybrid Phages:

The first step in the construction of gene fusions is the transposition of the two genes into juxtaposition. This is then followed by selection of deletions which permit the transcription of the lacZ gene from the promoter of interest. Since both the lacZ and polA genes were available on phages it was decided to bring the two genes together on a phage using in-vitro techniques.

The isolation of the trp-lac fusion strains in which expression of lacZ is regulated by the trp operator-promoter was described in the introduction. In fusion W205 (Mitchell et al, 1975), expression of beta-galactosidase is maximal indicating that all transcription stop signals and polarity had been removed. The insensitivity to the normal lac regulation mechanisms indicated that both the lac promoter and operator had also been removed by this fusion. This was confirmed by mapping the end-points of the fusion (Schrenk & Miller, 1974). This fusion had been transferred onto a phage (Barnes et al, 1974) and carried the lac and trp genes in the 'l'-orientation, i.e. transcribable from the lambda P_L promoter. The lacZ gene was located within the b2 region close to the tail gene J. The precise location is defined by the λlac5 transducing phage (Ippen et al, 1971). Consideration of the restriction sites in this region of the chromosome indicated that the only R.HindIII targets present were in the trpB and trpD genes (Hopkins et al, 1976).

The availability of a phage carrying this W205 trp-lac fusion (phage JDW36) presented the possibility of using in-vitro techniques to fuse lacZ to polA. The hybrid polA-lacZ phage was made as depicted in Fig 6.1. JDW36, carrying the W205 trp-lac fusion and having only
Fig 6.1: Genesis of the 'Lac-polA' Hybrid Phage D44
one H.HindIII target, was restricted with H.HindIII to provide the left arm of the required recombinant. NEM920 is a phage carrying the polA gene (actually it carries the polA1 allele) in the '1'-orientation and was used to provide the central polA fragment and also the right arm of the recombinant. This phage was only partially restricted as it was hoped that the polA fragment would remain attached to the phage right arm and hence the orientation of the polA gene in the final recombinant would not be altered. The DNAs were mixed and after ligation, recombinants were recovered by transfection of AA125. (suppressor-free)

Examination of the structure of the two parental phages showed that the right arms could be distinguished by virtue of JDW36 being red⁺ whilst NEM920 is red⁻. Thus a screen for Red⁻ phage (by failure to plate on lig-ts at 37°C) served to identify recombinant phages which carried the required right-arm. The left arms could be distinguished on the basis of JDW36 carrying the lacZ gene. However, it was thought unwise to make use of any selection which demanded expression of the lacZ gene from either of the polA or P_L promoters in case such a phenotype required any mutational changes in the phage. This cautious approach was adopted in spite of the high probability that the lacZ gene would be transcribed from P_L.

To circumvent the necessity to select for the Lac phenotype an alternative was used. The λpolA phage used as donor carried an amber mutation in a head gene (gene W). Therefore any recombinant phage which carries the left arm of the λpolA phage, and hence the Wam mutation, will fail to plate on a suppressor-free strain. It was for this reason that the transfection host used to recover the ligation mix was suppressor-free. Only phage DNA carrying the left arm of JDW36 could be recovered by transfection.
The above selection and the following screen for Red\textsuperscript{-} phage served to isolate recombinants carrying the required left and right arms of the hybrid phage leaving only the problem of screening for phage which also carried the polA fragment. This was achieved by virtue of the observation that Red\textsuperscript{-} phage fail to form plaques on a host that is deficient in polA (Zissler \textit{et al.}, 1971a). However, if the phage itself carries the polA gene then the infected host becomes phenotypically PolA\textsuperscript{+} and the Red\textsuperscript{-} phage will now form plaques. This characteristic was used during the original isolation of the polA recombinant (Kelley \textit{et al.}, 1977). Since the donor of the polA fragment carried the polA\textsuperscript{1} allele, the test for PolA\textsuperscript{+} was performed using the PolA\textsuperscript{-} strain feb\textsubscript{10} which carries an amber suppressor.

Out of 625 independent plaques recovered from the ligation reaction, 96\% (598) were parental JDW36 phage; the remaining 27 (4\%) were Red\textsuperscript{-} and hence had the left arm of JDW36 and the right arm of NEM920, 3 (0.5\%) of these also contained the polA gene.

These three phage with the required characteristics were examined genetically and confirmed to be Red\textsuperscript{-} imm\textsuperscript{21} and not imm\textsuperscript{21-ts}. Since these phages plated well on feb\textsubscript{10} (sup\textsuperscript{+}) but poorly on strain polA\textsuperscript{1} (suppressor-free) it was concluded that the insert carried the polA\textsuperscript{1} allele. The presence of the correct left arm was confirmed by the finding that these phage were Lac\textsuperscript{+} on BBL plates supplemented with the indicator Xg. This probably reflects expression from a lambda promoter. They were also found to be able to complement trpA\textsuperscript{88}.

polA\textsuperscript{+} derivatives of these phages were prepared by selecting for plaques on polA\textsuperscript{1}. It is found that lambda phage carrying the polA\textsuperscript{1} allele are able to "pick-up" the wild-type allele from the host chromosome by recombination. Selection from lysates grown on C600 for phage able to plate on the polA\textsuperscript{1} strain enabled the isolation of polA\textsuperscript{+}
Fig 6.2: Effect of Orientation on the Restriction Pattern
derivatives of the original polA-lacZ hybrid phages. DNA was prepared from these phages and shown by electrophoretic analysis to contain the HindIII fragment carrying the polA gene.

Although the λpolA parent (NEM920) had been only partially restricted, in order to try to maintain the original orientation of the polA gene, there was still the possibility that the orientation had changed. Since it was desired to construct a phage in which lacZ was transcribed from the polA promoter a method was required to confirm the orientation of the polA gene in these recombinants.

b) Orientation:

One of the simplest methods of distinguishing the orientation of a restriction fragment is to use another restriction enzyme that cuts the fragment asymmetrically. Under these conditions the restriction pattern will depend on the orientation of the fragment. Figure 6.2 illustrates this point. The difference between the sizes of bands c & d and c' & d' is clearly apparent.

Restriction analysis of DNA from phages λpolA" and λpolA¹ (NEM822 and NEM920) showed that the restriction endonucleases R.EcoRI, R.BamHI and R.HpaI did not cut the polA fragment (data not shown). R.HindII and R.HpaII cut the fragment many times but no difference could be seen in the restriction pattern of the two phages using 1% agarose gel electrophoresis. However, digestion with R.AvaI (Hughes, 1977) did show a distinct difference in the restriction pattern obtained with the two phages.

Fig 6.3 shows the restriction pattern obtained for these phages. Tracks 1 and 2 correspond to λpolA" and λpolA¹, respectively. The restriction pattern for the l-orientation phage is reproduced to the left of the photo. All of the bands are common to the two phages with
Fig 6.3: *AvaI* Digests on polA-lacZ Hybrid Phages

Track 1 = λpolA<sup>c</sup>

" 2 = λpolA<sup>l</sup>

" 3 = λlac-polA #86

" 4 = λlac-polA #50

varying bands
the exception of bands d and f which move in the predicted pattern of Fig 6.2. The sizes of each restriction fragment was estimated by scanning the gel negative with a densitometer to enable the precise estimation of the migration distance of each band. By the use of size standards run on the same gel (tracks not shown) the migration distance can be equated to size. The standards used were λ+ DNA digested with R.EcoRI; R.HindIII + R.EcoRI; R.EcoRI + R.BamHI. From these results and the restriction map of lambda for R.AvaI, a restriction map (Fig 6.4) was constructed for these phages. The results of double digests of R.AvaI + R.HindIII and R.AvaI + R.EcoRI confirmed this map. It can be seen that R.AvaI makes two cuts within the polA fragment. In the 'l'-orientation, fragment f (3.9kb) is replaced by a 4.7kb fragment in the 'r'-orientation phage.

