Apl: a multifunctional repressor and excisionase essential for prophage induction of temperate coliphage 186

A thesis submitted for the degree of

Doctor of Philosophy

at the

University of Adelaide

by

Michael Robert Reed

B.Sc. (Hons.)

Department of Biochemistry

October, 1994

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due reference is made in the text.

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Summary

Temperate coliphage 186 is an SOS inducible member of the P2 family of bacteriophages. The gene control strategies 186 has developed are similar to that of an unrelated coliphage, lambda, but the mechanisms by which these strategies are achieved are different and less complex. This thesis examines some of these mechanisms in detail and discusses the advantages this simplicity has to the development of 186.

As a temperate, integrating phage 186 can develop in one of two ways. The decision to enter one of these pathways is made at a transcriptional switch following infection. Lytic development is the default developmental state and results in replication of the phage DNA, synthesis of phage structural proteins, assembly of mature phage particles and lysis of the host cell. In about 10% of infections, lysogeny is entered, where the lytic functions are repressed by the 186 CI repressor, and the phage genome is integrated into the host chromosome by site-specific recombination at regions of the phage and bacterial chromosomes known as attachment sites. This reaction requires the phage *int* gene product. Lysogeny is an extremely stable state, and the phage genome (in this state known as a prophage) is passively replicated with the host chromosome. If the SOS functions of the lysogen are turned on (in response to DNA damage), lytic repression is removed (derepression), the phage DNA is excised from the host chromosome (which also requires Int) and lytic development is re-entered, a process known as prophage induction. The developmental state of the phage (determined at the transcriptional switch) is obviously intimately linked with the state of the phage DNA within the host cell (integrated during lysogeny and excised during lytic development - the recombinational switch). Strategies must therefore have evolved to ensure that these two switches are coordinated and are thrown simultaneously. The 186 *apl* gene is shown here to be an integral part of this coordination during prophage induction.

The transcriptional switch of 186 is comprised of two face-to-face promoters which produce transcripts that overlap by 60 nucleotides. The first two genes of the lytic operon (expressed from promoter P_R) are the *apl* and *c*II (required for establishment of lysogeny) genes. The lysogenic operon (expressed from promoter P_L) contains the *c*I (lytic repressor) and *int* genes. Apl contains a helix-turn-helix (HTH) DNA binding motif and represses transcription from both P_R and P_L. This is similar to the lambda *cro* gene product. Repression by Cro is required for efficient prophage induction, which it achieves by repressing lysogenic transcription. Repression of lytic transcription by Cro is essential for lambda lytic development.

Chapter Two describes the phenotypes of an *apl*⁻ phage and the binding sites for Apl on the 186 genome. It was shown that Apl is required for prophage induction, and that the majority of this requirement is for excision of the prophage from the host chromosome. Another component of the *apl*⁻ induction defect was an apparent inability of the prophage to derepress lytic transcription efficiently, presumably a Cro-like effect, as a result of the inability of Apl to repress lysogenic transcription from P_L. In support of these functions, Apl was found to bind to the phage attachment site and between the P_R and P_L promoters. There was no apparent preference for binding to either promoter, which is contradictory to previous *gal*K reporter studies which indicated that Apl was repressing P_L in preference to P_R. Apl was proposed to bind as a highly cooperative complex and a number of direct repeat DNA sequences were predicted to be Apl recognition sequences. This Chapter introduces two main areas of Apl biology that are addressed in greater detail in the following two Chapters. First, the role of Apl in excision was addressed in Chapter Three and second, the role of P_R and P_L repression by Apl in 186 development was determined in Chapter Four.

To show a direct role for Apl in excision, an *in vivo* plasmid assay system was developed. This system also confirmed the requirement for 186 Int in both integration and excision reactions and showed that *E. coli* IHF was required for efficient integration but not excision. IHF binding sites at the phage attachment site were identified by DNase I footprinting and Int binding sites were predicted by sequence comparison to the closely related phage, P2. Comparison of protein binding sites at the lambda, P2 and 186 attachment sites demonstrated a remarkable degree of conservation of the arrangement of these sites, indicating similar mechanisms exist for recombination in each phage.

To resolve the apparent inconsistancy of the Apl footprint at the P_R-P_L region showing no promoter preference whereas previous reporter studies had shown a preference for P_L repression, a *lac*Z reporter system was developed to determine the sensitivity of both promoters to Apl repression. This showed that, contrary to the previous reporter studies, but in agreement with the footprinting studies, both promoters were repressed equivalently by Apl. The fact that P_R is much stronger than P_L, however, means that it is always more active and therefore able to sustain lytic development, even when repressed by Apl. The role of Apl repression was addressed in Chapter Two with an *apl*⁻ phage; but this study was complicated by the severe excision defect of the phage. As a consequence, phenotypes of this phage due to a loss of repression of the two promoters could not be separated from those due to the loss of excision. To overcome this problem, operator mutants were isolated which inhibited Apl repression of either or both promoters. These mutants demonstrated that there were determinants of Apl

repression specific for each promoter, despite the fact that they were both repressed equivalently. These phage mutants were used to study the role of repression by Apl in various aspects of 186 development. This showed that Apl repression of P_L is required for efficient prophage induction. This role is similar to that of lambda Cro and presumably is required to prevent expression of CI in the inducing lysogen. The role of P_R repression by Apl is unclear as this function slightly impairs lytic development. These mutants also provided insight into the mechanism of Apl binding as they presumably defined nucleotides important for binding to and repression of both promoters.

Publications

Dodd, I.B., Reed, M.R. & Egan, J.B. (1993) The Cro-like Apl repressor of coliphage 186 is required for prophage excision and binds near the phage attachment site, *Mol. Microbiol.*, **10**, p.1139-1150.

Posters

Reed, M.R. and Egan, J.B. (1993) Repression and excision roles for the *apl* gene of temperate coliphage 186, *The Organisation and Expression of the Genome*, **15**, Lorne, Australia.

Reed, M.R. and Egan, J.B. (1994) The Apl protein of temperate coliphage 186 is an excisionase and differentially represses lytic and lysogenic transcription, *The Organisation and Expression of the Genome*, **16**, Lorne, Australia.

Reed, M.R. and Egan, J.B. (1994) The Apl protein of temperate coliphage 186 is an excisionase and a lambda cro-like repressor, *The Australian Society for Biochemistry and Molecular Biology Annual Conference*, Gold Coast, Australia.

Communications

Reed, M.R., Dodd, I.B. and Egan, J.B. (1993) The *apl* gene of temperate coliphage 186 plays a role in repression and recombination. *The Australian Society for Biochemistry and Molecular Biology Annual Conference*, Adelaide, Australia.

Reed, M.R., Dodd, I.B. and Egan, J.B. (1993) Dual role of Apl - repressor and excisionase, *The fourth international meeting on P4, P2 and related bacteriophages*, Oslo, Norway.

Reed, M.R., Dodd, I. and Egan, J.B. (1993) The *apl* gene of temperate coliphage 186 plays a role in repression and recombination, *Molecular Genetics of Bacteria & Phages*, Cold Spring Harbor, New York.

Submitted or in preparation

Reed, M.R., Pell, L.M. and Egan, J.B. (1994) Determinants of directionality in coliphage 186 site-specific recombination: Apl is the excisionase.

Reed, M.R. and Egan, J.B. (1994) Lysogenic and lytic repression by Apl in temperate coliphage 186.

Acknowledgements

I would like to thank everyone who has made my time here in Adelaide such a rich and rewarding one. Thanks to Professors G.E. Rogers, J.B. Egan and Dr. B.K. May for maintaining and organizing the Biochemistry Department at such a high standard and providing the opportunity for me to work there. To Barry Egan, my supervisor, thanks for creating such an intellectually stimulating environment in which to develop as a scientist and for your support, advice, licorice (BLECCCH!!) and your uncanny ability to lose doughnut bets.

To the people in Lab 19 who made life fun (most of the time) and put up with my dreadful singing: in particular Ian Dodd, for always having time to listen and for being a 186 encylopaedia and for being Doddsy; Tina Couch, for her friendship, late night company and gift of the gab (yarp?); Justin Dibbens, for being a legend; Petra Neufing, for always seeing the silver lining and Keith Shearwin, for showing us proteins aren't that scary.

Thanks to everyone in and out of the Department, especially Andrew, Rebecca, Tim, Tim, Mad Dog, Pauly, Julie, Linda, Dave, Lesley, Michael, Carl, Rick, Tim, Sally, Emma, Gayathri, "B.J., Paul, Gerry" (the oresome foresome), Carla, Crock and Beck for all the extracurricular activities, and Pete Rathjen for all the references, and Gus (the mighty F.F.) for showing me where it's at on those lazy Brisbane nights.

A very big and special thanks to my bestest friend, Fi, for being there, supporting me, lending me her wheels and providing constant (un)reality checks.

Finally, to Mum and Dad, words cannot express the gratitude I feel for all of your support, influence, patience and love these last 26 years. Thank you.

"....scholarship....must indeed have farflung grazing grounds, and in pursuit of a subject which interests no one but himself a scholar can accumulate knowledge which provides colleagues with information as valuable as that stored in a dictionary or an archive." Herman Hess, The Glass Bead Game, p.63.

".....Lambda is in the poo....." J.Barry Egan, Lorne Genome Conference, 1994.

CONTENTS

CHAPTER ONE

Introduction

Temperate bacteriophages are small. Compared to human beings, they are insignificant. They cannot develop outside a bacterial cell and when inside such a cell only have one developmental decision to make. So why study them? Because they *are* small, and because they develop inside simple cells and because they have a very simple developmental lifestyle. These factors all contribute to our ability to study a developmental switch at a level unimaginable in other, more complicated, systems. The vast contribution phage lambda has made to our understanding of gene control and development is a testament to the benefits of studying bacteriophages (Hendrix *et al.*, 1983; Ptashne, 1992).

The mode of development chosen by temperate, integrating phages, such as lambda and 186, basically depends on the expression of one of two operons, and the various players involved in the control of expression of those operons can be (relatively) easily characterized. This thesis describes the functions of a gene, called *apl*, which is one of these players in the development of 186. Like lambda, 186 infects and develops inside *Escherichia coli*, the best characterized cell in existence. In doing so it utilizes the gene expression machinery of the bacterium and thus, knowledge of the bacterium contributes greatly to our understanding of the phage. The phage itself has a small (30 kb) double stranded DNA genome which is easily manipulated. Regulatory control elements and expression patterns are easily identified and studied both *in vivo* and *in vitro*. As already mentioned, the development of the phage is very simple. It can develop in one of two ways, with the decision being determined by a single genetic switch. Once the mode of development has been chosen, a defined and invariable cascade of gene expression events occurs which utilizes a great number of control strategies to ensure the correct timing of expression.

The small size of the 186 genome and the fact that it is so highly evolved means it contains little functional redundancy. Discrete parts of the developmental process, from control of gene expression to recombination and DNA replication can be isolated, studied and related directly back to the whole phage. These attributes contribute to our ability to perform sophisticated and often elegant studies which can look at events from the molecular level to the whole organism and relate them back to each other. There are very few systems where this can

be done so easily and it has been a great experience to work with one and be able to contribute so much to the understanding of its development in so short a time.

1.1 TEMPERATE COLIPHAGE DEVELOPMENT AND GENOME ORGANIZATION

Temperate, integrating coliphages appear to have fundamental developmental control strategies in common. This is not particularly surprising as they all infect the same organism and have a similar developmental decision to make. The strategies they employ precisely program a cascade of gene expression events and determine the life cycle of the phage (Fig. 1.1). When a phage infects an *E. coli* cell, it injects its genome into the cell, where it is circularized, and early transcription occurs from the lytic promoter(s). Lytic development can be regarded as the default developmental state. Expression of lytic genes will continue unless the phage receives a signal telling it to stop. Early transcription then leads to middle gene expression, which usually results from antitermination of the early transcript and/or activation of another (middle) promoter. This results in expression of the phage DNA replication genes, required for replication of the phage genome. The final stage in lytic development is late gene expression in which the phage morphogenic proteins and the proteins required for phage assembly and host cell lysis are synthesized. Late expression is magnified by a gene dosage effect as a result of the replicating phage genome. The phage are then assembled and the host cell lysed, producing about 100-200 progeny phage for each lytically infected cell.

If the phage receives a signal diverting it from lytic development, it enters lysogeny. In this mode of development, a lysogenic promoter expresses the immunity repressor, which blocks lytic transcription, and the phage DNA is integrated into the host chromosome. This state is extremely stable. However, if the repression of lytic transcription is removed (derepression), the phage DNA will be excised from the host chromosome where it can then undergo normal lytic development. Although these events are common to most temperate, integrating phages, the gene control strategies used by each phage to coordinate such events are diverse, and in some cases, quite complex.

As the pattern of gene expression follows similar patterns in these phage, so too does the organization of their genomes. The early region contains the early lytic, lysogenic and recombination genes, the middle region contains the replication genes, and the late region contains the morphogenic and cell lysis genes. 186 is no exception, and the following Section outlines its genome organization and the expression strategies it uses in the various stages of development.

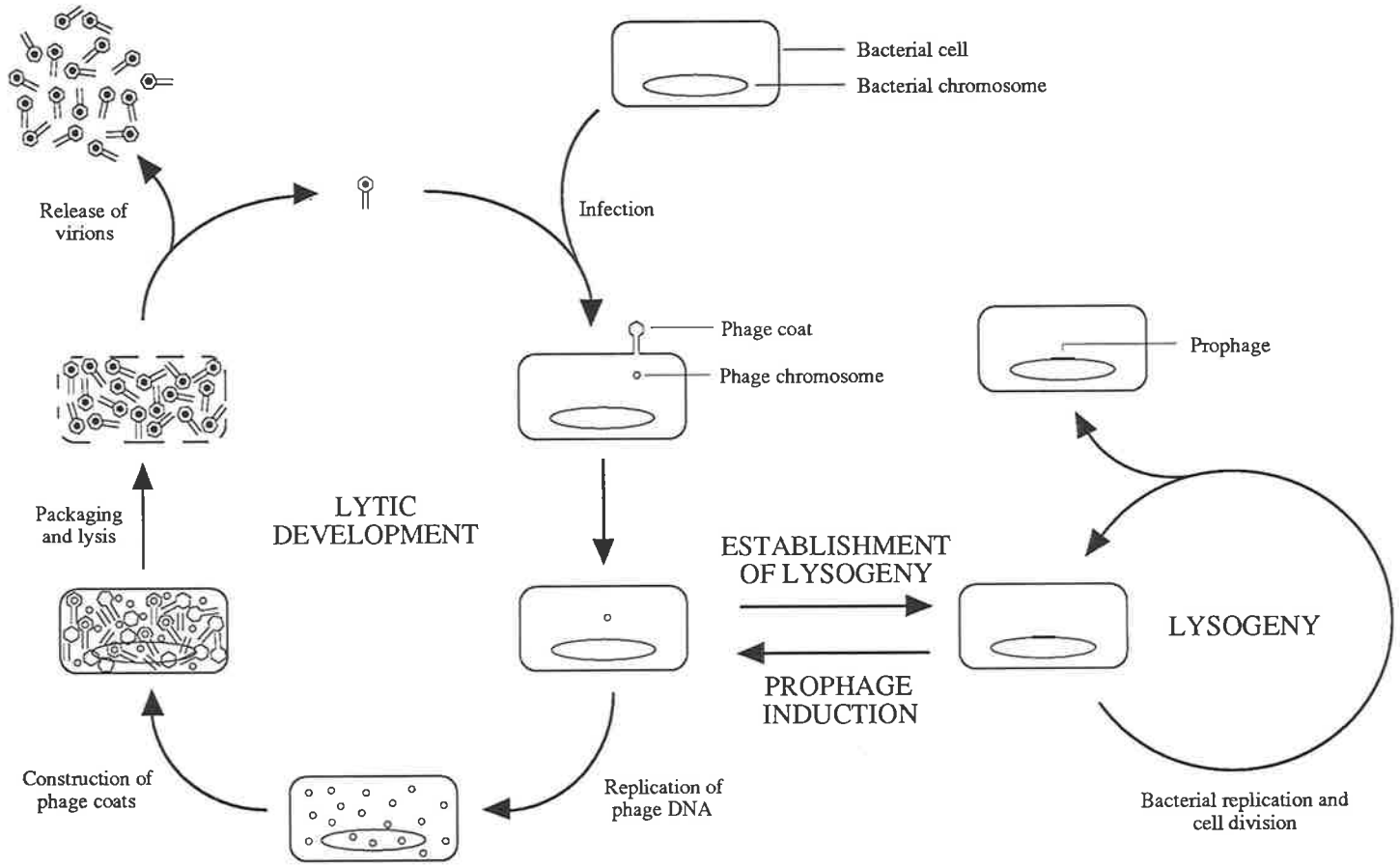


Figure 1.1 Life cycle of temperate, integrating bacteriophages

Following adsorption of the phage to the host cell, the phage DNA is injected into the host where it circularizes and early transcription begins. If the phage is to develop lytically, a coordinated series of events then occurs: the phage DNA is replicated, the structural proteins synthesized and assembled into phage coats, the replicated DNA is packaged into these coats, the host cell lysed and phage are released. If the phage is to enter lysogeny, lytic functions are repressed and the phage DNA is integrated into the host cell chromosome and becomes a prophage. The prophage is passively replicated with the host DNA and remains stably integrated until it is induced. During induction, lytic repression is removed (derepression) and the prophage is excised from the host chromosome, where it re-enters the lytic cycle.

1.2 186 DEVELOPMENT

Before any discussion concerning the development and gene control strategies of a phage, it should be remembered that most of the information has and will be obtained in optimal laboratory environments. In other words, phage are propagated in rich media at 30-37°C with vigorously aerated cultures. In the wild, however, such conditions are rarely encountered and so various strategies employed by the phage and the host cell, which may seem redundant in the conditions we use, may be relevant in other contexts.

1.2.1 Lytic development

Upon entering a cell, lytic development of 186 initiates with rightward transcription from two promoters, P_R and P_B (Fig. 1.2 and 1.3.A). Transcription from P_B expresses the late activation gene, *B*, and will be discussed below. Transcription from P_R is terminated by a cluster of three rho-independent terminators (collectively named T_R) just downstream of the *dhr* gene (Pritchard & Egan, 1985; Richardson, 1987; Richardson *et al.*, 1989; R. Jarvinen, unpublished data). Transcription of the early lytic operon (P_R-*apl*-*c*II-*fil*-*dhr*-T_R) results in the expression of four genes, the first two of which (*apl* and *c*II) are important for this discussion. These two genes each code for proteins that contain helix-turn-helix (HTH) DNA binding domains and act as transcriptional regulators. Apl represses lysogenic transcription from the leftward promoter, P_L, and also down-regulates its own transcription from P_R (Dodd *et al.*, 1990), with the biological significance of these two activities discussed in greater detail in Chapters Two and Four. CII activates a leftward promoter (P_E) upstream of P_L and is required for the establishment of lysogeny (Camerotto, 1992; Lamont *et al.*, 1993; P. Neufing, unpublished data; see below).

Middle transcription (Fig. 1.3.B) results in the expression of genes *CP79* to the replication gene, *A* (Hocking & Egan, 1982; Richardson *et al.*, 1989; Sivaprasad *et al.*, 1990), and is an extension of the P_R transcript as the result of an antitermination event at T_R (Richardson, 1987; R. Jarvinen, unpublished data). When antitermination occurs, the P_R transcript is processed by RNase III within the *dhr* gene and the early part of the message is rapidly degraded (Richardson, 1987). The antitermination event is apparently mediated by a phage RNA species derived from sequences well downstream of T_R (R. Jarvinen, unpublished data).

Late gene expression (Fig. 1.3.C) is dependent on expression of the *B* gene (Dibbens & Egan, 1992a). The B protein activates transcription from a number of late promoters which express the structural, DNA packaging and host cell lysis genes (Finnegan & Egan, 1981;

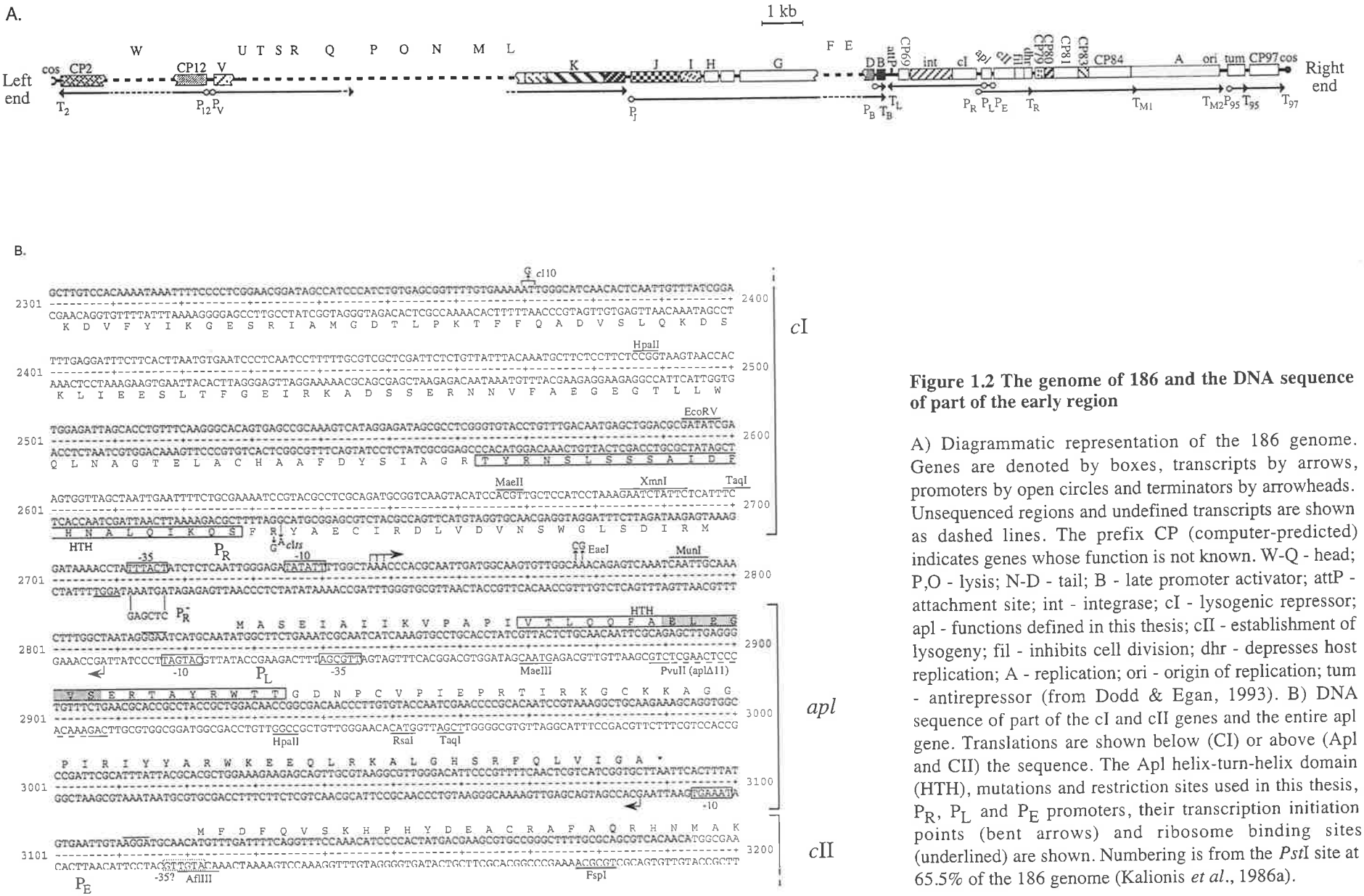


Figure 1.2 The genome of 186 and the DNA sequence of part of the early region

A) Diagrammatic representation of the 186 genome. Genes are denoted by boxes, transcripts by arrows, promoters by open circles and terminators by arrowheads. Unsequenced regions and undefined transcripts are shown as dashed lines. The prefix CP (computer-predicted) indicates genes whose function is not known. W-Q - head; P,O - lysis; N-D - tail; B - late promoter activator; attP - attachment site; int - integrase; cI - lysogenic repressor; apl - functions defined in this thesis; cII - establishment of lysogeny; fil - inhibits cell division; dhr - depresses host replication; A - replication; ori - origin of replication; tum - antirepressor (from Dodd & Egan, 1993). B) DNA sequence of part of the cI and cII genes and the entire apl gene. Translations are shown below (CI) or above (Apl and CII) the sequence. The Apl helix-turn-helix domain (HTH), mutations and restriction sites used in this thesis, P_R, P_L and P_E promoters, their transcription initiation points (bent arrows) and ribosome binding sites (underlined) are shown. Numbering is from the *Pst*I site at 65.5% of the 186 genome (Kalionis *et al.*, 1986a).

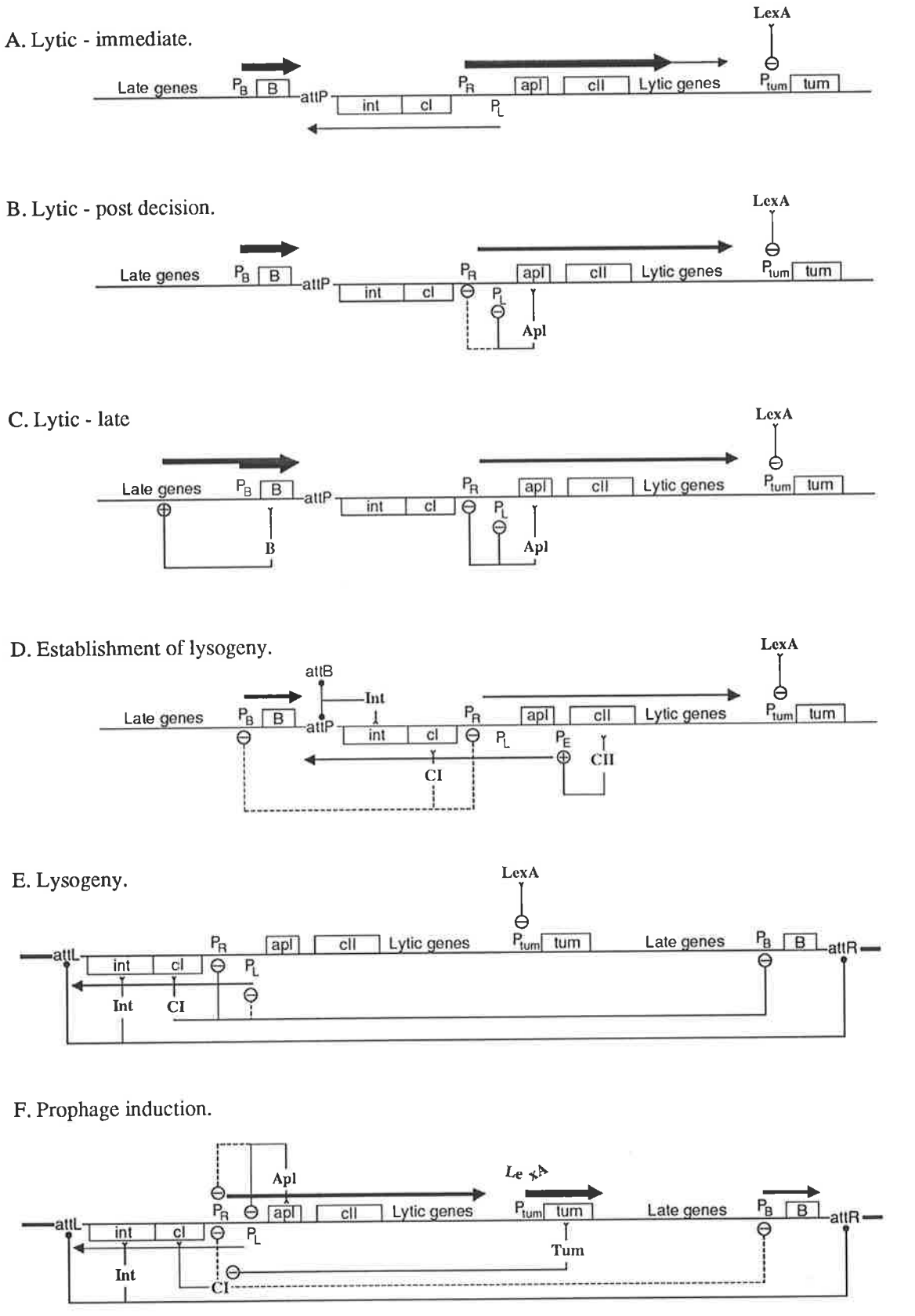


Figure 1.3 A model of the control of the 186 lysis-lysogeny system

Representation of the control strategies expected to be operating at six stages in the 186 life cycle. Details are given in the text. The diagram is not drawn to scale. Transcripts are shown as arrows with the thickness approximately indicating relative promoter strength. The inhibitory or stimulatory effects of gene products are shown by lines ending with circles containing negative or positive signs, respectively (modified from Dodd, 1993).

Kalionis *et al.*, 1986b; Dibbens & Egan, 1992a). The P_B promoter is controlled by the CI repressor and as such is transcribed early in lytic development (Dibbens & Egan, 1992b; Richardson, 1993). However, activation of late promoters is not seen until late in lytic development (Kalionis *et al.*, 1986b; Richardson, 1993). The reason for this delay is unclear, but may be due to a requirement for an increased copy number of the *B* gene. *B* is also expressed from a late promoter (Kalionis *et al.*, 1986b) late in infection (probably P_J; Xue, 1993).

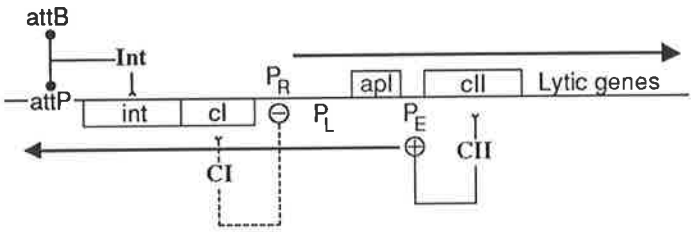
1.2.2 Establishment of lysogeny

The decision to divert from lytic development to establish lysogeny (Fig. 1.3.D) requires two things: i) repression of lytic transcription and ii) integration of the phage chromosome into the host chromosome. Expression of the lysogenic (leftward) operon achieves both of these requirements. The CI repressor apparently contains a variant HTH domain and is a DNA-binding protein produced from the first gene of the operon (Kalionis *et al.*, 1986a; Dodd, 1993). It binds at and represses the P_R and P_B promoters and so represses lytic transcription (Dodd *et al.*, 1990; Dibbens & Egan, 1992b; Dodd, 1993). The second gene expressed from the lysogenic operon is *int*, whose product is a member of the integrase family of site-specific recombinases (Argos *et al.*, 1986) and is required for both integration and excision of the phage chromosome from the host chromosome (Bradley *et al.*, 1975). A computer predicted open reading frame, *CP69*, is situated between the *int* gene and the phage attachment site and is translated, but its function is not known (Kalionis *et al.*, 1986a).

Transcription of the lysogenic operon can be initiated from two promoters (Fig. 1.2). In a lysogen (see below), the P_L promoter is active (Dodd *et al.*, 1990). This promoter is about 10-fold weaker than, and arranged face-to-face with, P_R, with the two promoters producing transcripts that overlap by 60 bp. During establishment, lysogenic transcription is thought to initiate primarily from the P_E promoter, which is located at the start of the *c*II gene, and is activated by CII (P. Neufing, unpublished data). CII binds to a sequence near the -35 region of P_E (Camerotto, 1992). Without CII, P_E is inactive, but in its presence is similar in strength to P_L; however, it is not affected by convergent transcription from P_R (P. Neufing, unpublished data), which appears to inhibit P_L transcription by over 90% (Dodd *et al.*, 1990). This advantage is presumably sufficient to allow synthesis of CI to levels adequate for P_R repression, and hence establishment of lysogeny.

The strategy used by 186 to establish lysogeny is very similar to that used by lambda (see Fig. 1.4), which also has a CII protein that activates a promoter (P_{RE}) required for

186



lambda

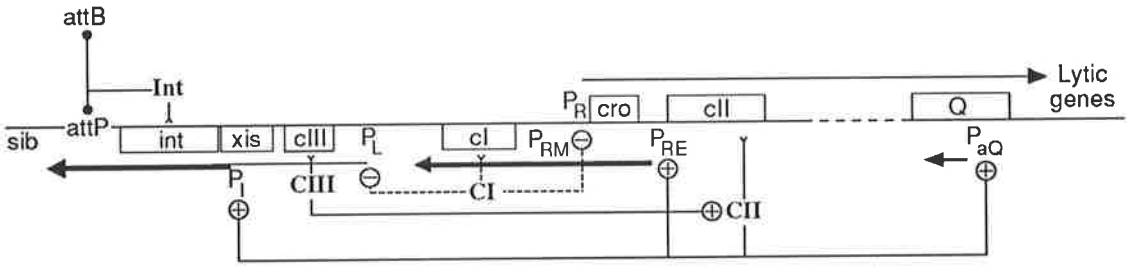


Figure 1.4 Comparison of the lysogeny establishment strategies of 186 and λ

Representation of the control strategies used by λ and 186 to establish lysogeny. Details are given in the text. The diagram is not drawn to scale and the symbols used are as in Fig. 1.3.

establishment. As in 186, the lambda *c*II gene is the second gene of the lytic P_R operon, with P_{RE} overlapping the start of the *c*II gene (Wulff & Rosenberg, 1983). Transcription from P_{RE} produces CI repressor and converges with transcription from P_R, with both of these functions contributing to P_R repression (Schmeissner *et al.*, 1980). Lambda Int, on the other hand, although required for establishment of lysogeny, is not situated on the lysogenic operon, but on a lytic operon transcribed from the P_L promoter. Transcription from this promoter is repressed by lambda CI, necessitating an alternative source of transcription for Int expression. This is supplied by transcription from another CII-activated promoter (P_I), located upstream of *int*, in the *xis* gene (Shimatake & Rosenberg, 1981). In addition to P_{RE} and P_I, another promoter, P_{aQ}, is also activated by CII (Stephenson, 1985; Hoopes & McClure, 1985). Transcription from this promoter inhibits *Q* gene expression, preventing expression of the lambda late genes.

Efficient activation of P_{RE}, P_I and P_{aQ} by CII, and hence establishment of lysogeny, is therefore dependent on the levels of CII within the cell. The lambda CIII protein (transcribed from the P_L operon), protects CII from proteolytic degradation when the infected cell is in a nutritionally poor state or during high multiplicities of infection (Wulff & Rosenberg, 1983). This makes sense, as a starving cell may not be able to support the enhanced metabolic processes required to support a lytic infection, and infection of one cell by a large number of phage would result in higher levels of CII and CIII proteins being produced. 186, on the other hand, does not have a CIII equivalent, and the effect of host genes and the multiplicity of infection on 186 development have not been examined.

Phage P2 is closely related to 186, although it has no *c*II gene equivalent, and the decision of this phage to enter lysogeny is thought to be a race between the lytic and lysogenic promoters, P_e and P_c (Saha *et al.*, 1987a). If this were the case, the decision to enter lysogeny would be statistical and not dependent on the state of the host cell or the multiplicity of infection. Such a mechanism would not be beneficial to the development of the phage, as the mode of development chosen by the phage may not be sustainable in any given infected cell. Although there is no evidence for this, it seems logical for the infecting phage to have some means of monitoring the state of the host cell such that the decision to enter lysogeny is not a random one. Perhaps the C repressor is stabilized or a new lysogenic promoter is activated by a host protein in starvation conditions, thus favouring lysogeny.

