ESTABLISHMENT OF LYSOGENY IN COLIPHAGE 186

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by

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CONTENT

SUMMARY

Coliphage 186 chooses to develop either lytically or lysogenically. The studies reported in this thesis were aimed at understanding how this phage establishes lysogeny.

The developmental decision in 186 occurs at the level of the lytic (p_R) and lysogenic (p_L) promoters.Transcription from either promoter is at the expense of the other. Thus transcription from p_L results in the production of the CI protein, which represses p_R, while transcription from p_R expresses the Apl protein which can repress p_L (Dodd *et al.*, 1990). Evidence from *galK* transcriptional promoter fusions has shown that the lytic promoter (p_R) is considerably more active than the lysogenic promoter (p_L) (Dodd *et al.*, 1990). Moreover, as a consequence of the face-to-face arrangement of p_R and p_L, p_L transcription is inhibited by converging p_R transcription. Yet during the establishment of lysogeny, the phage must theoretically progress from a state where p_L is repressed by Apl and interfered with by actively transcribing p_R, to the stable lysogenic state of autogenous control whereby CI repression of p_R allows p_L activity and thereby maintenance transcription of *c*I. The 186 *c*II gene has previously been shown to be required for the establishment of lysogeny and is expected to mediate this transition.

CII contains a potential helix-turn-helix DNA-binding motif suggesting that it may act as a transcriptional regulator. As a prelude to defining the mechanism of action of CII previous work had identified that CII could specifically bind to a minimal 165 bp DNA fragment of 186 which spanned the *apl*/*c*II intergenic region . In the present study, the CII DNA binding site within this region was identified by DNase I footprint. CII was found to bind to inverted repeat sequences separated by two turns of the helix which are located at the 5' terminus of the *c*II gene.

Location of the CII-binding site upstream of p_L suggested that CII may establish lysogeny by activating an alternative lysogenic promoter in this region. Results obtained from transcriptional *lacZ* reporter fusions confirmed that CII functions as a transcriptional activator and primer extension was used to map the start site of this CII dependent (p_E) transcript to the *apl*/*c*II intergenic region. Since results from transcriptional reporter fusions show that the p_E transcript extends into the lysogenic operon past p_R it is presumed that transcription from p_E expresses CI which leads to repression of p_R and relief of the inhibition of p_L by p_R, thus allowing maintenance transcription of *c*I. As the integrase gene (*int*) is part of the same operon as *c*I, it is also presumed that p_E transcription produces Int thus facilitating integration of the phage genome into the bacterial chromosome. It is of interest to note that p_E does not remain fully active throughout establishment since it is subject to direct negative feedback by CI.

In 186 both lysogenic promoters transcribe in the face of p_R yet while the p_R/p_L combination cannot establish lysogeny the $p_R/(p_L+p_E)$ promoter combination is proficient in establishment. Various single copy *lacZ* promoter fusions were constructed to determine why p_E+p_L is more proficient at establishing lysogeny in the face of p_R than p_L alone. Results from this study show that p_E is a significantly stronger promoter than p_L and is less inhibited by converging transcription from p_R. Since the activities of p_L and p_E are additive it was therefore expected that p_E+p_L would be even more proficient than p_E alone in extending transcripts beyond p_R. Contrary to expectation however, the p_E/p_R combination was able to extend a greater number of transcripts beyond p_R than the $p_R/(p_L+p_E)$ combination. Presumably, interfering complexes generated by the opposing transcription from p_R and p_L block the elongation of a small proportion of p_E transcripts.

Promoters initiating converging transcription such as p_R and p_E are expected to interfere with each others activities. p_R transcription has been shown to interfere with p_E but does p_E activity interfere with p_R transcription ? Results from *lacZ* reporter constructs monitoring p_R activity in the presence or absence of active CII indicate that p_E transcription is able to inhibit p_R transcription. This may be an efficient means for 186 to dampen p_R activity before CI represses p_R directly.

Establishment of lysogeny in 186 occurs in the presence of Apl. Since Apl binds in the p_R/p_L region and represses transcription from p_R and p_L it was of interest to determine how Apl would alter the flow of lytic and lysogenic transcription during the establishment of lysogeny. Results from transcriptional reporter studies used to address this question indicate that Apl acts in concert with p_E to increase *c*I transcription, by reducing interfering transcription from p_R.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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CHAPTER 1

Introduction

1.1 TEMPERATE PHAGES CHOOSE TO DEVELOP EITHER LYTICALLY OR LYSOGENICALLY

Upon infection of a bacterial host, temperate coliphages choose to develop either lytically or lysogenically (See Fig 1.1). Lytic development is characterised by viral DNA replication, expression and assembly of structural proteins and finally packaging and release of progeny virions by lysis of the host, while lysogeny describes a "dormant" prophage state in which the phage genome is integrated into the host chromosome and is replicated along with the DNA of its host.

These two independent developmental pathways are interconnected, with the phage being able to change from one cycle to another in response to specific environmental signals. The establishment of lysogeny is the transient process which occurs soon after infection and enables the transition from an early lytic type state to the lysogenic maintenance state. Prophage induction is the reverse process during which the prophage is excised from the bacterial chromosome and develops lytically. This thesis concerns itself with the mechanisms employed by 186 in establishing the prophage state.

In the temperate phages considered here the decision to undergo the lytic or lysogenic response occurs at the level of two promoters: with transcription from the lytic promoter resulting in expression of lytic functions and inhibition of lysogenic transcription and conversely transcription from the lysogenic promoter resulting in expression of lysogenic proteins and repression of lytic transcription Two groups of bacteriophages can be distinguished according to the relative position of their lytic and

lysogenic promoters. The lytic-lysogenic promoters of Lambda and lambdoid phages 434, ϕ80 and P22 are positioned back-to-back and initiate non-overlapping diverging transcripts while the functionally homologous promoters of non-lambdoid phages 186, P2, Mu and D108 are positioned face-to-face and initiate overlapping converging transcripts. The relative position of lytic and lysogenic promoters may be an important parameter in determining the mechanism these phage employ to establish lysogeny. How phages of each class are thought to establish lysogeny is considered in detail below.

1.2 ESTABLISHMENT OF LYSOGENY IN PHAGES WITH BACK-TO-BACK LYTIC/LYSOGENIC PROMOTERS

1.2.1 Establishment of lysogeny in Lambda

1.2.1.1 Early Lambda genes involved during lytic and lysogenic development

Immediately following injection of the phage chromosome into its host, the divergent phage promoters p_R and p_L are transcribed (Herskowitz and Hagen, 1980) (see Fig 1.2). Transcription from p_R expresses the Cro protein product which functions in repressing lysogenic development while the p_L transcript encodes N, a transcription anti terminator, which allows the p_L and p_R transcripts to extend beyond termination signals located after the *cro* and *N* genes (Friedman and Gottesman, 1983; Ptashne, 1986). The anti terminated p_R transcript in addition to Cro now also encodes the CII function, a labile protein required to establish lysogeny, the O and P proteins which direct phage replication and Q, a function which allows expression of DNA packaging and cell lysis genes (Oppenheim *et al.*, 1993). The anti terminated p_L transcript expresses the CIII protein, required to stabilize CII, as well as the excisionase Xis and the integrase Int which are both required during the excision of the prophage from the host chromosome (Oppenheim *et al.*, 1993). Under conditions which do not favour the

establishment of lysogeny (low cellular CII concentrations) expression of the above mentioned functions from p_R and p_L eventually commit the phage to lytic development (Wulff and Rosenberg, 1983).

Whereas lysis requires the expression of multiple functions from p_R and p_L, lysogeny is maintained by the expression from p_{RM} of only one function, namely CI. The CI protein maintains the prophage state by repressing lytic transcription from p_R and p_L (Gussin *et al.*, 1983). Its role is hence diametrically opposed to that of Cro, which perpetuates lytic development by repression of p_{RM}. The opposing roles of CI and Cro thus commit the phage to one life cycle at the exclusion of the reciprocal life cycle.

1.2.1.2 CI and Cro perpetuate the lysogenic and lytic states respectively

CI and Cro control transcription by binding to operator regions at p_{RM}/p_R (O_R) and p_L (O_L). Although CI and Cro bind to the same operator sequences, the outcomes differ because CI and Cro have different relative affinities for the same operator sequences (Ptashne *et al.*, 1982).