The three polA-lacZ phages were digested with R.AvaI and the restriction pattern of two of them is shown in Fig 6.3 tracks 3 and 4. From this it can be seen that the two polA-lacZ phages are in different orientations. This is shown most simply by the inset diagram which shows the bands that vary in size. Track 1 in conjunction with the restriction map (Fig 6.4) shows that the 4.7kb fragment is characteristic of polA phages which have the polA fragment inserted in the 'r'-orientation. As can be seen from track 4 this 4.7kb band is present indicating that in isolate #50 the polA gene is also inserted in the 'r'-orientation. Track 3, isolate #86, shows the presence of a 3.9kb fragment which is characteristic of phage in the 'l'-orientation (Fig 6.3 track 2, and Fig 6.4). The third phage isolated was also in the l-orientation.

Interestingly the hybrid phage with the polA gene in the 'r'-orientation formed smaller plaques on feb10 than the other two hybrid phages. The reduction in plaque size is presumed to be due to
Fig 6.4: *EcoR I* Restriction Map for λpolA Phages
decreased polA expression. The explanation is possibly that P_L transcription in opposition decreases the expression of the polA gene by convergent transcription (see Chapter 5).

Subsequent work showed that the two R.Aval targets in the polA fragment were also recognized by the enzyme R.XhoI (Gingeras et al., 1978) whose recognition sequence (CTCGAG) is a subset of that of R.Aval.

It was later shown that the endonuclease R.BglII also cuts the polA fragment at a single site. This site has not been accurately mapped but is probably located to the right of both R.XhoI targets in the 1-orientation phage. R.Sall, R.KpnI, and probably also R.PstI, fail to cut the polA fragment. Isolate #86 which was shown to have the polA gene in the 1-orientation is stock number D44 and was the progenitor of all of the phage and lysogens to be described in the following sections.

Whilst the hybrid polA-lacZ phage (D44) carries the lacZ gene in the same orientation as polA there is no guarantee that it will be expressed from the polA promoter. A failure to express beta-galactosidase could be due to two factors - either the presence of a transcription termination site at the end of the polA gene or else to polarity effects due to out-of-phase reading of the trpA gene present by virtue of the use of the W205 trp-lac fusion. To check this point the integration-defective polA-lacZ hybrid phage was converted to att^+ by crossing to h^80 att^\lambda sus-gam imm^\lambda (NEM74) and selecting for h^\lambda imm^\lambda recombinants on C600(\lambda imm^21). These were then screened for Red^+ on lig-ts. In Red^+ recombinants the recombinational event must have occurred to the right of the polA fragment and to the left of att. This att^+ derivative was shown to be Lac^+ but complementation of trpA88 was very weak. A lysogen of this
integration-proficient derivative was constructed in strain N261 (lacZam) and tested on Lac-MacConkey agar plates for its Lac phenotype. This showed that it was only weakly Lac+ (colonies were faintly reddish after 36 hours incubation whereas a Lac+ strain is bright red after overnight incubation). There are many possible explanations for this weakness:

1) presence of a leaky transcriptional termination signal after the polA gene and before lacZ.

2) polarity effects due to out-of-phase reading of the lac and trp genes.

3) presence of another, weak, promoter between polA and lacZ.

4) The power of the polA promoter is a lot weaker than anticipated.

To circumvent these problems deletions entering the polA and trp genes were selected to ensure expression of lacZ from the polA promoter. Absence of a functional polA gene was also required to prevent the phage producing polymerase I protein which would influence the cellular level of this protein. Since it was suggested that the cellular level of polI is responsible for regulation of the polA gene this expression from the phage is highly undesirable.

To overcome this problem it is ideally necessary to construct the fusion such that most of the polA gene has been deleted.

c) Construction of Gene Fusions:

Treatment of E.coli with various mutagens can result in the production of deletions. In general this method is rather unsatisfactory and suffers from the disadvantage that it is not possible to control the occurrence of secondary mutations. A more satisfactory approach is to construct a selection for the spontaneous
occurrence of deletions. Casadaban (1976a) isolates deletion mutants, which result in gene-fusions, by selecting heat-resistant derivatives of Mu-gts lysogens (see Chapter 2, section III).

In this study the genes to be fused were located on a lambda transducing phage. There are therefore two very simple approaches for producing deletions. The first method is to use restriction enzymes to delete the DNA between two restriction targets in-vitro. The dearth of restriction sites within the polA fragment and the adjacent trpA gene makes this approach untenable. The second approach is to make use of an observation of Parkinson & Huskey (1971) that wild-type lambda is extremely sensitive to chelating agents whereas derivatives with a smaller genome size are more resistant. Selection for phage able to form plaques on agar plates containing pyrophosphate therefore serves to isolate phage which have "acquired" a deletion. Since the phage isolated are plaque-forming the deletion must have occurred in a non-essential region of the phage chromosome - the most probable location being either within the E.coli DNA or in the lambda N-operon. Phage that have lost the ability to complement polA must have deleted at least a part of this gene. If they are still Lac' then the deletion must stop short of lacZ. The size of D44 is about 86% wild-type which makes it very resistant to pyrophosphate and therefore very difficult to select deletions. To increase the size of the phage genome, D44 was crossed with h80 cI57 (NEM125) selecting on C600(λimm21) to construct phage D48, a cI57 min+ derivative of the polA-lacZ hybrid. This phage has the appropriate sensitivity to pyrophosphate for a nearly wild-type (97%) genome.

D48 (λlacZ-trpA polA1 (att-red)Δ cI57) was plated for single plaques on C600. These were then individually picked into 1ml of phage buffer plus chloroform. 0.1ml samples were preadsorbed to C600
plating cells and then plated onto BBL plates containing pyrophosphate at a concentration of 7 or 7.5mM (this concentration was found by experiment to give optimal killing of parental phage and yield of deletion phages). One plaque from each of the selection plates (each plate from a separate plaque) was picked, purified and tested for its PolA phenotype. Out of 44 independently isolated deletions 8 were PolA− and of these, 4 were still Lac+. These four phage will be referred to as fusions #3, 13, 14 and 24 (stock numbers D52-55, respectively).
Throughout this section, phage D48 will be referred to as the parent polA-lacZ hybrid phage and strains D52-D55 will be abbreviated to fusions #3, 13, 14 and 24, respectively. These phage are integration-deficient and carry the c1857 repressor allele. All operations were performed at 32°C.

Complementation of trpA88 was found to be weak for the parent phage. However, fusion #24 complemented trpA88 strongly and must therefore have an intact trpA gene (i.e. the fusion-deletion must stop short of trpA). The other three fusions fail to complement and hence the deletions must have, at least, entered into the JrSA gene.

a) LacZ Expression:

To compare the derivative fusions with each other and with respect to the parent hybrid phage, lysogens were constructed in strain C38, lacZ_{415}(\lambdaimm^{21}). This strain was used for two reasons: firstly, the M15 deletion removes part of the lacZ gene so that there is no beta-galactosidase expression from the host chromosome, and secondly, the prophage provided an extensive region of homology to facilitate integration of the fusion phages into the host chromosome. The resultant lysogens, strains C48-052, were tested for their Lac phenotype on Lac-MacConkey agar plates. In agreement with previous results the parent hybrid phage was only weakly Lac\(^+\). The fusions, however, were all strongly Lac\(^+\) indicating good expression of lacZ in the lysogenic state. It was therefore concluded that in all four fusions the lacZ gene had been fused to a good promoter. The steady-state level of beta-galactosidase in these strains was determined (Table 6.1).
### TABLE 6.1 - ENZYME EXPRESSION IN LYSOGENS OF THE polA-lacZ FUSIONS

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fusion</th>
<th>Generation Time (Mins)</th>
<th>Enzyme Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>C38</td>
<td>(-)</td>
<td>85</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>C48</td>
<td>parent</td>
<td>110</td>
<td>93</td>
</tr>
<tr>
<td>C49</td>
<td>#3</td>
<td>110</td>
<td>290</td>
</tr>
<tr>
<td>C50</td>
<td>#13</td>
<td>110</td>
<td>410</td>
</tr>
<tr>
<td>C51</td>
<td>#14</td>
<td>120</td>
<td>730</td>
</tr>
<tr>
<td>C52</td>
<td>#24</td>
<td>110</td>
<td>920</td>
</tr>
</tbody>
</table>