1.2.3 Maintenance of lysogeny

Maintenance of lysogeny (Fig. 1.3.E) requires the stable repression of lytic functions following establishment. During lysogeny, replication of the phage genome occurs passively with host cell DNA replication, as the prophage is colinear with it. As stated above, the CI repressor is produced from a transcript originating at the P_L promoter, which is the predominant phage promoter active in a lysogen (Kalionis *et al.*, 1986a). CI is a DNA binding protein and represses the P_R and P_B promoters (Dodd *et al.*, 1990; Dibbens & Egan, 1992b; Dodd, 1993). A consequence of CI repression of P_R is that P_L activity is increased, presumably due to removal of P_R inhibition of P_L (Dodd *et al.*, 1990).

The presence of CI in a lysogen renders that cell immune to superinfection by another 186 phage. These repressors are therefore known as immunity repressors. As soon as the superinfecting phage enters the cell, its lytic promoters are repressed so it cannot develop. Mutant phage able to infect a lysogen are known as virulent phage and in 186, the mutations responsible for this phenotype map to the CI operators required for P_R repression (Lamont *et al.*, 1989). These mutations affect CI binding to (Dodd, 1993), and repression of, P_R, such that a superinfecting virulent phage is able to develop lytically in a lysogen.

While the face-to-face arrangement of P_R and P_L may allow CI to indirectly activate P_L transcription, it also creates a problem for P_R repression. Every time a transcribing RNA polymerase from P_L proceeds across the CI binding region, bound CI will probably be displaced. This will expose P_R for occupation by another polymerase. The mechanism used to ensure CI repression is reestablished in these situations is not known, although the following model has been proposed. Dodd (1993) has shown that in addition to binding to sites over the P_R and P_B promoters, CI also binds to sites flanking P_R, in the *c*I gene and *apl-c*II intergenic region. Integration host factor (IHF), an *E. coli* DNA bending protein, binds between the CI binding site in the *c*I gene and P_R (D. Reynolds, unpublished data). The presence of one or both of the CI flanking sites improves CI repression of P_R about three-fold (determined by *lac*Z reporter assays; I. Dodd, unpublished data). It is therefore envisaged that CI bound at P_R and the flanking sites interact, with IHF facilitating the interaction with CI bound at the *c*I site (although IHF involvement in repression is not known). These interactions would keep the local concentration of CI high and hence allow reoccupation of the P_R site by CI once the transcribing polymerase has passed. The interactions occurring between the various bound CI proteins may also enhance P_R repression by creating a DNA structure unfavourable for RNA polymerase binding or elongation, as DNA structure is known to play an important part in the initiation of transcription (Pérez-Martín *et al.*, 1994).

The presence of the flanking sites may also be an anti-virulence mechanism. Such a mechanism has been proposed for the lambdoid phage HK022 (Carlson & Little, 1993). In this phage, there is a binding site for the immunity repressor immediately dowstream of the *cro* gene as well as two sites overlapping the lytic P_R promoter. A virulent phenotype will not occur unless two of the binding sites are mutated. Such a system will obviously decrease the spontaneous occurrence of virulent mutations, which jeopardize the existance of a lysogen. If a lysogen can easily mutate to virulence, then that phage will immediately infect and destroy other lysogens in its vicinity. Any mechanism which reduces the frequency of such an event will therefore be beneficial to lysogeny maintenance. Only the P_R region of the 186 virulent mutants has been sequenced (Lamont *et al.*, 1988), so the existance of mutations in the flanking sites (or even at P_B) is not known but is currently under investigation (I. Dodd, pers. comm.).

The CI binding site in the *apl-c*II intergenic region overlaps the -10 region of the P_E promoter and it is thought that CI bound at this site will repress P_E transcription, as well as contributing to P_R repression. A reason for such repression is not clear. Perhaps continued transcription from P_E once lysogeny has been established (and before CII has been degraded) is harmful. Alternatively, repression of P_R by CI could be affected by the increased amount of leftward transcription, or excess Int, also expressed from leftward transcription, may be harmful to a lysogen. It may also prevent P_E transcription in an induced lysogen, which would prevent its own transcription.

The lambda CI repressor binds as a dimer to adjacent sites at two regions of the lambda chromosome and represses the two lytic promoters, P_R and P_L (Johnson *et al.*, 1979). The cooperative binding of two dimers (to sites O_R1 and O_R2) to repress P_R also activates the divergent lysogenic P_{RM} promoter, and hence stimulates its own transcription (Meyer *et al.*, 1980; Meyer & Ptashne, 1980). At higher concentrations of CI, a third adjacent site (O_R3) is non-cooperatively occupied and represses its own transcription from P_{RM} (Maurer *et al.*, 1980). This arrangement regulates the levels of repressor in a lysogen and ensures the stability of the lysogenic state.

1.2.4 Prophage induction

Prophage induction (Fig. 1.3.F), or the movement from lysogeny to lytic development, requires the removal of lytic repression and excision of the prophage from the host chromosome. 186 is rare among P2-like phages in that it is SOS inducible (Woods & Egan, 1974). SOS induction of 186 is dependant on the *tum* gene, transcribed from the P_{95} promoter

(Lamont *et al.*, 1989). P_{95} is under LexA control, so under SOS-inducing conditions, LexA is cleaved and *tum* expressed. Tum induces the prophage by inactivating the CI repressor (Brumby, 1994) and recent studies have shown that Tum inhibits CI binding at P_R *in vitro*, although the nature of this interaction is unknown (K. Shearwin, unpublished data). Following inactivation of CI, the prophage excises from the host chromosome and re-enters lytic development. Apl plays an integral part in the induction process after derepression of a 186 lysogen, which will be discussed in later Chapters.

Prophage induction of lambda is also SOS-dependant, although the mechanism is different to that of 186. The lambda CI immunity repressor is analagous to the LexA protein in that it is cleaved following the activation of SOS functions (Roberts & Devoret, 1983). When this occurs, transcription from the lytic promoters P_R (from which CI repression is preferentially removed), then P_L, commences (Johnson *et al.*, 1981). The *cro* gene is the first gene of the P_R operon. It binds to the same operators as the CI immunity repressor, but with a reverse order of affinity. As such, its effects are opposite to that of CI. Cro occupies O_R3 first, which results in repression of the lysogenic P_{RM} promoter.

P2 is non-inducible and does not have a system to respond to SOS damage.

1.3 Coordination of transcriptional and recombinational switches

The different modes of temperate phage development centre around different states of the phage DNA within the host cell. As such, the decision to enter a developmental state, which is made at a transcriptional switch, must be coordinated with a recombinational switch which determines the state of the phage DNA within the cell. During lytic development, the phage DNA must be separate from the host chromosome (excised) to allow its replication and packaging, whereas during lysogeny the phage DNA must be colinear with the host chromosome (integrated). Different phage have developed different strategies to ensure the correct timing of recombination with the change from one developmental state to another. These strategies are discussed throughout the rest of this thesis.

1.4 The scope of this thesis

Prior to the commencement of my work, it was known that Apl repressed transcription from the 186 P_R and P_L promoters (Dodd *et al.*, 1990). This function, its genetic location and possession of a HTH DNA-binding domain was similar to that of lambda Cro. Subsequent work with an *apl*$^-$ phage, however, demonstrated some functional distinctions between Apl and Cro (Dodd *et al.*, 1993; see Chapter Two). First, Apl was not essential for lytic development

and second, it was required for prophage induction, with the bulk of this requirement being for prophage excision. The excision defect is similar to that noted for a P2*cox*⁻ phage (Lindahl & Sunshine, 1972) and it was reported towards the end of my studies described here that Cox is directly required for P2 excision, as determined by an ***in vitro*** recombination assay (Yu & Haggård-Ljungquist, 1993a). As with Apl and lambda Cro, Cox also has been shown to repress lytic and lysogenic transcription (Saha *et al.*, 1987b).

Two major questions concerning Apl therefore presented themselves: 1) Is the role of Apl in 186 prophage excision direct and analagous to lambda Xis and P2 Cox, or merely a consequence of Apl repression of P_L (P_L transcription passes through the 186 attachment site and so may influence recombination)? 2) What is the mechanism and role of P_R and P_L repression by Apl?

The obvious place to start was to determine the sites of action of Apl on the 186 genome by DNA binding studies. These results are presented in Chapter Two and show that Apl binds to both the P_R-P_L region and at the phage attachment site, causing a significant structural distortion to the DNA in the process. The fact that Apl binds to the 186 attachment site supports a direct role for Apl in excision. This was addressed in detail in Chapter Three, which describes the development of an *in vivo* plasmid recombination system and shows such a direct role for Apl. The lambda Cro-like repression role of Apl is addressed in Chapters Two and Four. In Chapter Four, the repression of both promoters by Apl is characterized using *lac*Z reporter assays and operator mutants defective in repression of one or both promoters by Apl. Chapter Five discusses the consequences of the functional duality of Apl and the simplicity this imparts to 186 development.

CHAPTER TWO

2.A. The Cro-like Apl repressor of temperate coliphage 186 is also required for prophage excision

This Section is a reprint of a paper describing the phenotype of an *apl*$^-$ phage and DNA binding studies used to determine Apl's sites of action on the 186 chromosome. The construction of and work with the *apl*$^-$ phage was performed by Ian Dodd. The creation of the Apl overexpressing strain and the DNA binding studies were performed by me. The computer-assisted determination of the Apl and Cox recognition sequences was performed jointly by Ian Dodd and myself. The sections of this paper (Introduction, Results, Discussion, Experimental Procedures) will be referred to as Sections 2.A.1-4 of this thesis. Figures 1-5 and Tables 1-2 will be referred to as Figures 2.A.1-5 and Tables 2.A.1-2, respectively, of this thesis. (There is a typographical error in the legend to Fig. 2.A.2.B: pMRR4, not pMRR3, was used for the *att*P footprints).

I.B. Dodd, M.R. Reed and J.B. Egan. The Cro-like Apl repressor of coliphage 186 is required for prophage excision and binds near the phage attachment site. *Molecular Microbiology, v. 10 (5), pp. 1139-1150, December 1993.*

2.B. The mechanism of DNA binding by Apl

This Section is an addendum to the preceding paper, presenting some DNA binding data not shown in it and expanding on areas of the discussion concerning the mechanism of DNA binding by Apl and its recognition sequences.

2.B.1 Introduction

Chapter 2.A described DNase I footprints at the 186 attachment site (*att*P) and the P_R-P_L region using Apl-enriched cleared cell lysates. The mechanism of DNA binding by Apl was discussed and two possible models proposed. It seemed fairly clear from the DNase I footprint that the DNA was being bent by Apl and there are a number of ways in which this could be achieved. The bend could be a naked DNA loop created by the interaction of bound Apl molecules at the base of the loop. A similar model to this has Apl also bound at the apex of the loop. Finally, Apl could be binding across the whole region with the DNA bent by and around the protein. This last model was favoured due to the nucleosome-like nature of the footprints and the presence of the predicted Apl recognition sequences across the whole region. The fact that the minimal sequence required for Apl binding has not been determined weakens this argument.

Other DNA binding experiments, such as gel retardations, were described but not presented in the previous Chapter, so here I will be presenting these along with a retardation using purified Apl, which was isolated near the end of my research by K. Shearwin (unpublished data), and provides convincing evidence for the model of DNA binding by Apl favoured in Chapter 2.A, and hence strengthens the argument for the location of multiple recognition sequences across the region protected by Apl.

2.B.2 Results

2.B.2.1 Preparation of Apl extracts

Most of the DNA binding studies used Apl-enriched cell extracts as the source of Apl. A time course of Apl induction showed that expression reached its maximum level between 30 and 60 minutes after addition of IPTG (data not shown), with Apl contributing about 10% of the total cell protein. SDS-PAGE of the extract showed that about half of this protein was soluble (Fig.

2.B.1). The size of about 10 kD seen in Fig. 2.B.1 agrees with the molecular weight of Apl, predicted to be 9.77 kD from its amino acid sequence (Kalionis *et al.*, 1986a).

Towards the end of my PhD, Keith Shearwin partially purified Apl (to about 90% purity, as judged by SDS-PAGE) and gel filtration chromatography through Sephacryl S-200 suggested that it exists in a monomer:dimer equilibrium in solution (K. Shearwin, unpublished data).

2.B.2.2 Identification of Apl DNA binding regions by gel retardation

Gel retardation assays using end-labelled restriction digests of clones containing DNA fragments from the *Pst*I.1-*Bgl*II.4244 region of 186 (See Fig. 2.A.1) showed that only two small regions were consistantly retarded by the Apl-enriched cleared lysate. An example of this is the *Hpa*II digest of the 186 *Xho*I.629-*Bgl*II.4244 fragment (Fig. 2.B.2). Some fragments were end-labelled better than others, an anomaly which was often seen in these experiments and the reason for it is not clear. Nevertheless, Apl-specific retardation of the 677 bp *att*P-containing and 447 bp P_R-P_L-containing fragments and no others was evident. The AplΔ11 control showed no retardation of any fragment.

The 447 bp (P_R-P_L) *Hpa*II fragment was isolated and a two-fold dilution series performed with increasing amounts of the Apl extract (Fig. 2.B.3). There were three types of retarded product. At low levels of extract (0.5 and 1 μg), a small fraction of the DNA was slightly retarded (R1) while at the highest level of extract (4 μg), all of the DNA was retarded to a greater extent (R3) than the R1 species, indicating the formation of a higher order Apl-DNA complex. At intermediate concentrations of extract (2 μg), the transition from the R1 to the R3 complex was marked by various smeared products (R2), suggesting the presence of unstable Apl-DNA complexes. No significant retardation was seen with the AplΔ11 extract. Retardations with an isolated *att*P fragment were not performed with the Apl extract.

2.B.2.3 Gel retardation of *att*P using purified Apl

The use of partially purified Apl (K. Shearwin, unpublished data) in a retardation of an *att*P fragment provided a number of insights into the mechanism of Apl binding. The smeared R2 transition products seen when using the Apl extract with the P_R-P_L fragment (Fig. 2.B.3) were not apparent when partially purified Apl was used (Fig. 2.B.4.A); rather, discrete retarded bands were present (R2A and R2B). This suggests that a number of intermediate Apl-DNA complexes formed in the transition from the R1 to the R3 complex. The amount of unbound

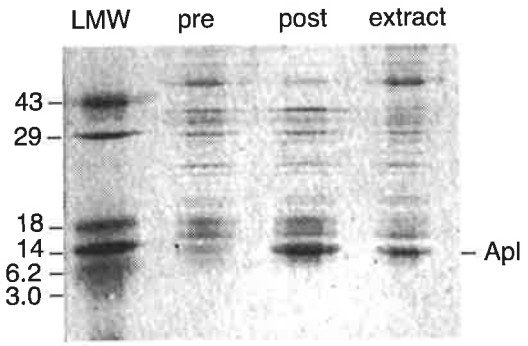


Figure 2.B.1 Induction of Apl overexpression

The Apl over-expressing strain (BL21(lDE3)pMRR1) was induced with 0.4 mM IPTG as described in Section 6.7.1. Pre-induced (pre) and post-induced (post) culture samples and soluble protein (extract) were visualized by electrophoresing through a discontinuous SDS polyacrylamide gel with a 15% separating gel (Section 6.7.3). Pre- and post-induced culture samples were prepared by centrifuging a 200 µl sample and resuspending in 20 µl of protein sample buffer (Section 6.4.6.3). The soluble protein was prepared by diluting 10 µl Apl extract (Section 6.7.1) into 10 µl of 2 x protein sample buffer. Prior to loading the gel, samples were heated at 95°C for 3 min then placed on ice to cool. Protein size standards (BRL low molecular weight markers) and the position of Apl protein migration are shown.

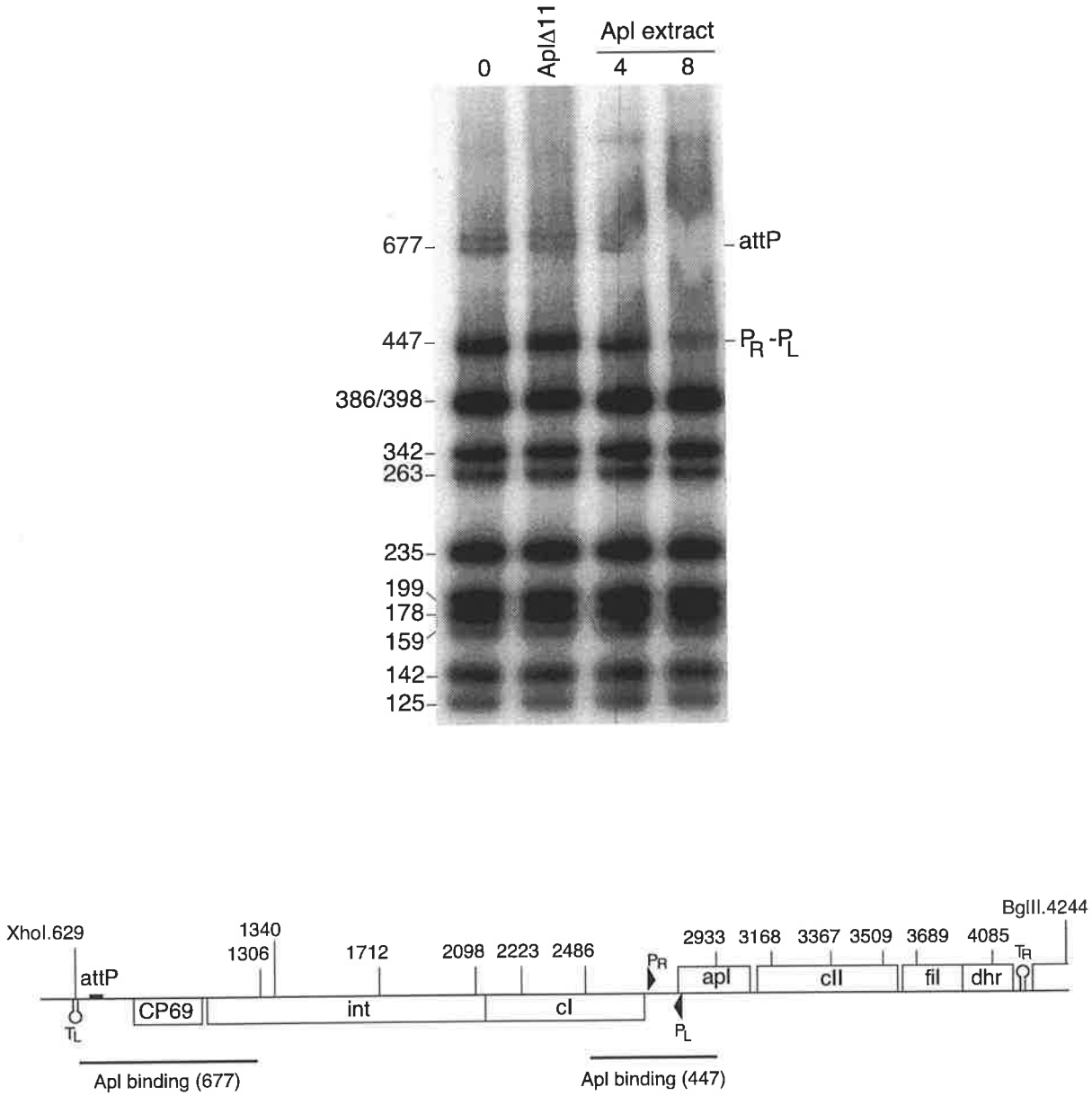


Figure 2.B.2 Apl extract retardation of a *Hpa*II digest of the *Xho*I.629-*Bgl*II.4244 fragment

The gel retardation assay is described in Section 6.7.4. A *Hpa*II digest of the 186 *Xho*I.629-*Bgl*II.4244 fragment was 3' end-labelled using [α-^{32}P]-dCTP in a Klenow end-fill reaction (Section 6.6.11). Approximately 1 nM of DNA was incubated with increasing amounts (4 or 8 µg total protein) of Apl extract or 8 µg AplΔ11 extract for 20 min at room temperature before electrophoresis through a non-denaturing 6% polyacrylamide gel at 4°C. End-labelled *Hpa*II fragments of pUC19 DNA (prepared similarly to the retardation fragments) were used as molecular weight markers (not shown). Fragment sizes (in bp) and retarded fragments (P_R-P_L and *att*P) are shown. The 67 and 34 bp fragments are not shown but were not retarded. The location of the *Hpa*II sites on the *Xho*I.629-*Bgl*II.4244 fragment is shown below the retardation. Numbering is from the *Pst*I site at 65.5% of the 186 genome (Kalionis *et al.*, 1986a).

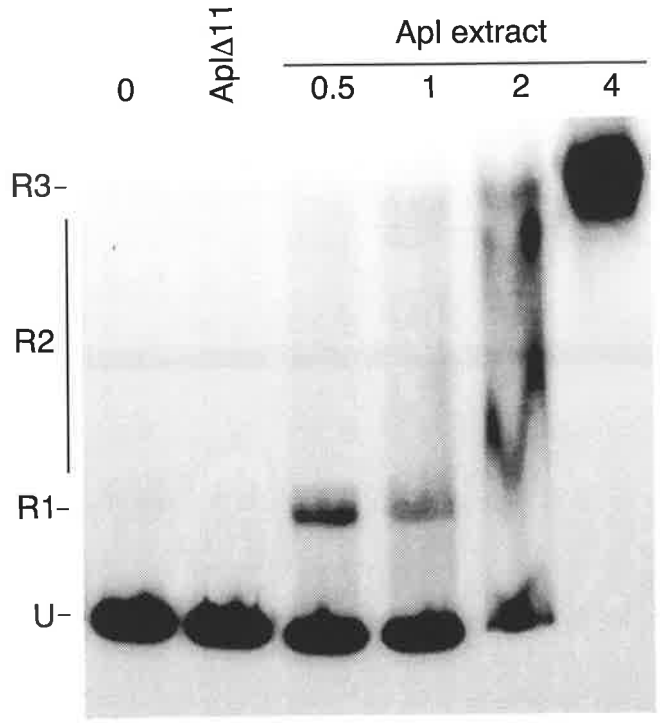


Figure 2.B.3 Retardation of the P_R-P_L fragment by the Apl extract

The gel retardation assay is described in Section 6.7.4. The 3' end-labelled 447 bp *Hpa*II fragment was prepared using a Klenow end-fill reaction with [α-^{32}P]-dCTP (Section 6.6.11) on a *Hpa*II digest of pEC603 and isolated from a non-denaturing 6% polyacrylamide gel (Section 6.6.8). Increasing amounts (0.5, 2 or 4 µg of total protein) of Apl extract or 4 µg AplΔ11 extract were incubated for 15 min at room temperature with approximately 0.5 nM of end-labelled 447 bp fragment prior to electrophoresis. Unbound (U) and retarded (R1 - R3) products are shown.

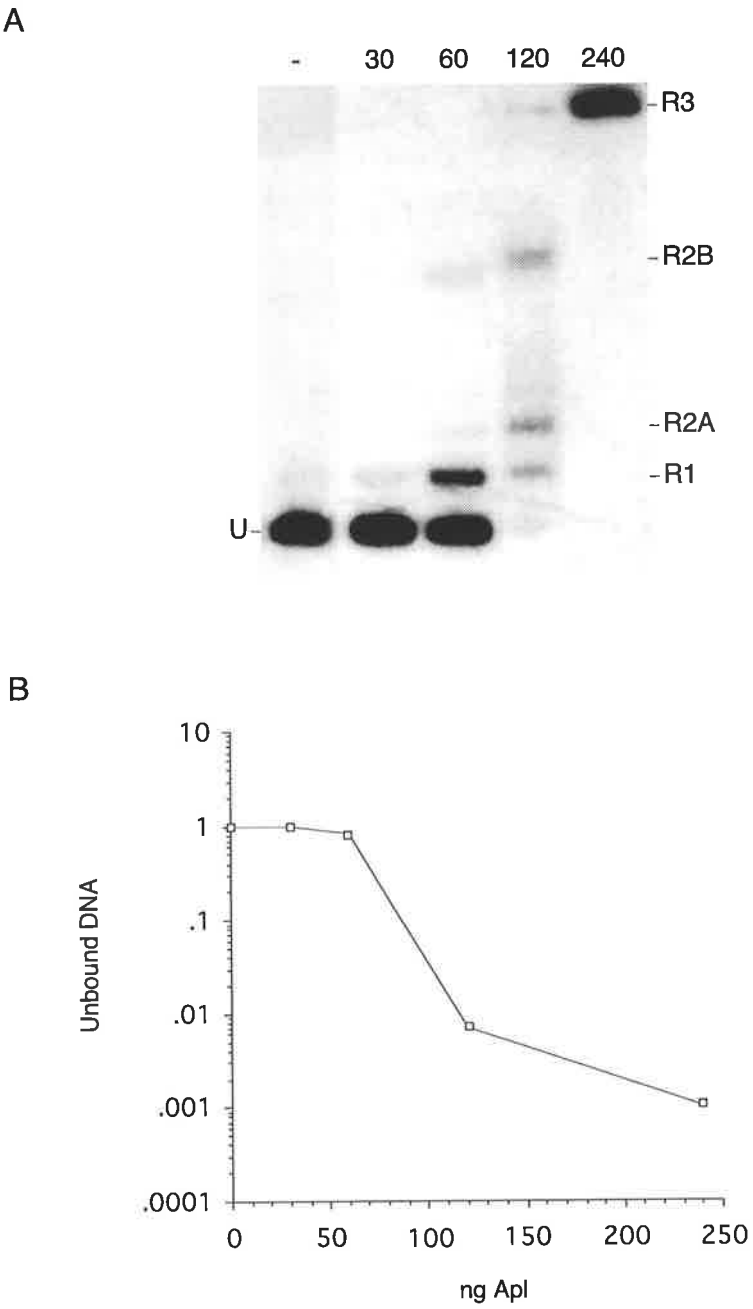


Figure 2.B.4 Purified Apl retardation of the *att*P fragment

A) The gel retardation assay is described in Section 6.7.4. The *att*P fragment was created by kinasing oligonucleotide 63 with ^{32}P-γ-ATP (Section 6.6.12) and using it in a PCR (Section 6.6.15) with oligonucleotide 60 on wild type phage DNA. The product was eluted from a 6% non-denaturing polyacrylamide gel and about 1 nM incubated for 15 min at room temperature with increasing amounts (in ng) of Apl protein. Unbound (U) and retarded (R1-R3) products are shown. B) The graph represents the percentage of unbound probe (determined by scanning laser densitometry) as a function of increasing amounts of Apl.

DNA (as determined by scanning laser densitometry) at each concentration of Apl is plotted in Fig. 2.B.4.B, showing that the interactions occurring in the transition stage were highly cooperative as an eight-fold increase in Apl concentration resulted in an 800-fold increase in the amount of retarded product. A retardation with a P_R-P_L fragment and partially purified Apl was not performed.

2.B.3 DISCUSSION

This Discussion will be broken into two main parts. Both are extensions of similar sections in Chapter 2.A and take into account the added data of this Chapter. The first part will discuss the mechanism of DNA binding by Apl, looking first at DNA bending and the role it may play in Apl action and second at the molecular mechanism of Apl binding. The second part will look at the identification of the Apl recognition sequences. Although significant repeats were predicted in Chapter 2.A, their validity was not known.

2.B.3.1 Structure of the Apl-DNA complex

2.B.3.1.1 The role DNA bending by Apl

The DNase I footprints (Fig. 2.A.2) with Apl extract indicated the DNA was being bent by Apl with the intense nature of the protections and enhancements suggesting that it was a very sharp bend. Confirmation of DNA bending by Apl could be achieved using a circular permutation gel retardation assay (such as the pBend2 system of Kim *et al.*, 1989). This procedure is based on the observation that a DNA fragment containing a bend in its centre will migrate more slowly through a polyacrylamide gel than the same fragment with a bend near its end. By altering the position of the binding site for a DNA bending protein within a DNA fragment, the location and approximate degree of bending induced by that protein can be determined in a gel retardation assay (Thompson & Landy, 1988). A problem with this assay is that a simple bend cannot be distinguished from a more complex structure where the DNA is wrapped 360° around the protein and exits at an angle. Therefore, a nucleosome-like structure which bends the DNA by 460° (*i.e.* wrapped once around a core then exiting at an angle of 100°) will not be distinguished from a simple 100° bend in the DNA. In the case of Apl, if it is assumed that the protein-DNA complex contains a number of Apl binding sites (see below), determining the degree of bending by Apl bound to a single site and multiplying this by the number of sites in the structure would give an estimate of the total bend induced by the complex. However, if the

bend is primarily induced by cooperative interactions between bound Apl proteins, this value would be an underestimate.

If it is assumed that Apl is bending the DNA, what is the role of this in Apl's function? Lambda Xis bends DNA (Bushman *et al.*, 1984) to facilitate competitive and cooperative interactions between other proteins bound to either side of it. Presumably, Apl will play a similar role at the 186 attachment site (see Chapter Three). The role of Apl bending in transcriptional repression of P_R and P_L is not as clear, however. Although repressor proteins often overlap promoters and repress transcription by steric occlusion of the RNA polymerase, some repressors, such as LacI (Straney & Crothers, 1987), KorB (Williams *et al.*, 1993) and GalR (Kuhnke *et al.*, 1989), can bind simultaneously with the polymerase. Repression in these cases occurs by repressor-induced alterations in the DNA structure inhibiting subsequent transcription steps by the RNA polymerase. It is conceivable that Apl is having such an effect, as discussed below.

2.B.3.1.2 The mechanism of DNA binding by Apl

The smeared pattern of retarded P_R-P_L DNA (R2) seen in the transition from the R1 species to the higher order R3 complex of a retardation with the Apl extract (Fig. 2.B.3) indicates that these complexes are unstable. Presumably, such a pattern would also be seen with retardation of an *att*P fragment as well. This instability appears to be a function of the extract, as using partially purified Apl resulted in discrete retarded bands appearing (products R2A and R2B; Fig. 2.B.4.A). If the complexes in this transition stage were less stable than the R1 or R3 complexes, then non-specific competition by host proteins in the extract may enhance this instability, resulting in the smeared pattern. Obviously, this depends on binding by Apl to the *att*P site and the P_R-P_L region being similar, and the similarity of the predicted recognition sequences and the footprints at both sites suggests that this is the case.

Two models for the mechanism of Apl binding were proposed in Chapter Two. The model of a naked loop tethered by interacting Apl molecules at the base of the loop is unlikely, with the retardation of the *att*P fragment by the partially purified Apl providing a strong argument against it. The fact that discrete products were apparent in the transition stage of this retardation indicates that increasing numbers of Apl proteins were binding to a number of sites on the DNA. If a naked loop was being formed, these transition products would not be expected.

The transition products formed with purified Apl also provide a means to look at the steps involved in Apl complex formation. DNase I footprinting was not a sensitive enough probe to examine these steps. Using a methylation interference approach (Wissmann & Hillen, 1991), the purine nucleotides which interact with Apl could be determined. As this technique involves the isolation of DNA fragments from the bound and unbound fractions of a retardation gel, the different transition products seen in a pure Apl retardation could be isolated and the regions of the DNA bound by Apl in each of these complexes determined. If each product represented a single complex and not a family of different complexes, the various Apl-DNA interactions occurring in these complexes could be determined. The centre and degree of bending occurring with each product could also be estimated by performing a circular permutation assay at an appropriate Apl concentration (*e.g.* 120 ng of purified Apl in Fig. 2.B.4). The use of interference techniques is a logical extension of this work as they will not be sensitive to the structural distortions of the DNA by Apl. Another alternative is to use different DNA cutting reagents to examine Apl-DNA interactions. An example of this is the hydroxyl radical (Tullius & Dombroski, 1986), which attacks the DNA at the sugar in the minor groove. The small size of the radical results in a higher resolution footprint than that obtained with DNase I. I performed some preliminary hydroxyl radical studies with the Apl extract but had limited success, presumably due to the high levels of glycerol and other cellular components present in the extract quenching the radicals (data not shown). Use of pure Apl should eliminate these problems.

Another factor to be taken into consideration is the involvement of host factors in cooperative or competitve binding with Apl. For *att*P, this is discussed further in Chapter Three. At the P_R-P_L region, the DNase I enhancements which extend into P_L may be the result of a host protein (possibly RNA polymerase) binding cooperatively with Apl to P_L. This could be analogous to the LacI repressor, which binds cooperatively with RNA polymerase, yet inhibits transcription initiation by altering the DNA structure of the promoter (Straney & Crothers, 1987). It is proposed that this type of arrangement allows the *lac* operon to be switched on rapidly in the presence of inducer. As soon as inducer binds to and inactivates the repressing LacI molecule, the RNA polymerase is ready to transcribe without having to bind to the promoter first, thereby reducing the response time. The benefit of such an arrangement with Apl at P_L is not apparent.

2.B.3.2 Apl recognition sequences

While Apl recognition sequences were predicted in Chapter Two, there was no direct evidence which supported them being the minimal sites for Apl binding. As discussed above, the most likely model for Apl binding is that a number of molecules bind across the region protected in the DNase I footprint. This model for Apl binding implies that Apl will be bound inside the bend; in other words, the DNA will be curved around the outside of the Apl proteins. As Apl contains a HTH DNA-binding motif, it presumably interacts with the DNA similarly to other HTH-containing proteins for which the DNA-protein co-crystal structures have been determined (such as the lambda Cro and *E. coli* CAP proteins; Brennan *et al.*, 1990; Schultz *et al.*, 1991). In these cases, the second α-helix of the motif inserts into the major groove of the DNA and interacts with the bases in the groove, with the particular combination of bases (*i.e.* the nucleotide sequence) providing the specificity for the interaction. Therefore, if Apl is bound to the inside of the bend, then the sequences where Apl binds to the DNA must have the major groove located on the inside of the bend as well. If this is the case, then the minor groove at these sequences must be located on the outside of the bend. As the DNase I enhancements of the Apl footprint are located in the widened minor groove on the outside of the bend, the major groove will be on the inside at these positions. The enhancements occurred within the predicted Apl recognition sequences (Fig. 2.A.3), so the major groove of these sequences will be located on the inside of the bend, supporting them as being sites of Apl binding.

Although the evidence for the predicted Apl recognition sequences is quite strong, the fact that these sequences are not highly conserved at both the P_R-P_L and *att*P regions is concerning. This may reflect the mechanism of Apl binding. It is possible that the cooperative nature of Apl binding negates the requirement for highly specific recognition sequences across the entire binding region. The most conserved sequences between the *att*P and P_R-P_L regions would therefore be located at the initial site of occupancy by Apl (perhaps seen as the R1 product of Fig. 2.B.3 and 2.B.4.A). Such sequences are located at the centre of the footprints at both regions, the same location as the strongest enhancements and protections (possibly the centre of the Apl-induced DNA bend). As the Apl concentration increases, cooperative interactions would result in Apl binding to the weaker adjacent sites, increasing (or creating) the bend in the DNA centred at the initial binding site. This may also explain the extent of the DNase I footprints beyond the predicted recognition sequences, as binding could occur at sequences bearing little similarity to a high affinity Apl site. This binding model is illustrated in Fig. 2.B.5.