Increasing amounts of CI will bind first to O_{L1} and O_{R1} then O_{L2} and O_{R2} and finally O_{L3} and O_{R3} (Schlief, 1987). CI binding at O_R controls transcription from p_R and p_{RM} and is particularly important in perpetuating the lysogenic state. CI binding to O_{R1} and O_{R2} is co-operative and not only represses p_R, by occlusion of RNA polymerase from this promoter, but also stimulates transcription from p_{RM} because CI, bound at O_{R2}, forms favourable contacts with RNA polymerase at p_{RM} (Bushman *et al.*, 1989; Ishihama, 1993) which result in increasing the isomerisation rate at p_{RM} (Gussin *et al.*, 1983). The binding of CI to O_{R3} is non co-operative and occurs at higher CI concentrations compared to binding at O_{R1} and O_{R2}. Association of CI with O_{R3} results in repression of p_{RM} and therefore negative autoregulation of CI expression (Herskowitz and Hagen, 1980).

Figure 1.1

The lifecycle of temperate phage

Upon infection of its host, temperate bacteriophage make a choice between lytic and lysogenic growth. Lytic development is characterised by replication of the phage DNA injected during infection, construction of phage coats, packaging and release of progeny virion, while lysogeny is characterised by integration of the phage genome into the bacterial chromosome. The phage enters the lysogenic pathway soon after infection by the process of establishment of lysogeny and exits that mode of development at any time by the process of induction.

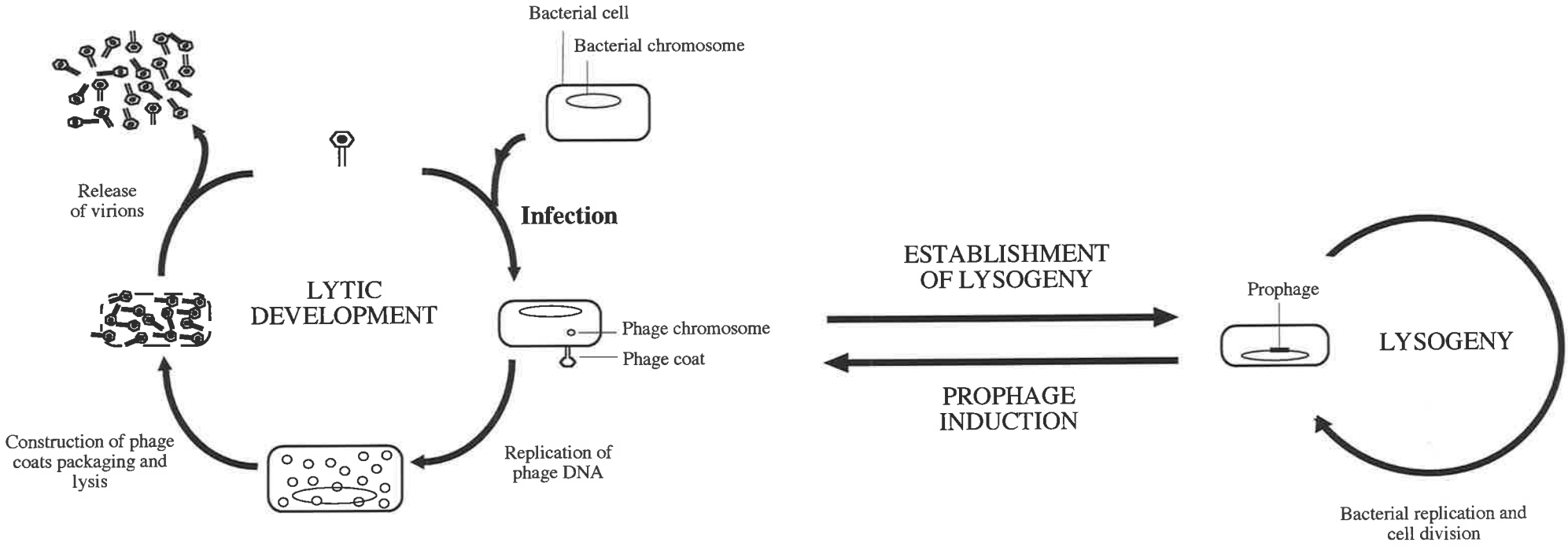
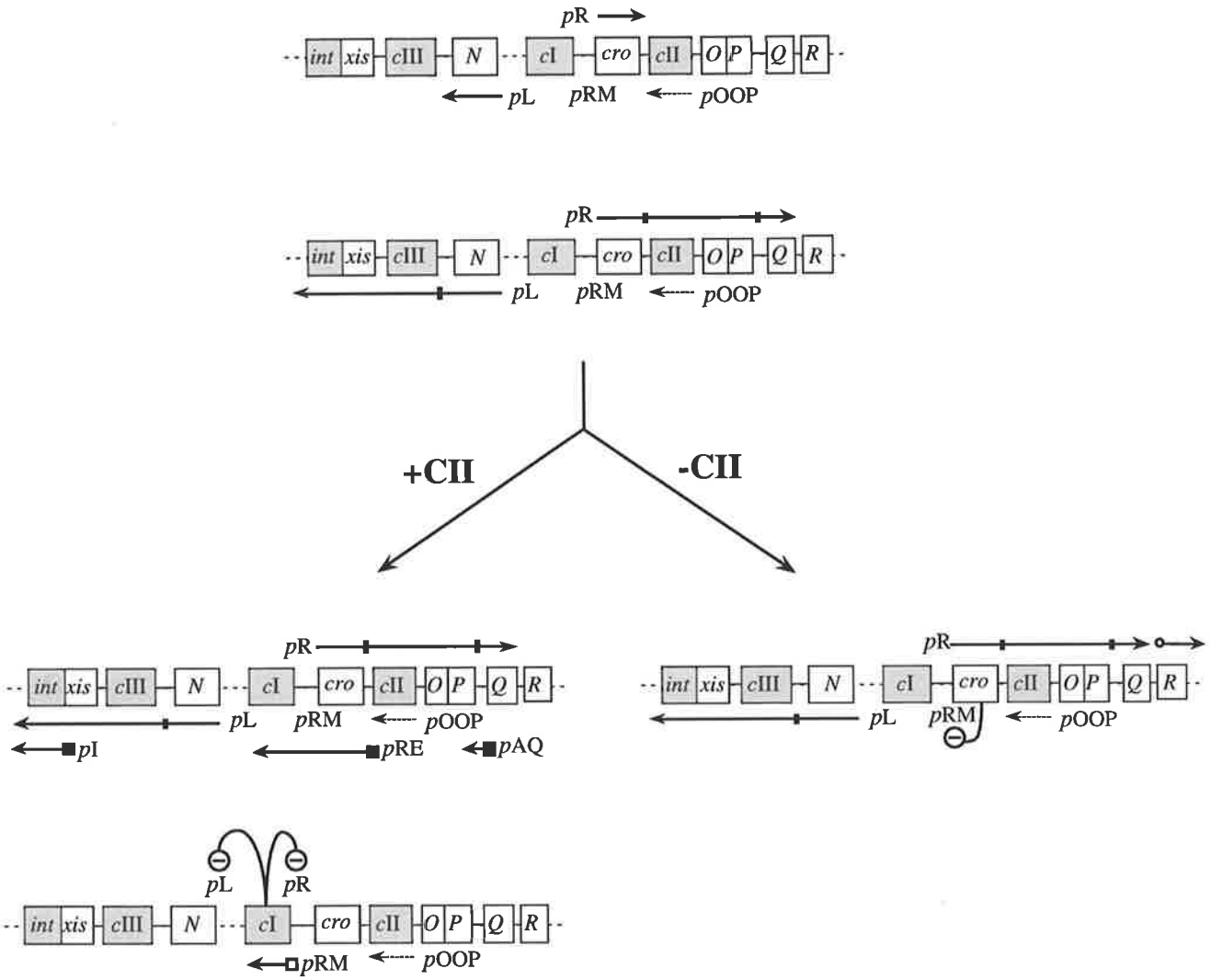

Bacterial cell
Bacterial chromosome
Release
of virions
Infection
LYTIC
DEVELOPMENT
Phage chromosome
Phage coat
Construction of phage
coats packaging and
lysis
Replication of
phage DNA
ESTABLISHMENT
OF LYSOGENY
PROPHAGE
INDUCTION
Prophage
LYSOGENY
Bacterial replication and
cell division

Figure 1.2

Regulation of early Lambda gene expression

The regulation of early Lambda gene expression is described in detail in the text. In this figure genes are boxed and lysogenic genes are displayed in grey background. Dotted lines between genes represent genes which were omitted in this region. Transcripts are represented by arrows with the dotted arrow showing the location of the LexA dependent p_{OOP} transcript. Slashes in arrows indicate locations of the N anti termination sites. Open circles, filled and open squares represent the Q activated $p_{R'}$ promoter, the CII dependent promoters and the lysogenic maintenance promoter respectively.



LYSOGENY

LYSIS

The relative affinities of Cro at O_R are opposite to those of CI. Cro associates first with O_{R3} and at increasing concentrations will subsequently bind to O_{R2} and O_{R1}. Unlike CI, Cro does not bind co-operatively. Cro represses p_{RM} at low concentrations by binding to O_{R3} and is able to repress p_R at increased concentrations when it also associates with O_{R1} (Gussin *et al.*, 1983). Cro and CI thus perpetuate the lytic or lysogenic state by repression of p_{RM} and p_R respectively and autoregulate their own expression.