**NOTES:**
1) Strain C38 is lacZ<sub>M15</sub>(λimm<sup>21</sup>)
2) Media = glucose ACH thiamine Vogel & Bonner salts at 32°C
3) Units = nano.moles o-nitrophenol/min/mg protein
The *polA-lacZ* parent phage gives quite a significant level of beta-galactosidase expression. That this is due to the phage is clearly shown by the inability to detect any enzyme activity in strain C38 which is lacking the *lacZ* phage. As expected the fusion phages show a significantly higher level of expression than the parent. The stimulation varied from 3-fold (fusion #3) to 10-fold for fusion #24. It might be expected that if all fusions had resulted in the fusion of *lacZ* to the *polA* promoter then the level of expression should have been the same in all cases. This is not necessarily true. Fusions between the *trp* and *lac* operons resulted in a 60-fold variation in the rate of *lacZ* expression (Reznikoff *et al.*, 1974). In many cases, decreased expression was attributed to deletion of the *lacZ* ribosome binding site or polarity.

b) *Restriction Analysis:*

The four fusion strains were physically characterized by analysis of their DNA with restriction endonucleases.

Digestion with R.*Hind*III confirmed that in all cases the deletions extended from within the *polA* fragment leftwards deleting the R.*Hind*III target.

Digestion with R.*Ava*I enabled the sizes of the deletions to be estimated. The restriction pattern for the parent and the four fusions is shown in Fig 6.5. Of particular significance are bands h and c. Band h corresponds to the DNA between the two R.*Ava*I cuts in the *polA* fragment. Should any of the fusions remove one or both of these targets then this band will disappear. Band c corresponds to the DNA between the R.*Ava*I target in *lacZ* and that in *polA*. By measuring the reduction in size of this band in the fusion phages the sizes of the deletions can be determined. As before, fragment sizes
Fig 6.5: *AvaI* Digests on *polA-lacZ* Fusion Phages

**TRACK**

1 2 3 4 5

- 6.7kb
- 6.1kb
- 3.2kb
- 2.9kb
- 2.0kb

Track 1 = Parent
2 = Fusion #3
3 = Fusion #13
4 = Fusion #14
5 = Fusion #24
were measured against a calibration curve prepared by digestion of lambda DNA.

With fusion #3, fragment c reduces from 6.1kb to 2.9kb indicating that this deletion is 3.2kb long. This is the only change in the restriction pattern. In the case of fusion #13, track 3, band h is missing. This indicates that one of the two R.AvaI targets in the polA fragment has been deleted. Since band e is present, only one of the R.AvaI targets has been deleted. Band e corresponds to the DNA between the right-hand R.AvaI target in polA and the R.AvaI target in the lambda N-operon. From this and the reduction in size of band c, this deletion can be calculated to be 4.6kb long - the longest of the deletions obtained. Fusions #14 is similar to #3 but is slightly shorter. The restriction pattern for fusion #24 is significantly different from that of the other fusions and the parent phage. The absence of both bands h and e indicate that both of the R.AvaI targets in polA have been deleted. These bands together with c were replaced by a fragment of 6.7kb indicating that this fusion has deleted about 4kb of DNA. A digest of fusion #24 with R.XhoI shows this phage has only one target (the site in lambda) confirming that both of the R.AvaI/XhoI targets in polA have been deleted.

The results of the restriction analysis and the following transduction analysis are presented in fig 6.6.

c) Transduction Analysis:

The end-points of the deletions in the fusion strains were defined by transduction of trpA and polA mutants (Table 6.2).
### Table 6.2 - Transduction Frequencies for polA-lacZ Fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fusion</th>
<th>$\text{trpA9761}$</th>
<th>$\text{trpA23}$</th>
<th>polA1</th>
<th>feb10</th>
</tr>
</thead>
<tbody>
<tr>
<td>D48</td>
<td>parent</td>
<td>530</td>
<td>nt</td>
<td>42000</td>
<td>75000</td>
</tr>
<tr>
<td>D52</td>
<td>#3</td>
<td>62</td>
<td>2</td>
<td>69</td>
<td>50</td>
</tr>
<tr>
<td>D53</td>
<td>#13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>D54</td>
<td>#14</td>
<td>72</td>
<td>3</td>
<td>150</td>
<td>68</td>
</tr>
<tr>
<td>D55</td>
<td>#24</td>
<td>970</td>
<td>800</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Transduction frequencies expressed as transductants/$10^9$ pfu phage
This marker maps very late in the trpA gene (Imamoto & Yanofsky, 1967). The results show that the hybrid parent phage and fusion #24 (which was TrpA+ by complementation) were both able to transduce the trpA9761 marker to Trp+. Fusions #3 and #14, although unable to complement trpA, were able to transduce trpA9761 and hence in these cases the fusion-deletion must stop short of this lesion. Therefore the lacZ mRNA ribosome binding site must be intact and hence the lacZ messenger must be translated normally. Fusion #13, however, is unable to transduce trpA9761 indicating that this deletion extends past this marker and may delete the entire trpA gene. In this case there must be some doubt as to whether or not the deletion may have cut into the lacZ ribosome binding site. This could be a reason for the lower rate of expression of lacZ in this fusion (Table 6.1).

trpA23

The amino-acid sequence of the alpha-subunit of tryptophan synthetase (Yanofsky et al., 1967; Li & Yanofsky, 1972) shows that the trpA gene is 804 base-pairs long. The sequence for trpA23 shows this lesion maps 174 base pairs from the C-terminus of the gene.

Fusion #24, as expected, transduced this strain at a very high frequency. The other three fusions, however, were negative. In the case of fusions #3 and #14 the transduction frequency was not zero, but since these figures were based on two or three colonies, respectively, they are probably not significant. However, it cannot be excluded that the deletions stop very close to trpA23 but leave it intact. In such a situation the transduction frequency would be extremely low.
Fig 6.6: Location of Deletions in polA-lacZ Fusions

FUSION Deletion

#3 3.2kb
#13 4.2kb
#14 2.9kb
#24 4kb

Restriction Sites

A = AvaI
H = HindIII
X = XhoI

A = AvaI
H = HindIII
X = XhoI

Location of Deletions in polA-lacZ Fusions
polA1

As described in the introduction, \textit{polA}^- mutants are sensitive to the alkylating agent methyl methane-sulphonate (MMS). Therefore resistance to MMS serves as a very useful selection for the transduction of a \textit{polA}^- mutant to PolA+. Transduction frequencies were corrected for the spontaneous reversion to PolA+ (as measured by transducing \textit{polA1} with \textit{\lambda lac5}).

The results show an extremely high transduction rate with the parent phage. This was expected since the phage is \textit{polA}^+ and therefore all lysogens will be PolA+. Hence the transduction frequency will be the same as the lysogenization frequency. Fusions #3 and #14 also transduce \textit{polA1} at a significant but much lower frequency. This indicates that these deletions stop short of \textit{polA1}, i.e. \textit{polA1} maps to the right of the end points of these fusions. The longer fusions, #13 and #24, however, do not transduce \textit{polA1} and this marker therefore must lie under these deletions.

\textit{feb10}

This strain is phenotypically very similar to \textit{polA1} (Zissler et al. 1971a). Transduction with the fusion phages gave similar results to those for \textit{polA1}. The only possible difference was that the transduction frequencies for fusions #3 and #14 were slightly lower than with \textit{polA1}. This could mean that the \textit{polA10} (\textit{feb10}) mutation maps closer to the end-points of the deletions than does \textit{polA1}.

\textit{polA6} and \textit{polA107}

These strains could not be transduced to MMS resistance since the level of spontaneous reversion to MMS resistance was 2-3 orders of magnitude higher that the transduction frequencies.
Although the transduction studies served to define the left-hand end-points of the fusions, the failure of the transduction of polA107 and polA6 meant that the right-hand end-points were defined only relative to polA1. Fusions #13 and #24, which delete polA1, might remove the entire polA gene and thereby couple lacZ to another promoter entirely.