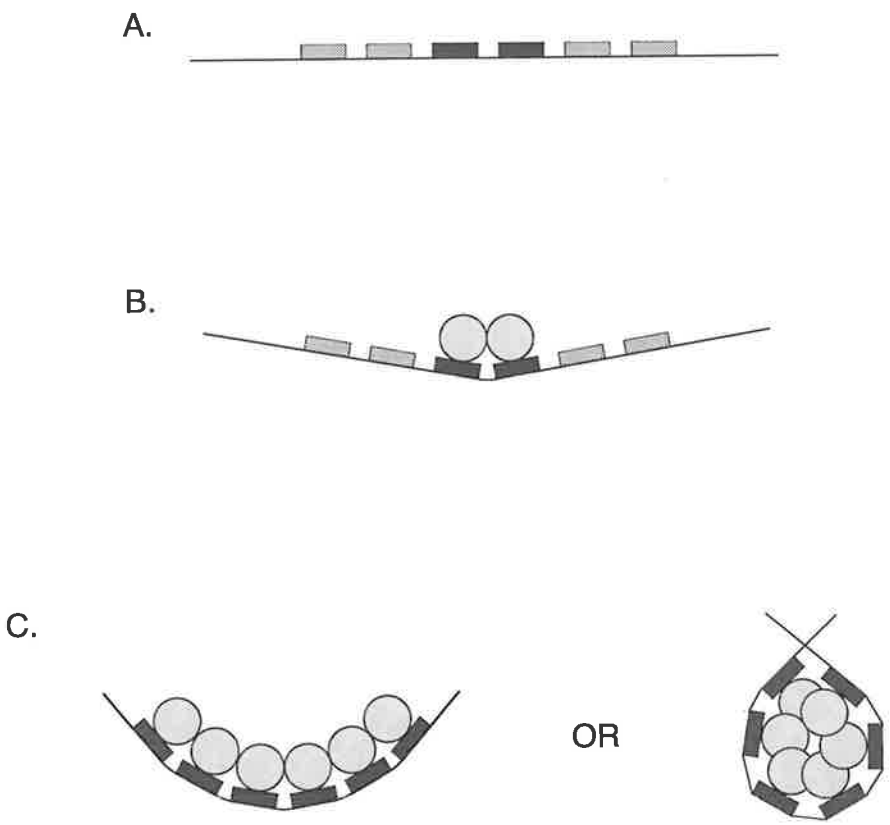


Figure 2.B.5 Model for Apl binding

The Apl bindng region is comprised of a relatively high affinity binding site flanked by lower affinity sites (A). At low concentrations of Apl, the high affinity site is occupied, possibly inducing a slight bend in the DNA (B). At high concentrations of Apl, cooperative interactions with Apl bound at the high affinity site results in the lower affinity sites becoming occupied, inducing a large bend in the DNA or creating a nucleosome-like structure (C). The Apl molecule is represented as a dimer, although this is not known.

Definition of the recognition sequences for Apl binding is obviously going to require alternative techniques to the ones used here. As discussed, the simplest and most obvious is to use methylation protection and interference studies which will probe major groove contacts and will enable the identification of the bases important for Apl recognition and binding. Apl operator mutants, such as those described later in Chapter Five, will also contribute greatly to this pursuit.

2.B.3.3 Conclusions

The use of cleared lysates enriched for a particular protein by overexpression of that protein can always be criticized due to the presence of an excess of other cellular proteins. Even if negative controls are used, it can be argued that any activity is due to the protein of interest combining with other host factors. While most of the studies described in this Chapter are subject to such criticism the use of the purified Apl now available will eliminate these. In saying this, however, it does not seem likely that any of the conclusions made in this Chapter will be seriously challenged because of the use of crude extracts.

Extra experiments, such as methylation interference footprinting and binding studies with operator mutants, are required to determine the bases important for Apl recognition. This information will allow the determination of a single Apl binding site and the number of these sites at each region, and as a result will greatly improve our understanding of the mechanism of Apl binding and action. The resolution of the structure of the Apl-DNA complex is a difficult problem. From this work it seems clear that a number of Apl molecules are binding to and inducing a bend in the DNA, which discounts the possiblity of a naked loop of DNA being created. The role of this structure at *att*P is likely to be analagous to that of lambda Xis (see Chapter Three), however, its significance at the P_R-P_L region is unclear.

CHAPTER THREE

Requirements for directionality in site-specific recombination of coliphage 186: Apl is the excisionase

The work in this Chapter was submitted as part of a paper to the *Journal of Molecular Biology*.

3.1 INTRODUCTION

In the Campbell model, the integration of temperate phage DNA into the host chromosome occurs by site-specific recombination between the phage (*att*P - denoted POP') and bacterial (*att*B - BOB') attachment sites. Each of these sites is comprised of a common sequence (O) flanked by arm sequences specific for each site (P, P', B, B'; Campbell, 1992). Integration generates a composite site at either end of the prophage genome (*att*R - POB' and *att*L - BOP'), comprised of one bacterial arm, the common sequence and one phage arm. Excision of the prophage regenerates *att*P and *att*B. The integration and excision reactions require phage and host functions, with regulation of the phage functions providing directionality to the reaction.

The site-specific recombination system of lambda has been well studied. The expression and function of the proteins involved is tightly regulated, with integration and excision both requiring the phage Int protein and the host integation host factor (IHF; a heterodimer of HimA and HimD subunits), and excision additionally requiring the phage Xis protein (for review see Landy, 1989). Int binds to nine different sites with two different recognition sequences (arm-type and core-type; de Vargas *et al.*, 1988) and catalyzes the DNA strand exchange reaction. It also interacts with the other proteins, both cooperatively and competitively, to regulate the recombination reactions. Xis facilitates excision by interacting cooperatively with Int and assisting DNA loop formation through DNA bending (Nunes-Düby *et al.*, 1989; de Vargas & Landy, 1991). The *E. coli* Fis protein enhances excision at low Xis levels and is probably required for excision *in vivo* (Thompson *et al.*, 1987; Ball & Johnson, 1991a; Ball & Johnson, 1991b). The coordinated control of *int* and *xis* gene expression tightly couples the developmental decision to enter the lysogenic or lytic pathways with the recombinational decision to integrate or excise.

The recombination system of P2 is similar to that of lambda. The P2 Int protein and *E. coli* IHF are required for integration and excision, with the Cox protein providing directionality as the excisionase (Yu & Haggård-Ljungquist, 1993a; Yu & Haggård-Ljungquist, 1993b). In contrast to lambda, however, *E. coli* Fis is not required and does not enhance excision (Yu & Haggård-Ljungquist, 1993a).

Little is known about site specific recombination in 186. Its *int* gene is a member of the integrase family of site-specific recombinases (Argos *et al.*, 1986) and is required for both integration and excision (Bradley *et al.*, 1975). Excision also requires the *apl* gene product (Chapter Two). However, as Apl represses P_L, and therefore *int* transcription and transcription through the phage attachment site (Fig. 3.1), it is possible that the Apl requirement for excision might be indirect, as a result of Apl affecting *int* transcription or transcription through *att*P. The bacterial attachment site for 186 is located in a putative novel isoleucine tRNA gene, closely related to *ile*X, that maps to the 57 min region of the *E. coli* chromosome (Woods & Egan, 1972; Reed *et al.*, submitted; Fig. 3.1 & 3.2.B).

To analyze the directionality determinants for 186 site-specific recombination, an *in vivo* plasmid assay system based on that of Leong *et al.* (1985a) for lambda, was devised for 186. The recombination plasmid (pATT) contains the phage and bacterial attachment sites arranged such that recombination between the two sites inverts the DNA segment between them. The orientation of the inverted segment, and therefore the recombination reaction, can be monitored using two restriction sites, one within the inverted fragment and the other in the uninverted region of the plasmid. This system demonstrated that Apl was playing a direct role in the excision reaction and that the Int expressed during lysogeny may be sufficient for prophage excision. It also showed a partial requirement for IHF in integration, but no role for Fis. Comparison of 186 protein binding sites at *att*P with those of lambda and P2 demonstrated a great deal of similarity and site-specific recombination is predicted to be similar, at least mechanistically, in all three phages.

3.2 RESULTS

3.2.1 Construction of a plasmid for monitoring 186 site-specific recombination *in vivo*

Site-specific recombination with a temperate phage such as 186 results either in the integration of the phage DNA into the host chromosome to form a single DNA molecule, or in excision of the prophage from the host chromosome to give two separate DNA molecules. However, if

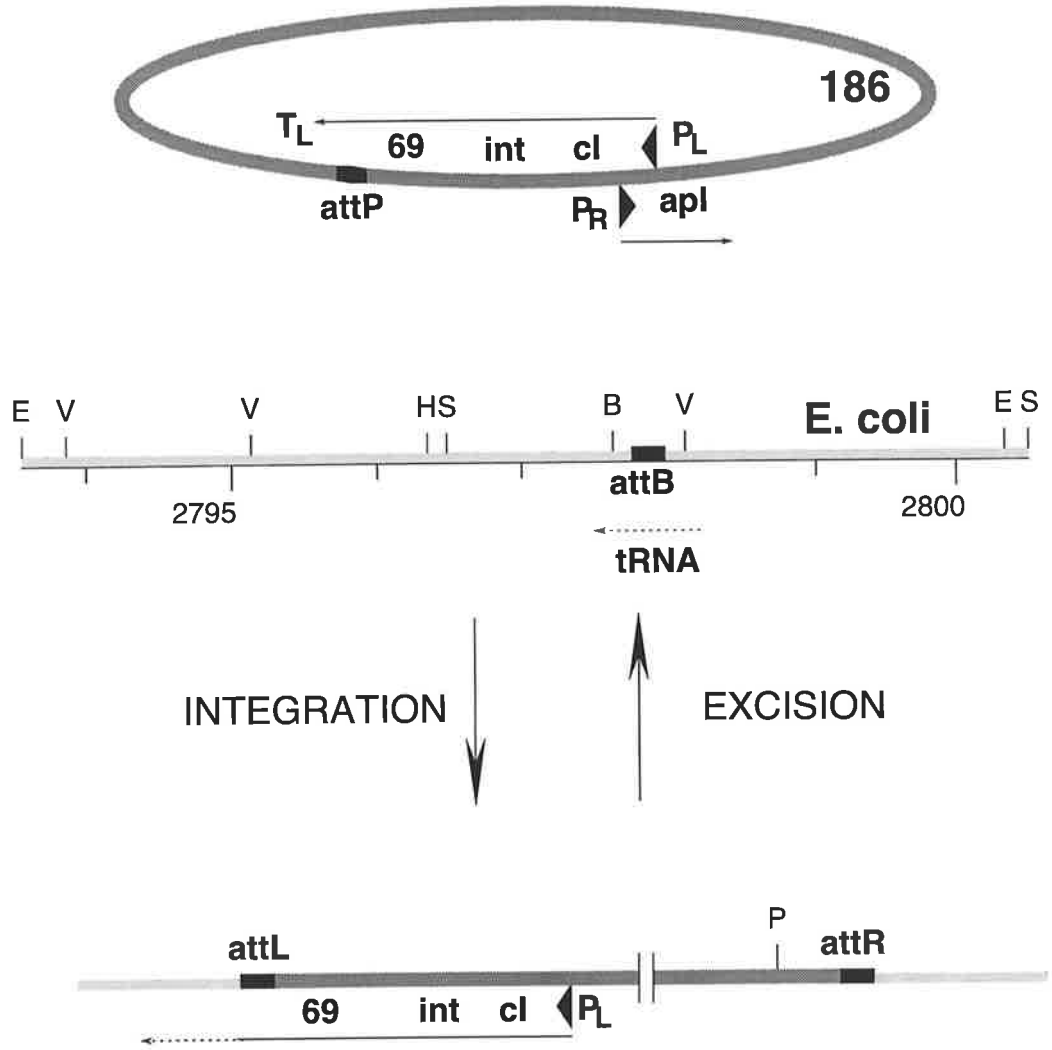


Figure 3.1 Prophage integration/excision and the restriction map of the 186*att*B region

186 integrates by site-specific recombination into a putative isoleucine tRNA gene. The various attachment sites are shown by black boxes. The lysogenic promoter (P_L), the T_L terminator and genes *c*I, *CP69* and *int* , the lytic promoter (P_R) and the *apl* gene, the *Pst*I restriction site at 65.5% (Kalionis *et al.*, 1986a) and Kohara positions across the *att*B region are shown. The P_R promoter is not active in a lysogen and the 3' end of the P_L transcript in a lysogen is not known. E - *Eco*RI; B - *Bgl*I; H - *Hin*dIII; P - *Pst*I; S - *Sac*I; V - *Eco*RV. The Figure is not drawn to scale.

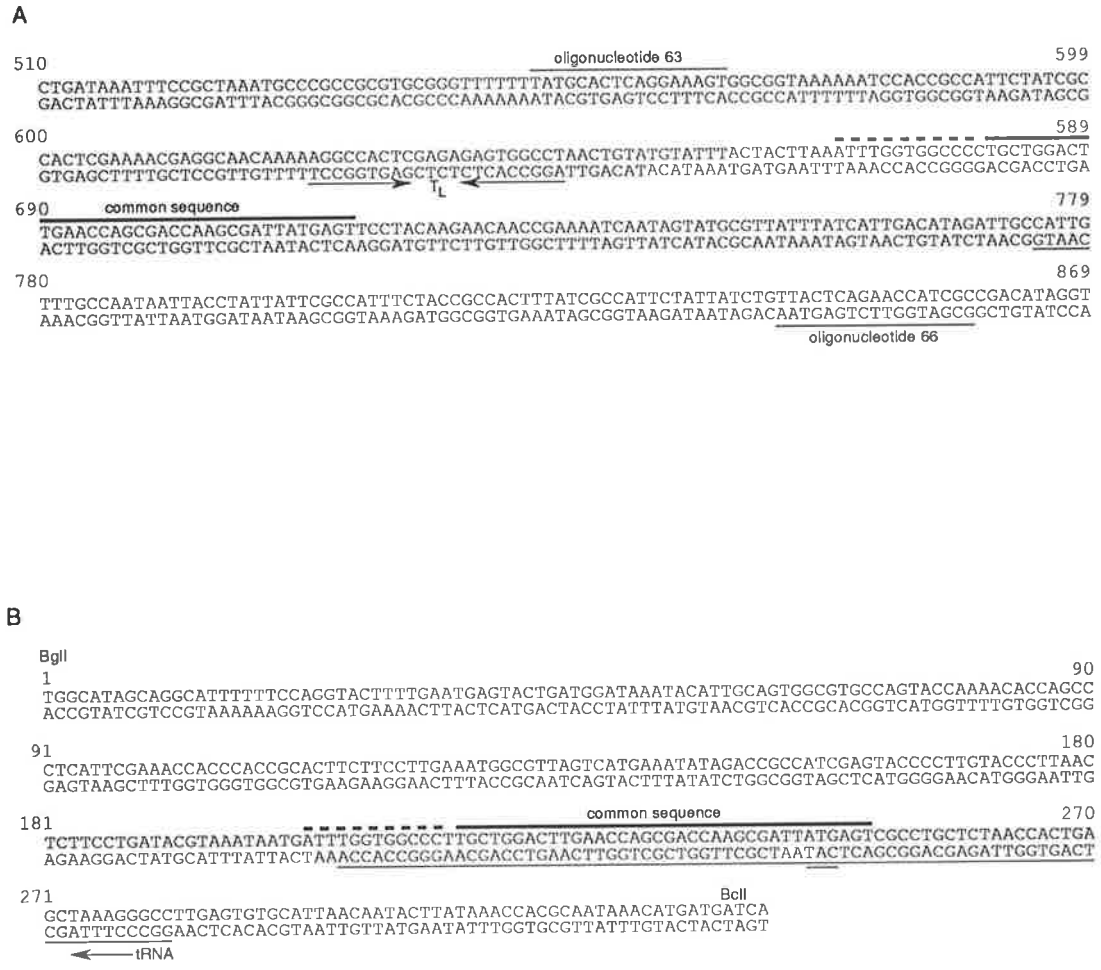


Figure 3.2 Phage and bacterial attachment sites of 186

A) the *att*P common sequence (thick black line) is located 681-716 bp from the *Pst*I site at 65.5% on the 186 map (Kalionis *et al.*, 1986a). A 12 bp extension in identity between *att*P and *att*B following a single bp mismatch is shown as a dashed line to the left of the common sequence. The lysogenic terminator (T_L) is shown, as are the locations of oligonucleotides 63 and 66. B) the *att*B common sequence (thick black line) is located in the 3' half of a putative Ile tRNA gene (underlined). The UAC anticodon is double underlined. The *Bgl*I and *Bcl*I restriction sites are shown. Numbering is from the *Bgl*I site.

*att*P and *att*B (or *att*L and *att*R) are aligned on the same DNA molecule in an inverted orientation, recombination results in the inversion of the DNA fragment between the two sites. This rationale was used to create an *in vivo* plasmid assay system to determine the phage functions required for 186 site-specific recombination. The system is similar to that of Leong *et al* (1985a), but is comprised of two plasmids, one carrying the phage and bacterial attachment sites and the other carrying the *int* and *apl* genes (or *apl*Δ11). This system ensures that expression of the 186 recombination genes (*int* and *apl*) is independent of any transcription across the two attachment sites.

The attachment site plasmid (pATT) is a tetracycline resistant pACYC184 derivative carrying 307 bp *att*P and 333 bp *att*B fragments in inverted orientation with respect to each other (see Fig. 3.3.A). The orientation of this fragment can be monitored using two *Eco*RI restriction sites, one located within the inverted fragment, the other in the vector backbone. Restricting the excised form of the plasmid (pATTx) with *Eco*RI generates fragments of 4041 bp and 457 bp, whereas in its integrated form (pATTi) fragments of 3691 bp and 807 bp are generated. The Int+Apl and Int+AplΔ11 plasmids (pMRR16 and 17) are derived from pMRR13. They have a ColEI origin and carry a β-lactamase gene for selection on ampicillin, so are compatible with pATT. The *int* and *apl* genes are arranged as an IPTG-inducible operon with each gene being translated from a T7 ϕ10 ribosome binding site. This plasmid was created in an attempt to tightly control the expression of both genes, so that the response to increasing levels of expression of the proteins could be determined (primarily when assaying the effects of host factors). With the operon organization of the two genes, there should be similar levels of expression of both.

3.2.2 Apl is directly required for prophage excision

Despite attempts to control Int and Apl expression, recombination was observed in the absence of inducer (IPTG). Induction with 1 mM IPTG increased expression of the two proteins (as determined by SDS-PAGE) but did not alter the outcome of the recombination reactions (data not shown). This inability to control *int* and *apl* expression resulted in the pATT plasmid being exposed to the effects of Int and Apl immediately following transformation and so kinetic studies could not be performed. When pATTx was introduced into C600 pMRR17 (Int+AplΔ11), it was completely converted to the integrated form (Fig. 3.3.B, track 1) and when pATTi was introduced into C600 pMRR16 (Int+Apl), about 80% was converted to the excised form (Fig. 3.3.B, track 2). In the absence of Int, no recombination was seen (data not

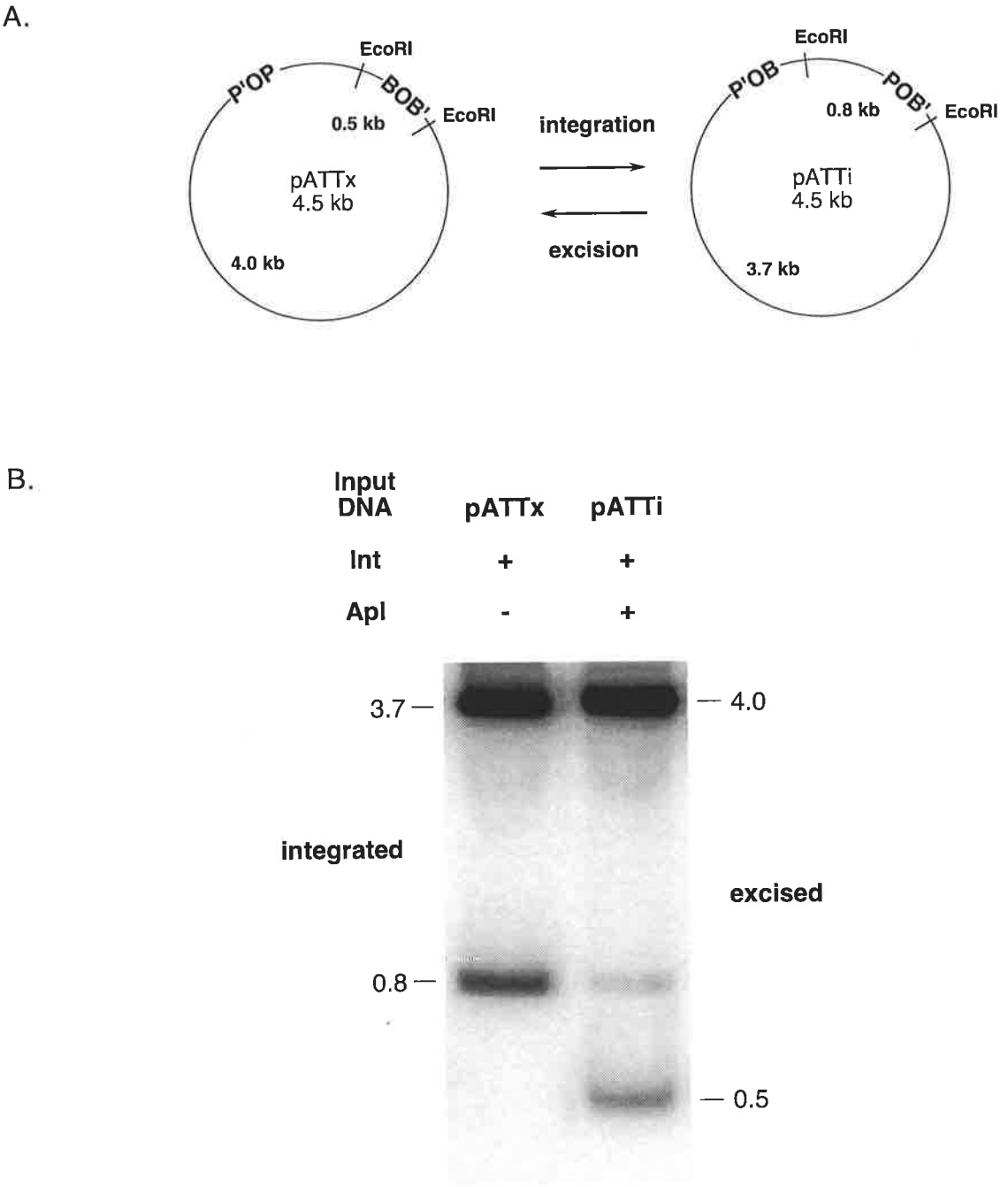


Figure 3.3 Control of directionality in 186 recombination

A) Diagrammatic representation of the plasmid recombination assay, showing the integrated (pATTi) and excised (pATTx) forms of pATT and the sizes of the fragments generated following restriction with *Eco*RI. The Figure is not drawn to scale. B) pATT DNA in either the integrated form (pATTi) or the excised form (pATTx) was used to transform C600 carrying plasmids pMRR16 (Int+Apl) or pMRR17 (Int+AplΔ11; shown as no Apl). Transformants were streaked for single colonies, grown overnight, subcultured into fresh medium then grown to late exponential phase and plasmid DNA isolated. The Figure is a phosphorimage of a Southern hybridization of ^{32}P-labelled pATTx to *Eco*RI restriction digests of the isolated DNA electrophoresed through a 2% TAE-agarose gel (Section 6.6.10.2). The presence of Int and Apl and the initial form of the plasmid (input DNA; pATTx - excised; pATTi - integrated) are shown.

shown). Int+AplΔ11 expression had no effect on pATTi, whereas Int+Apl expression resulted in about 20% of pATTx being converted to the integrated form (data not shown). If pATT (in the presence of Int and Apl) was isolated from early exponential phase cells, less than 5% of the plasmid was in the integrated form (data not shown), suggesting that, as the cells approach stationary phase, the excision reaction is inhibited.

3.2.3 Role of host factors in 186 recombination

The assay system was also used to determine if the *E. coli* host factors IHF and Fis are required for the recombination reaction. This was done by assaying integration and excision of the recombination plasmid in IHF and Fis deficient derivatives of C600 (C600 Δ*him*A::*cat* and C600 Δ*fis*::*Km*). Fig. 3.4 shows that IHF is required for efficient integration but not excision. Tracks 1 and 2 show the integration reactions, starting with pATTx in the presence of pMRR17 (Int+AplΔ11). In the IHF^+ strain, all of the plasmid was converted to the integrated form, but in the IHF^- strain, only about 60% was converted. Tracks 3 and 4, on the other hand, show that the proportion of integrated plasmid converted to the excised form in the presence of pMRR16 (Int+Apl) is the same in the IHF^+ and IHF^- strains (about 80%). Similar experiments with the Fis^- strain showed no effects of Fis on either recombination reaction (data not shown).

3.2.4 IHF binding at 186 attachment sites

Having demonstrated a requirement for IHF in efficient 186 integration, I determined its binding sites at *att*P using DNase I footprinting. Gel retardation assays with pure IHF (data not shown) demonstrated IHF binding to *att*P, with two retarded products being observed, suggesting the possibility of at least two binding sites. A gel retardation assay was also performed on the 333 bp *Bgl*I-*Bcl*I *att*B fragment but IHF binding to this fragment was not detected (data not shown).

DNase I footprinting using pure IHF at *att*P identified two separate IHF binding sites of apparently equal affinity (Fig. 3.5). One was located to the right of the common sequence (H') and overlapped the left edge of the Apl binding region while the second protection was adjacent to the left end of the common sequence (H; see Fig. 3.7).

3.2.5 A 186 lysogen constitutively expresses integrase

Following prophage induction of a 186 lysogen, the expression of *apl* will repress transcription from P_L. However, the P_L operon contains the *int* gene, the product of which is required for excision, so a mechanism must exist to provide Int for an inducing lysogen. One possibility is

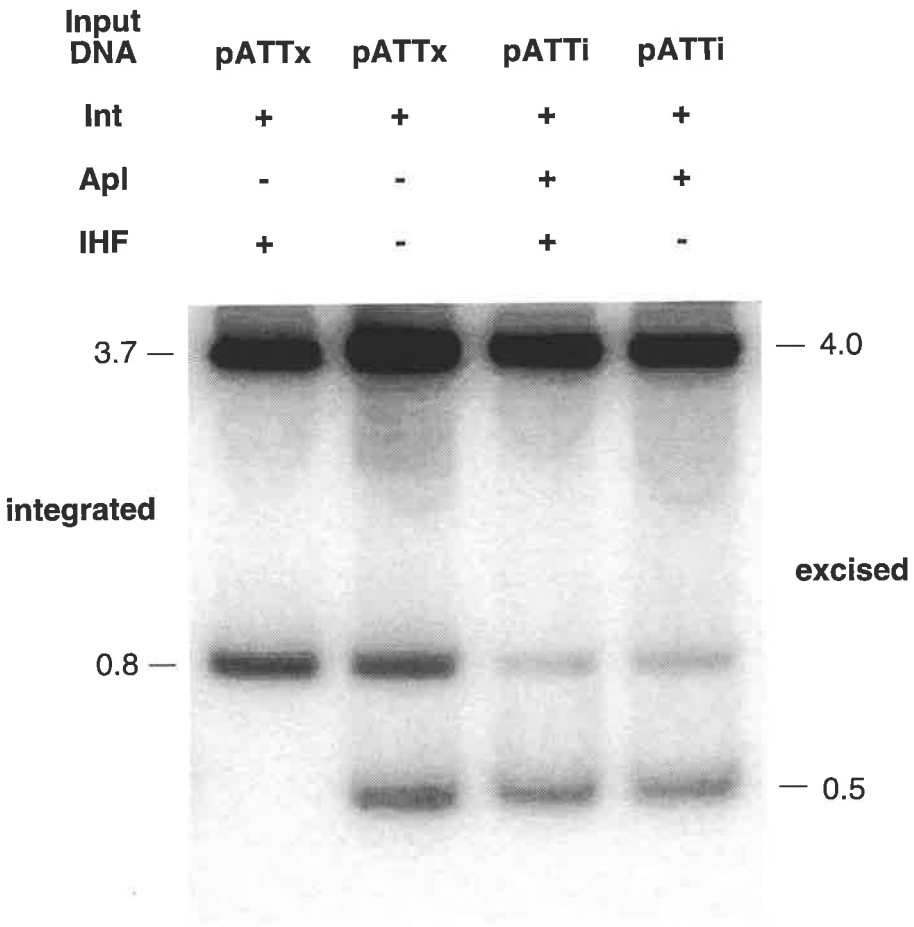


Figure 3.4 Role of IHF in 186 recombination

This experiment is similar to that described in Fig. 3.3.B, except plasmid DNA was isolated from C600 or C600 Δ*him*A::*cat*. The presence of Int, Apl and IHF and the initial form of the plasmid (input DNA; pATTx - excised; pATTi - integrated) is shown.

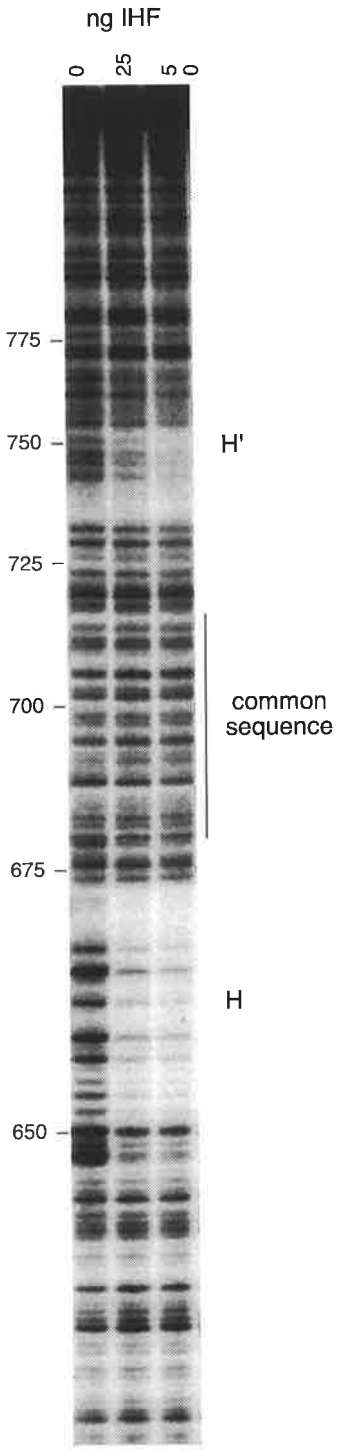


Figure 3.5 DNase I footprint of IHF at 186 *att*P

Singly end-labelled *att*P DNA was prepared by 5' end-labelling oligonucleotide 63 (see Fig. 4.2) with ^{32}P-γ-ATP (Section 6.6.12) and using it in a PCR with oligonucleotide 66 (Section 6.6.15) and the product isolated from a 6% non-denaturing polyacrylamide gel (Section 6.6.8). Increasing amounts of pure IHF were then incubated with the end-labelled fragment before partial DNase I digestion (Section 6.7.5) and electrophoresis through a 6% denaturing polyacrylamide gel (Section 6.6.6.2). Lane 1, DNase I reaction without IHF; lanes 2-3, DNase I reactions with 25 or 50 ng IHF. The common sequence of *att*P and the two IHF binding sites, H and H', are shown. Numbering is from the *Pst*I site at 65.5% (Kalionis *et al.*, 1986a).

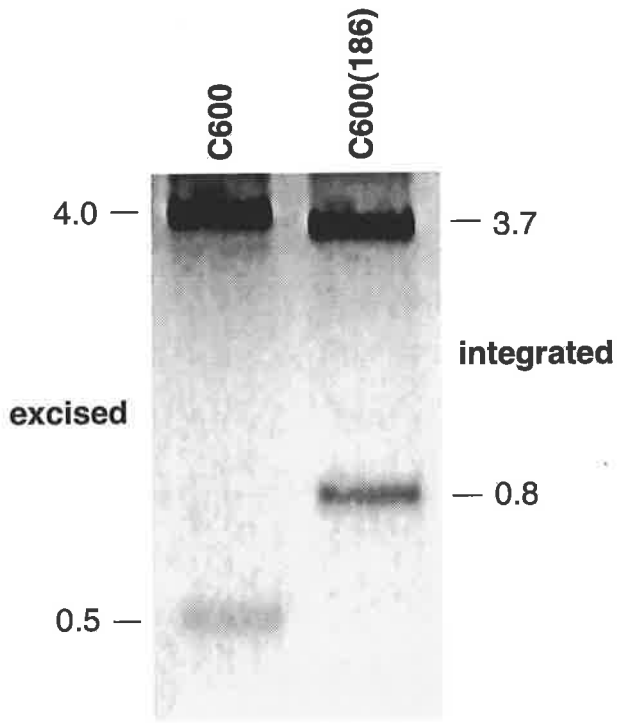


Figure 3.6 A 186 lysogen contains integrase activity

*Eco*RI restriction digests of pATT plasmids isolated from C600 and C600(186) strains were electrophoresed through a 2% TAE-agarose gel and stained with ethidium bromide. The plasmid was initially in the pATTx (excised) form.

that sufficient Int for excision persists from that expressed in the lysogen prior to induction, since the lysogenic transcript from P_L includes *int*. To test for the presence of Int in a lysogen, pATTx was introduced into a wild type 186 lysogen to determine whether integration could occur. *Eco*RI restriction of pATT isolated from an overnight culture of this strain showed that this is indeed the case, with all detectable pATT in the integrated form (Fig. 3.6).

3.3 DISCUSSION

3.3.1 Protein binding sites at *att*B and *att*P and requirements for 186 integration and excision

In lambda, the intasome complex formed during site-specific recombination is comprised of a number of DNA binding proteins contributed by the phage and the host which interact while bound to the attachment sites (Landy, 1989). The phage-encoded Int protein catalyzes the DNA strand exchange reaction and has two distinct DNA binding domains which recognize and bind to different DNA sequences (de Vargas *et al.*, 1988). Core sites are located in the common sequence of the attachment sites and are the sites of strand exchange, while the arm type binding sites are located in the DNA flanking the *att*P common sequence and have a higher affinity for Int than the core sequences (Ross & Landy, 1982; de Vargas *et al.*, 1988). The ability of a single Int protein to bind both sites simultaneously allows for the creation of a protein bridge between attachment sites (de Vargas *et al.*, 1988; Richet *et al.*, 1988; Kim *et al.*, 1990; Kim & Landy, 1992).

The use of the recombination assay confirmed genetic studies which showed that 186 *int* is required for integration and excision (Bradley *et al.*, 1975). Although Int binding sites were not determined, comparison of the location of the Int sites in the P2 *att*P sequence with similar regions in the 186 *att*P sequence resulted in the identification of five highly conserved direct repeats in similar locations (consensus: 5' YAYCGCCAYTY 3'; Fig. 3.7). These sites all had perfect matches to the consensus and no other sequences meeting this criterion were identified in this region. The sites were named P1, P2, P3, P'1 and P'2, respectively, with all except P1 being located on the same (upper) strand. Int core sites were predicted to be 5' YYGCTGG 3', present as inverted repeats separated by 7 bp (C and C' at *att*P and B and B' at *att*B) located at the left end of the common sequence (Fig. 3.7).