1.2.1.3 CII mediates the transition from the "lytic" type state to the lysogenic state

The lytically encoded CII protein is the master regulator which mediates the transition from a lytic type state, that exists immediately after infection, to the lysogeny maintenance state (Ho and Rosenberg, 1982). CII mediates its function by activating leftward transcription from p_{RE}, which spans the *cro* /*c*II intergenic region, resulting in the synthesis of CI protein and the establishment of lytic repression (Schmeissner *et al.*, 1980;1981; Shimatake and Rosenberg, 1981). Since p_{RE} is not only three fold more active than p_R *in vivo*, but also reduces p_R transcription by one half (Schmeissner et al., 1980), presumably because p_R and p_{RE} are located face-to-face, CII activation of p_{RE} also serves to reduce expression of lytic functions. Moreover, the placement of p_{RE} beyond *cro* has been speculated to play a role in the differential expression of antagonistic proteins (Cro and CII) from p_R (Spiegelmann *et al.*, 1972; McAdams and Shapiro, 1995). These authors suggest that production of antisense *cro* RNA from p_{RE} might reduce expresssion of the *cro* gene product during the establishment of lysogeny, presumably resulting in derepression of p_{RM}.

In addition to activating p_{RE}, CII also activates transcription from p_I which produces Int protein (Court *et al.*, 1977) and p_{AQ} which produces an anti-sense Q RNA and

decreases expression of the *Q* gene, whose product is required for transcription of late lytic genes (Hoopes and McClure, 1985; Ho and Rosenberg, 1985).

The CII binding sites, the affinity of CII for these binding sites and the location of these binding sites within the promoter region are conserved between p_{RE}, p_I and p_{AQ}. CII binds to TTGC(N6)TTGC direct repeats with the 6N between half sites comprising the -35 region (Ho and Rosenberg, 1982). Since CII associates to a tetramer in solution it is thought that two subunits, as part of a CII tetramer, contact this DNA binding sequence (Ho *et al.*, 1982).

Methylation protection studies carried out with CII and RNA polymerase have shown that CII and RNA polymerase interact with the DNA in the -35 region of p_{RE} by contacting opposite sides of the DNA helix effectively sandwiching the DNA between them (Ho *et al.*, 1983). The exact nature of interactions between CII and RNA polymerase required for activated transcription still remain to be understood. The *rpoA341* mutation, which changes lysine located at position 271 in the carboxy terminus of the alpha subunit of RNA polymerase (αCTD) to glutamate, has been found to reduce CII activated p_E transcription *in vivo* from 1649 to 38 β-galactosidase units (a 43 fold reduction in activity) (Obuchowski *et al.*, 1997). Taken on its own this evidence suggests that residue 271 of αCTD is a crucial contact site of CII on RNA polymerase. However, RNA polymerase in which the C terminal domain of the alpha subunit has been deleted up to residue 235 has been shown *in vitro* to reduce the ability of CII to activate p_E only 5 fold (Gussin *et al*, 1992). This latter evidence suggests that CII interations with αCTD while a major contributor are not exclusive for CII dependent activation. Strictly speaking these different results may simply reflect a difference in the sensitivity of the *in vitro* and *in vivo* systems used and it would therefore be of interest to see the effect of the *rpoA341* mutation *in vitro*.. Assuming that the two systems are comparable, the above results may suggest that the lysine to glutamate change at 271 induces a local conformational change which not only impedes

CII/αCTD interations but also impedes access of CII to other sites on RNA polymerase required for activation. It has already been shown that the CRP activator bound over the -41 region of the *gal* promoter and the bacteriophage Mu Mor protein bound over the -43.5 postion of p_m, contact not only αCTD but also sites on the σ^{70} subunit of RNA polymerase (Kumar *et al.*, 1994; Zou *et al.*, 1992; Zhou *et al.*, 1994; Artsimovitch *et al.*, 1996). Identification of RNA polymerase mutations, which perturb CII activated transcription, and isolation of CII mutations, which suppress these activation defects would contribute to the λCII activation field considerably.

1.2.1.4 Control of CII

CII activation of p_{RE}, p_I and p_{AQ} is able to override the bias favouring lytic development caused by the greater transcriptional activity of p_R compared to p_{RM}. An effective switch mechanism however, must be able to commit the phage to the appropriate lifecycle, be it either lytic or lysogenic, in response to its environment. Lambda achieves this switch by making its life cycle decision directly dependent on the cellular concentration of CII. It is therefore not surprising that CII expression is highly controlled at the level of transcription, translation and post-translationally.

1.2.1.4.1 At the level of transcription . Cro and CII are antagonistic functions and, although both are expressed from p_R, they are transcribed at different levels before the appearance of the N protein product. The t_{R1} Rho-dependent terminator located in the *cro*/*c*II intergenic region ensures that, in the absence of N, CII, and presumably all downstream early lytic genes, are transcribed 50 to 40% less relative to Cro (Rosenberg and Court, 1979; Dambly-Chaudiere *et al.*, 1983). Although the significance of its function is unknown, t_{R1} clearly controls *c*II transcription during the early infective stages.

1.2.1.4.2 Post transcriptionally . *c*II mRNA levels are reduced by expression of an antisense mRNA from the p_{OOP} promoter. p_{OOP} initiates a leftward transcript in the *c*II/*O* intergenic region which is complementary to 55 bases of the 3' end of *c*II mRNA. The *OOP* and *c*II RNA hybrid is subsequently cleaved in an RNase III dependent manner and the *c*II mRNA to the 5' side of the cleavage site is further degraded by nucleolytic processes (Krinke and Wulff, 1990). These events result in reducing *c*II mRNA levels several fold (Krinke *et al.*, 1991).

Somewhat surprisingly this reduction in CII expression does not effect the frequency of lysogeny as a phage carrying a p_{OOP} inactivating mutation was measured to lysogenise its host at the same frequency as a wild type counterpart. It is thought that *OOP* RNA accumulates to functionally significant levels only after the lytic lysogenic decision has been made and therefore does not participate in the decision as such (Krinke *et al.*, 1991). Instead, *OOP* RNA has its most pronouced affects during prophage induction, with a two fold decrease in burst size observed when a p_{OOP} mutant prophage is induced with UV compared to the wild type control. UV inactivation of the *E. coli* LexA protein which weakly represses p_{OOP} is thought to account for this observation (Krinke *et al.*, 1991).

1.2.1.4.3 At the level of translation . Expression of a *c*II *lacZ* gene fusion but not of a *c*II *lacZ* operon fusion is significantly reduced in an IHF$^-$ deficient host, suggesting that IHF enhances *c*II translation (Oppenheim *et al.*, 1993). It is thought that IHF mediates this function by binding just upstream of the ribosome binding site of *c*II although the exact mechanism whereby IHF, a DNA binding protein, affects translation is unknown (Mahajna *et al.*, 1986). The cellular level of IHF can vary quite dramatically (Goosen and van de Putte, 1995) and it therefore seems likely that the CII cellular concentrations will reflect these fluctuations in IHF concentrations. Since there is an absolute requirement for IHF during integration (Nash and Robertson, 1981), it

has been suggested that IHF control of *c*II expression is one way for the phage to co-ordinate the transcriptional and recombinational switches (Miller, 1981).

1.2.1.4.4 Post translationally . The CII protein is processed post-translationally by the removal of both the N-formylmethionine and the second amino acid valine predicted from the DNA sequence of the *c*II gene (Ho and Rosenberg, 1982). The removal of these first two amino acids seems to be an important event in the maturation of the CII protein as the *can1* mutation, which has a Val to Ala change in the second amino acid codon that confers resistance to processing, encodes a more stable CII protein compared with wild type (Wulff *et al.*, 1980). How the processing of CII exactly affects its turnover remains to be elucidated.

Although the level of CII present in an infected cell is controlled at multiple stages of CII synthesis (transcription, translation, processing), the rate of CII turnover is by far the most significant control mechanism. The independently isolated *hflA* and *hflB E. coli* mutations, identified because Lambda plates on these hosts with a high frequency of lysogeny, (Belfort and Wulff, 1973; Gautsch and Wulff, 1974) encode functions which specifically degrade CII. The *hflA* locus has been identified to code for three protein products HflX, HflK and HflC (Cheng *et al.*, 1988) and the *hflB* locus encodes a previously identified gene, *ftsH* ,which gives rise to an essential inner membrane zinc-binding protein with ATPase activity (Herman *et al.*, 1993; Herman *et al.*, 1995). In addition to the negative regulation described, CII is also positively regulated by the activity of the phage CIII protein which has been shown to protect CII from proteolysis (Hoyt *el al.*, 1982; Herman *et al.*, 1997).