The results presented earlier (Table 6.1) showed that the level of lacZ expression in fusions #3 and #13 was less than for the other two fusions. In the case of #13 this could be due to deletion of the lacZ ribosome binding site and for #3 could be due to polarity effects. The fusions of interest were therefore #14 and #24.

To map these fusions with respect to the polA markers A6 and A107 a method was required to increase the frequency of PolA+ transductants above the background reversion frequency. The method chosen was to construct lysogens of the polA-lacZ fusion phages in the polA- hosts. "Homogenetization" between the polA- lesion on the chromosome and the fragment of polA left on the polA-lacZ fusion will result in the production of cells which are now Pol+, assuming that the polA-lacZ fusion still retains the wild-type allele.

The original fusions were integration deficient and hence would not integrate at the normal lambda attachment site. It was therefore thought best to convert the fusions to att+. At the same time the immunity was changed to that of phage 21. This was for two reasons: firstly, lysogens could now be grown at 37°C (since the original fusion phages were cI857, lysogens had to be grown at 32°C) and secondly, it was desired that the fusions would subsequently be converted to att+ clind-. The use of an att+ imm21 intermediate would greatly facilitate this.
**TABLE 6.3 - FREQUENCY OF HOMOGENETIZATION**

<table>
<thead>
<tr>
<th>Host</th>
<th>Fusion #14</th>
<th></th>
<th>Fusion #24</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
<td>Stimulation</td>
<td>Frequency</td>
<td>Stimulation</td>
</tr>
<tr>
<td>polA107</td>
<td>18,000</td>
<td>3600</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>polA1</td>
<td>1,400</td>
<td>700</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>polA6</td>
<td>420</td>
<td>70</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

**Notes:**
1) Methyl methane-sulphonate resistant colonies per $10^6$ cells
2) Enhancement of methyl methane-sulphonate resistant colonies over that in non-lysogenic parent.
The conversions of fusions #14 and #24 to att\(^+\) imm\(^{21}\) were performed in-vitro using phage NEM540. Digestion of the fusion phages with R.HindIII generates a left arm containing the lambda head and tail genes plus the polA-lac\(^Z\) fusions. Similarly, digestion of NEM540 gives a right arm extending from the R.HindIII site just left of att and containing att\(^+\) imm\(^{21}\). Ligation of these left and right arm fragments should generate a phage carrying the polA-lac\(^Z\) fusion and at the same time being att\(^+\) imm\(^{21}\). Accordingly, the two phage DNA's were mixed, restricted, ligated, and recombinants recovered by transfection of AA125. 20-30% were found to be Lac\(^+\). All were found to be Red\(^+\) and hence have the right arm of NEM540.

The att\(^+\) derivatives were then used to lysogenize polA1, A6 and A107; the lysogens formed were verified to be still PolA\(^-\) by their sensitivity to MMS.

The frequency of homogenetization (Table 6.3) was measured as described in the Methods. In the case of fusion #24 the level of PolA\(^+\) cells was very low for all three polA mutations. Since the same levels were obtained for the non-lysogenic parents this indicates that all three polA mutations lie under the deletion. Thus there is no conclusive evidence that this deletion stops within the polA gene and that lac\(^Z\) is being read from the polA promoter.

The results for fusion #14 clearly show a high level of homogenetization of the polA1 marker. Since it is known that this fusion is able to transduce polA1 this serves as a positive control. With polA107 there was a much higher level of homogenetization than with polA1. This was expected since polA107 maps nearer to the N-terminus than polA1 (Kelley & Grindley, 1976). With the polA6 lysogen the result was not as clear. A higher level of PolA\(^+\) cells was found in a culture of the lysogen than in the non-lysogen, but the
enhancement was nowhere near as high as for the other two polA mutants. The simplest explanation would be that the polA6 mutation does not lie under the deletion but that it maps very close to the right-hand end-point. Therefore there is no conclusive evidence that fusion #14 cuts into the polA gene and couples the lacZ gene to the polA promoter. However, since the fusion is PolA it is highly probable that it does cut into the polA gene. The similarity of the beta-galactosidase enzyme levels for fusions #14 and #24 (Table 6.1), however, suggests that lacZ is being read from the same promoter in both fusions. This would have to be the polA promoter.
a) **Self-Regulation by polI**

The original objective of this study was to determine whether or not the expression of polA is regulated by the cellular level of polymerase I. By using a polA-lacZ fusion the rate of synthesis of beta-galactosidase should be dependent on the activity of the polA promoter. If the level of polI in the cell influences the expression of the polA gene then the rates of synthesis of beta-galactosidase in a polA+ and polA- host should be different since there is a 100-fold difference in the cellular level of polI.

Whilst it would be possible to measure enzyme synthesis after infection of these hosts with the \( \lambda \) polA-lacZ fusion phages this approach was not adopted for 4 reasons:

1) the discontinuity of infection might interfere with cellular regulation;
2) since polymerase I is involved in replication the infecting phage would replicate to differing degrees in the two hosts;
3) phage lysates contain high levels of beta-galactosidase activity which would necessitate either purifying the phage or washing the infected cells prior to assay;
4) lambda gene expression might influence cellular regulation and hence the results obtained would reflect an abnormal situation.

For these reasons it was decided that the best approach was to construct lysogens of the fusion phages in the required hosts and then to measure the steady-state level of beta-galactosidase.

polA- only contains about 1% the normal level of polI activity, but apart from its increased sensitivity to ultra-violet light is
completely viable. An isogenic polA+ strain was constructed by P1 transduction of polA1 to methyl methane-sulphonate resistance. The resultant strain was designated C53 and will be referred to as "polA+".

The original polA-lacZ fusion phages have two disadvantages as far as the formation of lysogens is concerned. Firstly, they are integration-deficient which means that integration, when it does occur, will be via host rec-mediated recombination against the homology on the chromosome. This occurs at much lower efficiency and means that the chromosomal location of the prophage will be indeterminate. These transducing phages carry the host polA, trp and lac regions of the chromosome and hence could integrate into any of these sites. This difference in location would introduce artifacts. For example, integration into trp would place expression of the phage lacZ gene under the control of the trp promoter. Thus the cellular level of beta-galactosidase would reflect expression from the trp promoter rather than the polA promoter. The second disadvantage is that the phage carry the c1857 mutation which renders the phage repressor temperature sensitive. Thus all lysogens would have to be made and used at 32°C. To circumvent these problems, att+ clind- derivatives of the polA-lacZ fusion phages were constructed. The reason for the ind allele was to completely block prophage induction.

Since fusions #3 and #13 produce considerably less beta-galactosidase than the other two fusions they were not used. In the cases of fusions #14 and #24, the mapping studies (see earlier) failed to conclusively demonstrate that the right-hand end points of the fusions stopped within the polA gene. From the size of the polA gene it is impossible that neither of the fusions (#14 and #24) would have coupled the lacZ gene to the polA promoter. It was therefore
decided to use both fusions.

The required phages were constructed using the att~imm~ derivatives of fusions #14 and #24 prepared earlier (section IIId). These were converted to att~imm~ by crossing to att~imm~ selecting Lac+ Imm- recombinants on strain C38, lac-15(limm-21), on X-g-indicator plates. Blue plaques were picked and purified on N259 in order to modify them against the host K-restriction system (strain C38 is deficient in modification). The att~imm~ derivatives of fusions #14 and #24 are strains D61 and D62, respectively.

Lysogens in the polA1 and polA+ hosts were made as described in Methods and were verified to be mono-lysogenic (only one prophage/chromosome) by the ability of λ21 to form plaques on these strains. Although polA1 lysogenic for fusion #14 was checked in this way, the lysogen of fusion #24 failed to plate λ21. Since later results have confirmed that the fusion #24 lysogen is mono-lysogenic it seems that the explanation must be associated with the nature of the λ21 virulence (Sly & Rabideau, 1969). This mutation leads to repressor-insensitive expression of the lambda replication functions but it is not until replication has titrated out the o1 repressor that the N gene and late functions can be expressed. Since this phage is phenotypically Red~ early in infection (due to o1 repression of P_L), replication in a polA- host will be poor and titration of the repressor may not be possible. Hence it is possible that under these circumstances late functions will not be expressed, no progeny will be produced and the phage will be unable to form a plaque. If this is so then the ability of the fusion #14 lysogen to plate λ21 might suggest that some polA activity is still present in this fusion.