Prophage excision in lambda requires Xis (Hoess *et al.*, 1980; Abremski & Gottesman, 1982) which binds between an Int arm site and the core and bends the DNA (Yin *et al.*, 1985). Xis is required to create an excision-competent structure at *att*R, a process which involves

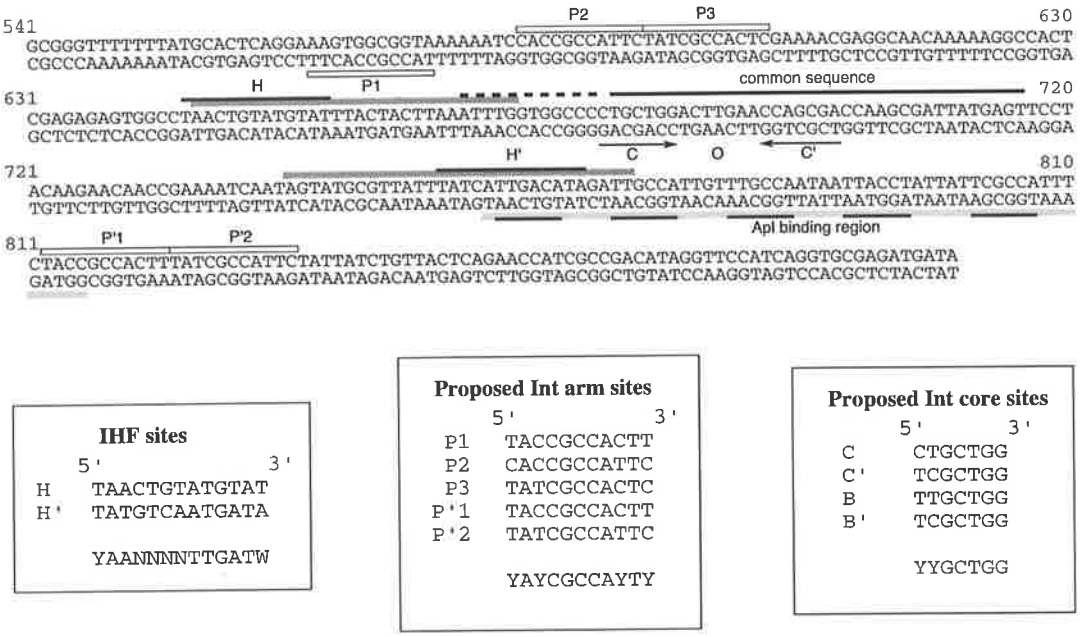


Figure 3.7 Protein binding sites at 186 *att*P

The 186 Apl protein binds 44-99 bp to the right of the common sequence, denoted by the light shaded line, with the predicted Apl recognition sequences shown as darker boxes (Chapter Two). The IHF binding regions (dark shaded lines) and consensus sequences (H, H' - darker boxes) and the predicted Int binding sites and consensus sequences are shown (arm sites - open boxes P1, P2, P3, P'1 and P'2; core sites - arrows C and C'). The common sequence (thick black line) and 12 bp homology (dashed line) are also shown. Numbering is from the *Pst*I site at 65.5% (Kalionis *et al.*, 1986a).

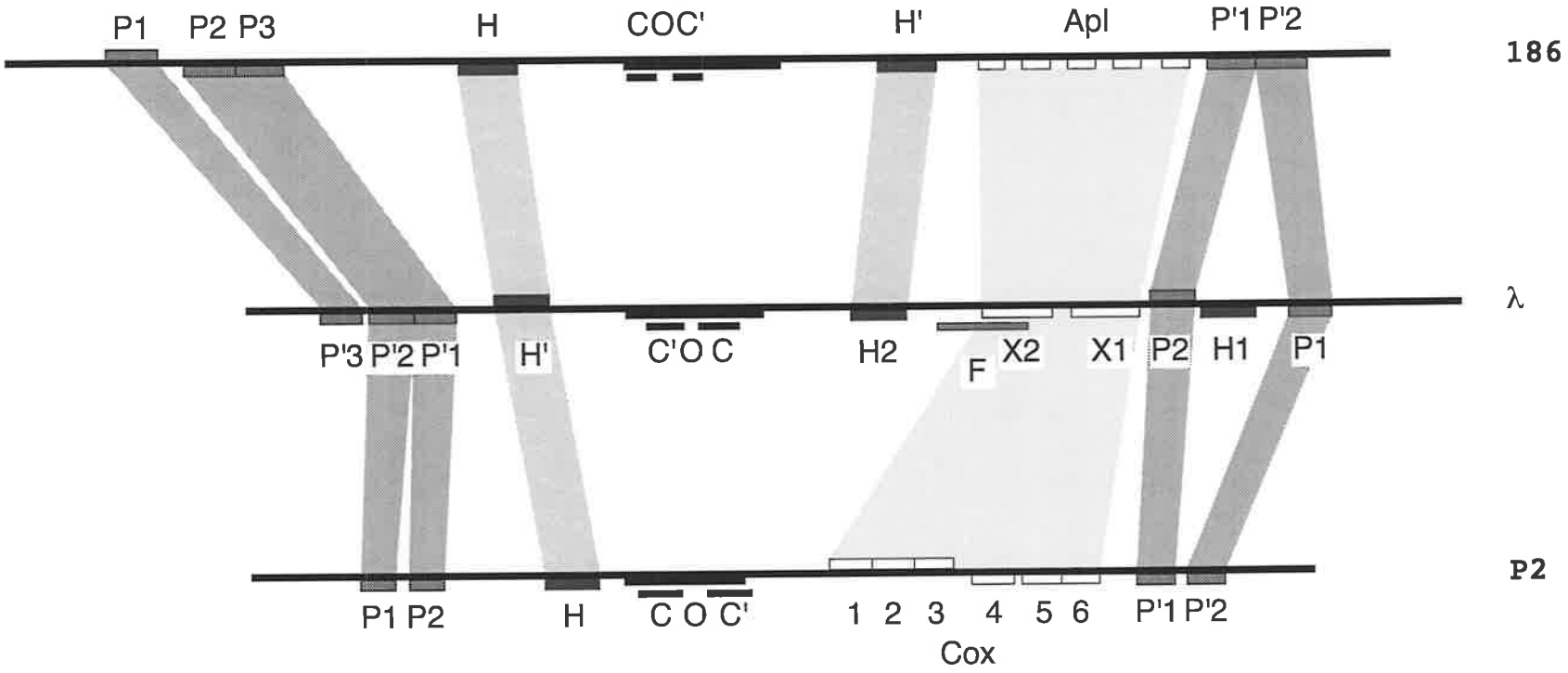


Figure 3.8 Comparison of protein binding sites at 186, lambda and P2 *att*P

The attachment sites from the three phage are aligned according to the location of their excisionase binding sites. The lambda *att*P was inverted so that the P sites are now on the right of the core sequence. The positional conservation of most of the binding sites can clearly be seen. The sites are drawn to scale. 186 Int binding sites are predicted by comparison with P2 Int binding sites. The alternative Cox sites predicted in Chapter Two overlap Cox boxes 4, 5 and 6 (Yu & Haggård-Ljungquist, 1993).

facilitating cooperative interactions between DNA-bound proteins (Nunes-Düby *et al.*, 1989), as well as interacting cooperatively with a neighbouring Int protein bound at the P2 site (see Fig. 3.8; Bushman *et al.*, 1984; Numrych *et al.*, 1992). Xis also inhibits integrative recombination (Nash, 1975; Abremski & Gottesman, 1982). This is due to Xis bending the DNA into an unfavourable conformation (de Vargas & Landy, 1991) and inhibiting Int and IHF binding at P1, P'3 and H1 (Fig. 3.8), all of which are required for integration. Such a control mechanism would prevent integration of the phage DNA following the decision to enter lytic development or during prophage induction.

In recombination, P2 Cox seems to act similarly to that of lambda Xis. It binds between an Int arm site and the core sequence, and, although not demonstrated, Yu & Haggård-Ljungquist (1993a) suggest that Cox bends the DNA. Cooperative interactions between Cox and Int were not shown, although Cox binds adjacent to an Int site (P'1; Fig. 3.8; Yu & Haggård-Ljungquist, 1993a).

The Apl footprint at the 186 attachment site indicates the DNA is being bent by Apl and, as occurs in P2 and lambda, it binds between an (predicted) Int arm site and the core sequence (Fig. 3.7). With the use of the recombination plasmid, I have shown that Apl is acting directly as an excisionase in an event that is independent of transcription of the lysogenic operon. As with P2 Cox, Apl is therefore playing two distinct roles: the role of an excisionase similar to lambda Xis and the role of a repressor similar to lambda Cro.

The host-encoded IHF protein is required for site-specific recombination in a number of temperate phages, including lambda (Gottesman & Abremski, 1980), P2 (Yu & Haggård-Ljungquist, 1993b) and HP1 (Hwang & Scocca, 1990). Replacing lambda *att*P IHF binding sites with intrinsically bent segments of DNA, or with the binding sites for other DNA bending proteins (such as HU or CRP), creates partially functional attachment sites (Goodman & Nash, 1989; Goodman *et al.*, 1992). This indicates that IHF is acting by bending the attachment site DNA to facilitate DNA-bound protein-protein interactions and has little or no direct role in the recombination reaction (de Vargas *et al.*, 1989). IHF is required for both integration and excision of P2 with the one IHF site identified at P2 *att*P being located on the opposite side of the core sequence to the Cox binding sites (Yu & Haggård-Ljungquist, 1993a).

The plasmid recombination assay system demonstrated a partial requirement for IHF in 186 integration. DNase I footprinting using purified IHF identified two binding sites at the phage attachment site, one on either side of the common sequence. These two sites have been

designated H and H'. The site predicted in Chapter Two only partially overlapped the IHF-protected region at H'. H contains a sequence that is a 6/9 match with the IHF consensus binding sequence YAANNNNTTGATW (Leong *et al.*, 1985b), whereas H' contains a 7/9 match to this sequence (Fig. 3.7). Neither site scores well with the Goodrich *et al.* (1990) IHF site weight matrix.

Should the predicted binding sites for 186 Int prove correct, a comparison of the protein binding sites at 186, lambda and P2 attachment sites demonstrates striking similarity between the three systems (Fig. 3.8). The basic structure of having higher affinity Int arm sites flanking the core sites, with the binding sites of DNA bending proteins (such as IHF, Apl, Xis and Cox) being located between these arm sites and the core sites, is basically the same. Kim & Landy (1992) show that, for lambda, the protein-DNA complex at the P' arm of *att*L (comprising one IHF and three Int arm binding sites) is more stable than at *att*R (P), and suggest that the P' arm has a primarily architectural role in the structure of the recombination complex. This non-excisionase arm of the three phage is similar, suggesting that such an architectural role may be common to them all. The most striking difference between the three attachment sites occurs at the excisionase arm, which Kim & Landy (1992) suggest is less stable than the other arm and has a regulatory role in the recombination reaction, controlled by the relative concentrations of the proteins that bind to it (Int, Xis, IHF and Fis). Interaction of the two arms would form a stable complex competent for recombination. The paucity of binding sites in the excisionase arm of the 186 and P2 attachment sites suggests that the regulation of the recombination reactions in these phage may be less complex than that of lambda. Despite this, it seems clear that while the regulation of the recombination reactions in the three phage may be different, the basic recombination mechanism will be similar and share common themes.

3.3.2 Apl as the coordinator of transcriptional and recombinational switches during 186 prophage induction

Prophage induction requires the coordination of the lysis-lysogeny transcriptional switch and the integration-excision recombinational switch. The derepression of lytic transcription must coincide with excision of the prophage from the host chromosome. 186 has developed a simple mechanism for such coordination by utilizing the product of the *apl* gene, the first gene of the lytic operon, both as a Cro-like repressor and as an excisionase. Derepression of the lytic operon in a prophage produces Apl which prevents further production of CI from the lysogenic

operon by repressing P_L and also acts at the prophage attachment site, in conjunction with Int, to excise the prophage. A potential problem with this model is that Int is required for excision but its transcription is repressed by Apl. Introducing the recombination plasmid to a 186 lysogen demonstrated that sufficient Int was present for integration. As Int would be required for excision for only a short period following derepression, it seems likely that the Int persisting in the induced lysogen would be sufficient.

P2 has a similar genetic arrangement to that of 186. Despite this, 186 induces efficiently whereas P2 prophage induction is very inefficient. Temperature induction of a P2 lysogen with a temperature sensitive repressor results in less than 1% of lysogens producing phage (Bertani, 1968). Supply of Int to a prophage overcomes this defect (Bertani, 1970; Ljungquist & Bertani, 1983), so it appears that Int levels are too low for excision during prophage induction. In contrast to 186, the amount of Int activity in a non-induced P2 lysogen is insufficient for integration (Bertani, 1970). Therefore, the efficient excision of 186 and the poor excision of P2 may be due simply to a difference in steady-state levels of Int activity in the lysogen. Int expression is regulated in a complex manner involving transcription termination, inhibition of translation initiation by secondary structure in the mRNA and posttranscriptional autoregulation by the Int protein (Yu *et al.*, 1994). These factors presumably all contribute to the low levels of Int in a P2 lysogen. A mutation altering the eighth amino acid of Int (*nip*1), which improves P2 excision (Calendar *et al.*, 1972; Bertani, 1980), appears to act by altering the mRNA structure of *int*, thereby reducing the inhibition of translation initiation (Yu *et al.*, 1994).

3.3.3 Conclusions

While the arrangement of protein binding sites at the attachment sites of lambda, P2 and 186 is similar, for 186 and P2 it is less complex, as there are fewer IHF and no Fis binding sites. This simplicity is also reflected in the arrangement of the phage genes used in the recombination reactions. The complex genetic arrangement of such genes in lambda requires complex control mechanisms to ensure the correct recombination genes are expressed at each stage of development. The coordination of recombination with development is greatly simplified in 186 (and P2) by having the *int* and immunity repressor genes expressed from the lysogenic transcript and by having Apl (and Cox) combine the roles of excisionase and Cro-like repressor.

CHAPTER FOUR

Characterization of the role and mechanism of Apl repression of P_R and P_L

4.1 INTRODUCTION

4.1.1 The role of Cro-like repressors in phage development

The genetic arrangement and development of most temperate phages adheres to a similar format. The decision to enter lytic or lysogenic modes of development is basically a choice between transcription from one of two promoters. If lysogeny is chosen, the phage will integrate into the host chromosome and a repressor will inhibit lytic transcription. If lytic development is chosen, a cascade of highly coordinated gene expression events results in phage DNA replication, structural protein synthesis, phage assembly and host cell lysis. During prophage induction (the transition from lysogenic to lytic development), a lytic gene represses lysogenic transcription, thus enforcing the decision to enter lytic development. In most cases, at higher concentrations, this repressor also blocks lytic transcription. In lambda, this repressor is called Cro (Eisen *et al.*, 1970), in P2 Cox (Lindahl & Sunshine, 1972), in Mu Ner (Wijffelman & van de Putte, 1974) and in 186 Apl (Dodd *et al.*, 1990).

Common features of these repressors include their location as the first gene of the lytic transcript, their possession of a HTH DNA binding motif, their small size and their ability to repress both lytic and lysogenic transcription. Despite these similarities, the functions these repressors have evolved to perform are quite diverse, due to the different gene control strategies employed by each phage.

4.1.1.1 Lambda Cro

Lambda Cro is the paradigm for such repressors as it has been the most extensively studied. It binds to both the right (O_R) and left (O_L) operators of the lambda genome, where it represses transcription from three promoters (Gussin *et al.*, 1983). Although binding to O_L, and subsequent repression of the lytic P_L promoter, is important for lytic development, for the

purposes of this discussion I will focus primarily on the role of Cro binding to O_R. O_R is located between the divergent P_R and P_{RM} promoters and is comprised of three protein binding sites (O_R1, O_R2 and O_R3), each recognized by Cro and the CI repressor (see Fig. 4.1.A). At low concentrations, Cro occupies O_R3 and represses transcription from the lysogenic P_{RM} promoter (which transcribes *c*I), but at higher concentrations, O_R2 and O_R1 are occupied to repress lytic transcription from P_R (which transcribes *cro*; Meyer *et al.*, 1980). On the other hand, in a lysogen, CI occupies O_R1 and O_R2 cooperatively and coordinately to repress P_R (Meyer *et al.*, 1980), with occupation of O_R2 stimulating P_{RM} transcription (Meyer & Ptashne, 1980). If CI levels increase, O_R3 becomes occupied and represses P_{RM} (Meyer *et al.*, 1980).

The role of Cro binding to O_R3 (and hence P_{RM} repression) is required for efficient prophage induction. Johnson *et al.* (1981) state that a prophage, carrying an O_R3 mutation that blocks Cro binding, induces poorly in response to UV induction. Presumably, this is due to continued expression of the P_{RM} operon which would contribute new repressor to the depleted pool in the induced lysogen (RecA cleavage of CI following UV induction is rarely complete; Bailone *et al.*, 1979), which then inhibits lytic transcription and hence lytic development. Furthermore, ectopic expression of Cro in a lambda lysogen induces the prophage. This effect is dependant on an intact O_R3 and is explained by Cro repressing P_{RM} resulting in reduction of CI levels and subsequent derepression (Johnson *et al.*, 1981).

In addition to its role in derepression, Cro has an essential role in lytic development. This requirement is complex and not completely understood and requires either fully active or fully inactive CI repressor.

In **cI$^+$cro$^-$** conditions, Eisen *et al.* (1970) concluded that the inability of the phage to develop was due to the inability of Cro to repress P_{RM}, with the resulting increase in "maintenance" expression of CI channeling all of the infecting phage into lysogeny. However, subsequent studies (Galland *et al.*, 1975; Folkmanis *et al*, 1977; Jones & Herskowitz, 1978) showed that this assumption was incorrect and that the inability of the phage to develop was primarily due to excess production of CII from the P_R promoter. This excess CII would produce high levels of CI repressor, by activating P_{RE}, which would block lytic development.

Jones & Herskowitz (1978) tested the frequencies of lysogenization of λ^+, λcro^- and $\lambda cro^- cII^-$ phage and showed that a λcro^- phage lysogenized about 10-fold more efficiently than wild type (which was 6.8%). This increase was *c*II dependent, as the $\lambda cro^- cII^-$ phage

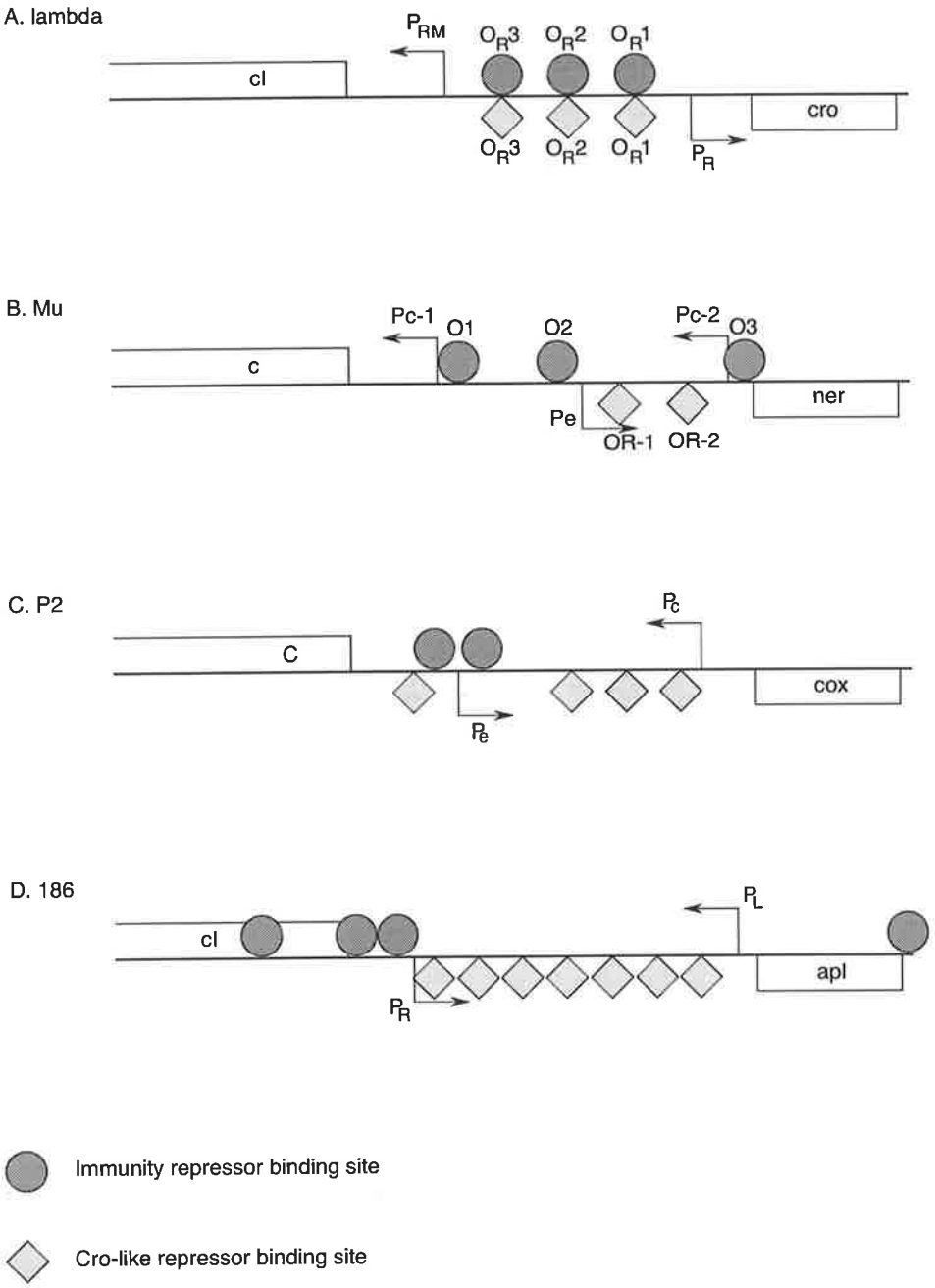


Figure 4.1 Transcriptional switch regions of four phage

Diagrammatic representation of the lysis-lysogeny switch regions of lambda (A), Mu (B), P2 (C) and 186 (D). The various promoters and the binding sites for the immunity and Cro-like repressors are shown.

lysogenized at a frequency about 30-fold less than wild type (0.22%). Therefore, the *c*I$^+$*cro*$^-$ phenotype is *c*II-dependent.

Contrary to the studies of Jones & Herskowitz (1978), it was found by Galland *et al.* (1975) and Folkmanis *et al.* (1977) that a *cro*$^-$ phage did not increase the frequency of lysogenization. The reason for this difference is not clear and is not discussed by Jones & Herskowitz (1978). The inability of the *cro*$^-$ phage to form a plaque in these studies appeared to be due to a failure in lytic development at a stage where the alternative state of lysogeny could not be established. In other words, the expression of CII in a *cro*$^-$ phage does not build up to active levels until late in infection. The subsequent activation of *c*I (and *int*) expression, although inhibitory to lytic development, is unable to establish a stable lysogen. Folkmanis *et al.* (1977) suggest this may be due to the phage DNA undergoing rolling-circle replication which could inhibit prophage integration.

In ***c*I$^-$*cro*$^-$** conditions, lytic development is inhibited by a phenomenon called the Tro effect, which is comprised of two components (Eisen *et al.*, 1975; Folkmanis *et al.*, 1977; Georgiou *et al.*, 1979). The first acts *in trans* to inhibit host cell DNA, RNA and protein synthesis, and results from overexpression of the *ssb* and *N* genes, both expressed from P_L (Georgiou *et al.*, 1979; Court & Oppenheim, 1983). The other component acts *in cis* to inhibit phage growth, suggested to be due to overexpression of the P_R operon (Georgiou *et al.*, 1979). The *cro*$^-$ effects are rescued by intermediate levels of CI (*i.e.* λ*c*I857ts grown at 37-40°C), which partially represses P_R and P_L to levels which still allow lytic development (Folkmanis *et al.*, 1977).

4.1.1.2 Non-lambdoid phage

The transcriptional lysis-lysogeny switches of Mu, P2 and 186 differ from that of lambda in that the lytic and lysogenic promoters are face-to-face and produce transcripts which overlap by 30-60 nucleotides. This adds another level of complexity to the control of the two promoters. Converging transcription means that each promoter can exert a direct effect on the other and independent control of one promoter by a repressor protein must be maintained as transcription from the other proceeds through the repressor binding site. As well as the face-to-face arrangement of the two promoters, the mechanism of their control by the Cro-like and immunity repressors is also different. Unlike lambda, the two repressors recognize separate operator sequences and in 186 and P2, the Cro-like repressors have another role as the phage excisionase (Chapter Four; Yu & Haggård-Ljungquist, 1993a).

Mu Ner. The transcriptional switch of Mu is comprised of the face-to-face Pe (lytic) and Pc-2 (lysogenic) promoters, which produce transcripts overlapping by 36 nucleotides (Fig. 4.1.B). The C immunity repressor is also transcribed from a promoter about 200 bp downstream of Pc-2, called Pc-1, although the role of this promoter is not known as it is much weaker than Pc-2. C binds to three operator sites O1, O2 and O3, which overlap the Pc-1, Pe and Pc-2 promoters, respectively, with O1 and O2 being occupied first (Krause & Higgins, 1986). The Mu Ner protein binds to two adjacent sites, OR-1 and OR-2, located between the Pe and Pc-2 promoters (Tolias & Dubow, 1986). These sites slightly overlap the C binding sites O2 and O3, respectively, suggesting the two proteins may compete for binding.

Ner represses transcription from both Pe (Wijffelman & van de Putte, 1974) and Pc-2 (van Leerdam *et al.*, 1982), with overexpression blocking Mu infection. This was due to repression of early transcription from Pe and spontaneous phage mutants which overcome this inhibition were isolated and found to map in OR-1 (van Leerdam *et al.*, 1982; Goosen & van de Putte, 1984), suggesting they are Ner operator mutants. As with lambda Cro, Ner is essential for lytic development, as a *ner* frameshift mutation is lethal. Deletion of the Pc-2 promoter suppresses this effect, although a frameshift mutation in the *C* gene did not, suggesting that it is the unrepressed activity of the Pc-2 promoter which interferes with phage development (Goosen & van de Putte, 1986).

P2 Cox. The genetic arrangement of the P2 transcriptional switch is very similar to that of 186 (Fig. 4.1.C). The face-to-face P_e (lytic) and P_c (lysogenic) promoters produce transcripts which overlap by about 30 bp. Expression from P_c produces the C repressor and the integrase. The C protein binds to two direct repeats at the P_e promoter (Saha *et al.*, 1987a). At low levels it represses P_e and activates P_c (probably indirectly, by reducing transcriptional interference from P_e, and at high levels it represses P_c (Saha *et al.*, 1987a). P2 Cox is transcribed from P_e and binds to the region between P_e and P_c (Saha *et al.*, 1989) and at the phage attachment site (Yu & Haggård-Ljungquist, 1993a). Similar to Apl, Cox is directly required for prophage excision (Yu & Haggård-Ljungquist, 1993a) and is a repressor of lytic and lysogenic transcription (Saha *et al.*, 1987b). Operator mutants (*cor*) which inhibit Cox repression of lytic transcription in P2 virulent deletion mutants have been isolated (de Vries *et al.*, 1991) and map to the binding sequences predicted by (Saha *et al.*, 1989) and also to those predicted in Chapter Two.

As with 186 Apl, mutations in the *cox* gene have no effect on lytic development (Lindahl & Sunshine, 1972). However, the frequency of lysogenization of a *cox* mutant was found by Bertani (1980) to be increased, although Lindahl & Sunshine (1972) found no such effect. The expression of Cox from a plasmid in a P2 lysogen increases spontaneous induction, similarly to lambda Cro, so, as expected, Cox is able to derepress a lysogen (Saha *et al.*, 1987b).

4.1.2 The role of Apl in 186 development

As described in Chapter One the 186 transcriptional switch is comprised of two face-to-face promoters producing transcripts which overlap by 60 bp (Fig. 4.1.D). Convergent transcription from the 10-fold stronger lytic P_R promoter appears to inhibit transcription from the lysogenic P_L promoter by about 90% (Dodd *et al.*, 1990). In addition to this control, the first gene of each transcript is a transcriptional repressor which controls transcription from both promoters. The *c*I gene is the first of the P_L operon and its product, the CI immunity repressor, blocks transcription from the lytic promoter, P_R, in a lysogen (Dodd *et al.*, 1990). It also increases P_L acitivity, presumably as a result of the decrease in transcriptional interference from P_R, and hence effectively enhances its own expression. The first gene of the lytic operon is *apl*. Apl binds to the region between the P_R and P_L promoters with no obvious preference for binding to either (Chapter Two). This is inconsistent with the *gal*K reporter studies of Dodd *et al.* (1990), which indicated that P_L was more sensitive to Apl repression than P_R. Such differential repression is seen with the lambda Cro repressor at the lambda P_R and P_{RM} transcriptional switch (Meyer *et al.*, 1980). In the case of Cro, however, this difference is the result of the differential affinity of Cro for its binding sites at either promoter. To resolve this apparent inconsistency, I developed a *lac*Z reporter system and assayed P_R and P_L activity in the presence of increasing amounts of Apl supplied *in trans* from an IPTG-inducible expression plasmid. These studies showed that, as the footprint suggested, P_R and P_L were repressed equivalently, although P_R remained about 100-fold stronger. It seems likely that the *gal*K result (Dodd *et al.*, 1990) was spurious.

Understanding the roles of Apl repression of P_R and P_L in 186 development using the 186*apl*Δ11 phage was complicated by the dual functions of Apl as a repressor and an excisionase (Chapter Two); especially during prophage induction where Apl is having its greatest effect. Separation of the excision and repression functions was therefore required. To do this I isolated operator mutants which blocked Apl repression of either or both promoters. The fact that these mutants could be isolated meant that, despite the equivalent repression of P_R

and P_L by Apl, there are DNA determinants specific for repression of each promoter. In these mutants, binding of Apl to the attachment site, and thus excision, should be unaffected. Similarly to lambda Cro, Apl repression of lysogenic transcription (from P_L) was found to be required for efficient prophage induction, presumably by aiding derepression. The inability of Apl to repress P_R resulted in an increase in lytic burst size similar to that seen with the *apl*Δ11 phage (Chapter Two). The significance of these roles to 186 development is further discussed.

4.2 RESULTS

4.2.1 Apl repression of P_R and P_L

4.2.1.1 Plaque morphology of 186 with increasing Apl concentration

Plating phage lambda on indicator strains expressing various levels of lambda Cro results in different plaque phenotypes depending on the concentration of Cro (Roberts *et al.*, 1979; Meyer *et al.*, 1980). At low levels (with little or no Cro-mediated repression), the plaques are turbid. At intermediate levels (where P_{RM}, but not P_R, is repressed; the "anti-immune" phenotype), the plaques are clear, as repressor can not be expressed in a lysogen (Eisen *et al.*, 1970; Calef *et al.*, 1971). At high levels (with both P_{RM} and P_R repressed), no plaques form, as lytic transcription is repressed (Roberts *et al.*, 1979). A similar experiment was performed with 186 and Apl. The *gal*K results of Dodd *et al.* (1990), which indicated that P_L was more sensitive to Apl repression than P_R, led me to believe that 186 would behave similarly to lambda, with a turbid-clear-no plaque progression with increasing Apl. It was possible that the Apl footprint did not reflect the *in vivo* situation, where other (host?) factors could result in P_L being preferentially repressed. The role of Apl as an excisionase would presumably enhance a clear plaque phenotype.

C600 cells containing pMRR20 (a tightly controlled IPTG-inducible Apl expression vector), grown to mid-logarithmic phase in TB medium supplemented with various concentrations of IPTG (0, 0.2, 0.4, 0.6, 0.8 and 1 mM), were used as indicator bacteria. About 200 186^+ phage were mixed with these bacteria, plated on TB plates containing the appropriate concentration of IPTG and incubated overnight at 37°C. Plaques were always turbid, but at 0.8 mM IPTG they became smaller. At 1 mM IPTG, infection was blocked. Surprisingly, no transition through a clear plaque stage was observed with increasing IPTG. Therefore, there was no evidence of preferential P_L repression by Apl which necessitated a reexamination of Apl's repression characteristics.

4.2.1.2 Reporter studies on Apl repression of P_R and P_L

Initially, the promoters were assayed as multicopy plasmid constructs, but the β-galactosidase units from these strains varied substantially from day to day. It was found that when present as single-copy chromosomal constructs, this variation was reduced dramatically. Therefore, single-copy derivatives of the plasmid constructs were used in these assays. Interestingly, it was found that the multicopy constructs were more sensitive to Apl repression. The reason for this is unclear, but is presumably due to nature of the reporter DNA (plasmid versus chromosomal) or that the chromosomal insertion site of the constructs is, for some reason, less accessible to Apl than a plasmid.

Single-copy P_R-*lac*Z and P_L-*lac*Z reporter constructs were created and assayed for β-galactosidase activity in the presence of Apl. Cultures were grown in M9CAA medium and Apl expression induced with IPTG from pMRR20 (the Apl expression plasmid used in the plating assays above). The stringent control of Apl expression in this system was demonstrable, as no Apl-dependent repression of either P_R or P_L was detectable at low (≤ 0.01 mM) concentrations of IPTG. By increasing the amount of IPTG and assaying for β-galactosidase expression, the response of each promoter to increasing amounts of Apl was determined. The assays were performed with the *Eco*RV.2593-*Pvu*II (*apl*Δ11).2888 fragment.

Figure 4.2 shows the results of these experiments. Each data point represents the average of six independent assays performed on the same day, with the standard deviations not extending beyond the data points. The top and bottom curves represent the activity of P_R and P_L, respectively, in the presence of the other promoter. P_R activity is about two orders of magnitude greater than that of P_L at all Apl concentrations. The most striking feature of these curves is that P_R and P_L are repressed similarly by Apl. In other words, in contrast to lambda Cro and the results of Dodd *et al.* (1990), Apl does not preferentially repress P_L over P_R, which agrees with the footprinting and plaque morphology results. If anything, P_R is repressed to a greater extent than P_L, although the absolute activity of P_R is much greater. To obtain an accurate picture of the sensitivity of both promoters to Apl repression, the concentration of Apl required to cause 50% of maximal repression needs to be determined. This cannot be done with this data as the maximally repressed activity of both promoters was not determined. To do this, another (more active) Apl expression plasmid would need to be used. Nevertheless, at the highest concentration of Apl used, P_R was repressed by about 95% and P_L by about 85%

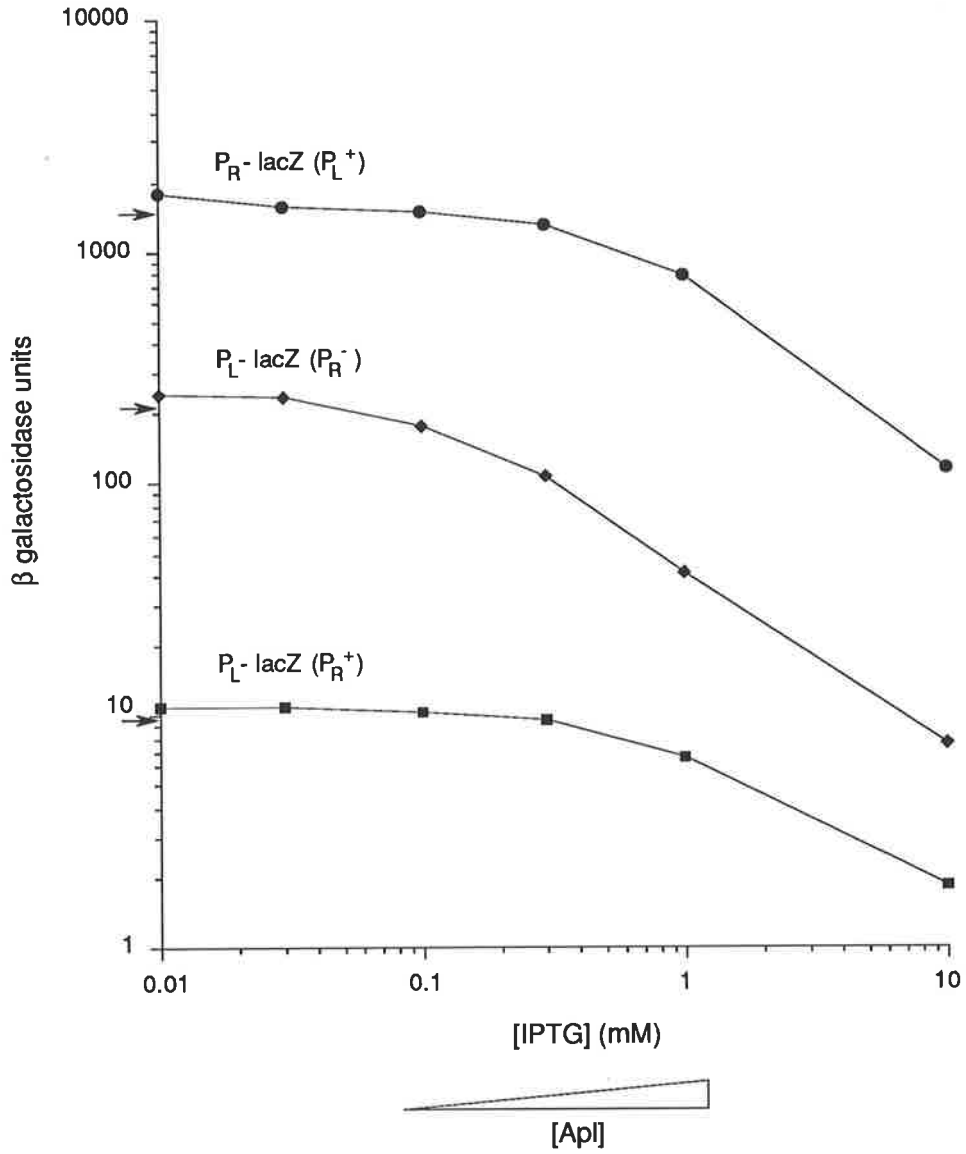


Figure 4.2 P_R and P_L activity as a function of Apl concentration

The single copy constructs used in this experiment were MC1061.5 lysogens of λMRR1 (P_R-lacZ P_L^+), λMRR2 (P_L-lacZ P_R^-) and λMRR3 (P_L-lacZ P_R^+). Apl was supplied from pMRR20 and its expression induced with IPTG. The increasing levels of IPTG reflect increasing amounts of Apl. Overnight cultures grown in M9CAA and 50 μg ml^{-1} kanamycin were diluted 10^{-3} into the same medium containing various concentrations of IPTG. These cultures were incubated at 37°C with shaking until an A_{600} of 0.2-0.6 was reached and the β-galactosidase levels determined (Section 6.7.6.3). The basal levels of transcription of each promoter (*i.e.* transcription at 0 mM IPTG) are indicated by arrows on the y axis. Each data point represents an average of at least six independent assays with the standard deviations not extending beyond the data points.