FtsH has been shown to specifically degrade the heat shock sigma factor σ^{32}, CII and CIII, with CII proteolysed most efficiently, followed by σ^{32} and then CIII (Herman *et al.*, 1995; Shotland *et al.*, 1997). Although CIII itself is degraded by FtsH, CIII is thought to inhibit FtsH activity, thereby protecting both CII and σ^{32} from proteolysis

(Herman *et al.*, 1993; 1995; 1997; Shotland *et al.*, 1997). Vivid proof of the CIII mediated σ^{32} stabilisation comes from the observation that overexpression of CIII induces the heat shock response (Bahl *et al.*, 1987; Kornitzer *et al.*, 1991a). How CIII inhibits FtsH is still unknown. What has been established however is that CIII stabilises CII and σ^{32} by different pathways (Shotland *et al.*, 1997). The protection that CIII confers on CII does not occur by interference with ATPase activity of FtsH (Kunau *et al.*, 1993; Tomoyasu *et al.*, 1993) nor does it require DnaK, a molecular heat shock chaperone, to which CIII binds avidly and which is required in the σ^{32} degradation pathway (Tomoyasu *et al.*, 1995); in fact CII protection only requires residues 16 to 37 of the CIII core region (Oppenheim A.B., personal communication). Although CIII mitigates the damaging effects of FtsH degradation of CII, the exquisite sensitivity of Lambda to the physiology of its host is still essentially dependent on the cellular level of FtsH available to degrade CII. In this regard it is important to note that FtsH levels fluctuate in the cell; specifically FtsH has been shown to be positively regulated at the level of transcription by σ^{32} (Herman *et al.*, 1995).

The FtsH pathway described in this section it seems can account for at least two well documented responses of Lambda to changes in its immediate environment, specifically its response to (i) starvation of the host bacterium and (ii) multiplicity of infection. When the host is starved prior to infection, the lysogenic response is favoured (Kourilsky, 1973; Wulff and Rosenberg, 1983). Because proteolysis of CII by FtsH is ATP dependent (Tomoyasu *et al.*, 1995; Shotland *et al.*, 1997) and because the starved host is presumed to be ATP depleted, it may be envisaged that in the starved host FtsH mediated CII proteolysis is reduced, and as a consequence a lysogenic outcome is favoured. The frequency of lysogeny is also influenced by the multiplicity of infection, with low multiplicity of infection favouring a lytic outcome and high multiplicities favouring lysogeny (Boyd, 1951; Lieb, 1953). It has been suggested that at high multiplicities the incidence of multiple infection of a single host is increased, resulting in the additive expression of CIII as p_L of all infecting phages is

transcribed (Kourilsky, 1974; Reichardt, 1971). CII is thus more efficiently protected from FtsH proteolysis by CIII which ultimately results in increasing the frequency of lysogeny.

1.2.2 Establishment of lysogeny in other phages with back to back lytic/lysogenic promoters

Although there is no doubt that Lambda is the most extensively studied phage of its family, establishment of lysogeny has also been characterised in other lambdoid phages including 434, ϕ80, HK022 and the *Salmonella typhimurium* specific phage P22. While these three lambdoid phages only share a limited degree of nucleotide sequence homology with Lambda, their genomes are organised very similarly, with genes of similar function occupying corresponding chromosomal locations (Campbell, 1994). More specifically, these three phages have conserved the back-to-back arrangement of promoters corresponding to Lambda p_R and p_{RM}, and their Cro and CI analogues, like Lambda, maintain lytic or lysogenic transcription respectively by binding in reverse order of affinity to three operator sites at O_R and O_L (although differences in relative binding affinities and co-operativity of the binding of repressor to corresponding operator sites have been noted) (Cam *et al.*, 1991; Ogawa *et al.*, 1988a, 1988b; Retallack *et al.*, 1993).

It is interesting to note that the distance separating the divergent promoters analogous to Lambda p_R and p_{RM} is not conserved between these phages (see Fig 1.3). Whereas in Lambda, ϕ80 the predicted -35 regions of p_R/p_{RM} are positioned 12 and 10 bp apart respectively, in 434 they coincide and in HKO22 and P22 the -35 regions of p_R and p_{RM} overlap by 4 bp (Carlson, 1993; Ogawa *et al.*, 1988a, 1988b; Bushman, 1993; Oberto *et al.*, 1989). Although the consequences of overlapping divergent transcription on transcription from p_{RM} in particular have not been looked at in P22, they have been noted in phage 434. In 434, deletion of p_R or repression of p_R by Cro (on an O_{R3}-

template) raises basal p_{RM} transcription 2 fold, suggesting that p_R activity interferes with p_{RM} transcription (Bushman, 1993). In contrast p_{RM} activity in Lambda is unaffected when p_R contains a mutation which reduces polymerase binding 20 fold (Gussin, 1983). These facts imply that, immediately post infection and prior to establishment of lysogeny, p_{RM} promoters, whose polymerase recognition sequences overlap with p_R, are inhibited by p_R transcription and repressed by Cro, whereas the p_{RM} promoters of phage with non-overlapping divergent p_R and p_{RM} are only repressed by Cro. Although the biological significance of this additional regulatory loop is unknown it does not seem to effect the way P22, 434, ϕ80 and HK022 establish lysogeny since these phages, as discussed below, would seem to employ a very similar mechanism to Lambda to establish lysogenic transcription.

1.2.2.1 Establishment of lysogeny in phage HK022

The CII protein of HK022 has 29% amino acid identity with Lambda CII (Oberto *et al.*, 1994) and mediates its function by binding to p_E (Oberto *et al.*, 1989a) and p_I (Yagil *et al.*, 1989) in analagous locations to Lambda, although a p_{AQ} analogue has yet to be found in HK022. The CII DNA binding sequence at p_I is identical in Lambda and HK022 however, HK022 CII binds to a sequence at p_E which has a 7 out of 8 bp match with the λ CII DNA binding sequence (Oberto *et al.*, 1989). Like in Lambda, the HK022 direct repeats also center over the -35 of the corresponding promoters (Oberto *et al.*, 1989). In lambda the *ctrl* mutation consists of a single base pair change in the CII DNA binding determinants which converts the wild type Lambda CII binding site to an HK022 p_E type CII binding sequence. CII dependent *ctrl* p_{RE} activity has been found to be half that of the wild type promoter (Wulff and Mahoney, 1987). It is not known whether the HK022 CII activation at this promoter is affected similarly or whether HK022 CII can better accommodate such a deviation from the consensus binding site.

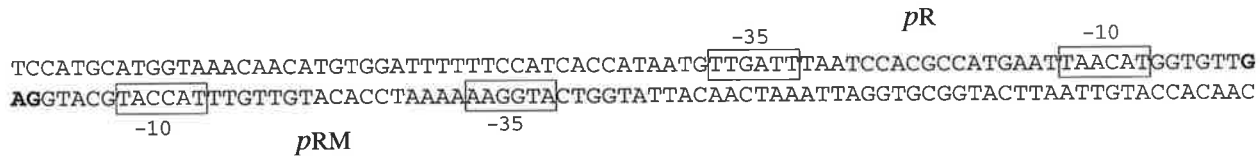
Figure 1.3

Sequence of the lytic/lysogenic promoter regions of lambdoid phages λ, ϕ80, 434, HK022 and P22

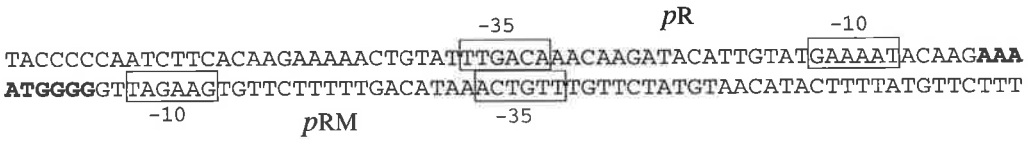
The transcription start sites are denoted bold letters (if they have been identified) and the -10 and -35 hexamers are boxed. Details of λ, ϕ80, 434 and HK022 promoter regions are taken from Gussin *et al.*, 1983; Ogawa *et al.*, 1988; Bushman, 1993; Carlson and Little, 1993. The P22 promoter region was compiled from Bushman, 1993 and Oberto *et al.*, 1989.