The steady state levels of beta-galactosidase in each of the four lysogens constructed were determined at least twice.
### TABLE 6.4 - EFFECT OF polA1 ON polA TRANSCRIPTION

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Lysogen Fusion</th>
<th>Expts</th>
<th>Enzyme Units</th>
<th>Ratio A1:A⁺</th>
<th>Generation Time(Mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C60</td>
<td>polA⁺</td>
<td>#24</td>
<td>4</td>
<td>338 ± 25</td>
<td>1.119 ± 0.20</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>C59</td>
<td>polA⁺</td>
<td>#24</td>
<td>3</td>
<td>503 ± 31</td>
<td>2.49 ± 0.20</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>C55</td>
<td>polA⁻</td>
<td>#14</td>
<td>2</td>
<td>281 ± 13</td>
<td>1.23 ± 0.12</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>C54</td>
<td>polA⁻</td>
<td>#14</td>
<td>2</td>
<td>345 ± 17</td>
<td>1.23 ± 0.12</td>
<td>48 ± 0</td>
</tr>
<tr>
<td>C53</td>
<td>polA⁺</td>
<td>-</td>
<td>2</td>
<td>1.7 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53</td>
<td>polA⁺</td>
<td>IPTG</td>
<td>1</td>
<td>11500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
1) Growth medium: Vogel & Bonner salts + glucose, ACH, thiamine
2) IPTG induction performed in glycerol minimal medium using 1mM IPTG
3) Enzyme Units = nano.moles o-nitrophenol/min/mg protein
The results (Table 6.4) for the non-lysogenic polA+ strain C53 show that the contribution of the host chromosomal lac operon is negligible under the growth conditions used.

For each of the fusions there is a consistently higher rate of enzyme synthesis in the polA1 host than in the polA+ host. For the short fusion (#14) the effect is small but still significant. This suggests that in a polA1 host the polA promoter is slightly more active than with a polA+ host. Since the strains are isogenic it is unlikely that this effect could be due to variation in the efficiency of translation of the lac specific mRNA. The growth rates of all of the strains were identical which eliminates any gross metabolic abnormality. The level of active polI enzyme is only about 1% normal (DeLucia & Cairns, 1969) in a polA1 host and therefore if the level of active polymerase I enzyme was the regulator of polA expression it would have been expected that the effect would be more pronounced.

There are two possibilities - firstly, only a part of the polA gene product might be involved in self-regulation. It is known that in a polA1 host the level of the associated 5'-3' exonucleolytic activity is about normal (Lehman & Uyemura, 1976). Also, since fusion #14 leaves most of the gene intact it is possible that although the remnant protein is inactive with respect to polI activity it could still be active with respect to regulation. The second possibility is that polA is not self-regulating but that the cell "adapts" to a lack of PolI enzyme and in so doing alters the concentration of the true regulator of polA expression resulting in a slight increase in the transcription of polA.

An increased rate of enzyme synthesis is again observed in the polA1 host from the fusion with the long deletion (#24). Here not only is the effect somewhat more pronounced but the levels are higher
by about 20% than the corresponding figures for fusion #14.

It is interesting to compare these results with those obtained by Kelley et al. (1977) for the synthesis of polI protein after infection with the polA phages (Fig 6.7). Here it was shown that infection of a polA1 host gave an approximately 40% higher total yield of polI protein.

Table 6.4 shows that the steady-state level of beta-galactosidase is 25% (fusion #14) and 50% (fusion #24) higher in a polA1 host. This is in close agreement with the above result and strongly suggests that lacZ expression is accurately monitoring transcription of the polA gene.

The rate of expression of beta-galactosidase in these fusions is approximately 3% that of the fully induced lac operon. Since there are about 20,000 molecules of beta-galactosidase in a cell when lacZ expression is fully induced (Cohn, 1957) this implies that the level of synthesis from the fusions would be equivalent to 600 molecules of beta-galactosidase per cell. Assuming that the polA mRNA is translated at equal efficiency to lacZ this would indicate a steady-state level of 600 molecules of polymerase I enzyme per cell. It has been estimated that the steady-state level of poll is about 400 molecules/cell (Kornberg, 1974). Thus the power of the polA promoter, as estimated in these gene fusions, is very close to that required to explain the cellular level of polymerase I and suggests that translational regulation does not occur. It further implies that translation of the mRNA is efficient (>60% that of lacZ). The close compatibility of these figures is again suggestive that lacZ is expressed from the polA promoter. Also, the similarity in the rates of expression of lacZ in these two fusions suggests that in both cases transcription is from the same promoter. From the size of the cloned
Table 6.5 - EFFECT OF GROWTH RATE ON polA EXPRESSION

| Fusion Source | Source | ACH | Generation Time(Mins) | Enzyme Synthesis | Ratio  
|---------------|--------|-----|-----------------------|-------------------|--------
| Glycerol -    | -      | 153 ± 18 | 515 ± 38             | 1.22 ± .09        |
| Glycerol +    | +      | 118 ± 3  | 460 ± 13             | 1.09 ± .03        |
| Glucose -     | -      | 73 ± 1   | 423                  | 1                 |
| Glucose +     | +      | 59 ± 2   | 423                  | 1                 |
| Glycerol -    | -      | 149 ± 18 | 608 ± 9              | 1.42 ± .02        |
| Glycerol +    | +      | 107 ± 13 | 508 ± 3              | 1.18 ± .01        |
| Glucose -     | -      | 71 ± 4   | 416 ± 26             | 0.97 ± .06        |
| Glucose +     | +      | 60 ± 1   | 429 ± 1              | 1                 |

Notes:
1) Fusion strains #14 = D61; #24 = D62. Lysogens in W3110
2) ACH = acid-hydrolysed casein = casamino acids
3) Units of enzyme synthesis are nano.moles o-nitrophenol/min/mg protein. All results are the mean of 2 independent experiments.
4) Ratio expressed relative to cells in glucose + ACH
b) Effects of Growth Rate

The results of the previous sub-section suggest that polymerase I has very little effect on its own synthesis. If expression of polA is truly constitutive, then the rate of synthesis of polI (and hence beta-galactosidase for these fusions) will be dependent on the gene copy number and consequently the rate of synthesis will depend on the cell growth rate.

To examine this proposal the level of beta-galactosidase under steady-state conditions was measured for cells growing at different growth rates. Lysogens in W3110 of fusions #14 and #24 (phage strains D61 and D62, respectively) were made and checked to ensure they were mono-lysogenic. W3110 was used since it is a standard wild-type strain. The results (Table 6.5) show that whilst the growth rate varied by a factor of 2.5, the rate of enzyme synthesis varied by a maximum of only 40%. It therefore appears that polA expression is possibly coupled to the cell growth rate. The slightly higher levels of enzyme in glycerol medium could be due to increased efficiency of translation of lacZ mRNA.

c) Effect of Cadmium

It has been found that treatment of cells with cadmium results in a rapid loss of viability (Mitra et al., 1975). This loss appears to be a consequence of the accumulation of single-stranded breaks in the chromosomal DNA (Mitra & Bernstein, 1978). Polymerase I is known to be a zinc metalloenzyme (Springgate et al., 1973) and replacement of the zinc moiety by cadmium results in the inactivation of the enzyme. If this occurred in-vivo as a consequence of cadmium treatment then
the explanation for the accumulation of single-stranded nicks could be an inactivation of polI and the consequent inability to repair the breaks left during replication.