As already mentioned, it was suggested by Dodd *et al.* (1990) that P_R has a large inhibitory effect on P_L activity. However, this experiment was not properly controlled, as the comparison between P_L in the presence and absence of P_R was determined by removing a DNA fragment containing P_R. Therefore, the inhibitory effect was a function located on the removed DNA fragment, but not necessarily P_R. I therefore assayed P_L activity from the same DNA fragment in the presence of wild type P_R and P_R in which the -35 region was mutated by site-directed mutagenesis to create a *Xho*I site (CTCGAG). This mutation abolished P_R activity, as determined by the β-galactosidase activity from the mutated promoter fused to a *lacZ* gene (data not shown). With P_R inactive, P_L transcription increased 10-fold, confirming that this inhibition was dependant on P_R activity. In the absence of P_R (Fig. 4.2, middle curve), although the absolute activity of P_L is greater, it is also more sensitive to Apl repression. The efficiency of P_L repression by Apl therefore appears to be inhibited by P_R activity.

These data show that, in the presence of the other promoter, both P_R and P_L are repressed equivalently. Given this characteristic, Apl repression may be an all or nothing process, where binding to the entire region is required for repression of both promoters. Alternatively, sites specific for repression of each promoter but independant of the repression status of the other promoter may be occupied at similar concentrations of Apl. To determine this and to examine the role repression of each promoter plays in phage development, I isolated Apl operator mutants defective in repression of P_R and P_L.

4.2.1.3 Isolation of Apl operator mutants deficient in P_R repression

As high levels of Apl block infection of 186, presumably by repressing transcription from P_R (similar to the action of high levels of Cro on lambda development), a selection for spontaneous mutant phage able to infect cells expressing these levels of Apl was performed. These mutants should contain mutations in operators for Apl repression of P_R, as did mutants isolated in a similar experiment with Mu Ner (van Leerdam *et al.*, 1982). The Apl-expressing plasmid used was pMRR22, which expresses Apl constitutively from the P_{tet} promoter of pACYC184. The *apl* gene in this plasmid is translated from the T7ϕ10 RBS of pET3a (Studier *et al.*, 1990), thus providing high levels of Apl. A control strain, carrying a plasmid expressing AplΔ11 (pMRR23), did not inhibit 186 infection.

When isolating these phage, I was unsure of the nature of the mutations that would be obtained, so various mutant backgrounds (*e.g.* cI^-, *vir*) were used to see if they affected the

frequency or type of mutation. High titre phage stocks of 186$^+$, 186*c*Its, 186*c*I10 *vir*118 and 186*c*I10 were plated on LE392 carrying pMRR22. A total of 16 spontaneous phage mutants were isolated at a frequency of 10^{-8}-10^{-9}. The different parental phage backgrounds did not alter this frequency. As expected, the phenotype (denoted *goa*, for *g*row *o*n *A*pl) was mapped to the small *Xho*I.629-*Bgl*II.4244 fragment, which contains the early control region. None of the mutant phage (except those with a *vir*118 background) were able to plate on a 186 lysogen, and so were not virulent. The P_R-P_L region of all the mutants was sequenced (Fig. 4.3). The *goa* mutations fall into two classes. One class is comprised of small deletions in the middle of the P_R-P_L region, while the other class is an identical C to T change (independently isolated eight times) located between the -10 and +1 regions of P_R. Interestingly, this substitution occurs in the same region as the site III *vir* mutations (Lamont *et al.*, 1988), and occurred at the same position as one of the three changes in *vir*121 (Fig. 4.3). Subsequently, the *vir*121 mutant was found to have a *goa* phenotype. Although not shown, it will be assumed for the purposes of this Chapter that the *goa* mutations are interfering with DNA binding by Apl.

The efficiency of plating of many of the mutants in the presence of Apl was determined (Table 4.1). Included in this was a phage carrying an *Eae*I restriction site created by site-directed mutagenesis to introduce a restriction site between P_R and P_L (Dodd *et al.*, 1990). This change is a two base substitution located within the five bases deleted in the *goa*8 mutation and may therefore alter an Apl binding site (Fig. 4.3). Also included was the *vir*121 mutant (see above) and the *vir*100 mutant, which contains a substitution next to the *vir*121 site III position. Two levels of Apl were tested; a high level provided by pMRR22 (used to isolate the *goa* mutants), and a lower level provided by pMRR24, which contains *apl* expressed from its native promoter (P_R) and ribosome binding site and decreases the plaque size (but not the plating efficiency) of a wild type phage. As expected, the *goa* mutants and *vir*121 all plated at high efficiency on cells expressing high levels of Apl. The *vir*100 and *Eae*I mutations had plating efficiencies that were higher than the wild type phage but much lower than the *goa* mutants (and *vir*121). This suggests that the *Eae*I and *vir*100 mutations slightly reduced P_R repression by Apl. On lower levels of Apl, all mutants (including *vir*100 and *Eae*I) had normal sized plaques, in contrast to the smaller plaques of a wild type phage.

4.2.1.4 Effect of *goa* mutations on P_R and P_L repression by Apl

Characterization of the operator mutants described in the preceding Section was performed by using *lac*Z reporter assays to determine the effect each mutant had on Apl repression of P_R and

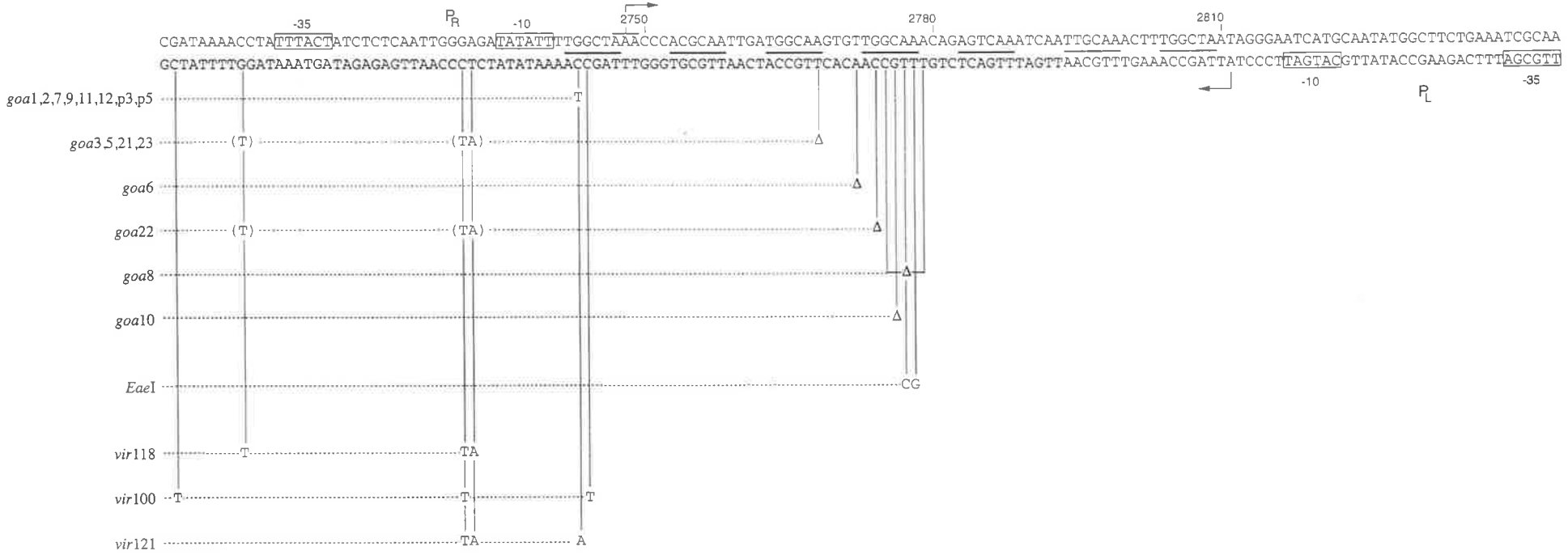


Figure 4.3 DNA sequences of *goa*, *Eae*I and *vir* mutations.

The DNA sequence changes of the three types of mutations are shown, together with the predicted ApI recognition sequences (thick horizontal lines), the P_R and P_L promoters (shaded boxes) and their respective initiation points (bent arrows). Numbering is from the *Pst*I site at 65.5% (Kalionis *et al.*, 1986a). The *goa* mutations were isolated and sequenced in this work. *Goa*s 1-3 and 5-9 were isolated from a cI^+ background, 10-12 from a *c*I10 background, 21-23 from a *vir*118 background (with the *vir* changes shown in brackets) and p3 and p5 from a *c*Its background. The *Eae*I mutation was created by Dodd *et al.* (1990) and the *vir* mutations were isolated and sequenced by Lamont *et al.* (1988).

Phage	high Apl[a]	low Apl
wild type	10^{-8}-10^{-9}	0.95[b]
*goa*2	0.54	1.10
*goa*3	0.80	1.03
*goa*6	0.67	1.02
*goa*8	0.87	0.94
*goa*10	0.72	1.01
*Eae*I	10^{-5}-10^{-6}	0.97
*vir*100	10^{-3}	1.08
*vir*121	0.61	0.93

[a] very small, irregular shaped plaques

[b] small plaques

Table 4.1 Plating efficiencies of 186 operator mutants on Apl expressing strains.

Wild type and mutant phage were plated on indicator strains carrying pMRR22 (high Apl) or pMRR24 (low Apl). Plating efficiency is expressed as the ratio of the number of plaques forming on the Apl expressing strains to the number of plaques forming on an AplΔ11 (pMRR23) expressing strain.

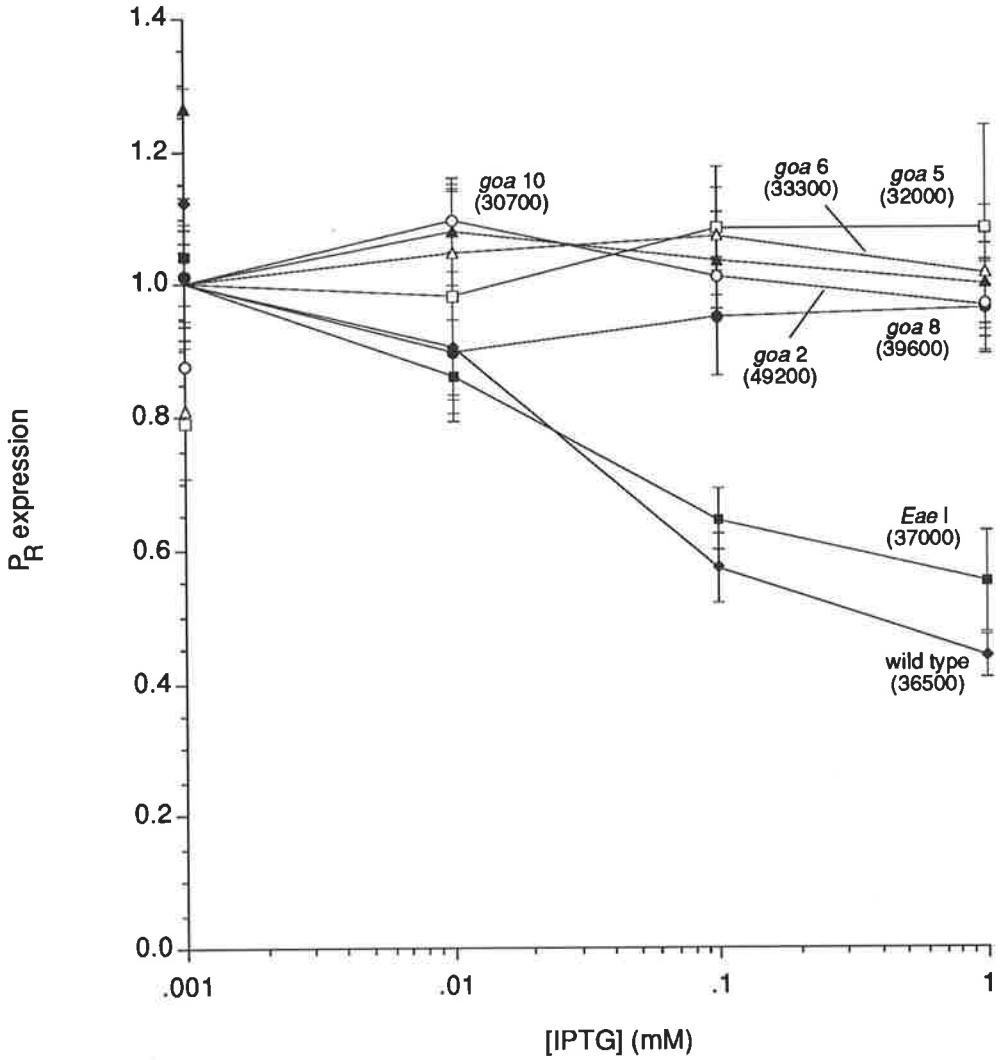


Figure 4.4.A P_R activity of the *goa* and *Eae*I mutations as a function of Apl concentration

P_R activity was assayed in strains carrying pMRR26 (P_R-*lac*Z) or mutant derivatives thereof, in the presence of various levels of Apl or AplΔ11 (supplied by IPTG induction of pMRR20 or pMRR21, respectively) as described in Fig. 4.2 (with 50 μg ml^{-1} ampicillin to select for the reporter plasmid). Normalized expression factors (units in presence of Apl/units in presence of AplΔ11; see text) were normalized to 1.0 for the lowest IPTG concentration. The average raw expression factors obtained at the lowest IPTG concentration are shown on the y axis. Each data point represents the average of 6 independent assays with the error bars representing the standard deviation (these are approximately equal to the 95% confidence limits of the means as determined by the student's t-test). The basal levels of promoter activity (0 mM IPTG) are given in brackets. Units have been multiplied by 10 to account for the decrease in transcription caused by the lambda t_{R1} terminator in pMRR9T (see Section 6.7.6.1).

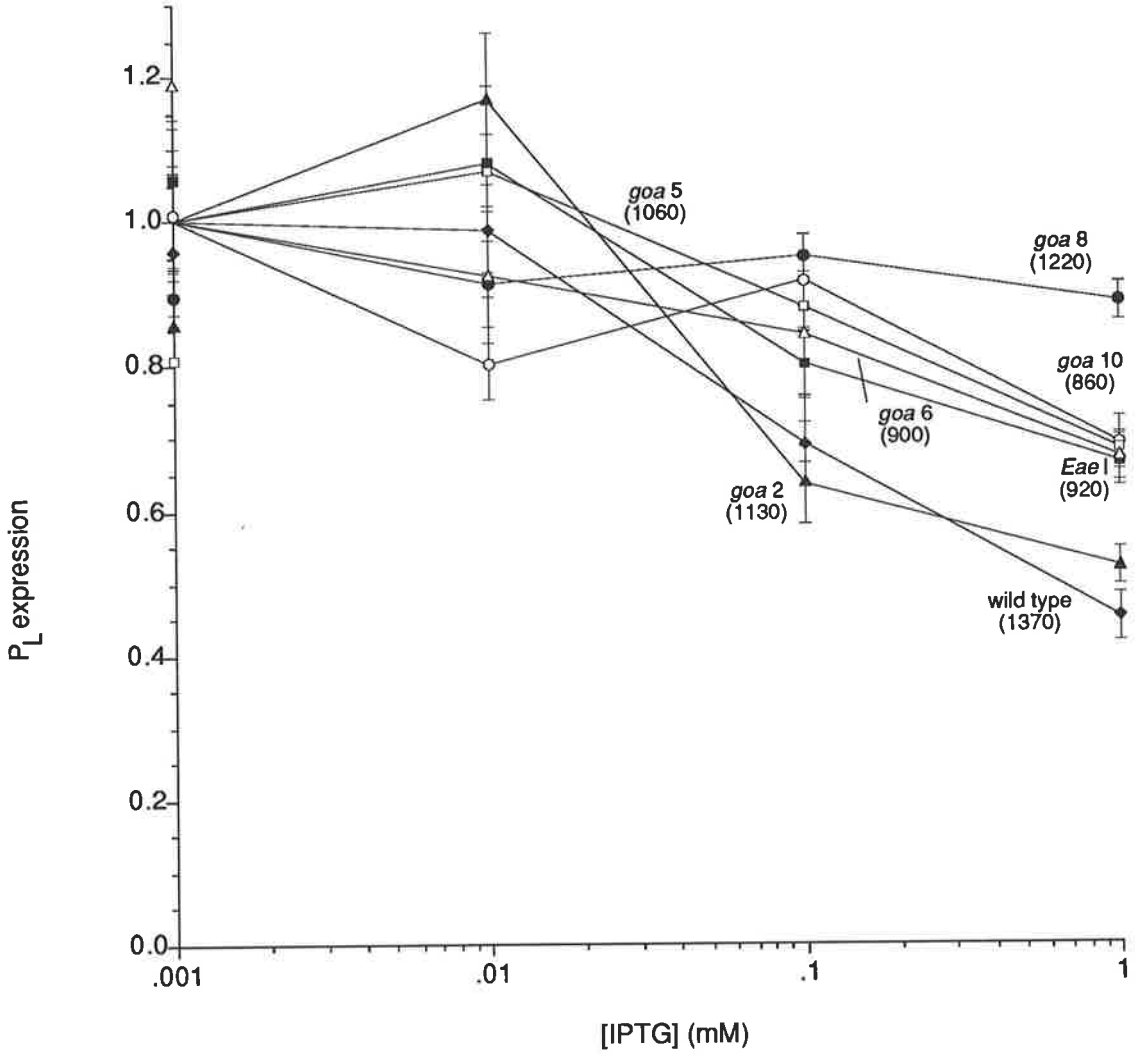


Figure 4.4.B P_L activity of the *goa* and *Eae*I mutations as a function of Apl concentration

This experiment was performed as described in the legend to Fig. 4.4.A, except P_L activity was assayed in strains carrying pMRR27 (P_L-*lac*Z) or mutant derivatives thereof.

P_L. This was done as a prelude to determining the biological phenotypes of the mutants in the phage. The assays were performed using multicopy plasmid reporter constructs, which, as stated above, gave variable results due to day to day variation. The source of this variation is not known, although was presumably due to differences in the copy number of the plasmids, perhaps caused by unknown differences in the media and reagents used each day. The number of assays performed made copy number determination of each one unfeasible and time constraints did not allow the creation of single copy constructs. However, the multicopy data do allow a reasonable measure of Apl repressibility once these variations are accounted for. Cultures containing the reporter plasmid with the Apl expression plasmid (pMRR20) were assayed at all IPTG concentrations in parallel with cultures containing the AplΔ11 expression plasmid (pMRR21) as a control.

To isolate the effects of Apl on the reporter constructs from non-Apl dependant variations (*e.g.* the copy number of the reporter plasmid), the data was treated in two ways. At each IPTG concentration, the *lac*Z units for a reporter plasmid in the presence of the Apl plasmid was divided by the value for the same reporter plasmid in the presence of the AplΔ11 plasmid, assayed in parallel with the Apl-containing cultures, to give a raw expression factor. The raw expression factors for each reporter construct in the absence of Apl induction (lowest concentration of IPTG) should be 1.0, but were not (see Fig. 4.4), suggesting non-Apl-dependant variation between reporter plasmid activity in the presence of pMRR20 versus pMRR21 (although, generally, these differences were less than those obtained when the same constructs were assayed on different days). Therefore, the raw expression factors for each reporter construct (at each concentration of IPTG) were normalized so that the uninduced expression factor was 1.0.

The effect of the *Eae*I and representative *goa*mutations (except *goa*22, as it is from a *vir*118 background so the extra mutations could effect the interpretation of the results) on the repression of P_R and P_L by Apl is shown in Fig. 4.4 with the normalized expression factors represented as a function of increasing IPTG (Apl/AplΔ11) concentration. Basal (unrepressed) levels of P_R and P_L activity for each mutant (shown in brackets on Fig. 4.4, with variations between constructs presumably experimental) were assayed on the same day to reduce day to day variation, and did not vary greatly.

As expected, Apl repression of P_R was severely disrupted by all of the *goa* mutations while the *Eae*I mutation did not significantly affect P_R repression. The effects of the mutations

on P_L repression were more complex than P_R. Repression of P_L is completely abolished by *goa*8, the 5 bp deletion, and was not affected at all by the *goa*2 mutation, the single base substitution near P_R. In the remaining *goa* mutations (the single nucleotide deletions) and the *Eae*I mutation, P_L repression was reduced by about 50% at the maximum level of Apl induction (1 mM IPTG).

4.2.2 The biological roles of P_R and P_L repression by Apl

The reporter studies of the *goa* and *Eae*I mutations showed that there were three classes:

i) Those affecting repression of P_R only (*e.g. goa*2)

ii) Those affecting repression of P_L only (*e.g. Eae*I)

iii) Those affecting repression of both P_R and P_L (*e.g. goa*8).

This section will examine the effects these mutations have on lytic development and UV induction and spontaneous induction of a prophage.

All of the mutant phage assayed were $c\mathrm{I}^+$. The *Eae*I mutation was introduced into 186$c\mathrm{I}^+$ in a similar manner to that described for creation of the *apl*Δ11 phage in Section 2.A.4. All of the mutant phage gave rise to plaques on C600 that were indistinguishable from wild type, suggesting that lytic development was not seriously impaired. Stable lysogens were easily obtained, so the establishment and maintenance of lysogeny also did not appear to be seriously affected by the mutations.

4.2.2.1 Effect on lytic development

Lytic burst sizes of the mutants were determined by single-step growth experiments (Fig. 4.5 and Table 4.2 column 3). The inability of Apl to repress P_L (186*Eae*I) had no effect on lytic development, whereas the inability of Apl to repress P_R (186*goa*2 and 186*goa*8) resulted in a 1.7-1.8 fold increase in burst size. This is similar to the increase seen for the 186*apl*Δ11 phage (Chapter Two).

4.2.2.2 Effect on prophage induction

As 186$c\mathrm{I}^+$ lysogens are inducible by the SOS functions of *E. coli*, the ability of the *goa* and *Eae*I lysogens to induce in response to UV irradiation was tested and compared to wild type (Table 4.2, column 4). As the induction assay used here is a combined measurement of induction efficiency and lytic development, any effects the mutants have on lytic development should be taken into account when analyzing the induced burst size. The adjusted value (Table

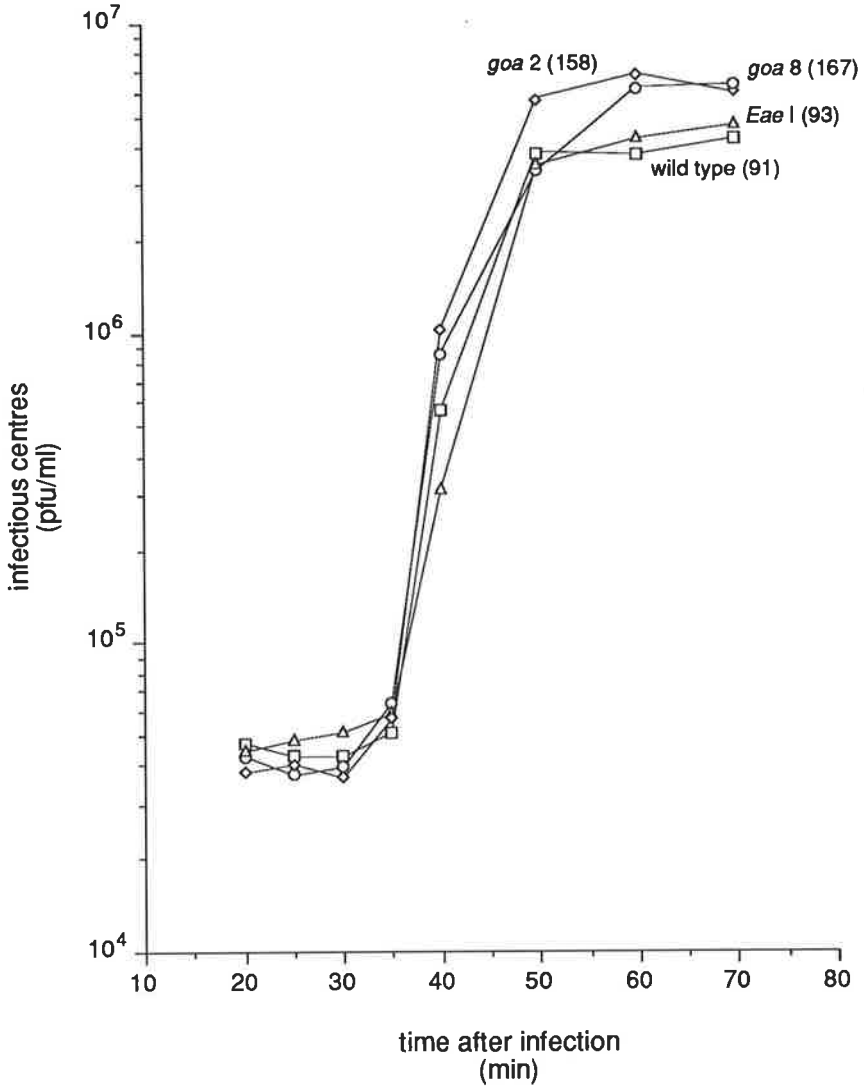


Figure 4.5 Effect of *goa* and *Eae*I mutations on 186 phage bursts

Burst assays were performed as described in Section 2.A.4, except phage were added at a m.o.i. of approximately 0.01 and, following adsorption, the infection mix was diluted 10^{-2}, 10^{-3} and 10^{-4} into fresh, pre-warmed LB. The time is taken from when the phage were added to the bacteria. Burst sizes were calculated by averaging the last two (60 and 70 min) titres (post-burst) and dividing by the average of the first three (20, 25 and 30 min) titres (pre-burst). Similar burst sizes were obtained in duplicate experiments. The low pre-burst titres (less than 10% of the added phage) are due mainly to poor 186 adsorption in these conditions.

Phage/ Prophage	Repression defect	Lytic Burst Size	U.V. Induction	Adjusted U.V.I.	Spontaneous Induction	Adjusted S.I.	Apl induction [IPTG] 0 mM	1 mM
186+	None	1.00	1.00	1.00	1.00	1.00	-	-
186*Eae*I	P_L	1.02	0.46	0.45	0.37	0.36	-	-
186*goa*2	P_R	1.74	1.83	1.05	2.80	1.61	1.0	40
186*goa*8	P_R & P_L	1.84	0.42	0.23	0.33	0.18	1.0	3.1

Table 4.2 Biological phenotypes of *goa* and *Eae*I mutants

Lytic burst, UV induction and spontaneous induction sizes are expressed relative to the wild type values. Lytic burst analyses were performed as described in Fig. 4.6.

UV inductions were performed by subculturing overnight cultures (grown in LB) of C600 lysogens of the various mutant phage 10^{-2} into M9CAA and grown to an A_{600} of approximately 0.6 (M9CAA was used as it absorbs less UV radiation than LB). A sample of the culture was diluted 10^{-5} and assayed for colony forming units (Section 6.5.6) present in the pre-irradiated culture. This was between 1.5×10^8 and 3.1×10^8 cfu ml^{-1}. Another sample of the culture was treated with chloroform to lyse any cells present and assayed (Section 6.5.5) for the number of pre-induced plaque forming units present in the culture (data not shown). A final sample (6.4 ml at a depth of 1 mm in a glass petri dish) of the culture was exposed to 15 J m^{-2}, subcultured 1/200 into fresh LB and grown for two hours to allow the lysogens to induce. A non-irradiated sample was also grown the same way. Following the two hour incubation, a sample of the culture was chloroform treated and assayed for plaque forming units. UV induced wild type lysogens typically produced about 10^1 pfu cfu^{-1}(pre-irradiated), whereas the non-induced wild type lysogens typically produced about 10^{-5} pfu cfu^{-1}(pre-irradiated). This value is 100 fold lower than those obtained for the spontaneous inductions (see below) because the cfu are were assayed before the 10^{-2} dilution. This was repeated five times for each mutant lysogen (with a wild type lysogen induced concurrently to account for day to day differences in induction), and the value obtained for each mutant lysogen divided by the value obtained for the wild type lysogen induced on that day. The values are averages of the five independant inductions, with standard errors of no more than 5%.

Spontaneous inductions were performed by diluting overnight cultures (grown in LB) of C600 lysogens of the various mutant phage 10^{-2} into fresh LB and growing to an A_{600} of approximately 0.6. Free phage were removed by centrifuging the bacterial pellet, washing and resuspending in an equal volume of LB. A sample was then subcultured 10^{-2} into fresh LB and grown for two hours at 37°C. A sample was then chloroform treated and assayed for plaque forming units and another sample assayed for colony forming units. Wild type lysogens typically spontaneously produced about 10^{-3} pfu cfu^{-1}. The data is presented as for the UV induction, each value being an average of five independant assays.

Adjusted values were obtained by dividing the induction values for the particular phage by the lytic burst size, as the induction experiment is assaying lytic development as well as efficiency of induction. The lytic development effects are therefore negated by this calculation.

Apl induction of 186 mutant lysogens was performed by subculturing overnight cultures of C600 lysogens of 186*goa*2 and 186*goa*8, carrying Apl or AplΔ11 expression plasmids (pMRR20 or pMRR21, repectively) grown in M9CAA and 50 µg ml^{-1} kanamycin, 10^{-2} into fresh media and grown to an A_{600} of about 0.6. Free phage were removed by centrifuging and washing in M9CAA and then a sample diluted 10^{-2} into fresh media supplemented with 50 µg ml^{-1} kanamycin and 0 or 1 mM IPTG (to induce expression of the Apl proteins). Cultures were then incubated at 37°C for two hours and a sample chloroform treated and assayed for plaque forming units. A sample of the pre-induced culture was assayed for colony forming units. The values are expressed as a ratio of phage produced in the Apl expressing strain for each cfu to the phage produced in the AplΔ11 expressing strain for each cfu. Phage produced in the induced (1 mM IPTG) AplΔ11 strain did not differ from those produced in the non-induced strain (0 mM IPTG), indicating IPTG was not affecting induction and so the 0 mM readings were normalized to 1.0.

4.2, column 5) was therefore obtained by dividing the induction burst size by the increase in lytic burst size (Table 4.2, column 3). As with lambda Cro, the inability to repress lysogenic transcription (*Eae*I and *goa*8) results in a decrease in induction burst size, with *goa*8 having the greatest effect. The inability to repress P_R (*goa*2), on the other hand, had no effect on induction.

When performing the UV inductions, it was noticed that the pre-induced phage titre of the mutant lysogens sometimes differed to that of wild type (data not shown). This suggested that spontaneous induction (and therefore the stability) of the lysogens was affected, so this characteristic was quantitated. For the wild type phage, the frequency of lysogens that spontaneously induced was in the order of 10^{-3} pfu cfu^{-1} (see legend to Table 4.2). Table 4.2 (column 6) shows the level of spontaneous phage production of the *goa* and *Eae*I mutations as compared to wild type, and column 7 shows the spontaneous phage production values adjusted to allow for changes in lytic development. The induction of the *Eae*I and *goa*8 mutants was impaired to an extent similar to that seen with UV induction. The *goa*2 lysogen produced more phage than wild type, suggesting that this mutation reduced the stability of the lysogen. I suspect that the *goa*2 mutation may affect CI binding to P_R (see Discussion).

4.2.2.3 Ability of Apl to induce a 186 prophage

The ectopic expression of lambda Cro and P2 Cox repressors in their respective lysogens will induce the prophage by preventing transcription of the immunity repressor gene (Johnson *et al.*, 1981; Saha *et al.*, 1987b). This was also demonstrated for 186 Apl (Table 4.2, columns 8 and 9). Apl induction of a *goa*2 (P_R repression inhibited) and a *goa*8 (P_R and P_L repression inhibited) prophage was compared to test the importance of Apl repression of P_L in the induction. A background in which P_R is insensitive to Apl was needed for this experiment because Apl would otherwise inhibit lytic development. *Goa*2 and *goa*8 lysogens carrying pMRR20 (Apl) or pMRR21 (AplΔ11) were grown with 0 or 1 mM IPTG to induce expression of the Apl proteins. The phage produced are expressed as a ratio of Apl-induced lysogens to AplΔ11-induced (control) lysogens. Induction of *apl* in the 186*goa*2 lysogen produced about 13-fold more phage than the 186*goa*8 lysogen, demonstrating the requirement for Apl repression of P_L for this effect.

4.3 DISCUSSION

4.3.1 The role of Apl repression in 186 development

Prior to this work, the role of Apl repression in 186 development was basically unknown. Studies with an *apl*Δ11 phage (Chapter Two) showed that it may have had a role in derepression of the prophage during induction, however, this interpretation was affected by the predominant excision defect of these phage. Previous studies suggested that Apl preferentially repressed lysogenic transcription from P_L, but also down-regulated lytic transcription from P_R to a lesser extent (Dodd *et al.*, 1990). This was in conflict with the footprint data for Apl at the P_R-P_L region, which showed no preference for binding to either promoter (Chapter Two). The work in this Chapter has resolved most of these questions. P_R and P_L are repressed equivalently by Apl, in agreement with the footprint but contrary to the earlier studies (Dodd *et al.*, 1990). The isolation of operator mutants which blocked Apl repression of the P_R or P_L or both promoters allowed the role Apl repression of both promoters plays in 186 development to be determined as well as providing information on the binding characteristics of Apl. As is the case with lambda Cro, Apl repression of lysogenic transcription is important for efficient derepression during prophage induction. The role of P_R repression, however, is less clear.