λ

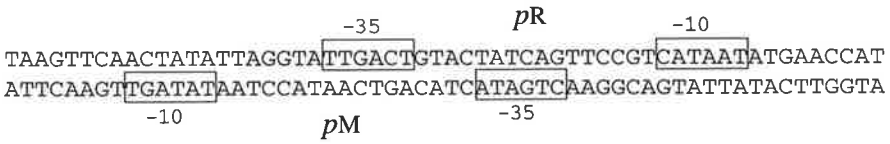
ϕ80



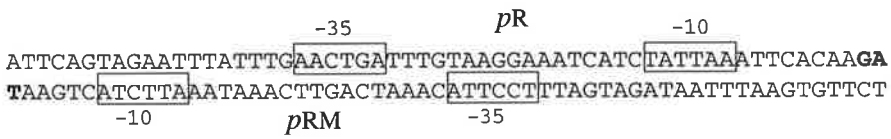
434



HK022



P22



It seems that, like Lambda, HK022 may control CII expression with p_{OOP}. The p_{OOP} promoter sequence which includes a potential LexA binding site is very well conserved between the two phages (Oberto *et al.*, 1994). The predicted amino acid sequences of HK022 CIII and Lambda CIII also show significant homology suggesting that HK022 CIII may function similarly to Lambda CIII (Oberto *et al.*, 1989). Furthermore, initiation of translation of the CIII gene seem to require a similar mRNA structure in both HK022 and Lambda (Kornitzer *et al.*, 1991b)

1.2.2.2 Establishment of lysogeny in Salmonella typhimurium specific phage P22

C1 of P22 is the CII homologue of Lambda and these two proteins share 50% amino acid identity (Ho *et al.*, 1992). Out of the 9 residues which make up the HTH motif 7 of these are conserved between the two phages (Ho *et al.*, 1992). C1 activates transcription from p_{RE} and p_{a23}, by binding to DNA sequences which share a 7/8 bp match and an 8/8 bp match with the λCII DNA binding sequences respectively (Ho *et al.*, 1992). The P22 promoters p_{RE} and p_{a23} thus appear to be counterparts of λp_{RE} and λp_{AQ}. In constrast to λCII, C1 binds to an additional sequence, which shares a 7/8 bp match with the λCII DNA binding sequence, in an open reading frame upstream of *xis* and *int* , activating a promoter p_{aI} transcribed in the opposite direction to λp_I (Herskowitz and Hagen, 1980). The functional significance of p_{aI}, if any, remains to be determined.

Although P22 C1 and λCII are significantly homologous proteins and bind to almost identical sequences, complementation studies as well as transcriptional reporter studies indicate that they function very selectively on their cognate phage genomes and exhibit little if any cross-reactivity. The poor activation of λCII on P22 p_{RE} sequence can only partially be accounted for by the single base pair deviation from the primary CII recognition sequence. The basis by which this *in vivo* promoter recognition specificity is achieved is still under investigation (Ho *et al*, 1992)

1.2.2.3 Establishment of lysogeny in phage ϕ80

Very little is known about how ϕ80 establishes lysogeny. A putative candidate for the *c*II gene has been identified and is located in the second ORF of the early lytic trancript. Although there is no significant homology between Lambda and ϕ80 CII at the amino acid level the assignment of ϕ80 *c*II seems to be correct since an amber mutation in the *c*II gene forms clear plaques suggesting that the mutation affects lysogenisation (Ogawa *et al.*, 1988b). Potential CII binding sites have yet to be identified (Ogawa *et al.*, 1988a). Reminiscent of HK022 and P22, ϕ80 has also conserved a short sequence associated with the OOP transcription start point and a LexA binding site in the same region as λ p_{OOP} (Oberto *et al.*, 1994).

1.3 ESTABLISHMENT OF LYSOGENY IN PHAGES WITH FACE-TO-FACE LYTIC/LYSOGENIC PROMOTERS

The non-lambdoid phages considered below belong to two distinct classes: Mu and D108 represent the Mu-type phages whilst P2 and 186 are members of the P2-like family. Reminiscent of their lambdoid counterparts, expression of the lytic transcript of non-lambdoid phages results in the lytic response to the exclusion of lysogeny and expression of the lysogenic transcript commits the phage to the corresponding lifecycle and excludes the lytic response. Where non-lambdoid and lambdoid phages mainly differ is in the arrangement of their lytic and lysogenic promoters:back-to-back for lambdoid phages, face-to-face for the non-lambdoid phages considered here (see Fig 1.4).

The case of 434, where p_R seems to inhibit p_{RM} activity as a consequence of the overlap of promoter recognition sequences, has already been highlighted. Since a face-to-face arrangement of a strong lytic and a weak lysogenic promoter is an extension of

the 434 situation, it seems likely that a face-to-face arrangement would further amplify the transcriptional inhibition imparted on the lysogenic promoter. Since lysogeny is maintained by transcription from the lysogenic promoter, a face-to-face arrangement presents additional challenges for establishing lysogenic transcription. Although it is largely unclear how non-lambdoid phages overcome this problem, what is known about these mechanisms is presented below.

1.3.1 Establishment of lysogeny in phage Mu

Bacteriophage Mu makes its developmental decision at the level of transcription with expression from p_E resulting in lytic development and p_C activity resulting in lysogeny (Krause and Higgins, 1986; Goosen and van de Putte, 1987). As is the case with the functionally equivalent promoters in P2 and 186 (Saha et al., 1987; Dodd *et al.*, 1990), the Mu p_C promoter is significantly less active than p_E (Alazard *et al.*, 1992; van Rijn *et al.*, 1988) and, due to the face-to-face arrangement of p_E and p_C, p_C is also presumably inhibited by transcription from p_E.

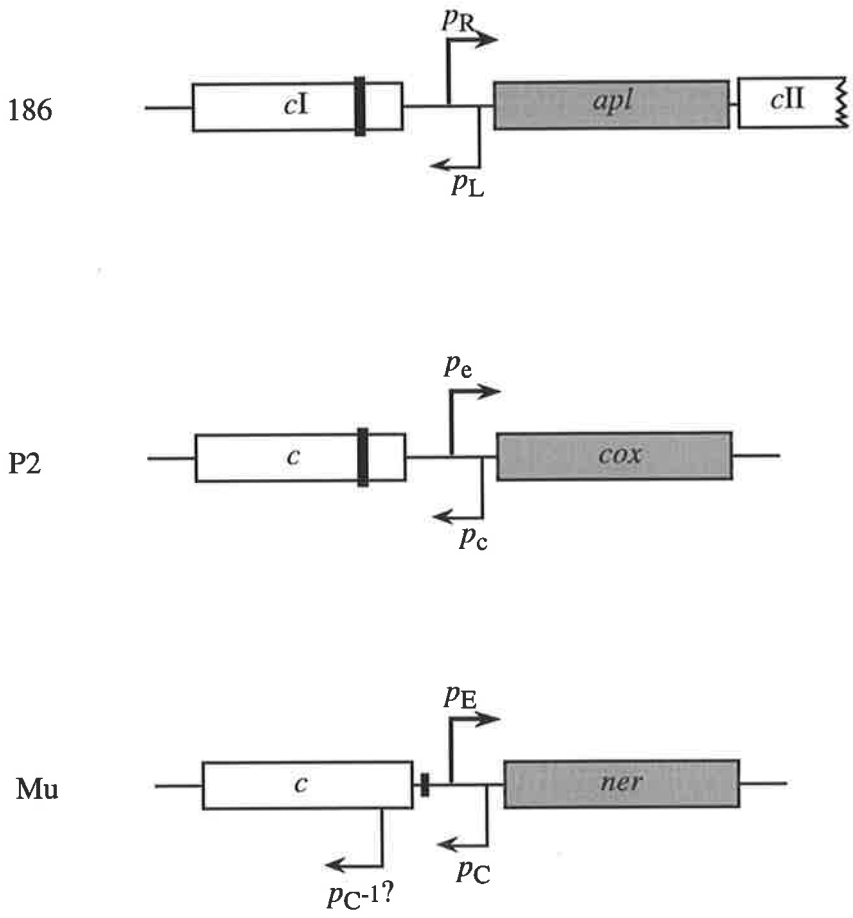
Mu does not seem to encode a CII-like function and the mechanism employed by this phage to establish lysogeny is not understood. It has been suggested that the host protein FIS (Factor for Inversion Stimulation) may help Mu to establish lysogeny. FIS improves binding of C to its operators over p_E when the C concentration is low (Betermier *et al.*, 1993). As the concentration of FIS varies greatly during the cell cycle, reaching a peak during early log phase in rich media (Nilsson *et al.*, 1992; Thompson *et al.*, 1987), it is predicted that Mu would lysogenise its host with the greatest frequency at this point in the cycle (Betermier *et al.*, 1993). This hypothesis however still remains untested.

It has also been suggested that establishment of lysogeny in Mu is influenced by the host protein IHF (Integration Host Factor) as well as changes in DNA topology. On a

Figure 1.4

The lytic-lysogenic regulatory regions of 186, P2, Mu and D108

Genes involved in lysogeny are represented as white filled rectangles while lytic genes are shaded. Transcription start sites are denoted by arrows. The location of the controversial P_{C-1} promoter of Mu is indicated by a dotted arrow and a question mark. IHF sites are indicated by darkly fill rectangles. This diagram is not drawn to scale. Refer to the text for a more complete description.