If expression of polA is affected by a specific regulator then it might be expected that a complete inactivation of polI activity, and accumulation of a lethal quantity of single-strand nicks, would lead to an induction of polI synthesis. Consequently, the effect of 3\(\mu\)M cadmium chloride on the expression of polA was determined. Beta-galactosidase synthesis from polA-lacZ fusion #24 was used as the monitor of polA expression in W3110(D62), growing on glucose minimal medium. In this experiment the result of cadmium addition was an increased cell generation time from 73 to 95 minutes. At the same time the differential rate of enzyme synthesis (increase in enzyme activity/increase in cell protein) increased by a corresponding proportion.

This suggested that the cell might respond to polI deprivation by a reduction in the growth rate rather than by induction of polI synthesis. Although growth rate, in itself, does not affect polI expression, it is possible that polI is synthesized at a high rate for a short period of the cell cycle. The effect of cadmium might be to prolong this period of synthesis.

However, this result must be interpreted with extreme caution. A second experiment failed to show any effect of cadmium at all. The original report of the cadmium effect indicated that 3\(\mu\)M cadmium was sufficient to completely stop cell growth. This certainly did not occur here. The explanation could be that the growth medium used was Vogel & Bonner salts which contain a high level of citrate. It is probable that the citrate was chelating most of the added cadmium. To circumvent this a citrate-free medium (as used in the cadmium
experiments, Mitra et al. (1975), but containing 50mM phosphate to aid growth) was made-up. Using W3110(D62), cadmium was again shown to have an effect on the growth rate. However, a repeat experiment failed to show any significant effect, even at 30µM cadmium.

Because of the gross lack of reproducibility, this line of investigation was not pursued. It should be noted that even Analar reagents may contain zinc contamination at levels of <5ppm which corresponds to about 1µM. It has been shown that this concentration of zinc is sufficient to completely counteract the effect of cadmium (Mitra et al., 1975).

d) Involvement of Other Genes

The properties of the polA1 mutation were described in Chapter 2. This mutation has been found to be inviable in combination with any of the rec mutants and also with uvrE. In polA1 cells there is still a residual polymerase activity which suggests that cell viability is dependent on polymerase I. This is supported by the lack of absolute defective mutants (except of the conditional-lethal type) and the currently held idea of the role of polI in DNA replication.

If expression of polA is regulated it should be possible to isolate regulatory mutations which are deficient in expression of polA. These would be expected to produce lower levels of polI than normal with the consequence that the cells would become sensitive to ultra-violet irradiation (c.f. polA1). It was therefore thought possible that some of the ultra-violet sensitive mutants might represent this type of mutation.

Although it has not been reported whether the combination uvrD3 / polA1 is viable it is found that uvrD3 is dominant over the wild-type allele. A non-inducible repressor of polA expression would be
### TABLE 6.6 - EFFECT OF uvrD

<table>
<thead>
<tr>
<th>Host</th>
<th>Fusion</th>
<th>Generation Time (Mins)</th>
<th>Enzyme Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>uvrD⁺</td>
<td>#14</td>
<td>60</td>
<td>690</td>
</tr>
<tr>
<td>uvrD⁻</td>
<td></td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>uvrD⁺</td>
<td>#24</td>
<td>60</td>
<td>700</td>
</tr>
<tr>
<td>uvrD⁻</td>
<td></td>
<td>64</td>
<td>400</td>
</tr>
<tr>
<td>recA</td>
<td>#24</td>
<td>78</td>
<td>470</td>
</tr>
<tr>
<td>rec⁺</td>
<td></td>
<td>83</td>
<td>520</td>
</tr>
</tbody>
</table>

**Notes:**
1) Media = glucose, ACH, thiamine, Vogel & Bonner salts at 37°C
2) Enzyme Units = nanomoles o-nitrophenol/min/mg protein
expected to act in this manner.

*uvrD* is cotransducible with *metE* using P1. The donor strain was N14-4 (*uvrD3*) and the recipient AB2545 (HfrH *metE* polA1). Selection was for Met+ transductants, which were then screened for sensitivity to ultra-violet light. In addition to the *uvrD* transductant, an ultra-violet resistant derivative was also purified. This pair of strains should be isogenic with the exception of the *uvrD* mutation. The resultant strains were then lysogenized with the *polA-lacZ* fusion phages. As before, both fusions #14 and #24 were used and all lysogens were verified to contain only a single prophage.

The steady-state level of beta-galactosidase was measured as a monitor of *polA* expression (Table 6.6). All of the strains had approximately the same growth rate indicating that there is no gross abnormality associated with these strains. Interestingly the level of beta-galactosidase in the *uvrD* hosts was found to be only about 60% that of the *uvrD*+ hosts. This same effect was found for both of the *polA-lacZ* fusions used (#14 and #24).

Preliminary results indicate that *uvrE156* (mutU4) has the same effect on *polA* expression.

Using an almost isogenic pair of *recA*+ and *recA*− hosts (QR47 and QR48) the effects of this allele on *polA* expression was tested. In this case only lysogens of *polA-lacZ* fusion #24 were constructed. The results (Table 6.6) indicate that *recA* has no effect on *polA* regulation.
Currently one of the most popular and rewarding methods of studying gene regulation is by the construction of gene fusions to \textit{lacZ}. In this manner, the rate of expression of beta-galactosidase can be used as a convenient monitor of transcription from the promoter under study. The simplicity, convenience and sensitivity of the assay for beta-galactosidase makes it possible to study systems for which there is no suitable assay or in which the rate of expression is extremely low.

Perhaps the most versatile method of constructing gene fusions is that pioneered by Casadaban (see Chapter 2) using a \textit{lac::Mu} phage. However, this method is only readily applicable to genes which are not essential for cell viability and would therefore be difficult to apply to the regulation of the \textit{polA} gene. For these reasons the approach adopted was to construct the gene fusions on a lambda transducing phage using in-vitro techniques.

The construction of the gene fusions between \textit{polA} and \textit{lacZ} was described in section I. Availability of lambda transducing phages carrying \textit{polA} and \textit{lacZ} greatly facilitated the transposition of these genes into close proximity. The construction of the \textit{polA-lacZ} hybrid phage (Fig 6.1) made use of a phage carrying the \textit{trp-lac} fusion W205 in the 1-orientation. The advantage of this is two-fold: firstly, this fusion removed the \textit{lac} promoter thereby eliminating the normal control of \textit{lacZ} expression. Secondly, the presence of a R.HindIII target in \textit{trpR} makes it possible to insert the HindIII fragment carrying the \textit{polA} gene at this point. Assuming this fragment is inserted in the correct orientation, then the \textit{polA} promoter will be correctly positioned for transcription of the \textit{lacZ} gene.
Three phage were isolated which carried both the lacZ and polA genes. The orientation of the polA gene in these recombinants was determined by the use of restriction enzyme analysis. When two phage carrying an insert in opposite orientations are digested with a suitable enzyme the restriction pattern is found to differ (Fig 6.2). Finding a suitable enzyme for use with these phages proved to be difficult. Neither R.BamHI, R.HpaI, R.SalI, R.KpnI nor R.PstI were found to cut the polA fragment. However, R.AvaI was found to have two targets within this fragment and this allowed the orientation of these polA phages to be determined. The different restriction patterns for these two orientations are shown in the gel photo (Fig 6.3).

Part of the R.AvaI restriction map for the original polA phages is shown in Fig 6.4. The most important feature is that the 3.9kb fragment (f) is characteristic of phages carrying the HindIII polA fragment in the l-orientation whilst the 4.7kb fragment is an indicator of the r-orientation. This allowed the determination of the orientation of the three polA-lacZ hybrid phages. Two were found to yield the 3.9kb AvaI fragment and were therefore in the l-orientation.

It is interesting that the one phage that was found to have the polA gene in the r-orientation produced smaller plaques on a polA-host than the other two phages. Since expression of the lambda polA gene is essential for the replication of the phage on this host, this suggests that polA expression is reduced. The most likely explanation for this would be an interference with expression due to opposing transcription from the lambda Pl promoter (see Chapter 5). It was shown (Fig 5.4) that under the analogous situation of the trp operon in the r-orientation, there is very little expression of the trp genes until Pl transcription is turned-off by cro. In view of the
requirement for polymerase I for replication of lambda, the reduced synthesis early in the infective cycle would be expected to have a significant effect on phage replication. This would be reflected in the plaque size.