4.3.2 Apl operator mutants and the mechanism of repression by Apl

The DNA binding studies of Chapter Two and the single copy *lac*Z reporter studies of this Chapter showed that Apl is binding to and repressing the P_R and P_L promoters equivalently (see below). It was therefore possible that there were no binding sites unique for the repression of each promoter, so that the complete Apl-DNA complex was required for repression of both promoters. The isolation and characterization of Apl operator mutants showed that this was not the case, and that there are independent components of Apl repression of P_R and P_L. There are sites required only for P_R repression (*e.g.* defined by the *goa*2 mutation) and sites required only for P_L repression (*e.g.* defined by the *Eae*I mutation), which allowed the separation and independent study of P_R and P_L repression by Apl. These studies are discussed later.

The nature and location of the mutants provided some insights into the mechanism of Apl binding to the P_R-P_L region. In Chapter Two, two models for DNA binding (and bending) were discussed. The naked loop model was argued against and the operator mutants here provide convincing evidence for that argument. The model requires that Apl binds at the base of the loop, probably near each promoter, providing a clasp-like structure with the DNA looping out from it. The *goa*2 mutation would disrupt Apl binding to the P_R site. However, the

fact that P_L can still be repressed in this mutant means that P_L repression would not require the formation of the clasp (loop) structure. The fact that P_L repression by Apl is disrupted by the deletion *goa* and *Eae*I mutations, which should not affect binding to the P_L site, demonstrates the implausibility of this model. Instead, the model favoured in Chapter Two, with Apl bound across the whole region, is supported.

The deletion *goa* mutations mapped to the centre of the Apl binding region. It was proposed that Apl bound to this central region interacts cooperatively with other Apl molecules to fill the adjacent (lower affinity) sites. In support of this, these mutations disrupted both P_R and P_L repression. The deletion *goa*s would disrupt both the central site(s) and the spacing (cooperative interactions) across the P_R-P_L region, suggesting that disrupting one of these alone is not sufficient to disrupt P_R repression. The reason for this is not clear but may be a consequence of the high levels of Apl used to select the mutants being able to overcome such (weaker) mutations. The *Eae*I mutation had a significantly inhibitory effect on P_L repression but had a negligible effect on P_R repression by Apl, despite mapping in the 5 bp region deleted in the *goa*8 mutation. This indicates that the site it disrupts is more important for P_L repression than P_R repression. The *goa*2 mutation presumably defines a nucleotide crucial for Apl binding to a site specific for P_R repression and is located in the left-most recognition sequence predicted in Chapter Two. Its location between the -10 and +1 regions of P_R suggests that Apl repression of P_R occurs by steric exclusion of the RNA polymerase.

The Apl operator mutants isolated here show that there appear to be three components of Apl repression: 1) occupation of the central site(s), required for repression of both promoters 2) occupation of sites to the left for P_R repression and 3) occupation of sites to the right (presumably) for P_L repression. It would be informative to select *goa* mutants at lower levels of Apl to determine whether a broader range of (less severe) mutations could be obtained. More detailed DNA binding studies using pure Apl and the P_R-P_L region containing *goa* and *Eae*I mutations will undoubtedly lead to a better understanding of DNA binding by Apl and through this, its mechanism of repression.

4.3.3 The equivalent repression of P_R and P_L by Apl

To solve the apparent discrepancy between the *gal*K reporter studies of Dodd *et al.* (1990) and the DNase I footprint of Chapter Two, a *lac*Z reporter system was developed and used to show the activity of both P_R and P_L in the presence of increasing amounts of Apl. In agreement with the Apl footprint, these assays showed that Apl repression of both P_R and P_L is basically

equivalent. In other words, lysogenic transcription is not repressed before lytic transcription. The initial *gal*K reporter studies which showed a difference in repression of both promoters (Dodd *et al.*, 1990) used a different strategy to the one used here. In those studies, Apl was expressed *in cis* from the 186 P_R promoter and was part of the DNA fragment being assayed. It was shown that when P_R activity was assayed in the presence of Apl (*i.e.* the construct contained P_R-P_L-*apl*-*gal*K), it was 10% of the P_R activity assayed from a shorter fragment without *apl* (*i.e.* P_R-P_L-*gal*K). With P_L being assayed in the reverse orientation constructs, GalK units dropped from 6.6, in the absence of *apl*, to -2.2 in the presence of *apl* (after background GalK activity with the vector alone was subtracted), indicating complete repression by Apl. By comparison, when the single copy P_R-*lac*Z construct assayed here was 90% repressed by Apl (at about 9 mM IPTG), P_L activity was reduced by about 80%, which is weaker repression than in the *gal*K assays. It seems likely that the -2.2 GalK unit result is an artifact, possibly due to the reporter construct carrying a spontaneous mutation in the *gal*K reporter gene or P_L (I. Dodd, personal communication).

The mechanisms that gave the apparently simple kinetics of Apl repression of both P_R and P_L (Fig. 4.2), were probably quite complicated, involving a balance between three aspects: 1) Apl repression of both promoters, 2) P_R inhibition of P_L and 3) P_R interference of Apl repressing P_L (and P_R). The ability of Apl to repress P_L was adversely affected by the activity of P_R; that is, Apl had a 10 fold effect in absence of P_R at 10 mM IPTG, but a 5 fold effect in the presence of P_R. However, the basal activity of P_L was reduced by an order of magnitude with P_R present, so the absolute P_L activity was lower. Thus, although Apl is required for biologically effective repression of P_L, P_R is probably an essential co-repressor. The equivalent repression of P_R and P_L by Apl means that at any stage in phage development where Apl is repressing P_L, P_R must also be repressed. The 100-fold difference in promoter strength would allow sufficient P_R expression for lytic development, while P_L transcription was basically inactive.

The repression characteristics of P2 Cox are similar to those of Apl. The *cat* reporter assays of Saha *et al.* (1987b) examined the effect a single invariable level of Cox had on P_c (lysogenic) and P_e (lytic) transcription. These studies indicate that P_e transcription affects Cox repression of P_c, although this is not discussed by the authors. In the presence of P_e the absolute activity of P_c is reduced to very low levels by Cox. However, when P_e is not active, the fold decrease of P_c transcription is greater. In other words when considered independently,

P_c is more sensitive to Cox repression than P_e, but when both promoters are present, they are repressed equivalently. This means that if Cox repression of P_c is required for efficient derepression during prophage induction (as is lambda Cro binding at O_R3), then P_e must also be repressed during lytic development, as is the case with 186 and Apl.

4.3.4 The functions of P_R and P_L repression by Apl

The three classes of Apl operator mutants (inability to repress P_R - *goa*2, P_L - *Eae*I or both - deletion *goa*s) provided a powerful means for looking at the role of Apl repression in 186 development while leaving its excisionase function intact.

4.3.4.1 P_R repression

When Apl was unable to repress P_R, lytic development was improved, both in infection and prophage induction (Fig. 4.5 and Table 4.2), similarly to the *apl*Δ11 mutation. As this loss of repression appears to be simple to evolve (*e.g.* the single nucleotide substitution of *goa*2) and provides an obvious advantage to the phage, retention of P_R repression implies that it must be important for phage development in some conditions. In Chapter Two, it was suggested that P_R repression by Apl may provide optimal utilization of host cell resources during development in starvation conditions. In a starving cell (conditions not used in the laboratory), transcription and translation machinery would be limiting, so down-regulation of early lytic development would ensure that this machinery is optimally used for synthesis of phage proteins, and would not inactivate the cell prior to completion of the lytic cycle.

4.3.4.2 P_L repression

It was proposed in Chapter Two that efficient derepression of an induced prophage required Apl repression of P_L. Assaying prophage induction with mutants deficient in Apl repression of P_L supported this proposal and showed that P_L repression results in a five-fold increase in the induction size. This effect is similar to that seen with the O_R3 mutants of lambda, which block Cro repression of P_{RM} and induce poorly (Johnson *et al.*, 1981), although quantitative figures have not been published. The inability to repress the lysogenic promoter would allow expression of the CI repressor which would contribute to the partially inactivated pool in the induced lysogen and repress lytic transcription.

Ectopically expressed Apl was shown to be able to induce a 186 lysogen (Table 4.2) in a manner similar to the induction of lambda and P2 lysogens by Cro or Cox, respectively

(Johnson *et al.*, 1981; Saha *et al.*, 1987b). This property is dependent on the ability of Apl to repress P_L and is similar to lambda Cro induction, which requires an intact O_R3 (Johnson *et al.*, 1981). The induction was not very efficient (about 40 times better than spontaneous induction - actually closer to 20 times when the increase in burst size due to the lack of P_R repression is taken into account), which was presumably a result of the high levels of CI present. Normal prophage induction initiates with the inactivation of CI, then repression of P_L by Apl. If P_L is repressed first, the lysogenic concentration of CI will need to decrease to a level sufficient for P_R transcription to proceed, a process which depends on the half-life of the protein. As this degradation process will be relatively slow, P_R transcription, and therefore lytic development, will be somewhat inhibited, resulting in a reduced burst size.

4.3.5 An Apl-CI interaction?

The deletion *goa* and *Eae*I mutant lysogens had spontaneous induction rates lower than that of wild type (Table 4.2). This was most likely a result of the reduced ability of Apl to repress P_L, as the differences were similar to those seen in the UV induction (Table 4.2). The *goa*2 lysogen, on the other hand, had an adjusted spontaneous induction rate higher than that of a wild type lysogen, indicating the mutant lysogen was less stable. This is not a function merely of loss of P_R repression by Apl, as the *goa*8 lysogen was not affected, but was specific to the *goa*2 mutation. It seems likely then, that this is probably a consequence of the mutation affecting CI repression of P_R. Although *goa*2 does not have a virulent phenotype, it is located in the same region as the site III *vir* mutations (Fig. 4.3), with *vir*121 being a substitution of the same nucleotide. The site III mutations have been isolated only in conjunction with site I or site I and site II *vir* mutations, indicating that, independently, they do not disrupt CI repression of P_R sufficiently to cause virulence. However, it is presumed that they affect CI binding slightly, and hence prophage stability would be decreased.

If this operator sequence is required for binding by both Apl and CI, it is possible that binding of Apl could exclude CI, hence providing another mechanism for enforcing derepression. The stimulus which induces a 186 lysogen is unlikely to inactivate all of the CI repressor present in a lysogen. The opportunity for this residual CI to inhibit lytic transcription is therefore a problem during induction. As CI repression of P_R is far more efficient than that of Apl (Dodd *et al.*, 1990; I. Dodd, unpublished data), using Apl to exclude CI from P_R will result in increased P_R activity. Performing DNA binding studies with Apl and CI will determine whether any interaction between the two proteins is occurring.

4.3.6 Conclusions

For a phage whose mode of development basically depends on the activity of one of two promoters, understanding the factors which control these promoters is obviously important for an understanding of the phage's biology. The control of P_R and P_L by Apl is complex and not completely understood. The equivalent repression of both promoters by Apl means that both promoters will be repressed during lytic development. The fact that this repression is not complete and that P_R is a strong promoter means that sufficient P_R transcription occurs for lytic development to be successful. P_L repression by Apl is required for efficient prophage induction, presumably by preventing excess CI production. The reason for P_R repression by Apl is not clear. Since loss of this repression seems fairly easy to evolve (*e.g. goa*2 mutation) and since the lack of this repression appears to be an advantage under some conditions, it is expected that there are some conditions in which P_R repression is important.

CHAPTER FIVE
General Discussion

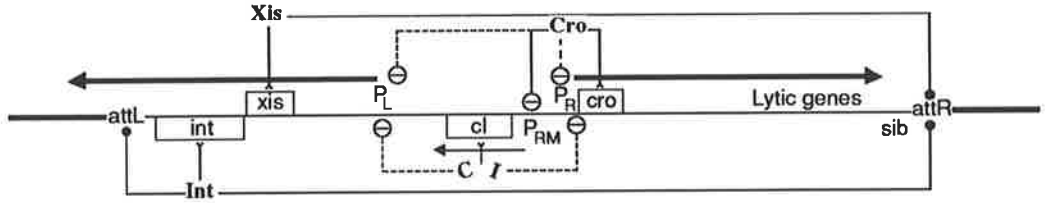
5.1 A SIMPLE STRATEGY FOR COORDINATING 186 TRANSCRIPTIONAL AND RECOMBINATIONAL SWITCHES

Productive development of temperate, integrating bacteriophages requires the coordination of the lysis-lysogeny transcriptional switch and the integration-excision recombinational switch. A phage which integrates during lytic development and excises during lysogeny will clearly not be viable, so different phage have evolved a number of different strategies to ensure this does not occur.

The paradigm for such phages is lambda, which has developed complex mechanisms to regulate its development. Contributing to this complexity is the location of *int* on the lytic P_L operon, directly downstream of *xis*. With this arrangement, lambda has had to develop a mechanism to express *int* without *xis* during the establishment of lysogeny and *int* with *xis* during prophage induction and to coordinate this control with the control of the lysis-lysogeny switch. If the decision to enter lysogeny is made following infection, CII will activate P_{RE} (Wulff & Rosenberg, 1983) and P_I (Shimatake & Rosenberg, 1981). P_{RE} transcribes the *c*I gene, the product of which represses P_R and P_L transcription, thus preventing expression of *cro* and *xis*, among other lytic genes. With P_L turned off, however, another source of Int transcription is required. The activation of the P_I promoter provides this. P_I is located in the *xis* gene and so its activity does not result in the expression of Xis. The Int required for integration of the prophage is thus independently supplied concurrently with the activation of repressor synthesis which results in the repression of lytic functions.

Prophage induction (Fig. 5.1.A) requires the derepression of lytic transcription. This results in the production of Cro from P_R, which blocks lysogenic transcription and repressor expression. Derepression of P_L results in the production of other lytic genes, but also of Int and Xis, both required for excision of the prophage. To ensure excess Int is not produced after excision, *sib* retroregulation degrades the P_L message from the 3' end so that *int* is degraded before *xis* and hence is translated less (Guarneros, 1988). The requirement of *sib* is questionable, as deleting it has no known adverse effect on phage development (Guarneros, 1988).

A. Lambda



B. 186

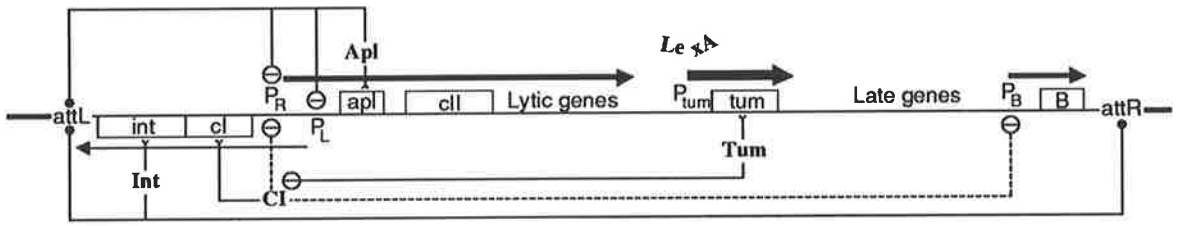


Figure 5.1 Comparison of lambda and 186 prophage induction strategies

Representation of the control strategies operating during prophage induction of lambda (A) and 186 (B). Details are given in the text. The diagram is not drawn to scale. Transcripts are shown as arrows with the thickness approximately indicating relative promoter strength. The inhibitory or stimulatory effects of gene products are shown by lines ending with circles containing negative or positive signs, respectively.

186 has developed a much simpler strategy for such coordination. This is primarily due to the *c*I repressor and *int* genes being transcribed from the lysogenic P_L promoter, while the Cro and Xis functions are combined in the one gene, Apl, transcribed from the lytic P_R promoter. During the establishment of lysogeny, CII, expressed from the P_R transcript, activates the P_E promoter, located upstream of P_L, which transcribes the *c*I and *int* genes. CI then represses lytic transcription while Int catalyses the integration of the prophage. Prophage induction is also simplified (Fig. 5.1.B). Derepression of lytic transcription in a prophage produces Apl which prevents further production of CI from the lysogenic operon by repressing P_L. Apl also acts at the prophage attachment site, in conjunction with the residual Int expressed from P_L in the lysogen, to excise the prophage.

5.2 THE STRUCTURE AND FUNCTION OF THE APL PROTEIN-DNA COMPLEX

The Apl protein is, on the surface, remarkable. Its ability to repress transcription and directly influence site-specific recombination has similarities to a global regulator protein, such as IHF. Obviously, it does not have the wide-ranging effects of such a host protein, but nevertheless, it is another example of the flexibility of biological systems. Another testament to this is that Apl is able to perform both of these apparently mechanistically distinct processes simply by binding to and bending DNA at two separate sites on the 186 genome. Repression of the P_R and P_L promoters may occur by simple steric occlusion of RNA polymerase, as the Apl binding region overlaps the transcription start point of each promoter. However, Apl creates a bend in the DNA that may also contribute to their repression. Excision presumably also utilizes a similar bent structure which could facilitate the interaction (or prevent non-productive interactions) of other proteins involved in the recombination reaction.

The bent nature of the DNA dominated the Apl footprints and provided no distinct picture of the bases important for sequence recognition by Apl. It seems likely that Apl initially recognizes and binds to a high affinity site, with subsequent highly cooperative interactions resulting in lower affinity sites being occupied. These interactions either increase or create a bend in the DNA. Such a model for Apl binding explains the difficulty in obtaining a significant consensus sequence for (high affinity) Apl binding sites, as the low affinity sites, by definition, would not conform to the high affinity sequence. Comparison of the sequences protected in the *att*P and P_R-P_L footprints suggests that this high affinity site is in the centre of the binding region, as the most conserved sequences between the two sites are located in this region. The Apl binding region at P_R-P_L is larger than that at *att*P but it is not known if this is the result of an increased number of binding sites (as suggested by the predicted recognition sequences) or increased spacing between the same number of sites. Further characterization

and isolation of operator mutants and detailed DNA binding studies using pure Apl are required to determine the mechanism of Apl binding, which will give a better insight into the repression and excision roles of Apl.

Site-specific recombination appears to be mechanistically similar in a number of phage systems. The phage integrase protein catalyses both integration and excision reactions, while another phage protein, the excisionase, is required for excision and therefore provides directionality to the reaction. Host DNA binding proteins also participate in the reaction. The role of all proteins, except Int, appears to be structural. In other words, they interact cooperatively or antagonistically with each other and bend the DNA to provide the correct DNA structure for the strand exchange reaction, while not actually taking part in it. Different combinations (and concentrations?) of these proteins regulate the structure of the "intasome" complex and, in so doing, the direction of the reaction.

Little is known about the mechanism Apl uses to foster excision, but it is presumably similar to that of the lambda Xis protein. This can be concluded for a number of reasons. They are both small proteins, and they both cause significant structural deformations to the DNA on binding. The location of their binding sites at their respective attachment sites is similar which indicates that, as with Xis, Apl binding to this site will foster the interaction of Int proteins bound to either side of it. The Apl binding region partially overlaps an IHF binding site so it is possible that some form of competitive interaction will occur between the two proteins, as is the case with Xis. The functional resemblance of Apl to a dedicated recombination protein, such as Xis, while also being a transcriptional repressor highlights the flexibility of this system.

Repression of P_R and P_L transcription by Apl is relatively straightforward. Footprinting showed that Apl binds between the two promoters with no preference for either promoter. This characteristic was also a functional one, as *lac*Z reporter studies showed that Apl repressed both promoters equivalently. The fact that P_R is much stronger than P_L means that in the Apl-repressed state P_R will be relatively active whereas P_L will be basically silent. Repression of P_L is required during prophage induction, presumably to prevent continued expression of the CI repressor, a function which may be enhanced by Apl excluding CI binding to P_R. As P_R and P_L are repressed equivalently by Apl, repression of P_L during induction means that P_R will also be repressed. In other words, the actvity of P_R utilized by the phage during lytic development is that which is "escaping" Apl repression. The amount of repression is not known, but quantitating the amount of P_R transcript produced in an inducing lysogen will help answer this. The reason for P_R repression is not clear and in contrast to lambda, which

requires lytic repression by Cro for productive development. It is obviously of value to the phage as a simple mutation (such as the *goa*2 substitution) abolishes P_R repression without affecting P_L repression and hence presumably would have been selected for. A better understanding of the reasons for repression of both promoters by Apl will require a more detailed study of their activity *in vivo*, involving direct analysis of the two transcripts isloated at various stages of development from phage carrying various types of Apl operator mutants. In this way, the promoter activities resulting in the phenotypes observed for these mutants can be determined.

5.3 CONCLUSIONS

The overriding theme of this thesis has been the simplicity generated by combining two functions critical for prophage induction into one DNA-binding protein, Apl. Although a simple concept, the logistics of evolving a single protein for both recombination and repression are quite complex. The excision role is presumably quite straightforward and similar to lambda Xis. Repression of P_R and P_L, on the other hand, must take into account the converging transcription of two promoters of completely different strengths. The strongest of these, P_R, which inhibits P_L activity and affects Apl's repression capabilities, also transcribes the *apl* gene. Thus, Apl repression involves a balance of all of these factors and others, including the presence of CI repressor and host factors. The Apl-DNA complex is formed highly cooperatively and induces a large structural deformation in the DNA which may contribute to both of its functions. The end result is that Apl allows a 186 prophage to induce simply and efficiently.

CHAPTER SIX

Materials and Methods

The Materials and Methods described in Section 2.A.4 covers the experiments performed in that Chapter.

6.1 BACTERIAL STRAINS

Strain	Genotype	Use	Reference
BL21	F⁻ *ompT* r_B^- m_B^- *lon*⁻	pET3a cloning	(Studier *et al.*, 1990)
BL21(λDE3)	λDE3 lysogen of BL21	Expression of pET clones. λDE3 carries a copy of the T7 RNA polymerase gene expressed from the P_{lacUV5} promoter	(Studier *et al.*, 1990)
C600	F⁻ *thr*-1 *leu*B6 *thi*-1 *lac*Y1 *ton*A21 *sup*E44 *hsd*R	General 186 work	(Appleyard, 1954)
C600 *fis*	C600 Δ*fis*::*Km*	Fis⁻ strain for use in recombination assays. Created by P1 transduction from WM2016	This work
C600 *him*A	C600 Δ*him*A::*cat*	IHF⁻ strain for use in recombination assays. Created by P1 transduction from HN1491	This work
C600(186⁺)	186⁺ lysogen of C600		This laboratory
C600(186*apl*Δ11)	186*apl*Δ11 lysogen of C600		This work
C600(186*cIts apl*Δ11)	186*cIts apl*Δ11 lysogen of C600		This work
C600(186*cIts*)	186*cIts* lysogen of C600		This laboratory
C600(186*Eae*I)	186*Eae*I lysogen of C600		This work
C600(186*goa*2)	186*goa*2 lysogen of C600		This work

Strain	Genotype	Use	Reference
C600(186*goa*3)	186*goa*3 lysogen of C600		This work
C600(186*goa*6)	186*goa*6 lysogen of C600		This work
C600(186*goa*8)	186*goa*8 lysogen of C600		This work
C600(186*goa*10)	186*goa*10 lysogen of C600		This work
DH5α	F⁻ *end*A1 *hsd*R17 ($r_K^-m_K^+$) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Nal^R) *rel*A1 Δ(*lac*ZYA-*arg*F) U169	Routine cloning and for propagation of plasmids for double standed sequencing	(Hanahan, 1983)
GM119	F⁻ *dam*3 *dcm*6 *met*B1 *gal*K2 *gal*T22 *lac*Y1 *tsx*7:*sup*E44	Used to prepare non-*dam* and non-*dcm* methylated DNA	(Yanisch-Perron *et al.*, 1985)
HN1491	N99 Δ*him*A::*cat*	IHF⁻ donor strain for P1 transductions	(Granston & Nash, 1993); gift from Howard Nash
JM101	F' *tra*D36 *lac*I^q Δ(*lac*Z)M15 *pro*AB /*sup*E *thi* Δ(*lac-pro*AB)	Propagation of M13 phage	(Yanisch-Perron *et al.*, 1985)
LE392	*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 *lac*Y1	Routine cloning	(Maniatis *et al.*, 1982)
MC1061.5	F⁻ *ara*D139 Δ(*ara-leu*)7696 Δ(*lac*)X74 *gal*U *gal*K *hsd*R2 (r_K^- m_K^+) *mcr*B1 *rps*L (Str^R)	Used as host for all *lac*Z assays	(Koop *et al.*, 1987)
MC1061.5(λMRR1)	λMRR1 lysogen of MC1061.5	LacZ assays	This work
MC1061.5(λMRR2)	λMRR2 lysogen of MC1061.5	LacZ assays	This work
MC1061.5(λMRR3)	λMRR3 lysogen of MC1061.5	LacZ assays	This work
WM2016	CSH50 Δ*fis*::*Km*	Fis⁻ donor strain for P1 transductions	(Koch *et al.*, 1988); gift from Walter Messer
XL1-Blue	F'::Tn10 *pro*A$^+$B$^+$ *lac*I^q Δ(*lac*Z) M15/*rec*A1 *end*A1 *gyr*A96 (Nal^R) *thi* *hsd*R17 ($r_K^-m_K^+$) *sup*E44	Routine cloning	(Bullock, 1987)

6.2 BACTERIOPHAGE

Bacteriophage	Description	Reference
186		
186^{+}	The phage we love and nurture	(Jacob & Wollman, 1956)
186*apl*Δ11	Carries a 15 bp deletion in the *apl* gene	This work
186*c*I10	Carries a missense mutation in the immunity repressor gene	(Huddleston, 1970); sequenced in this work
186*c*I10 *goa*10	Can form plaques on Apl-expressing strains	This work
186*c*I10 *goa*11	Can form plaques on Apl-expressing strains	This work
186*c*I10 *goa*12	Can form plaques on Apl-expressing strains	This work
186*c*I10 *vir*118	Can form plaques on 186 lysogens.	(Lamont *et al.*, 1988)
186*c*I*ts*	Also called 186*c*I*ts*p. Carries a temperature-sensitive immunity repressor	(Baldwin *et al.*, 1966; Woods & Egan, 1974)
186*c*I*ts apl*Δ11	186*c*I*ts* carrying a 15 bp deletion in the *apl* gene	This work
186*c*I*ts goa*p3	Can form plaques on Apl-expressing strains	This work
186*c*I*ts goa*p5	Can form plaques on Apl-expressing strains	This work
186Δ1*vir*121	Can form plaques on 186 lysogens and Apl-expressing strains	(Lamont *et al.*, 1988)
186*Eae*I	Carries a 2 bp substitution creating an *Eae*I site between P_R and P_L	This work
186*goa*1	Can form plaques on Apl-expressing strains	This work
186*goa*2	Can form plaques on Apl-expressing strains	This work
186*goa*3	Can form plaques on Apl-expressing strains	This work
186*goa*5	Can form plaques on Apl-expressing strains	This work
186*goa*6	Can form plaques on Apl-expressing strains	This work
186*goa*7	Can form plaques on Apl-expressing strains	This work
186*goa*8	Can form plaques on Apl-expressing strains	This work
186*goa*9	Can form plaques on Apl-expressing strains	This work

Bacteriophage	Description	Reference
186*vir*100	Can form plaques on 186 lysogens.	(Lamont *et al.*, 1988)
186*c*I10 *vir*118 *goa*21	Can form plaques on Apl-expressing strains	This work
186*c*I10 *vir*118 *goa*22	Can form plaques on Apl-expressing strains	This work
186*c*I10 *vir*118 *goa*23	Can form plaques on Apl-expressing strains	This work
lambda		
λMRR1	λRS45 derivative containing promoter and *lac* sequences from pMRR29	This work
λMRR2	λRS45 derivative containing promoter and *lac* sequences from pMRR30	This work
λMRR3	λRS45 derivative containing promoter and *lac* sequences from pMRR31	This work
λRS45	Vector for creating single copy *lacZ* constructs	(Simons *et al.*, 1987); see Fig. 6.4

6.3 PLASMIDS

Plasmid	Replicon	Description	Reference
pACYC177	p15A	General cloning	(Chang & Cohen, 1978)
pACYC184	p15A	General cloning	(Chang & Cohen, 1978)
p*att*B	p15A	pACYC184 derivative containing the end-filled 353 bp *Eco*RI-*Bam*HI (both pBluescript sites) fragment of pMRR5 ligated to the 3780 bp *Pvu*II fragment of pACYC184 (with the end-filled *Bam*HI site nearest the pACYC184 *Eco*RI site). The end-filled *Eco*RI site creates a new *Eco*RI site when ligated to the *Pvu*II site.	This work
pATTi	p15A	The "integrated" form of pATTx. It is the result of exposing pATTx to 186 Int and *E. coli* IHF and contains *att*L and *att*R in an inverted orientation	This work; see Fig. 3.3.A
pATTx	p15A	Used in the recombination assays (Chapter Three). Contains 186 *att*P and *att*B oriented such that site-specific recombination between the two sites inverts the DNA fragment between them. A DNA fragment containing all of the predicted binding sites at *att*P (see Fig. 3.8) was amplified by PCR using oligonucleotides 63 and 66 as primers and inserted into the end-filled *Tth*111I site of p*att*B (with the oligonucleotide 63 sequence nearest the *att*B insert)	This work; see Fig. 3.3.A
pBluescript	ColE1	General cloning, double-stranded sequencing and site-directed mutagenesis vector	Stratagene
pDM1.1	p15A	Kanamycin resistant pACYC177 derivative containing a 1.23 kb SalI fragment carrying *lac*I (*lac* repressor) expressed from the I^q promoter	(Lanzer & Bujard, 1988); from H. Bujard
pEC603	ColE1	pKO2 derivative carrying the *Hae*III.1721-*Hae*III.2998 fragment of 186*cIts*	(Dodd *et al.*, 1990)
pEC620	ColE1	pUC18 derivative carrying the *Xho*I.629-*Bgl*II.4244 fragment of 186*cIts* created as described in Chapter Two	This work
pEC621	ColE1	*apl*Δ11 derivative of pEC620, created as described in Chapter Two	This work
pEC625	ColE1	pBluescript SK^- with end-filled 186 *Mae*II.2666-*Mae*III.2868 fragment cloned into the *Sma*I site, created as described in Chapter Two	This work

Plasmid	Replicon	Description	Reference
pEC630	ColE1	pEC620 derivative with the *Nru*I.1473-*Bss*HII.3411 region replaced with that region from mEC4 (M13tg130 derivative carrying the *Sac*I.1403-*Bgl*II.4244 fragment with the *Eae*I mutation; see (Dodd *et al.*, 1990), so that this plasmid is now cI^+ and carries the *Eae*I mutation	This laboratory
pET3a	ColE1	Carries a T7 promoter and ribosome binding site that can be used for expression of proteins from inserted DNA fragments	(Studier *et al.*, 1990)
pKM2	ColE1	pKO2 derivative carrying the tR1 terminator of λ following the polycloning site	(de Boer, 1984)
pKO2	ColE1	GalK reporter plasmid	(de Boer, 1984)
plysS	p15A	pACYC184 derivative carrying the T7 lysozyme gene expressed from P_{tet}	(Studier *et al.*, 1990)
pMO1	ColE1	pBluescript SK^- derivative with the end-filled 2.8 kb *Bgl*I-*Eco*RI fragment of 186 *att*B inserted at the *Sma*I site. See Fig. 3.1	Reed *et al.*, submitted
pMRR1	ColE1	pET3a clone expressing Apl, created as described in Chapter Two	This work
pMRR2	ColE1	pET3a clone expressing AplΔ11, created as described in Chapter Two	This work
pMRR3	ColE1	pUC19 derivative carrying the 186 *Xho*I.629-*Bgl*II.4244 fragment, created as described in Chapter Two	This work
pMRR4	ColE1	pBluescript SK^+ derivative carrying the 186 *Hae*III.676-*Eco*RV.889 fragment, created as described in Chapter Two	This work
pMRR5	ColE1	333 bp *Bam*HI (pBluescript site)-*Bcl*I fragment from pMO1 into the *Bam*HI site of pBluescript SK^+ (with the *Bcl*I site nearest the *Sma*I site of pBluescript). See Fig. 3.2.B	This work
pMRR6	ColE1	2.5 kb *Bcl*I-*Eco*RI fragment obtained by religating pMO1 following removal of the 333 bp *Bam*HI-*Bcl*I fragment (see pMRR5)	This work
pMRR7	ColE1	P_R^- derivative of pMRR3 created by site-directed mutagenesis with oligonucleotide 39	This work
pMRR9	ColE1	pRS415 derivative containing translation stop codons from pKO2 and pUC19 poly-cloning site. Used for *lac*Z assays of P_L	This work; see Fig. 6.3

Plasmid	Replicon	Description	Reference
pMRR9T	ColE1	pRS415 derivative containing translation stop codons and T_{R1} terminator from pKM2 and pUC19 poly-cloning site. Used for *lac*Z assays of P_R	This work; see Fig. 6.3
pMRR11	ColE1	P_R^- derivative of pEC621, created by site-directed mutagenesis with oligonucleotide 39	This work
pMRR13	ColE1	pUHE24-2 derivative containing the 1.23 kb *Sal*I fragment from pDM1.1 inserted at the *Sal*I site. This fragment contains *lac*I under the control of the I^q promoter and is oriented so that transcription from the I^q promoter is in the same direction as the P_{A1} promoter of pUHE24-2	This work
pMRR14	p15A	Large end-filled *Bam*HI-*Ban*I fragment from pACYC177 ligated to the small end-filled *Xho*I-*Hin*dIII fragment of pMRR13, with the end-filled *Bam*HI site adjacent to the end-filled *Xho*I site. Replaces ColE1 *ori* of pMRR13 with lower copy number p15A *ori*.	This work
pMRR15	ColE1	End-filled 1140 kb *Bgl*II-*Bam*HI (*int*) fragment of pMRR18 inserted into the 4630 bp end-filled *Eco*RI-*Bam*HI fragment of pMRR13 with the end-filled *Bgl*II end ligated to the end-filled *Eco*RI end. This gives the structure: $P_{A1/O4/O3}$-T7 RBS-*int*-P_{lacIq}-*lac*I	This work
pMRR16	ColE1	Int/Apl expression plasmid created by inserting the end-filled 392 bp *Bgl*II-*Afl*III fragment from pMRR1 into pMRR15 cut with *Bam*HI and end-filled. This gives the structure: $P_{A1/O4/O3}$-T7 RBS-*int*-T7 RBS-*apl*-P_{lacIq}-*lac*I	This work
pMRR17	ColE1	Int/AplΔ11 expression plasmid created by inserting the end-filled 392 bp *Bgl*II-*Afl*III fragment from pMRR2 into pMRR15 cut with *Bam*HI and end-filled. This gives the structure: $P_{A1/O4/O3}$-T7 RBS-*int*-T7 RBS-*aplΔ*11-P_{lacIq}-*lac*I	This work
pMRR18	ColE1	Int expression plasmid created by inserting the *Nde*I-*Bam*HI fragment of the PCR product generated using oligonucleotides 70 and 73 into the *Nde*I and *Bam*HI sites of pET3a. The sequence of the *int* gene was confirmed by sequencing.	This work
pMRR20	p15A	*Mun*I.2791-*Afl*III.3119 fragment of pMRR3 inserted at the *Eco*RI-*Nco*I sites of pMRR14. Used for tightly controlled Apl expression	This work
pMRR21	p15A	*Mun*I.2791-*Afl*III.3119 fragment of pEC621 inserted at the *Eco*RI-*Nco*I sites of pMRR14. Used for tightly controlled AplΔ11 expression.	This work

Plasmid	Replicon	Description	Reference
pMRR22	p15A	End-filled small *Bgl*II-*Eco*RV fragment from pMRR1 ligated to the large *Eco*RV-*Nru*I fragment of pACYC184, with *apl* expressed from the P_{tet} promoter	This work
pMRR23	p15A	End-filled small *Bgl*II-*Eco*RV fragment from pMRR2 ligated to the large *Eco*RV-*Nru*I fragment of pACYC184, with *apl*Δ11 expressed from the P_{tet} promoter	This work
pMRR24	p15A	*Xmn*I.2684-*Fsp*I.3177 fragment from pMRR3 ligated to the large end-filled *Hin*dIII-*Nru*I fragment of pACYC184, with *apl* expressed from the 186 P_R promoter and translated from its own ribosome binding site	This work
pMRR26	ColE1	*Xmn*I.2684-*Rsa*I.2950 fragment of pMRR3 inserted at the *Sma*I site of pMRR9 with P_L expressing *lac*Z. Wild type parent clone for assaying P_L activity in *goa* DNA fragments	This work
pMRR27	ColE1	*Xmn*I.2684-*Rsa*I.2950 fragment of pMRR3 inserted at the *Sma*I site of pMRR9T with P_R expressing *lac*Z. Wild type parent clone for assaying P_R activity in *goa* DNA fragments	This work
pMRR29	ColE1	*Eco*RV.2593-*Pvu*II (*apl*Δ11).2888 fragment of pMRR3 inserted at the *Sma*I site of pMRR9T with P_R expressing *lac*Z	This work
pMRR30	ColE1	*Eco*RV.2593-*Pvu*II (*apl*Δ11).2888 fragment of pMRR11 (P_R^-) inserted at the *Sma*I site of pMRR9 with P_L expressing *lac*Z	This work
pMRR31	ColE1	*Eco*RV.2593-*Pvu*II (*apl*Δ11).2888 fragment of pMRR3 inserted at the *Sma*I site of pMRR9 with P_L expressing *lac*Z	This work
pRS415	ColE1	*lac*Z reporter plasmid	(Simons *et al.*, 1987); see Fig. 6.2
pUC19	ColE1	General cloning, double-stranded sequencing and site-directed mutagenesis vector	(Yanisch-Perron *et al.*, 1985)
pUHE24-2	ColE1	Contains a tightly controlled IPTG-inducible promoter	From H. Bujard; see Fig. 6.1

6.4 REAGENTS

6.4.1 Enzymes

Calf intestinal phosphatase: Boehringer Mannheim.