186
p_R
cI
apl
cII
p_L
P2
p_e
c
cox
p_c
Mu
p_E
c
ner
p_C
p_{C-1}?

relaxed template p_C is observed to be 1.7 to 2 fold more active than p_E whether IHF is present or not. In contrast, on a supercoiled template the reverse is true with p_E 2.5 fold more active than p_C in the absence of IHF, and 20 fold more active than p_C in the presence of IHF (Higgins *et al.*, 1989). Assuming that these *in vitro* observations mirror the *in vivo* situation, Mu may be able to establish lysogeny from p_C in response to changes in DNA topology with IHF acting to amplify small changes in supercoiling (Higgins *et al.*, 1989).

Mu may also establish lysogeny by transcribing C from a promoter, other than p_C, which is insensitive to p_E inhibition. Such an alternative weak promoter initiating in the *c* coding region ($p_{C\text{-}1}$, see Fig 1.4) has been noted by one group (Goosen *et al*, 1984). A weak promoter in an analogous position of the highly homologous Mu-like phage D108 has also been detected. Furthermore, RNA polymerase was able to form an open complex at this D108 promoter and express a truncated but functional form of the repressor (Kukolj and DuBow, 1992). It remains to be seen if this promoter is biologically significant in D108 and whether its function is retained in Mu.

1.3.2 Establishment of lysogeny in phage P2

In P2 the lysogenic promoter p_c controls synthesis of C, a repressor of the early lytic promoter and the integrase both of which are required for lysogeny (Saha *et al.*, 1987). The early lytic promoter p_e on the other hand results in the synthesis of protein A and B required for phage replication and Cox a repressor of p_c (Saha *et al.*, 1987) (see Fig 4). The intrinsic activity of p_c is 5 fold less than p_e, but 139 fold less when measured in the presence of converging p_e transcription (p_e transcription has only been measured in the presence of p_c). P2 does not encode a *c*II-like gene (Linderoth *et al.*, 1992) and Saha *et al.* (1987) have stated that the establishment of lysogeny reflects the relative activity of p_e and p_c. If frequency of lysogeny were simply a reflection of promoter strength, P2 would be expected to have a frequency of lysogeny of less than 1%.

However, Bertani (1962) measured that between 5 to 15 % of P2 infections give rise to lysogens. Little is known about how P2 overcomes the bias which favours lytic development, at the promoter level, in order to establish lysogeny at these frequencies.

The Cox gene product would seem to play a part in the lytic/lysogenic decision. A P2 phage which contains a Cox inactivating mutation, *cox3,* lysogenises its host 3-5 fold more efficiently than a wild type counterpart (Bertani, 1980). Furthermore, when a Cox overexpressing strain is infected with P2, the outcome is essentially lytic, with < 0.0001 % lysogens scored (Saha *et al.*, 1987). However, it is unclear how Saha *et al.* (1987) arrive at this number. If the frequency of lysogeny (fol) (colony forming units ÷ (colony forming units + infective centres) is determined from the number of colony forming units and the number of infective centres given, the presence of *cox* decreases the fol from 52 % to 28 %. This suggests that the presence of *cox* decreases the fol by a factor of approximately 2 and not 10^6. The antagonistic effect of *cox* on the frequency of lysogeny is also reflected at the promoter level, with lysogenic transcription measured from a p_C CAT reporter construct (in the presence of p_E) reduced by 58 % when *cox* is supplied in *trans* from a compatible *cox* expression plasmid. It must be noted however, that Cox does not exclusively antagonise lysogenic development but also antagonises lytic development. This is exemplified by a 12 to 30 % decrease in the efficiency of plating of P2 on hosts carrying a Cox expression plasmid and by a 61 % decrease in lytic transcription (Saha *et al.*, 1987).

The role of Cox in the P2 lytic/lysogenic decision is complex and not well understood. How important the absence of Cox is in the establishment of lysogeny remains to be convincingly determined. In that respect it would be informative to measure the fol of a P2 *Δcox* mutant.

Apart from Cox, the P2 lytic/lysogenic decision is also affected by IHF. The *E. coli* IHF protein, a ubiquitous host DNA bending protein, is required for the establishment

of lysogeny since P2 does not lysogenize IHF deficient strains (Saha *et al.*, 1990). IHF affects the P2 lytic/lysogenic decision by regulating the transcriptional activities of p_e and p_c in favour of p_c transcription (Saha *et al.*, 1990). In addition IHF facilitates the lysogenic state, since it is part of the structural component of the nucleoprotein complex formed at attP prior to integration (Yu and Haggard-Ljungquist, 1993). At the level of transcription, IHF is thought to mediate its function by binding to a site within the *c* coding region. The effect of IHF on p_e and p_c transcription was measured by CAT transcriptional reporter constructs in the absence of active C and Cox proteins. When these constructs were transformed into various IHF^- host mutants transcription from both promoters decreased, with p_c transcription affected more severely than p_e transcription (p_c transcription drops between 1.9 to 2.7 fold and p_e activity between 1 to 1.6 fold) (Saha, 1988). As a consequence the disparity between the trancriptional activities of p_e and p_c is greater in the IHF^- than in the wild type host. In the wild type host there is a 5 fold difference between the intrinsic activities of p_e and p_c while in the IHF mutant hosts there a 6 to 12 fold difference in the intrinsic activities of p_e and p_c, depending on the individual IHF point or deletion mutations tested. At the promoter level the presence of IHF therefore would seem to favour lysogenic transcription.

The recorded differences in promoter strengths between IHF^+ and IHF^- strains above are fairly small especially compared to the differences recorded between the various IHF^- strains. The spread in IHF^- values may indicate that the various IHF point mutations and the deletion tested inactivate IHF more or less efficiently. However, as the CAT units obtained for the two multicopy transcriptional reporter constructs measuring p_e and p_c activity were not corrected for copy number flunctuations between IHF^+ and IHF^- host strains, the differences observed may equally be due to a variable reduction of plasmid copy number in the IHF^- strains. Further transcriptional reporter studies using single copy constructs would therefore be informative.

A more dramatic effect of IHF has been observed when the chloramphenicol and kanamycin resistance genes are inserted on either side of the *c*-p_e-p_c-*cox* region of P2 yielding a construct where resistance to chloramphenicol and kanamycin is a measure of p_c and p_e transcription respectively. When this reporter contruct was transformed into various IHF$^-$ hosts only kanamycin resistant colonies were scored whereas in an isogenic IHF$^+$ strain both kanamycin and chloramphenicol resistant colonies were observed (at a frequency of 0.2 and 0.8 respectively) (Saha *et al.*, 1990). It must be noted that the distribution of 20 % "lytic" colonies and 80 % "lysogenic" colonies obtained in the IHF$^+$ strain is not consistent with the frequency of lysogeny of 5-15 % measured for P2 phage (Bertani, 1957; Bertani, 1962; Bertani and Bertani, 1971). This is claimed to occur because the physiology of infected and transformed cells differs (Saha, 1988). Importantly however, the absence of IHF only "lytic" colonies are scored. This is in essence consistent with the results obtained for the CAT reporter studies described above although the effect of IHF is much greater and the same result is obtained for the various IHF strains tested. If one assumes that results from these two experimental systems can be compared, it may be suggested that IHF acts by modifying the action of C and/or Cox yet only a marginally effects the activities of p_e and p_c directly.

The main criticism of these IHF experiments is that although IHF binds within the *c* coding region, the use of IHF$^-$ hosts does not prove that IHF itself alters the transcriptional flow. The effect may be due to a secondary mediator itself under IHF control. Studies with a P2 mutant in which the IHF site has been altered (while conserving the amino acid sequence and codon usage of the *c* gene) would be informative. While we await such experiments, it is not really clear by what mechanism P2 establishes lysogeny.

1.3.3 Establishment of lysogeny in 186

1.3.3.1 Early 186 genes involved during lytic and lysogenic development

In 186, the lytic-lysogenic decision occurs at the level of two promoters: the lytic promoter p_R and the lysogenic promoter p_L. Lysogenic transcription from p_L results in expression of CI which represses transcription from p_R, integrase (Int) and CP69 a protein of unknown function (Dodd *et al.*, 1990). Early lytic transcription from p_R on the other hand, produces the Apl protein which functions both as the 186 excisionase and a repressor of p_R and p_L (Reed *et al.*, 1997), the CII product which involved in the establishment of lysogeny (Lamont *et al.*, 1996), and Fil and Dhr which inhibit cell division and host replication (Richardson and Egan, 1989). Since transcription from p_L results in production of CI which represses p_R and transcription from p_R expresses the Apl protein which can repress p_L, transcription from either promoter is at the expense of the other.