One of the phages with the polA fragment on the 1-orientation was used for the construction of all of the phages and lysogens subsequently used. In the lysogenic state, an integration-proficient derivative of this phage was shown to be weakly Lac+. It is not known whether or not this reflects expression of lacZ from the polA promoter or another promoter located between polA and lacZ.

In view of the finding that infection of a polA1 host by the original λpolA phages resulted in a higher rate of synthesis of poll than infection of a polA+ host, it was suggested that poll might influence its own synthesis. The investigation of polA regulation was therefore directed to examining this proposal. In order to prevent the polA-lacZ phage from disturbing the cell it is important that it should not produce any poll protein. It was therefore necessary to couple lacZ expression to the polA promoter and at the same time delete as much of the polA gene as possible. This fusion was achieved by taking advantage of the sensitivity of lambda to chelating agents but the relative resistance exhibited by deletion derivatives. Four deletion phages were studied in more detail and their characterization was presented in section II.

The rate of expression of lacZ in the lysogenic state for each of the four fusions was compared with the parent hybrid phage (Table 6.1). Although the rate of beta-galactosidase synthesis was found to be substantially higher in all four fusions there was a 3-fold variation in the rate of expression. It might be expected that fusion of lacZ to a common promoter, in this case polA, would give the same
rate of beta-galactosidase expression, however, this is not necessarily so. With fusions between lacZ and trp, a 60-fold variation in the level of beta-galactosidase was found (Reznikoff et al, 1974). In some cases, a reduced expression can be the result of polarity effects due to translation terminating before lacZ. In many cases the reason of the depressed expression was found to be due to a decreased efficiency of translation. This could be the result of a partial deletion of the ribosome binding site. A further alternative is the production of a trp-lac fusion polypeptide of lower than normal activity. In the case of the W209 trp-lac fusion this resulted in a hybrid protein which was considerably more labile than normal.

With most gene-fusions to lacZ it is possible to verify that expression of lacZ is from the promoter of interest since some of the normal regulatory mechanisms will be known (e.g. an amino-acid biosynthetic operon will be repressed by the amino-acid product). In view of the fact that nothing was known about the regulation of polA expression it was not possible to immediately check whether lacZ transcription was being directed from the polA promoter. An initial characterization was therefore under-taken with a view to defining the end-points of these four fusions.

All four fusions differed in the size of the deletion (Fig 6.5). The presence of two R.Aval targets within the polA fragment enabled the division of the fusions into three categories according to how far right the deletions extended. From the restriction pattern all three categories were represented.

By using the fusion phages to transduce point mutations in trpA (the trp-lac fusion used to construct these polA-lacZ hybrid phages has an intact trpA gene) it was therefore possible to define the
Two mutations were used - \texttt{trpA}9761 and \texttt{trpA}23. The \texttt{trpA}9761 marker is located very close to the C-terminus of the gene. Only fusion #13 failed to transduce this mutation (Table 6.2) suggesting that in this case the entire \texttt{trpA} gene might have been deleted. It is possible that the ribosome binding site for \texttt{lacZ} might not be intact and this might account for the reduced expression in this fusion. Since the right-hand end-point of this fusion is defined by its deleting one but not both of the R.Aval targets in \texttt{polA} (see Fig 6.6), it is possible to locate this fusion quite accurately. From the size of the deletion and the physical structure of the phage it is apparent that the R.Aval target lies very close inside the deletion and the \texttt{trpA}9761 mutation will only just have been deleted.

The finding that both fusions #3 and #14 - the short fusions - are able to transduce \texttt{trpA}9761 but not \texttt{trpA}23 locates the left-hand end points of these fusions to a region of about 100 base-pairs within the C-terminus of the \texttt{trpA} gene.

The size of the \texttt{polA} fragment is 5.1kb (this was estimated by summation of the sizes of the three fragments produced by R.Aval digestion of the \texttt{polA} HindIII fragment), whilst the \texttt{polA} gene product is 109kd. This size of protein would require the size of the gene to be about 3kb. Although the right-hand end points of the fusions could be estimated, the position of the \texttt{polA} gene with respect to the restriction targets was not known. It was hoped that transduction of \texttt{polA} mutations would enable the position of the promoter to be defined and therefore establish that the fusions had coupled \texttt{lacZ} expression to the \texttt{polA} promoter. Three \texttt{polA} mutants were available that spanned the gene. \texttt{polA}107 maps within the N-terminal segment with \texttt{polA}1 located centrally and \texttt{polA}6 positioned in the C-terminal region.
Although methyl methane-sulphonate resistance is a clean selection for PolA^+ transductants it is found that the reversion rate is very high. This has greatly hampered standard genetic mapping of polA mutations. In this case it was found that transduction of polA6 and polA107 was totally obscured by the extremely high frequency of reversion. However, it did prove possible to transduce polA1 and also polA10 (feb10). The results were essentially the same for both mutations. Both fusions #3 and #14 were able to transduce these lesions indicating that these fusions stop short of these alleles. The longer deletions, #13 and #24, failed to transduce either of these mutations. Therefore the polA1 and polA10 mutations must lie within the interval defined by the end-points of fusions #3 and #13 which is a distance of about 1kb.

Although it is not possible to draw any quantitative conclusions about the relative efficiencies of transduction of these polA mutations, it is interesting that the transduction frequencies of polA10 by the fusions were lower than for polA1. This might suggest that feb10 lies closer to the end-points of fusions #3 and #14 than polA1. This would be supported by the finding that transduction was higher with fusion #14 which is slightly shorter than #3. However, transduction in general might be more efficient in the polA1 host.

To try to map the fusions with respect to the polA6 and polA107 markers, the frequency of homogenetization between the chromosome and the remnant of polA left on the phage (present as a prophage) was measured. With the short fusion #14 the frequency of homogenetization of the polA mutations showed a graduation according to their map position (Table 6.3). Thus the polA107 marker, which maps in the N-terminal segment of polA and hence furthest from the fusion end-points, gave the highest frequency. With polA6, however, the
result suggested that the A6 allele had not been deleted in the fusion phage. This unfortunately means that there is no conclusive evidence that the deletion in fusion #14 cuts into the polA gene. This, however, is very unlikely for two reasons. Firstly, the fusion derivative is PolA-. Although it is possible that this fusion could have resulted from a simultaneous acquisition of a deletion and a mutation in polA, it is more likely that the loss of the polA function is due to the deletion extending into the gene. Secondly, the rate of beta-galactosidase expression in this fusion is only slightly less than that of fusion #24 (Table 6.1) suggesting that both are expressed from the same promoter. However, it might be possible that, in this fusion, expression of lacZ could be from another promoter. However, if fusion #14 leaves the polA gene intact, then the entire gene must lie to the right of this deletion. From the locations of the right-hand end-points of these fusions this would mean that fusion #24 must stop within the polA gene and therefore lacZ will be expressed from the polA promoter in this case.

The homogenetization results for fusion #24 indicate that even the polA107 allele has been deleted. It is therefore possible that the polA promoter could also have been deleted and that expression of lacZ would then be from another promoter. If this was the case, however, from the size of the polA gene, fusion #14 must have cut into the polA gene and therefore have coupled lacZ expression to the polA promoter.

Consequently, either fusion #14 or #24 (or both) must have fused lacZ to the polA promoter. From the physical positions of the right-hand end-points of these two fusions it is impossible for neither to have coupled lacZ transcription to the polA promoter. It was for this reason that the following experiments on the regulation of polA were performed using both fusions. Since all the results with
these two fusions have been very similar, it suggests that both fusions have coupled the polA promoter to lacZ.

Whilst these "homogenetization-reactions" were not successful in defining the end-points of these fusions, the technique would find ready application for the deletion mapping of polA mutations. It has not proved a simple task to map polA mutations because of the high reversion rate. The application of λpolA deletion phages should greatly facilitate such mapping studies.