E. coli DNA polymerase I (Klenow fragment): BRESATEC (Australia).

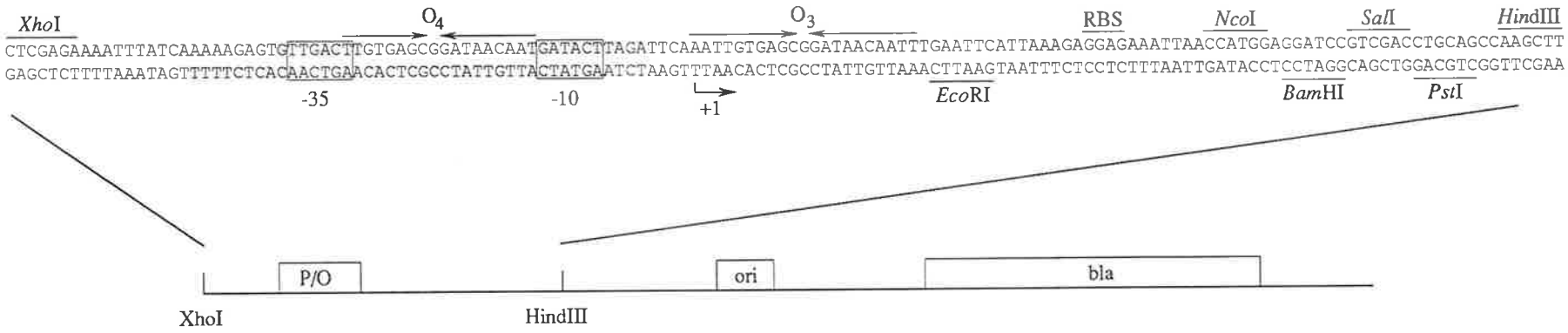


Figure 6.1 Map of pUHE24-2

This vector contains an artificially constructed promoter/operator (P/O), derived from the T7 promoter A1 (P_{A1} - an *E. coli* RNA polymerase promoter) combined with two high affinity *lac*I operators; one between the -10 and -35 regions of the promoter and one over the transcription start site. Other features of this plasmid include a ribosome binding site (RBS) and polycloning site downstream of the promoter/operator region, a ColE1 origin of replication and aβ-lactamase gene for selection on amplicillin. The promoter/operator nomenclature is as described by Lanzer & Bujard (1988). The size of the plasmid is 3.4 kb.

E. coli DNase I: Boehringer Mannheim. DNase I was stored as a 2 mg ml^{-1} solution in 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 100 µg ml^{-1} BSA, 50% glycerol at -80°C in small aliquots.

Lysozyme: Sigma Chemical Co.

Proteinase K: Boehringer Mannheim.

Restriction Endonucleases: New England Biolabs, Toyobo, Bethesda Research Labora-tories or Boehringer Mannheim.

RNase A: Sigma Chemical Co. 10 mg ml^{-1} stock solutions were heated at 95°C for 20 min to inactivate DNases.

T4 DNA ligase: Promega and BRESATEC.

T4 DNA polymerase: Promega.

T4 Polynucleotide kinase: Boehringer Mannheim.

Taq DNA polymerase: BRESATEC

6.4.2 Radiochemicals

Radiochemicals [α-^{32}P]-dCTP and [α-^{32}P]-dATP of specific activity 3000 Ci $mmol^{-1}$, [γ-^{32}P]-rATP of specific activity 4000 Ci $mmol^{-1}$ (radioactive concentrations of 5 mCi ml^{-1}) and [α-^{35}S]-dATP of specific activity 1000-1500 Ci $mmol^{-1}$ (radioactive concentration of 12.5 mCi ml^{-1}) were purchased from BRESATEC (Australia).

6.4.3 Chemicals

All chemicals were of analytical grade or of the highest purity available.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal): Sigma Chemical Co. Stock solutions at 20 mg ml^{-1} in dimethyl formamide were kept at -20°C.

8-hydroxy quinoline: Sigma Chemical Co.

Acetic acid: B.D.H. Labs., Australia.

Acrylamide: Sigma Chemical Co.

Agarose: Sigma Chemical Co.

Amine A: Humpko Sheffield, U.S.A.

Ammonium acetate: B.D.H. Labs., Australia.

Ammonium persulphate (APS): May and Baker Ltd. Stock solutions at 25% (w/v) in H_2O were prepared fresh on the day of use.

Ammonium sulphate: May and Baker Ltd.

Ampicillin (sodium salt): Sigma Chemical Co. Stock solutions (50-100 mg ml^{-1} in H_2O) were millipore filtered and stored at -20°C.

β-Mercaptoethanol: Sigma Chemical Co.

Bacto-tryptone, yeast extract and Bacto-agar: Difco Labs., U.S.A.

Boric acid: B.D.H. Labs., Australia.

Bovine serum albumin (BSA): Sigma Chemical Co. Kept as a 10 mg ml^{-1} solution in H_2O at -20°C.

Brij 58 (polyoxyethylene 20 cetyl ether): Sigma Chemical Co.

Bromocresol purple: B.D.H. Labs., Australia. Stock solutions were made to 1% (w/v) in ethanol.

Bromophenol blue: B.D.H. Labs., Australia.

Caesium chloride (CsCl): Bethesda Research Labs.

Calcium chloride ($CaCl_2$): Sigma Chemical Co.

Casein amino acids (vitamin free CAA): Difco labs, U.S.A..

Chloramphenicol: Sigma Chemical Co. Stock solutions (30 mg ml^{-1} in ethanol) were stored at -20°C.

Chloroform: B.D.H. Labs., Australia.

Coomassie blue G-250: Sigma Chemical Co.

Coomassie blue R-250: Sigma Chemical Co.

Deoxyribonucleoside triphosphates (dNTP): Sigma Chemical Co. Stock solutions at 20 mM (prepared in 5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) were kept at -20°C.

Di-potassium hydrogen orthophosphate (K_2HPO_4): B.D.H. Labs., Australia.

Di-sodium hydrogen orthophosphate (Na_2HPO_4): B.D.H. Labs., Australia.

Dialysis membrane (18/32): Union Carbide. Prepared by boiling 10 cm strips in 2% sodium bicarbonate, 1 mM EDTA for 10 min and storing in 20% ethanol.

Dithioerythritol (DTE): Sigma Chemical Co. Stored as a 0.1 M solution in H_2O at -20°C.

Dithiothreitol (DTT): Sigma Chemical Co. Stored as a 1 M solution in H_2O at -20°C.

Ethanol (95%): Redistilled before use.

Ethidium bromide: Sigma Chemical Co. Stored as a 10 mg ml^{-1} solution in H_2O in the dark at 4°C.

Ethylenediaminetetraacetic acid (EDTA): Disodium salt. Sigma Chemical Co.

Ficoll 400: Pharmacia Fine Chemicals.

Formamide: B.D.H. Labs., Australia. De-ionized and stored in the dark at -20°C.

Glucose: Ajax.

Glycerol: B.D.H. Labs., Australia.

Glycogen: Boehringer Mannheim.

Hydrochloric acid (HCl): B.D.H. Labs., Australia.

Isopropanol (IPA): May and Baker Ltd.

Isopropyl-β-D-thiogalactopyranoside: Sigma Chemical Co. Stock solutions (1 M in H_2O) were millipore filtered and stored at -20°C.

Kanamycin (sulphate): Sigma Chemical Co. Stock solutions (50 mg ml^{-1} in H_2O) were millipore filtered and stored at -20°C.

Low melting temperature (LMT) agarose: Bethesda Research Laboratories.

Magnesium acetate: Ajax.

Magnesium chloride: Ajax.

Magnesium sulphate: Ajax.

Manganese chloride: B.D.H. Labs., Australia.

Methanol: B.D.H. Labs., Australia.

Mixed bed resin (508-X8(D)): Bio-Rad Labs.

N, N'-methylene-bis-acrylamide (bis): Sigma Chemical Co.

N, N, N', N'-tetramethylethylenediamine (TEMED): Eastern Kodak Co.

Nitrocellulose: Schleicher & Schuell BA85 (0.45 μm).

Nonidet P40: B.D.H. Labs., Australia.

O-nitrophenyl-β-D-galactopyranoside (ONPG): Diagnostic Chemicals Ltd. Stored as a 4 mg ml^{-1} solution in 0.2 M phosphate buffer pH 7.0.

Phenol: AR grade, B.D.H. Labs., Australia. Redistilled and stored in the dark at -20°C. TE-saturated phenol was prepared as follows. To melted phenol was added 8-hydroxy-quinoline to 0.1% final concentration. Buffer equilibration was carried out by the addition of an equal volume of 1 M Tris-HCl (pH 8.0) and the mixture heated until the phenol and aqueous layers mixed. The phases were allowed to separate and the aqueous phase removed. Two volumes of TE was then added to the phenol phase, mixed and allowed to stand until the phases separated. This was repeated two more times. Equilibrated phenol was stored under TE and kept frozen in 10 ml aliquots at -20°C until required.

Phenylmethylsulfonyl flouride (PMSF): Sigma Chemical Co.

Polyethylene glycol (PEG) 8000: for phage preparations and general use was from Sigma Chemical Co.

Polyvinyl pyrrolidone: May and Baker Pty. Ltd.

Potassium acetate: B.D.H. Labs., Australia

Potassium chloride: B.D.H. Labs., Australia

Protein molecular weight markers: BRL low range molecular weight protein markers (ovalbumin 43 kD, carbonic anhydrase 29 kD, β-lactoglobulin 18.4 kD, lysozyme 14.3 kD, bovine trypsin inhibitior 6.2 kD and insulin α+β 3.0 kD).

rATP: Sigma Chemical Co.

Salmon sperm DNA: Sigma Chemical Co. Sonicated and stored as a 10 mg ml^{-1} solution in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA at -20°C - 4°C.

Sodium acetate: B.D.H. Labs., Australia.

Sodium carbonate: B.D.H. Labs., Australia

Sodium chloride: B.D.H. Labs., Australia

Sodium deoxycholate: Sigma Chemical Co.

Sodium dihydrogen phosphate: May and Baker Ltd.

Sodium dodecyl sulphate (SDS): Sigma Chemical Co.

Sodium hydroxide: Ajax.

Spermidine: Sigma Chemical Co.

Sucrose: Ajax

Tetracycline: Upjohn Pty. Ltd. Stock solutions (25 mg ml^{-1} in ethanol) were stored at -20°C.

Tetramethylammonium chloride (TMACl): Aldrich Chemical Co.

Thiamine: Sigma Chemical Co.

Thiourea: B.D.H. Labs., Australia.

Tri-sodium citrate (Na_3citrate): B.D.H. Labs., Australia.

Tris acetate: B.D.H. Labs., Australia.

Trizma base and Tris 7-9: Sigma Chemical Co.

Urea: Sigma Chemical Co.

Xylene cyanol: Sigma Chemical Co.

Zinc chloride: May and Baker Ltd.

6.4.4 Oligonucleotides

The oligonucleotides used during the course of this work for site-specific mutagenesis and DNA sequencing were constructed by BRESATEC (Australia) using an Applied Biosystems 380B DNA synthesiser.

Oligonucleotide	186 Sequence Coordinates[a]	Use	Sequence (5'-3')
12	2988-3004 (*r*-strand)	General sequencing and PCR of P_R-P_L region	TCGGGCCACCTGCTTTC
22	3189-3205 (*r*-strand)	General sequencing and PCR of *apl* gene	CCAGCTTCGCCATGTTG
26	2813-2831 (*r*-strand)	To create *Nde*I site (underlined) at *apl* initiation codon	GCCATATGGCATGATTCCC
34	2660-2689 (*l*-strand)	General sequencing and PCR of P_R-P_L region	ACATCCACGTTGCTCCATCCTAAAGAATCT
35	2843-2872 (*r*-strand)	General sequencing and PCR of P_R-P_L region	TAACGATAGGTGCAGGCACTTTGATGATTG
37	2918-2935 (*r*-strand)	General sequencing and PCR of P_R-P_L region	CGGTTGTCCAGCGGTAGG
38	2281-2298 (*l*-strand)	General sequencing and PCR of *c*I gene	GCCGTCAGACAATGATGC
39	2701-2731 (*l*-strand)	Creating a *Xho*I site (underlined) at P_R -35 region. Also creates a number of other restriction sites	GATAAAACCTACTCGAGATCTCTCAATTGGG
56		Amplifying inserts in λRS45, pMRR9 and pMRR9T clones (right side primer; see also oligonucleotide 57)	TTAACTGCGCGTCGCCGC
57		Amplifying inserts in λRS45, pMRR9 and pMRR9T clones (left side primer; see also oligonucleotide 56)	TGCCAGGAATTGGGGATC
63	552-568 (*l*-strand)	PCR primer to amplify *att*P (left side; see also oligonucleotide 66)	TATGCACTCAGGAAAGT

Oligonucleotide	186 Sequence Coordinates[a]	Use	Sequence (5'-3')
66	843-859 (*r*-strand)	PCR primer to amplify *att*P (right side; see also oligonucleotide 63)	GCGATGGTTCTGAGTAA
69	1601-1617 (*l*-strand)	Sequencing *int* following creation of *Nde*I site at its initiation codon	GTCGTGAGTTAGCCAAG
70	2110-2127 (*l*-strand)	Creating an *Nde*I site (underlined) at initiation codon of *int* gene (see also oligonucleotide 73)	GTTAACATATGACCGTCC
72	1347-1363 (*l*-strand)	Sequencing *int* following creation of *Nde*I site at its initiation codon	GCGCCATAACAATCACT
73	1079-1094 (*l*-strand)	Creating a tail (underlined) at the end of the *int* gene, containing a *Bam*HI site (see also oligonucleotide 70)	CGGATCCTAATCGAACTG ACAT
M13 Reverse Sequencing Primer (RSP)		Sequencing and PCR	CACACAGGAAACAGCTA TGGACCATG
T7 Primer		Used for PCR and sequencing from pET3a clones	AATACGACTCACTATAG
Universal Sequencing Primer (USP)		Sequencing and PCR	GTAAAACGACGGCCAGT

[a] where applicable

6.4.5 Growth media.

6.4.5.1 Liquid media

All media were prepared in glass distilled H_2O and were sterilised by autoclaving for 25 min at 120°C and 120 kPa.

2YT medium	1.6% Bacto-typtone, 1% yeast extract, 0.5% NaCl, pH 7.0.
10 x M9 salts	60 mM Na2HPO4, 60 mM KH2PO4, 0.5% NaCl, 1% NH4Cl.
LB medium (LB)	1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0.

M9CAA medium	1 x M9 salts, 0.1 mM CaCl2, 1 mM MgSO4, 0.2% glucose, 0.2% vitamin free CAA.
Minimal A medium	1.05% K2HPO4, 0.45% KH2PO4, 0.1% (NH4)2SO4, 0.05% Na3citrate and H2O to 1000 ml. This solution was autoclaved, cooled to 45°C and the following added from separately prepared sterile solutions; 10 ml of 20% glucose, 0.8 ml of 1 M MgSO4, 0.5 ml of 1% thiamine.
SOC medium	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl and H2O to 1000 ml. This solution was autoclaved, cooled and 10 ml of 2 M MgSO4 and 20 ml of 1 M glucose added.
TB medium (TB)	1% Bacto-tryptone, 0.5% NaCl, pH 7.0.

Antibiotic supplements to L, T and 2YT broth were ampicillin at 50-100 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, chloramphenicol at 30 μg ml^{-1} and tetracycline at 10-25 μg ml^{-1}. M13 minimal medium was supplemented with ampicillin at 50 μg ml^{-1}. M9CAA medium was supplemented with amplicillin at 50 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, tetracycline at 15 μg ml^{-1} and chloramphenicol at 30 μg ml^{-1}.

6.4.5.2 Solid media

L plates	1.5% Bacto-agar was added to L broth.
M9CAA plates	1.5% Bacto-agar was added to 1 x M9 salts and autoclaved. Following cooling to 45°C, $CaCl_2$ (100 mM), $MgSO_4$ (1 mM), glucose (0.2%) and vitamin free CAA (0.2%) were then added from separately prepared sterile solutions.
Minimal A plates	1.5% Bacto-agar was added to M13 minimal medium. Used for plating M13.
Soft agar overlay	1% Bacto-tryptone, 0.7% Bacto-agar, 0.5% NaCl, pH 7.0. Used for 186, λ and P1 platings.
T plates	1.5% Bacto-agar was added to T broth.
YT soft agar overlay	0.8% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.7% Bacto-agar, pH 7.0. Used for M13 platings.

Antibiotic supplements to L and T plates were ampicillin at 50-100 μg ml^{-1} and kanamycin at 50 μg ml^{-1}. M13 minimal plates were supplemented with ampicillin at 50 μg ml^{-1}. Plates were poured from 30 ml of the appropriate medium, dried overnight at 37°C and stored at 4°C.

6.4.6 Buffers and solutions

6.4.6.1 General DNA buffers

6 x agarose gel loading buffer	0.1% bromophenol blue, 0.1% xylene cyanol, 15% Ficoll 400, 20 mM EDTA, 0.2% SDS.
6 x PAGE loading buffer	30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol.
10 x polynucleotide kinase	500 mM Tris pH 7.9, 100 mM $MgCl_2$, 100 mM β-mercapto-ethanol, 100 mM EDTA. Stored at -20°C.
10 x SD restriction buffer	33 mM Tris acetate pH 7.85, 0.65 M potassium acetate, 0.1 M magnesium acetate, 4 mM spermidine, 20 µM DTE.
10 x T4 ligase buffer	10 mM ATP, 100 mM $MgCl_2$, 500 mM Tris pH 7.5, 25 mM DTT.
10 x TAE	0.4 M Tris-acetate, 0.2 M Na acetate, 10 mM EDTA, pH 8.2.
10 x TBE	0.89 M Tris-HCl, 0.89 M boric acid, 2.7 mM EDTA, pH 8.3.
20 x SSC	3.0 M NaCl, 0.3 M sodium citrate, pH 7.4 with concentrated HCl.
20 x SSPE	3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA, pH 7.0 with NaOH.
100 x Denhardts solution	2% Ficoll 400, 2% BSA, 2% polyvinyl pyrrolidone.
Colony cracking solution	50 mM NaOH, 0.5% SDS, 50 mM EDTA, 10% glycerol, 0.05% bromocresol purple.
Formamide loading buffer	95% Formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA.
GET buffer	50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0.
IAC	24 parts chloroform to 1 part isoamyl alcohol.
Oligo hybridization buffer	5 x SSPE, 5 x Denhardt's solution, 1% SDS, 0.05% sodium phosphate buffer.
PAGE elution buffer	0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA.
PCR buffer	67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.045% Triton X-100, 0.2 mg ml^{-1} gelatin.
Southern hybridization buffer	3 x SSPE, 1% BSA, 1% SDS, 100 µg ml^{-1} denatured sonicated salmon sperm DNA.
STENA	TENA plus 0.1% SDS (SDS helps redissolve the pellet).

TE	100 mM Tris-HCl, pH 8.0, 0.1 mM EDTA was used for general storage of DNA.
TENA	100 mM Tris pH 8.0, 0.1 mM EDTA, 0.3 M sodium acetate pH 5.2.
TMACl wash solution	3 M TMACl, 2 mM EDTA, 0.05 M Tris-HCl pH 8.0, 1% SDS. Stored in the dark at room temperature.

6.4.6.2 Phage solutions

PSB	10 mM Tris-HCl, pH 7.1, 10 mM $MgSO_4$ was used for preparation and storage of 186, λ and P1 phage.

6.4.6.3 Protein solutions

Bradford reagent	0.01% Coomassie Blue (G-250), 50% ethanol, 8% phosphoric acid.
Coomassie blue stain	0.125% coomassie blue R-250, 50% methanol, 10% acetic acid.
Destain solution	50% methanol, 10% acetic acid.
DNase I dilution buffer	100 mM KCl, 100 mM $MgCl_2$, 20 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0, 2 mM DTT.
DNase I stop buffer	In 100 µl, have 50 mM EDTA, 1% SDS, 0.4 mg ml^{-1} glycogen, 5 µl saturated ammonium acetate.
Footprint binding buffer	Same as the gel retardation binding buffer.
Gel retardation binding buffer	10 mM Tris pH 7.5, 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol, 50 µg ml^{-1} BSA, 1 µg ml^{-1} sonicated salmon sperm DNA.
Gel retardation loading buffer	1 x gel retardation binding buffer, 0.05% bromophenol blue, 0.05% xylene cyanol.
IHF retardation binding buffer	0.25 mg ml^{-1} BSA, 50 mM Tris-HCl pH 8.0, 20% glycerol and 5 mM EDTA.
Protein extraction buffer	100 mM Tris pH 7.9, 200 mM KCl, 0.1 mM DTT, 1 mM EDTA, 5% glycerol, 1 mM PMSF.
Protein sample buffer	63 mM Tris pH 6.8, 3% SDS, 10% glycerol, 0.75 M β-mercaptoethanol, 0.05% bromophenol blue.
SDS-PAGE buffer	25 mM Tris-HCl pH 8.3, 200 mM glycine, 0.1% SDS.
Z buffer	100 mM KCl, 100 mM sodium phosphate buffer pH 7.0, 2.7 mM β-mercaptoethanol, 1 mM $MgSO_4$.

6.4.7 DNA markers

DNA size markers were all purchased from BRESATEC (Australia) and 200 ng were routinely loaded on an agarose gel.

*Hpa*II digest of pUC19 DNA at 500 ng μl^{-1}. Fragment sizes in bp: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26.

*Hin*dIII/*Eco*RI digest of phage λ DNA combined with *Hin*dIII digest of phage λ DNA at 500 ng μl^{-1}. Fragment sizes in bp: 23130, 21226, 9416, 6557, 5148, 4973, 4361, 4277, 3530, 2322, 2027, 1904, 1584, 1330, 983, 831, 564, 125.

*Eco*RI digest of phage SPP-1 DNA at 500 ng μl^{-1}. Fragment sizes in bp: 7840, 6960, 5860, 4690, 3370, 2680, 1890, 1800, 1450, 1330, 1090, 880, 660, 480, 380.

6.5 PHAGE AND BACTERIAL PROCEDURES

6.5.1 Storage of bacterial and phage stocks

Bacterial stocks for short term storage were maintained on the appropriate plates at 4°C. Long term storage of bacterial cultures was at -80°C after addition of glycerol to a final concentration of 20%.

Low titre stocks of M13 recombinant phage were maintained in 2YT broth at -20°C.

Low titre 186 and λ phage stocks were mixed with a few drops of chloroform and stored at 4°C.

6.5.2 Growth of bacterial strains

Stationary phase bacterial cultures were prepared by inoculating broth with a single colony of bacteria from a plate stock or a loopful of bacteria directly from a glycerol stock, and incubating overnight with aeration at the appropriate temperature (usually 30°C or 37°C).

Log phase cultures and indicator bacteria were prepared by diluting a fresh stationary culture 50-200 fold into sterile broth and incubating with aeration at the appropriate temperature, until the required cell density was reached. Cell density was measured by observing the A_{600} using a Gilford 300 T-1 spectrophotometer. Indicator bacteria were chilled and kept on ice until required.

For most cultures LB medium was used, with the following exceptions. 186 indicator bacteria were grown in TB medium. Strains used for β-galactosidase assays and strains used for Apl induction of a 186 lysogen were grown in M9CAA medium. JM101 was grown overnight in Minimal A medium and subcultured into 2YT medium.

6.5.3 186 and lambda phage stocks

Low titre stocks (10^9-10^{10} pfu ml^{-1}) of 186 strains were prepared by heat induction, liquid infection (as described by Hocking & Egan, 1982) or plate lysate. Low titre stocks of lambda strains were prepared by plate lysate. For plate lysates, the appropriate indicator bacteria was grown in TB (186) or LB supplemented with 2% maltose (lambda) to A_{600} = 0.8 and placed on ice. Lambda indicator bacteria were then harvested by centrifugation and resuspended in half a volume of 100 mM $MgSO_4$. Indicator bacteria (200 µl) were then placed in a tube and phage added to a multiplicity of about 0.1. Lambda infections were allowed to adsorb for 20 min at room temperature and 186 infections were used immediately. The phage/cell mixture was then mixed with 3 ml of molten soft agar overlay and poured onto TB plates wetted with 1 ml sterile H_2O. Plates were incubated overnight at 37°C and the phage harvested by adding 3 ml PSB, leaving for 1-2 hours at room temperature and removing the liquid to a 10 ml Oakridge tube. Several drops of chloroform were then added and the tube vortexed and placed on ice for at least 30 min. Following centrifugation (12000 rpm, 4°C, 10 min, JA21), the supernatant was transferred to a McCartney bottle and several drops of chloroform added. Stocks were stored like this at 4°C.

6.5.4 M13 phage stocks

M13 phage stocks were prepared as follows. A fresh stationary phase culture of JM101 grown in M13 minimal medium, was diluted one hundred fold into 2YT broth, an M13 plaque toothpicked into the culture and incubated at 37°C with aeration for 5-7 hours. The cultures were then centrifuged in a bench centrifuge (6000 rpm, 10 min, room temperature) and the supernatants, containing the M13 single stranded DNA phage, carefully transferred to an Eppendorf tube and centrifuged (10000 rpm, 10 min, room temperature, Eppendorf centrifuge). The supernatant was stored at -20°C.

6.5.5 Phage assays

186 phage were assayed for plaque forming units (pfu) by mixing 0.1 ml of the phage diluted in PSB, 0.2 ml of exponential phase indicator bacteria (A_{600} = 0.6-1.0 in LB) and 3 ml melted (0.7%) soft agar overlay and pouring the mixture onto TB plates. The agar was allowed to solidify and the plates were inverted and incubated overnight at the appropriate temperature. Plaques were counted and scored as plaque forming units per ml (pfu ml^{-1}). λ phage were assayed similarly to 186, except that the indicator bacteria were grown in medium containing 0.2% maltose, and when at exponential phase were harvested by centrifugation and resuspended in half a volume of 10 mM $MgSO_4$. Phage were allowed to adsorb to the bacteria

at 37°C for 20 min prior to plating. P1 phage were also assayed similarly to 186, except the indicator bacteria were grown in medium containing 4 mM $CaCl_2$, phage were allowed to adsorb to the bacteria for 20 min at 37°C prior to plating, the soft agar was supplement with 4 mM $CaCl_2$ and plated on LB plates.

6.5.6 Bacterial assays

Bacterial viable counts were measured by diluting a culture of cells in the appropriate medium and spreading the appropriate dilution onto the appropriate plates. The plates were incubated at the appropriate temperature and the colonies were counted and scored as colony forming units per ml (cfu ml^{-1}).

6.5.7 Construction of bacterial strains

6.5.7.1 Construction of 186 lysogens

Phage were plated for single plaques on a lawn of the bacterial strain to be lysogenized and the plates incubated at the appropriate temperature overnight. The centre of a turbid plaque was streaked for single colonies on the appropriate plate and the plate incubated overnight. Single colonies were tested, along with the appropriate controls of a lysogen and a non-lysogen, for immunity to 186 by streaking across 10-30 µl dried streaks of 186*c*I10 and 186*vir*1 on a TB plate. A colony which was capable of growth over 186*c*I10 but not 186*vir*1 was considered to be lysogenic. This colony was purified two times by streaking for single colonies and several of these colonies tested for immunity to 186 as described above.

6.5.7.2 Transformation/transfection by $CaCl_2$/heat shock

For routine cloning events where high efficiency transformation was not required, preparing competent cells by treatment with $CaCl_2$ and heat shocking was used. Fresh competent cells were prepared from 2-50 ml cultures grown in L broth with aeration to A_{600} = 0.3-0.6. After chilling on ice for 10 min, the cells were harvested by centrifugation (5000 rpm, 10 min, 4°C, JA20 rotor or 4000 rpm, 10 min, room temperature, bench top centrifuge), resuspended in 1/10 vol ice cold 100 mM $CaCl_2$ and left on ice for 0.5-24 hours.

Alternatively, frozen competent cells were prepared by inoculating 500 ml of fresh LB with the appropriate bacterial strain and incubating at 37°C, with aeration, to an A_{600} = 0.7-1.0. The culture was chilled on ice for 10 min and the cells harvested by centrifugation (10 min, 5000 rpm, 4°C, JA10). The cells were then resuspended in 50 ml cold 0.1 M $CaCl_2$ and left on ice for a further 20 min. The cells were again harvested and resuspended in 5 ml cold 85 mM $CaCl_2$, 15% glycerol. Small (50 µl) aliquots were transferred to pre-chilled tubes and

stored at -75°C until use. Just prior to use, an aliquot was thawed on ice, 0.95 ml cold 0.1 M $CaCl_2$ added and the cells allowed to stand on ice for 5-10 min.

Plasmid/phage DNA or an aliquot of the reaction mix from a ligation reaction was added to 0.1 ml competent cells in chilled, sterile eppendorf tubes and kept on ice for 10-20 min. The cells were heat shocked by incubation at 37°C for 5 min or 45°C for 2 min and placed on ice for 2-5 min.

For plasmid transformations, L broth (1 ml) was added to each tube and the tubes incubated at the appropriate temperature for 15-60 min, to allow the expression of the antibiotic resistance gene(s) present on the plasmid. Usually, the whole transformation mix (concentrated by centrifugation) was spread onto the appropriate plates and incubated at the appropriate temperature overnight. For use of the *lacZ* blue/white colony screening of pUC and pBluescript plasmids, 7 µl of 100 mM IPTG and 40 µl of 20 mg ml^{-1} X-gal was spread onto the plates and allowed to dry before plating the transformation mix. For using *lac*Z blue/white colony selection of *lac*Z reporter constructs, 40 µl of 20 mg ml^{-1} X-gal was spread onto the plates and allowed to dry before plating the transformation mix.

For 186 transfections, indicator bacteria were added to the heat shocked cells and the mixture plated as for a phage assay (Section 6.5.5).

For M13 transfections, 0.2 ml log phase JM101 indicator bacteria and 3 ml of molten YT soft agar containing 7 µl of 100 mM IPTG and 20 µl of X-gal (20 mg ml^{-1}) were added to the heat shocked cells. The mixture was poured onto an L plate and the plates incubated overnight at 37°C. M13 recombinants usually appeared as uncoloured plaques whereas parental M13 phage plaques were blue.

6.5.7.3 Electrotransformation

Cells were made competent for electrotransformation by growing 500 ml cultures of the appropriate bacterial strain in LB broth, with aeration, to an A_{600} of 0.5-1.0. The cultures were then chilled on ice and the cells harvested by centrifugation (4000 rpm, 4°C, 10 min, JA10). Cells were washed once in an equal volume of cold H_2O, once in half of the original volume of cold H_2O and once in 1/50 of the original volume of cold 10% glycerol. The cells were then resuspended in 1/300 of the original volume of cold 10% glycerol, distributed into small (40 µl) aliquots in eppendorf tubes, frozen in liquid nitrogen and stored at -75°C.

To electroporate the cells, the aliquots were thawed at room temperature and 1-2 µl DNA (in low ionic strength medium *e.g.* TE or H_2O) added. The suspension was mixed vigourously by flicking the tube, then the cell/DNA mixture electrotransformed as recommended for the

electroporator used (Bio-Rad GenePulser). Following electroporation, 1 ml of SOC medium was added to the cells and they were incubated at the appropriate temperature for 30-60 min, to allow the expression of the antibiotic resistance gene(s) present on the plasmid. Usually, the whole transformation mix (concentrated by centrifugation) was spread onto the appropriate plates and incubated at the appropriate temperature overnight.

6.5.7.4 P1 transductions

This procedure is based on that described in Miller (1992). The donor strain was grown in LB broth supplemented with 4 mM $CaCl_2$ to A_{600} = 0.8 and P1*vir* phage added to 1 ml of cells in a tube at a multiplicity of addition of approximately 1 (moa = 1). The tube was incubated at 37°C for 20 min to allow adsorption of the phage, then mixed with 3 ml of molten soft agar overlay supplemented with 4 mM $CaCl_2$ and poured onto L plates. Plates were incubated face up overnight at 37°C, the phage were harvested by adding 3 ml PSB, leaving for 1-2 hours at room temperature and removing the liquid to a 10 ml Oakridge tube. Several drops of chloroform were then added and the tube vortexed and placed on ice for 30 min. Following centrifugation (12000 rpm, 4°C, 10 min, JA21), the supernatant was transferred to a McCartney bottle and several drops of chloroform added. P1 stocks were stored like this at 4°C. The P1 stock was passaged at least twice through the donor strain before use as a transducing stock.

For the transduction, 100 µl of fresh overnight (or stationary phase) cultures of the recipient strain grown in LB supplemented with 4 mM $CaCl_2$ were mixed with 100 µl of undilute, 10^{-1} and 10^{-2} dilutions of the P1 donor lysate in an eppendorf tube. After incubation at 37°C for 20 min to allow adsorption, 200 µl of 100 mM sodium citrate pH 7.0 was added to prevent further P1 infection. 100 µl of the mixture was plated on selective plates and incubated at the appropriate temperature overnight. Controls of uninfected recipient bacteria and phage alone (as background controls) were also plated. Transductants were subjected to at least two colony purifications.