Reminiscent of the situation in P2 and Mu, in 186 p_L is intrinsically 7 fold less active than p_R and is reduced a further 12 fold in the presence of p_R, giving an overall difference of 86 fold (Dodd *et al.*, 1990). Yet, during the establishment of lysogeny, the phage must theoretically progress from a state where p_L is repressed by Apl and interfered with by actively transcribing p_R, to the stable lysogenic state of autogenous control whereby CI repression of p_R allows p_L activity and thereby maintenance transcription of *c*I. This begs the question of how p_L is given a transient advantage over p_R to establish lysogeny.

1.3.3.2 The CII protein of 186 establishes lysogeny

To date bacteriophage possessing face-to-face promoters such as P2, Mu and D108 are thought not to require a Lambda CII-like function to establish lysogeny. This is in contrast to 186 where a search for additional phage encoded functions required during

lysogenic development identified a clear plaque mutant which was able to complement a mutation in the *c*I gene and therefore belonged to a separate complementation group to *c*I (Lamont *et al.*, 1993). This function, named *c*II, was mapped to the second open reading frame on the lytic transcript and was shown to be required to establish rather than maintain lysogeny (Lamont *et al.*, 1993). CII is predicted to encode a 18.7 kD protein which contains a potential Helix-Turn-Helix DNA binding domain (Dodd and Egan, 1990). The presence of this motif suggested that CII may bind to DNA and thereby function as a transcriptional regulator. As CII is required to establish lysogeny it is most likely to play a part in transiently redressing the flow of transcription at p_R and p_L to favour p_L transcription. CII may achieve this by either activating p_L or repressing p_R, or alternatively activating a second lysogenic promoter. Locating the CII DNA binding site was expected to help differentiate between these possibilities.

1.3.3.3 The scope of this thesis

Prior to my work, it had been shown that CII overexpressed in a crude cell extract was able to bind a minimal 165 bp fragment of DNA which spanned the 3' and 5' terminii of *apl* and *c*II respectively (Camerotto, 1992). To understand the mechanism employed by CII to establish lysogeny, I first defined the CII DNA binding determinants within this 165 bp minimal region. This work is reported in Chapter 2. As the location of the CII-binding site upstream of p_L is reminiscent of λCII, the hypothesis that 186 CII acts in a similar fashion was tested. Chapter 3 describes results obtained from single copy transcriptional reporter and primer extension studies which confirm the existence of a CII activated leftward promoter upstream of p_L.

Because 186 employs face-to-face promoters to maintain and establish lysogeny I asked why p_E but not p_L can overcome opposing transcription from p_R. Chapter 4 describes results from single copy transcriptional reporter studies used to address this question. Since establishment of lysogeny in the wild type phage occurs in the

presence of Apl work described in Chapter 5 looks at how Apl alters the flow of lytic and lysogenic transcription during the establishment of lysogeny.

CHAPTER 2

Identification of the CII DNA binding determinants

2.1 INTRODUCTION

*c*II is predicted to encode a product of 18.7 kD. Residues 23 to 43 of the inferred amino acid sequence of CII score highly in the Dodd and Egan (1990) weight matrix method for detecting helix-turn-helix DNA binding motifs, suggesting that CII may bind DNA and act as a transcriptional regulator. As a prelude to defining the mechanism of action of CII, its binding to DNA was confirmed. With CII predicted to affect the lytic/lysogenic decision in some way, restriction digests of the early region DNA of 186 (*Xho* I 629-*Bgl* II 4244) were initially tested for CII binding by gel mobility shift assay (Figure 2.1). The assays, using a CII enriched crude cell extract, were performed on *Hinf* I, *Hpa* II and *Taq* I restriction digests of DNA spanning the p_R and p_L region. The CII binding site was thus located within a 165 bp region (3003-3168) which encompasses the *apl*/*c*II intergenic region as well as portions of the 3' end of the *apl* gene and the 5' end of the *c*II gene (Camerotto, 1992 described in Neufing *et al.*, 1996). This chapter describes work which was carried out to further define the CII binding site.

2.2 RESULTS

2.2.1 Defining the CII-binding site

The DNase I footprint analyses, performed with purified CII protein (a gift from Keith Shearwin) on DNA fragments encompassing the 165 base pair region common to the fragments retarded in the gel mobility shift assays, are summarized in Figure 2.2.2. The pattern obtained with the *r*-(bottom) strand shows that CII protects a 35 bp region overlapping the *c*II gene (Figure 2.2.1). These protections are flanked by regions of

weak enhanced cleavages. The protections and flanking enhancements are symmetrically bisected by a central region of strong enhanced cleavage. The sequences protected by CII on both strands are associated with a 7 base perfect inverted repeat, $ATGTTTG(N)_{13}CAAACAT$, bisected by the central enhancements. These data suggest that these sequences are the DNA binding determinants for the CII protein. The centres of these repeats are 20 bp (approximately two turns of the helix) apart and so are located on the same face of the DNA.

2.2.2 The *c*IV476 mutation abolishes CII binding

Footprint analysis of the clear plaque mutant phage, *c*IV476, was performed to further confirm these sequences as CII operators. The possibility of this phage being a CII binding site mutant arose because it was consistent with the fact that the mutant, although its locus is sited within the *c*II gene, nevertheless complements cII mutants (Lamont *et al.*, 1993). The *c*IV476 mutation is a T to A change at position 3126 (Lamont *et al*, 1993), which alters the putative left CII binding sequence from ATGTTTG to ATGTTAG. Footprint analysis of the *l*-strand (data not shown) and the *r*-strand of the mutant DNA showed that CII binding was completely abolished (Figure 2.2.1). This supported the ATGTTTG boxes being CII binding determinants and also indicated that CII binding at the two boxes is not independent.

2.2.3 Identification of other putative CII DNA-binding sites in the 186 genome

As only the *Xho* I to *Bgl* II region of 186 was initially tested for CII binding, it is possible that CII mediates its action by binding to additional regions outside of this boundary. A search of the known sequence of 186 located three potential CII binding sites (within the *CP84* and *CP97* sequences and at the start of the *B* gene) with a significant match of 11 out of 14 bp with the inverted repeat of the 186 CII binding

Figure 2.1

Genetic map of 186 and summary of the CII gelshift status of the *Hinf* I, *Hpa* II and *Taq* I restriction fragments of the *Xho* I- *Bgl* II region of 186.

The 186 genome is represented at the top of the figure. Genes are denoted by boxes, transcripts by arrows, while promoters are represented by open circles and terminators by arrow heads. The function of various genes are indicated above the figure.

The *Xho* I-*Bgl* II region is shown in expanded view. In this bottom part of the figure, darkly shaded areas represent restriction fragments specifically retarded by a CII enriched crude cell extract, lightly shaded regions represent fragments not retarded by CII, while white areas represent fragments whose retardation status could not be determined from the data (Neufing *et al.*, 1996). The location of the CII binding site is indicated. The *Pst* I site at 65.5 % (Kalionis *et al.*, 1986a) is the reference point for all sequence co-ordinates in this thesis.