To examine the proposal that the cellular level of polI influences the rate of expression of the polA gene, lysogens of the polA-lacZ fusions #14 and #24 were made in an isogenic pair of polA1 and polA+ strains. Since the level of polI activity in polA1 cells is only about 1% normal, there should be a substantial difference in the rate of beta-galactosidase synthesis if polI does influence regulation.

It was found that the lysogen of fusion #24 in the polA1 host failed to allow λg17 to plate. Since this strain was later shown to be mono-lysogenic the failure to plate must be due to the nature of virulence by this mutation which is essentially due to repressor-insensitive phage replication. Since replication is poor in polA1 hosts it is probable that insufficient replication occurs to allow λg17 to form a plaque. The ability of the lysogen of fusion #14 in polA1 to allow λg17 to plate is therefore interesting. It suggests that some polymerase I activity might be being produced from this fusion. The results on the effect of the cellular level of polI on expression of beta-galactosidase for this fusion would be consistent with this. However, since this fusion phage is phenotypically PolA- the level of activity must be low.

The results (Table 6.4) show that the steady-state level of beta-galactosidase synthesis, presumably due to transcription from the
polA promoter, was consistently higher in the polA1 host as compared to the polA+ host. The effect, however, was not large, being only about 25%-50% for the two fusions. In view of the vast difference in polI levels in these two hosts, the cellular level of polI cannot be directly responsible for regulating polA expression.

Essentially this result raises two possibilities. Firstly, the level of the 5'-3' exonuclease in polA1 hosts is almost the same as in polA+ hosts. Thus it is possible that regulation of polA expression might be controlled by the level of this exo. It is possible that the N-terminal segment of the polI polypeptide could be responsible for interaction with the polA operator-promoter. This is somewhat consistent with the results. Fusion #14 leaves the N-terminal segment intact, and therefore in lysogens of polA1 there would be two gene-copies of the exo moiety whereas in lysogens of fusion #24, which deletes most of the polA gene, there will only be one gene copy. The stimulation of beta-galactosidase synthesis in fusion #24 is double that for fusion #14.

The second possible explanation for the very small effect of polA1 on transcription from the polA promoter could be that polymerase I does not control its own synthesis. Instead it would be proposed that the cell "adapts" to a lack of polymerase I function and in so doing the concentration of the true regulator alters resulting in the slight increase in transcription of polA. Exactly how this might be mediated is not clear, but the decreased rate of conversion of Okazaki fragments to high molecular weight DNA in polA1 cells could be the controlling factor. The finding that the lysogen of fusion #14 in polA1 allows λc17 to plate, whereas that of fusion #24 does not, suggests that the former lysogen is not as deficient in polI as the latter. This might explain why the stimulation of expression in
Fig 6.7: DNA polymerase I synthesis after infection with λpolA

fusion #14 is not as high as for fusion #24.

Following infection by the original λpolA strains, Kelley et al (1977) showed that the rate of polymerase I synthesis was higher in a polA1 host. Their results are reproduced in Fig 6.7. From this it is apparent that the initial rate of synthesis is about 60% greater in the case of the polA1 host and the total yield about 40% greater. These figures compare very closely with the results obtained for fusion #24 which suggests that the results obtained with this polA-lacZ fusion is representative of expression from the polA promoter. With the shorter fusion (#14) the results are similar to that of fusion #24 which again would suggest that this has also fused lacZ expression to the polA promoter. These results also indicate that the higher rate of polI synthesis on infection of a polA1 host (Fig 6.7) is due to an increased rate of transcription from the polA promoter and is not due to trivial effects due to phage infection.

Comparison of the steady-state level of beta-galactosidase in lysogens of these fusion strains with the level of expression from the lac promoter enables an estimate to be made of the power of the polA promoter. With fusion #24 (in a polA+ host) the level is about 3% that for lac, and slightly less than this for fusion #14. This corresponds to about 600 molecules of beta-galactosidase per cell which is in very close agreement with the estimated 400 molecules of polI per cell (Kornberg, 1974). This again suggests that expression of beta-galactosidase is directed by the polA promoter. It also shows that the efficiency of translation of the polA transcript must be high and therefore suggests that translational control is very unlikely.

In some systems (e.g. trp) it is found that the steady-state level of enzyme remains constant even when the cells are growing at significantly different rates. This regulation seems to be achieved
by varying the rate of initiation at the promoter. If polA expression was regulated co-ordinately with the cell cycle it would be expected that the level of polI would be higher in faster growing cells since these are replicating at a high rate. Constitutive expression, i.e. totally unregulated, would be expected to give the opposite result. Lysogens of fusions #14 and #24 in W3110 were used to determine the steady-state level of beta-galactosidase in different growth mediums (Table 6.5). A 2.5-fold variation in the growth rate had only a marginal effect (20% or 40%) on the steady-state level of beta-galactosidase. It is possible that the slightly higher levels in glycerol medium could be due to the result of an enhanced efficiency of translation perhaps resulting from an increased stability of the lacZ mRNA. It is therefore possible that under these circumstances, measurement of beta-galactosidase may not be representative of polA expression. The results seem most compatible with regulation of polA maintaining a constant steady-state level over a wide range of growth rates by a general effect on transcription initiation.

Cadmium has been shown to be toxic to E.coli. This appears to be due to the accumulation of single strand nicks in the bacterial DNA. It was thought possible that this effect of cadmium might be the result of inactivating the zinc metallo-enzyme polymerase I. It is known that polI deficient strains show a reduced rate of conversion of Okazaki fragments into high molecular weight DNA. A total inactivation of polI might block this completely with the consequent accumulation of single-strand breaks in the chromosome and cell death.

If this was the case then it is possible that the cell might respond and induction of polA transcription might ensue. The results suggested that this probably does not happen but that the cell generation time increases. Lack of reproducibility makes any
conclusions impossible, nevertheless cadmium does appear to have some effect on polA expression. The only way to repeat this experiment would be to remove all traces of heavy-metal ions from the medium. This would lead to two problems. Firstly E. coli requires trace amounts of these ions for some enzymes, e.g. iron for cytochrome e, zinc for polI, and these would have to be added. Secondly leaching of these ions from the glassware and adsorption to the glassware would be a serious problem.

The results for the effect of recA on the rate of expression of polA (Table 6.6) indicate that recA is probably not involved in the regulation of polA. recA is involved as the primary component of the SOS repair system which is responsible for the repair of very heavily damaged chromosomal DNA. In this role it appears to be responsible for inducing the synthesis of many components which cooperate to prevent cell death. Since polI does function as a DNA repair enzyme it might have been thought that recA would have had a regulatory role in polA expression. However, the action of recA in the SOS pathway is only manifested when the DNA is heavily damaged and it is therefore possible that induction of polA by recA might only occur under such circumstances. Mitomycin C causes an induction of at least some of the components of the SOS pathway; however, it does not appear to have any significant effect on polA expression as indicated by these polA-lacZ fusions (data not presented).

The effect of uvrD on regulation of polA (Table 6.6) shows that the rate of synthesis of beta-galactosidase is two-fold lower in the mutant uvrD3. It is not clear, however, whether this represents an inhibition by the uvrD3 gene product or a requirement of active uvrD for polA expression. In either case it does suggest that uvrD may be involved in polA regulation. It is, however, not possible to exclude
the possibility that the effect on polA expression could be indirect.

Preliminary results have also indicated that \( uvrE \) has a similar effect on polA expression to that of \( uvrD3 \). The lower rate of expression of polA in the mutant could be responsible for the inviability of the combination of \( uvrE \) and polA1 mutations (Siegel, 1973).

There are two approaches to continuing this study of polA regulation. Firstly, since polI is involved in DNA replication it is possible that its synthesis could be coupled to the cell cycle. This could be tested by the use of synchronous cell cultures. The major problem with this is that perturbations caused by the synchronization procedure can lead to misleading conclusions.

The second approach would be to use these polA-lacZ fusions to select for polA regulatory mutations by utilizing the wealth of experience in selecting lac mutants.
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