6.6 DNA TECHNIQUES

6.6.1 DNA preparations

6.6.1.1 186 DNA

186 DNA was prepared by PEG precipitating a low titre phage stock as follows: PEG 8000 (10% w/v final) and NaCl (1.25 M final) were added to 10-50 ml phage solution and incubated on ice for at least 30 min. Following centrifugation (15000 rpm, 4°C, 15 min, JA20), the supernatant was discarded and the pellet resuspended in 500 µl TE. The supernatant was then

extracted twice with chloroform, to remove the PEG, then twice with phenol and once with IAC (Section 6.6.2) to release the phage DNA. The DNA was then ethanol precipitated (Section 6.6.3) and resuspended in about 100 µl TE.

6.6.1.2 M13 single stranded DNA

M13 single-stranded DNA was prepared from M13 phage stocks (Section 6.5.4). Phage were precipitated by mixing 1 ml of stock with 1 ml of PEG solution (20% PEG w/v, 2.5 M NaCl). Phage particles were allowed to precipitate for 15 min at room temperature and the phage pellets collected by centrifugation (14000 rpm, 5 min, room temperature, Eppendorf centrifuge) and the supernatants withdrawn by aspiration. Tubes were briefly recentrifuged again and any traces of the supernatant removed. The phage pellets were resuspended in 200 µl of TE, phenol extracted (Section 6.6.2), the DNA ethanol precipitated (Section 6.6.3) and the pellet dissolved in 25 µl of TE.

6.6.1.3 Plasmid/M13 RF form DNA mini-preps

Small scale preparations of plasmid DNA were performed similarly to the alkaline lysis method of Sambrook *et al.* (1989). Cells from 1.5 ml overnight cultures of bacteria, grown with aeration in medium containing the appropriate antibiotic(s), were harvested by centrifugation in eppendorf tubes and resuspended in 100 µl GET buffer. 200 µl of 0.2 M NaOH/1% SDS was added and the tubes placed on ice for 10 sec-2 min. 150 µl ice cold 3 M sodium acetate pH 4.6 was then added, the tubes vortexed and centrifuged (15000 rpm, 5 min, room temperature, Eppendorf centrifuge) and the supernatant extracted with a half volume each of phenol and IAC. The DNA was precipitated by addition of two volumes of ethanol and the pellet dissolved in 20-50 µl TE containing 40 µg ml^{-1} RNase A.

For M13 RFDNA preparations, a fresh overnight culture of JM101, grown in minimal A medium, was diluted one hundred fold into 2YT broth, an M13 plaque toothpicked into the culture and incubated at 37°C with aeration for 5-7 hours. The culture was then treated as for the overnight plasmid culture described above.

6.6.1.4 Plasmid DNA purification by CsCl equilibrium density gradient centrifugation

Large quantities of DNA from a scaled-up mini-prep (Section 6.6.1.3) of a 100 ml overnight culture were purified, following ethanol precipitation by adding CsCl (0.95 g ml^{-1} w/v final) and ethidium bromide (0.2 ml of a 10 mg ml^{-1} solution per ml of CsCl solution). The solution

was then added to a 1.5 ml tube and centrifuged to equilibrium (80000 rpm, 20°C, overnight, Beckman TL100 rotor) in a Beckman TL100 ultracentrifuge.

Alternatively, DNA was isolated from a 500 ml overnight culture as follows. A fresh 2 ml overnight culture was used to inoculate 500 ml of fresh LB medium containing the appropriate antibiotic and grown overnight with vigourous aeration. Cells were harvested by centrifugation (5000 rpm, 4°C, 10 min, JA10 rotor) and resuspended in 2.75 ml 25% sucrose, 50 mM Tris-HCl pH 8.0 solution. This suspension was transferred to a 50 ml Oakridge tube and 4 mg of lysozyme was added. The tube was kept on ice for 5 min before addition of 3.75 ml of 0.1 M EDTA, pH 8.0 and kept on ice for another 10 min. After addition of 6 ml of Brij solution (1% Brij 58, 0.4% deoxycholate, 50 mM Tris-HCl pH 8.0, 25 mM EDTA) and gentle mixing, the tube was stored on ice for 10 min and centrifuged (30 min, 17000 rpm, 4°C, JA20 rotor). To the supernatant was added CsCl (0.95 g ml^{-1} w/v final) and ethidium bromide (0.2 ml of a 10 mg ml^{-1} solution per ml of CsCl solution). This solution was then placed in a 10 ml polycarbonate tube and centrifuged to equilibrium (45000 rpm, 20°C, 24-48 hours, Beckman Ti50 rotor) in a Beckman L8-70 ultracentrifuge.

Following ultracentrifugation, the bands of DNA could be visualized under subdued flourescent light and the lower of the two bands, containing the plasmid DNA, was collected by piercing the tube from the bottom and draining, or from the side with a syringe. The upper band contained chromosomal and nicked plasmid DNA. Ethidium bromide was removed by three extractions with isopropanol equilibrated with 5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The DNA solution was then diluted two-fold with H_2O and ethanol precipitated with 6 (original) volumes of ethanol. The pellet was dissolved in 50-500 µl TE and stored at 4°C.

6.6.1.5 Colony cracking

Initial identification of recombinant plasmids was sometimes performed using this procedure. Colonies containing putative recombinant plasmids were transferred using a toothpick to 20 µl of colony cracking solution and heated to 65°C for 20 min. This lyses the cells and denatures the plasmid DNA. The samples were then eletrophoresed through a TAE agarose gel (Section 6.6.5) and visualized by staining with ethidium bromide (4 mg ml^{-1} (w/v) in H_2O) and photographed under illumination by short wave UV light.

6.6.2 Phenol extraction of DNA solutions

DNA solutions were vortexed thoroughly with one volume of TE-equilibrated phenol and centrifuged (12000 rpm, 5 min, room temperature, Eppendorf centifuge or 12000 rpm, 5 min,

20°C, JA20 rotor for 50 ml Oakridge tubes). The aqueous phase was removed and the extraction repeated with an equal volume of IAC (see Section 6.4.6.1). The aqueous phase was then ethanol precipitated (Section 6.6.3).

6.6.3 Ethanol precipitation of DNA solutions

Generally, 1/10 volume of sodium acetate (pH 5.2) was added to the DNA solution and mixed by vortexing. Sometimes, for removal of unincorporated radionucleotides, a half volume of 7.5 M ammonium acetate was used instead of sodium acetate. Two volumes of ethanol were then added and the solution vortexed. Precipitation conditions varied, ranging from 1 min at room temperature (DNA minipreps) to overnight at -20°C. The DNA was collected by centrifugation (12000 rpm, 15 min, room temperature or 4°C, Eppendorf centrifuge, or 16000 rpm, 20 min, 4°C, JA20/JA21 rotor). The supernatant was removed by aspiration (the pellet sometimes rinsed in 70% ethanol (v/v)), dried under vacuum for 2-10 min, dissolved in TE or H_2O and stored at 4°C or -20°C.

6.6.4 Restriction digestion

Restriction digests either were carrried out as recommended by the manufacturer or in 1 x SD buffer, which was suitable for all enzymes used. Digestion times varied from one hour to overnight. Digests were checked by gel electrophoresis (Section 6.6.5 and 7.6.6.1).

6.6.5 Agarose gel electrophoresis

Agarose gel electrophoresis (1% to 3% w/v agarose in TAE, stored at 65°C) was carried out using horizontal minigels. A 1/6 volume of 6 x agarose gel loading buffer was added to the samples and electrophoresis carried out in TAE buffer at 75-150 mA.

DNA was visualised by staining gels with ethidium bromide (4 mg ml^{-1} (w/v) in H_2O). Gels were photographed under short wavelength UV light. Approximate concentrations of DNA solutions were determined by visual comparison of the intensity of the ethidium bromide stained bands with that of bands of known mass from tracks with DNA molecular weight markers.

6.6.6 Polyacrylamide gel electrophoresis

6.6.6.1 Non-denaturing gels

A 30% acrylamide stock solution (acrylamide:bis 30:0.8) was prepared by dissolving 146.1 g acrylamide and 3.9 g bis-acrylamide in 500 ml of glass distilled H_2O. The solution was de-ionised by adding 10% (w/v) of mixed bed resin and gently stirring the solution at room

temperature for 30 min. Mixed bed resin was removed by filtration. The acrylamide solutions were then de-gassed for 10 min using a vacuum pump and stored at room temperature in the dark. Gels ranged from 4%-8% acrylamide in 0.5 or 1 x TBE and 10% glycerol. To make 20 ml of 6% gel mix, 80 µl of freshly prepared 25% (w/v) APS and 20 µl TEMED was added. Gels were poured at room temperature, with polymerization of the gel solution occurring within 5-10 min. Polymerization was allowed to proceed for at least 30 min on the bench to ensure polymerization was complete. Gel dimensions were 20 x 40 x 0.05 cm or 15 x 15 x 0.05 cm. Gels were pre-electrophoresed for at least 30 min. For non-retardation gels, 1/3 to 1/10 volume of glycerol loading buffer was added to the samples.

6.6.6.2 Denaturing (sequencing) gels.

Stock gel solution (6% polyacrylamide; acrylamide:bis 19:1; 8 M urea in TBE) was prepared by dissolving 57 g acrylamide, 3 g bis-acrylamide and 480.5 g urea in 400 ml glass-distilled H_2O at room temperature. The solution was made to 900 ml with glass-distilled H_2O and was then de-ionised (see above). 100 ml of 10 x TBE was added and the solution de-gassed. Polymerisation was carried out by adding 400 µl of freshly prepared 25% (w/v) APS and 100 µl of TEMED, to 80 ml of gel stock solution. Gel dimensions were 20 x 40 x 0.04 cm or the BRL Life Technologies S2 sequencing system. Polymerisation was allowed to occur for 60 min at room temperature. Pre-electrophoresis was in 1 x TBE for 30 min at 800-1200 V. Electrophoresis was at 1000-2000 V. After electrophoresis, one of the glass plates was removed and the gel transferred to Whatman 3MM filter paper and dried under vacuum at 65°C. The gel was then autoradiographed (Section 6.6.7).

6.6.7 Autoradiography, phosphorimaging and autoradiograph scanning

Fuji RX medical X-ray film or Kodak XAR5 Scientific Imaging Film was used for autoradiography. Gels were exposed at room temperature (^{35}S and some ^{32}P) or at -80°C with a DuPont Hi plus-T intensifying screen (^{32}P). A Molecular Dynamics phosphorimager with Imagequant software was used for some analyses. For figure preparation, autoradiographs were scanned using a Molecular Dynamics laser scanning densitometer or an Apple Color OneScanner.

6.6.8 Isolation of DNA fragments from gels

DNA to be extracted from agarose for the purpose of cloning was visualized by staining the gel with ethidium bromide (4 mg ml^{-1} (w/v) in H_2O) and brief exposure to long wavelength UV light. Agarose containing the desired DNA fragment was excised from the gel with a sterile

scalpel blade and the DNA removed from the agarose slice by the Geneclean procedure (Bio 101, La Jolla, California) performed according to the manufacturer's protocol.

Polyacrylamide gel electrophoresis was used to purify small DNA fragments for cloning and radiolabelled DNA fragments for use in footprinting reactions and for some of the gel retardation assays. If the DNA was a fragment to be used for cloning, it was visualized by staining the gel with ethidium bromide (4 mg ml^{-1} (w/v) in H_2O) and brief exposure to long wavelength UV light. Acrylamide containing the desired DNA fragment was excised from the gel with a sterile scalpel blade and the DNA eluted from the acrylamide slice by the method of Maxam & Gilbert (1980). Radiolabelled DNA fragments were visualized by autoradiography and purified as for the non-labelled fragments.

6.6.9 Cloning procedures

Vector plasmid or phage DNA that had been restricted and if necessary, end-filled, was purified by agarose electrophoresis (Section 6.6.8). The DNA fragments to be cloned were isolated from agarose or polyacrylamide gels (Section 6.6.8).

Ligations were performed in 10 µl reactions containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2.5 mM DTT, 1 mM rATP and T4 DNA ligase (0.5 units for staggered end ligations or 1.0 unit for blunt end ligations). A vector-only control ligation was always included. The mixture was incubated for 1 to 16 hours at 14°C and then an aliquot used to transform bacterial cells (Section 6.5.7.2 or 7.5.7.3). The vector only control was included to determine background ligation levels.

Initial screens for the presence of insert DNA were performed by colony cracking when cloning high copy number and large (>10% of vector) inserts (Section 6.6.1.5) or by using PCR (Section 6.6.15) with low copy number or small (<10% of vector) inserts. The PCR was performed with primers flanking the insert DNA or with one primer located within the insert DNA fragment. A fraction of a colony containing a putative recombinant plasmid was added to the PCR tube, with the high temperatures of the PCR being sufficient to release DNA from the cells. Amplified products of the correct size were sometimes analyzed by restriction digestion to determine the orientation of the fragment. To confirm the identity of recombinant plasmids, DNA was prepared by the miniprep method (Section 6.6.1.3) and the identity and orientation of the insert DNA confirmed by diagnostic restriction analysis and sometimes by sequencing.

6.6.10 Hybridisation techniques

6.6.10.1 Hybridisation screening of bacterial colonies and M13 plaques

Colony lifts were carried out essentially as described by Sambrook *et al.* (1989), based on the procedure of Grunstein & Hogness (1975). Colonies to be tested were spotted onto an appropriate plate and a master plate. Positive and negative controls were spotted when possible. After overnight incubation, the plates were cooled to 4°C and the colonies transferred to a nitrocellulose filter (Schleicher and Schuell BA85 - 0.45 μm) by placing a dry filter on top of the agar plate. The filter was left on the plate for about 1 min and treated as described by Sambrook *et al.* (1989), which basically involved lysing the cells with SDS, denaturing the DNA with sodium hydroxide and neutralizing the filter with Tris-HCl. Following the neutralization step, the DNA was cross-linked to the filter using a Bio-Rad GS Gene Linker™. The filter was then soaked in 3 x SSC, 0.1% SDS at 65°C and colony debris scraped off.

Pre-hybridization was performed in 10 ml of oligo hybridization buffer for 1-2 hours in a petri dish at 42°C. After pre-hybridization, 50 ng ^{32}P-labelled oligonucleotide (Section 6.6.12) was heated to 95°C for 2 min, snap-chilled on ice and added to the pre-hybridization mix. Hybridization was carried out at the same temperature for 4-16 hours.

The solvent TMACl (tetramethylammonium chloride) was used for the stringent washing of filters. TMACl eliminates the preferential melting of AT versus GC base pairs, allowing accurate prediction of melting temperatures (Wood *et al.*, 1985). After hybridization, the filters were washed twice in 100 ml of 6 x SSC for 10 min at room temperature, rinsed in TMACl wash solution (3M TMACl, 2 mM EDTA, 50 mM Tris-HCl, pH 8.0, 1% SDS) at room temperature, dried and autoradiographed overnight. All colonies with homology to the oligonucleotide are detected at this point. For stringent washing (*e.g.* to identify mutant plasmids), the filters were washed in TMACl wash solution for 30 min at a temperature 2-5°C below the melting temperature of the mutant oligonucleotide and mutant DNA (Wood *et al.*, 1985). The filters were dried and autoradiographed overnight. Positive hybridising plaques or colonies were then taken from the master plate and purified by streaking.

M13 plaque lifts were performed the same way except filters were not soaked at 65°C or scraped prior to prehybridization.

6.6.10.2 Southern hybridization

Southern blots were performed by transferring DNA from TAE/agarose gels to Zetaprobe® membrane (BioRad) by capillary transfer in 0.4 M NaOH for 2 hours (Reed & Mann, 1985; Reed, 1991). The membrane was then neutralized in 2 x SSPE and prehybridized at 65°C for 1

hour in Southern hybridization buffer. The appropriate ^{32}P-labelled probe (labelled using the BRESATEC (Australia) Gigaprime DNA labelling kit) was added and hybridization allowed to proceed at 65°C for 4 hours. The filter was then rinsed at room temperature in 2 x SSC, washed once at room temperature in 2 x SSC/0.1% SDS for 15 min, and once at 65°C in 0.2 x SSC/1% SDS for 10 min. The filter was rinsed in 2 x SSC/0.1% SDS and exposed to a phosphorimage intensifying screen.

6.6.11 End-labelling and end-filling using the Klenow fragment of DNA polymerase I

DNA to be cloned, which required the ends to be blunt, was end-filled using the Klenow fragment of *E. coli* DNA polymerase I in a reaction containing 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 25 µM dNTPs (added from a stock containing 0.25 mM of each of the four dNTPs in 5 mM Tris-HCl pH 8.0, 1 mM EDTA) and 1 unit of Klenow fragment. The reaction was incubated at 37°C for 15-30 min and terminated by heating at 70°C for 15 min or by the addition of EDTA to a final concentration of 50 mM.

DNA restriction fragments to be used as radioactive size markers or for analysis of restriction patterns on polyacrylamide gels, were end-labelled by an endfilling reaction containing the restriction buffer, 1 unit of Klenow fragment and 25-50 µCi [α-^{32}P]-dCTP and/or [α-^{32}P]-dATP. After incubation at 37°C for 15 min, the reaction was terminated by heating to 70°C for 15 min or by adding EDTA to a final concentration of 50 mM. When a 3' overhang needed to be blunted or radiolabelled, or a blunt end needed to be radiolabelled, a chew-back fill-in reaction was performed by allowing 2 min of Klenow fragment activity before adding nucleotides.

For endlabelling fragments for use in gel retardation assays and DNase I footprinting reactions (and for some general labelling reactions), unlabelled dNTPs (other than the labelled dNTPs) were added to the reaction (0.025 mM final concentration) and for the last 5 min of the reaction, a 1/10 volume of a stock containing 0.25 mM of each of the four dNTPs (in 5 mM Tris-HCl pH 8.0, 1 mM EDTA) was added to ensure that all fragments were fully extended.

6.6.12 Kinasing of oligonucleotides

Oligonucleotides to be used for hybridisation screening or as primers in a PCR were labelled using T4 polynucleotide kinase and [γ-^{32}P]-rATP. Oligonucleotide (100 ng) was kinased in a 10 µl reaction containing 1 x polynuceotide kinase buffer, 50 µCi of [γ-^{32}P]-rATP and 5 U of T4 polynucleotide kinase. Incubation was at 37°C for 30 min and the reaction stopped by heat inactivation of the enzyme at 95°C for 5 min.

For oligonucleotides to be used as primers in mutagenesis reactions, the oligonucleotide was kinased in the same way, except for the substitution of 1 mM rATP (final concentration) for [γ-^{32}P]-rATP.

6.6.13 Oligonucleotide directed mutagenesis

The method used for oligonucleotide site-directed mutagenesis of M13 cloned DNA (creation of *apl*Δ11 and *apl Nde*I mutations) was derived from the procedures of Zoller & Smith (1983). The method used for oligonucleotide site-directed mutagenesis of double stranded plasmid DNA (creation of P_R^- mutations) was derived from the procedure of (Deng & Nickoloff, 1992). Before use in a mutagenesis reaction, a mutagenic oligonucleotide was tested for its ability to hybridize specifically to the region of interest by using the oligonucleotide as a primer in a sequencing reaction.

M13 single stranded DNA to be mutagenized (2 µl of preparation described in Section 6.6.1.2) was mixed with 4 ng of the cold kinased oligonucleotide containing the appropriate mutation (Section 6.6.12), and 2 ng of cold kinased universal sequencing primer, in a volume of 15 µl containing 5 µl of TM (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$) and 5 µl of 200 mM NaCl. The mixture was heated to 75°C for 5 min and cooled slowly to room temperature. After the annealing step, 20 µl of dNTP solution (0.25 mM of each dNTP), 10 µl of 10 mM rATP, 10 µl of 20 mM DTT, 27 µl H_2O, 4 U of Klenow fragment and 3.5 U of T4 DNA ligase were added (total volume of 85 µl) to the annealed DNA and the extension/ligation reaction was allowed to proceed at room temperature for 2 hours.

JM101 competent cells were prepared and 2-20 µl of the extended-ligated M13 DNA used to transfect them (Section 6.5.7.2), but without the addition of IPTG or X-gal to the agar before plating. Plaques obtained after this transfection were spotted onto master plates and replica plates seeded with a lawn of JM101 and the plates incubated overnight at 37°C. The phage were then tested for the presence of the desired mutation by plaque hybridization using the relevant oligonucleotide as a probe (Section 6.6.10). To confirm that the phage identified by plaque hybridization contained the correct mutation, the region was sequenced (Section 6.6.14).

Mutagenesis of double stranded plasmid DNA was performed as described by Deng & Nickoloff (1992) except only the mutagenic primer was used and the putative mutant colonies were identified by oligonucleotide hybridization and TMACl washing (Section 6.6.10).

6.6.14 DNA sequencing

DNA sequencing of the *apl*Δ11 and *apl Nde*I mutations was by the dideoxy chain termination method on single-stranded M13 templates (Sanger *et al.*, 1977), using Bresatec DNA ^{32}P/Klenow fragment sequencing kits and the protocol recommended by the manufacturer. The rest of the sequencing performed in this thesis was by using alkaline denatured double-stranded plasmid DNA templates, [α-^{35}S]-dATP and Sequenase v.2.0 (USB) using the protocol recommended by the manufacturer. Sequencing of some of the *goa* mutations was performed on PCR products using the Promega fmol™ DNA sequencing system with a ^{32}P-kinased primer (Section 6.6.12). The template DNA used in these PCRs was obtained from a single plaque added to the PCR tube. The high temperatures of the PCR release the phage DNA. Maxam and Gilbert purine (G+A) sequencing reactions (using the New England Nuclear DNA Sequencing System Kit (NEK-010)) were used as markers in the footprints of Chapters Two, Three and Five. Double stranded DNA sequencing reactions with Sequenase and a ^{32}P kinased primer were used as markers in the footprint of Chapter 4.

6.6.15 PCR amplifications

The following cycles were used for standard PCR amplifications: 2 min 94°C, 30 x (5 sec 94°C, 5 sec 55°C, 20 sec 74°C). The following cycles were used for PCR amplifications with mutagenic oligonucleotides (*e.g.* 70 and 73): 2 min 94°C, 30 x (5 sec 94°C, 5 sec 50°C, 60 sec 74°C). Each reaction contained PCR buffer (Section 6.4.6.1), 50 ng of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.1-0.3 U Taq polymerase in a total reaction volume of 50 µl. For phage DNA amplifications 1 µl of a 1 x 10^8 pfu ml^{-1} phage stock or single plaques were used as the source of template DNA. For prophage DNA amplifications, a fraction of a colony of the lysogen was used. For plasmid DNA amplifications, approximately 20 nM DNA or a fraction of a colony carrying the plasmid were used.

6.7 PROTEIN TECHNIQUES

6.7.1 Preparation of Apl extracts and estimation of protein concentrations

Overnight cultures of BL21(λDE3) plysS pMRR1 or BL21(λDE3) plysS pMRR2 in LB containing ampicillin (100 µg ml^{-1}) and chloramphenicol (30 µg ml^{-1}) grown at 37°C, were diluted 200-fold into 50 ml of the same broth and incubated with aeration at 37°C. At an A_{600} = 0.5, IPTG was added to 0.4 mM. After 2 hours the cultures were chilled and cells harvested by centrifugation (10000 rpm, 10 min, 4°C, JA20 rotor). The pellets were resuspended in 3 ml precooled protein extraction buffer and the cells disrupted by sonication (3 x 15 second bursts with a Branson Sonic Power Co. B-30 Sonifier Cell Disrupter. The lysates were cleared by

centrifugation (18000 rpm, 30 min, 4°C, JA21 rotor) and aliquots frozen in dry ice and stored at -75°C.

BSA standard solutions (containing 10-100 µg of BSA) and the protein extract, in a volume of 0.1 ml, were mixed with 1 ml of Bradford reagent and allowed to stand for 2-60 min. The A_{595} was then determined and the amount of protein in the extract determined by plotting a standard curve of the BSA A_{595} readings.

6.7.2 Preparation of SDS lysates

Cultures were induced as in Section 6.7.1. Bacterial culture (200 µl) was centrifuged (10000 rpm, 2 min, room temperature, Eppendorf centrifuge). The pellet was resuspended in protein sample buffer, heated at 100°C for 2 min and stored at -15°C.

6.7.3 SDS gel electrophoresis (SDS-PAGE)

The mini-Protean II discontinuous SDS gel system from Bio Rad was used as recommended with a 15% separating gel. The gels were stained for one hour at room temperature in 0.125% Coomassie blue R-250, 50% methanol, 10% acetic acid, destained for two hours in 50% methanol, 10% acetic acid and dried onto Whatman 3MM paper under vacuum at 65°C for one hour.

6.7.4 Gel retardation

Frozen protein extracts (Section 6.7.1) were thawed on ice and diluted in protein extraction buffer without PMSF on ice. A 10 µl binding reaction contained various amounts of purified protein or protein extract, approximately 1 nM radiolabelled DNA probe and gel retardation binding buffer. The reactions were incubated at room temperature for 15 min and loaded onto 6% non-denaturing polyacrylamide gels containing 10% glycerol and electrophoresed at 4°C in 0.5 x or 1 x TBE at 25 mA (Section 6.6.6.1). Following electrophoresis, the gels were vacuum dried onto Whatman 3MM paper and autoradiographed as required (Section 6.6.7). IHF retardations were performed similarly with pure IHF (a gift from Howard Nash), except IHF retardation buffer was used instead of gel retardation binding buffer.

6.7.5 DNase I footprinting

Generation of the singly end-labelled DNA fragments used in the footprinting reactions are given in the appropriate figure legends. About 1 nM labelled DNA was incubated with 2-20 µg protein extract in a 90 µl reaction containing DNase I footprint binding buffer at 30°C for 20 min before addition of 5 µl each of prewarmed 200 mM $CaCl_2$, 100 mM $MgCl_2$ solution

and 0.4 μg ml^{-1} DNase I. Reactions were then incubated for 2 min at 30°C and stopped by the addition of 100 μl of DNase I stop buffer. Addition of 400 μl ethanol and incubation on ice for 15 minutes was followed by centrifugation at 12000 rpm and 4°C for 15 min (Eppendorf centrifuge). The pellet was vacuum-dried and dissolved in 100 μl TENA. This was then extracted with an equal volume of phenol (Section 6.6.2) and ethanol precipitated (Section 6.6.3). Following centrifugation (12000 rpm, 15 min, 4°C, Eppendorf centrifuge), the pellet was vacuum-dried and dissolved in 3.5 μl H_2O and 1.5 μl formamide loading buffer and loaded onto a 6% sequencing gel (Section 6.6.6.2). Following electrophoresis, the gel was dried onto Whatman 3MM paper under vacuum and autoradiographed as required (Section 6.6.7). IHF footprinting was performed similarly except pure IHF was used with IHF binding buffer, instead of DNase I footprint binding buffer and the dried gel exposed overnight to a phosphorimage intensifying screen.

6.7.6 β-galactosidase assays of *lac*Z reporter constructs

6.7.6.1 Creation of *lac*Z reporter vectors

Previous promoter studies in our laboratory (Dodd *et al.*, 1990) had used the pK02 and pKM2 *gal*K reporter plasmids (de Boer, 1984). However, *lac*Z reporter constructs are more amenable to study as the β-galactosidase assay is simpler and cheaper. For this work, I created a *lac*Z reporter system to examine the kinetics of Apl repression of P_R and P_L. The reporter plasmid is based on the pRS415 plasmid (Simons *et al.*, 1987; Fig. 6.2) which contains the *lac*Z, *lac*Y and *lac*A genes downstream of a polycloning site (*Eco*RI-*Sma*I-*Bam*HI) that is used to insert promoter fragments. Upstream of the polycloning site are four adjacent T1 terminators from the *E. coli rrn*B ribosomal RNA operon (Brosius *et al.*, 1981) which block background transcription into the *lac*Z gene. The plasmid has a ColEI origin and a β-lactamase gene for selection on ampicillin. Translation stop codons are present in all three frames between the polycloning site and the *lac*Z gene, however, they are not adjacent.

In modifying this vector, I replaced the sequence between the polycloning site and the *lac*Z gene with a sequence from pKO2 (or pKM2) containing three adjacent stop codons in all three frames (see Fig. 6.3). This was done so that any translation termination effects on transcription do not vary with the reading frame (de Boer, 1984). The pKM2 fragment is identical to the pK02 fragment, except that it also contains the λ T_{R1} terminator, which reduces transcription levels by 90%, and so allows strong promoters (which normally produce lethal amounts of Lac proteins) to be assayed. As such, all P_R units have been multiplied by 10 to account for this termination. I then replaced the pRS415 polycloning site with that from

pUC19, which contains a larger number of restriction sites and so allows a larger number of "forced" clonings. Before this was done, the *Sal*I and *Bam*HI sites of pRS415 were destroyed by end-filling and religation so that these sites were unique in the polycloning sites of pMRR9 and pMRR9T.

6.7.6.2 Creation of single copy *lacZ* reporter constructs

The series of vectors creaed by (Simons *et al.*, 1987) also included phage λ vectors which could be recombined *in vivo* with pRS415 clones (λRS45; Fig. 6.4). Lysogens of these *ind*$^-$ phage can then be created so stable chromosomal copies of any construct can be made. As pMRR9 and pMRR9T had the pRS415 sequences required for recombination with λRS45, single copy versions of any constructs made with these vectors could also be created.

To create recombinant λ phage, plate lysates (Section 6.5.3) of λRS45 propagated on MC1061.5 carrying a pMRR9 or pMRR9T plasmid clone were made. 10^4-10^6 phage from this lysate were then plated on MC1061.5 in soft agar containing 40 µl of 20 mg ml^{-1} X-gal. Phage which have recombined with the plasmid clone, and hence carry a promoter expressing the *lac* genes, produce blue plaques. Such recombinants were obtained with a frequency of about 10^{-4}. A blue plaque was purified and the turbid centre (containing lysogens) was streaked for single colonies on L plates pre-spread with 40 µl of 20 mg ml^{-1} X-gal. Blue colonies (putative lysogens) were then purified. Multiple lysogens were commonly obtained by this method, so at least four independent lysogens were purified and assayed. As the number of prophages in a lysogen is directly proportional to the β-galactosidase units produced, the distinction of single and multiple lysogens is simple.

6.7.6.3 β-galactosidase activity assay

This procedure is identical to that described by Miller (1972). Overnight cultures (or glycerol stocks) of the appropriate *lacZ* reporter strain grown in M9CAA (plus appropriate antibiotics) were subcultured 10^{-3} into fresh media (containing appropriate concentrations of IPTG) and grown to an A_{600} = 0.2-0.7 at 37°C with aeration. Cultures were then placed on ice for 30 min-2 hours at which time the A_{600} was measured. Cultures were then kept on ice until assayed. 0.05 ml (strong promoters)-0.5 ml (weak promoters) of culture was added to Z buffer, in a glass tube, such that the total volume was 1 ml. Tubes were placed at 28°C and 30 µl of chloroform and 10 µl of 0.1% SDS were added. The tubes were then vortexed for exactly 10 sec and placed at 28°C for 5 min. 200 µl of 4 mg ml^{-1} ONPG was then added and the tubes briefly vortexed. When sufficient yellow colour had developed, the reaction was stopped by the addition of 500 µl of 1 M Na_2CO_3, the time recorded and the tube placed on

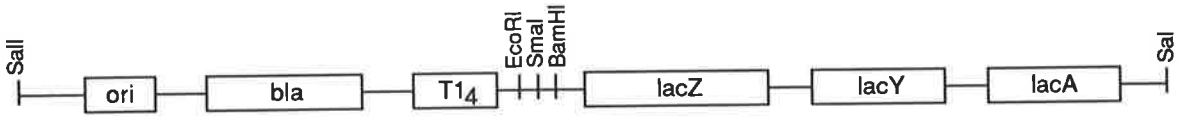


Figure 6.2 Map of pRS415

A full description of this plasmid is given in Simons *et al.* (1987). This figure shows the ColE1 origin of replication (ori), the β-lactamase gene for selection on amplicillin (bla), the lacZ, lacY and lacA genes, the rrnB T1 terminators ($T1_4$) and relevant restriction sites. The size of the plasmid is 10.8 kb. This Figure is not drawn to scale.

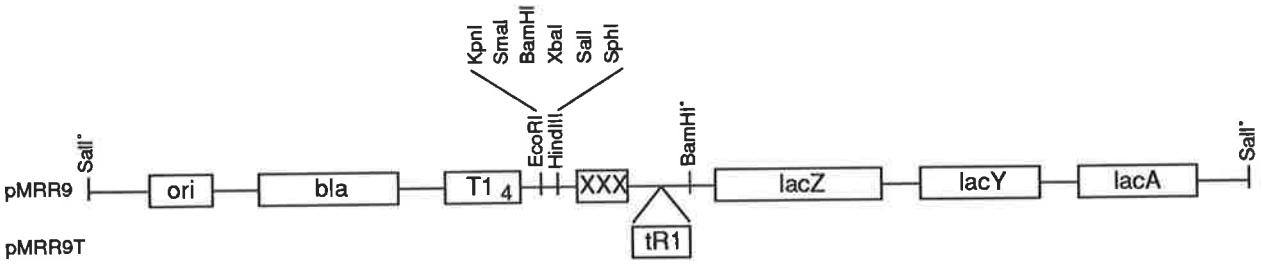


Figure 6.3 Maps of pMRR9 and pMRR9T

These vectors are derived from pRS415 and pKO2 or pKM2, respectively. pMRR9 was constructed by inserting the 184 bp *Sma*I-*Eco*RV fragment of pKO2, containing adjacent translation stop codons in all three reading frames (XXX; sequence TAACTAACTAA) at the *Sma*I site of pRS415 with the *Sma*I site of the pKO2 fragment closest to the *Eco*RI site of pRS415 (pMRR32). The *Bam*HI (BamHI° - pMRR34) and *Sal*I (SalI° - pMRR35) sites were then destroyed by end-filling and religation and the *Eco*RI-*Hin*dIII polycloning sequence of pUC19 was inserted at the *Eco*RI-*Hin*dIII sites of pMRR33 (the *Hin*dIII site is from the pKO2 fragment) to give pMRR9.

pMRR9T was constructed similarly to pMRR9 except the 584 bp *Sma*I-*Eco*RV fragment of pKM2, containing the adjacent translation stop codons in all three frames and the lambda t_{R1} terminator (tR1), was inserted at the *Sma*I site of pRS415 (pMRR33). BamHI° and SalI° intermediates are pMRR36 and pMRR37, respectively. This Figure is not drawn to scale.

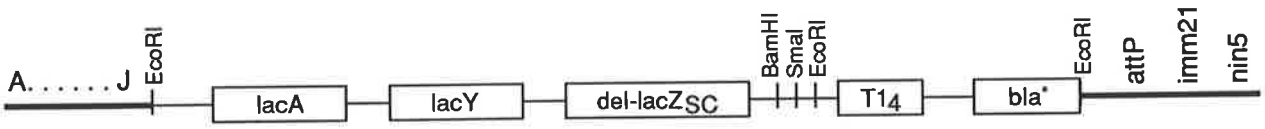


Figure 6.4 Map of λRS45

A full description of this phage is given in Simons *et al.* (1987). This Figure is not drawn to scale. Lambda DNA is represented by thick lines and plasmid derived DNA by thin lines. Relevant phage genes are shown above the thick line. Plasmid genes include a portion of the β-lactamase gene (bla°), a mutated *lac*Z gene which gives white plaques on X-gal (del-lacZ$_{SC}$), the lacA and lacY genes, the four tandem T1 terminators ($T1_4$) and relevant restriction sites.

ice. The solution was then transferred to an Eppendorf tube and centrifuged (14000 rpm, 3 min, room temperature, Eppendorf centrifuge) to remove cell debris. The A_{420} of the solution was then measured and the β-galactosidase units calculated by the following formula:

$$\text{Units} = 1000 \times (A_{420}/(\text{time} \times \text{volume} \times A_{600})).$$

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