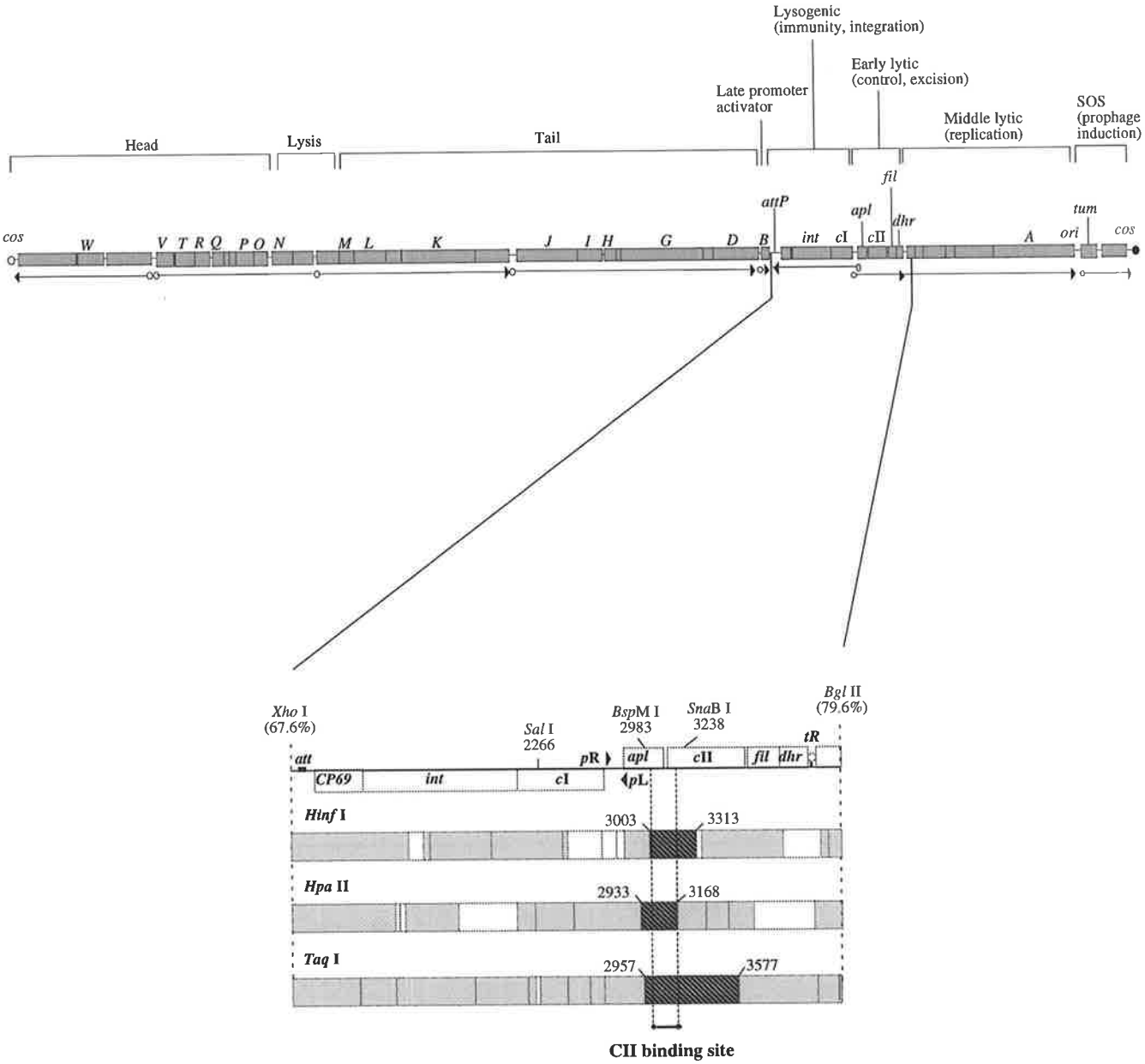

Lysogenic
(immunity, integration)
Early lytic
(control, excision)
Late promoter
activator
Middle lytic
(replication)
SOS
(prophage
induction)
Head
Lysis
Tail
fil
attP
apl
dhr
tum
cos
W
V T R Q P O N
M L
K
J I H
G
D
B
int
cI
cII
A
ori
cos
Xho I
(67.6%)
Sal I
2266
BspM I
2983
SnaB I
3238
Bgl II
(79.6%)
tR
att
pR
apl
cII
fil
dhr
CP69
int
cI
pL
Hinf I
3003
3313
Hpa II
2933
3168
Taq I
2957
3577
CII binding site

Figure 2.2.1 (Top)

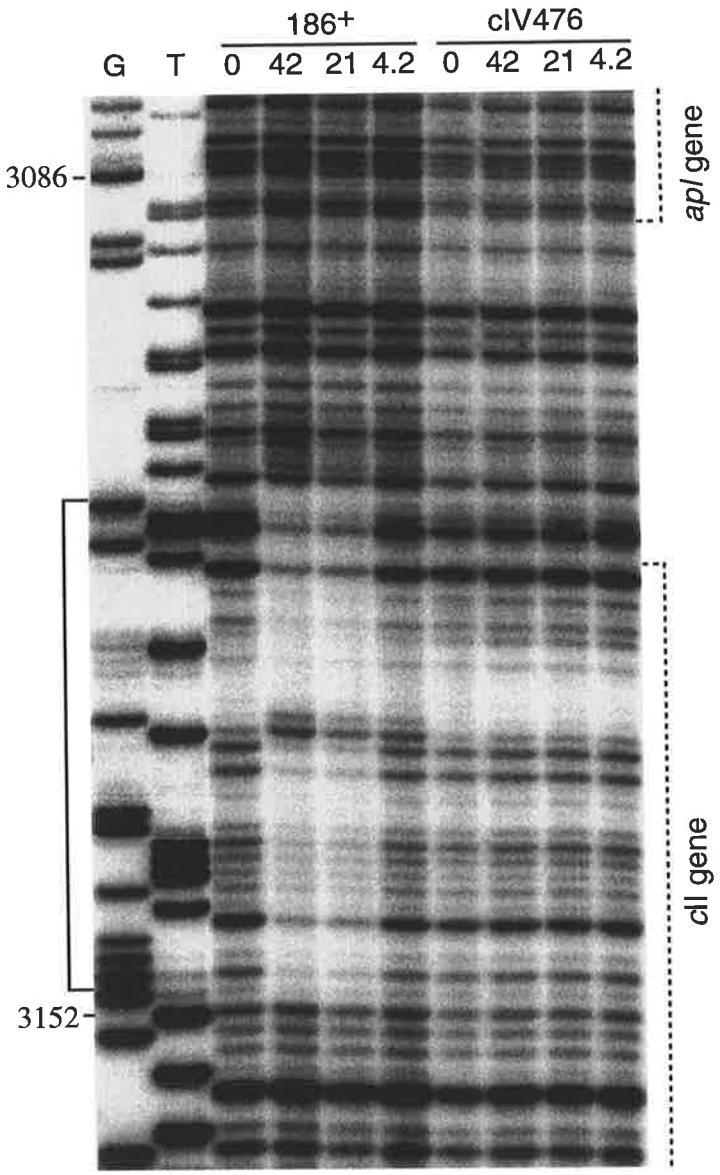
CII DNase I footprinting pattern on the *r*-(bottom) strand of wild type 186 (lanes 1-4) and 186*c*IV476 (lanes 5-8), encompassing the 165 bp CII binding region.

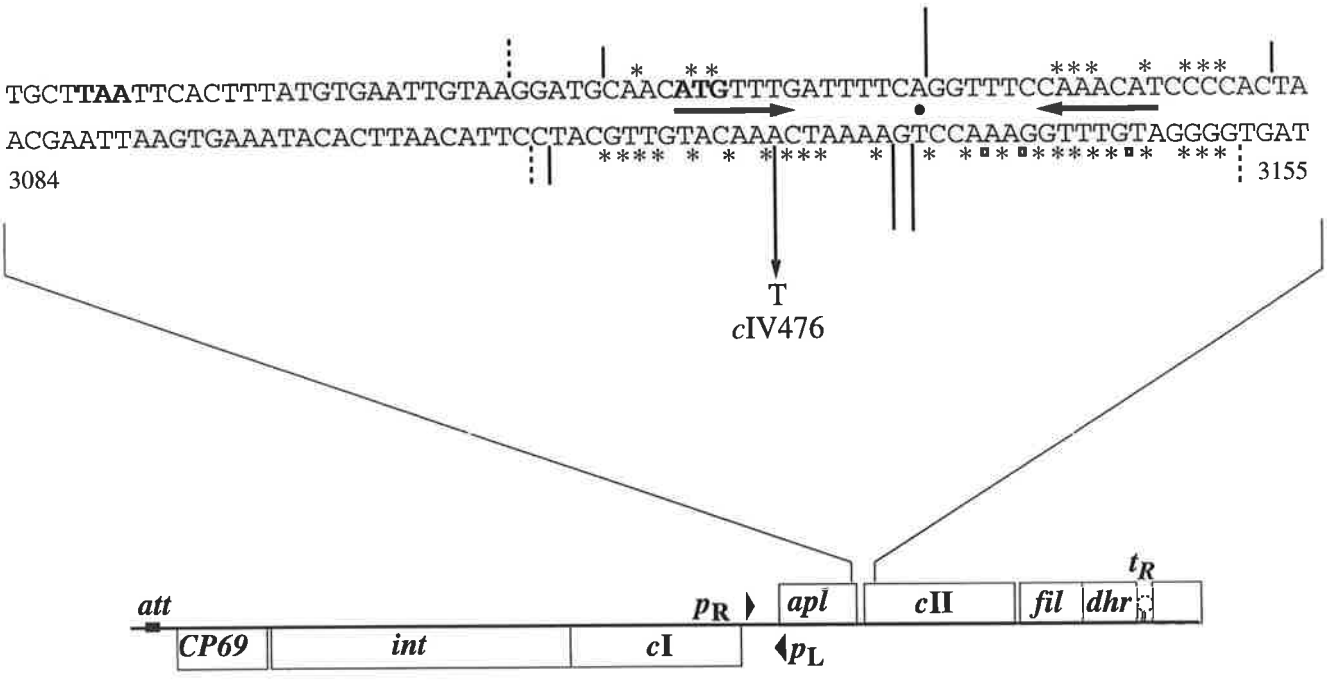
^{32}P end-labelled wild type DNA was generated from pEC622 or a 186*c*IV476 plaque by PCR with primer 34 and ^{32}P labelled primer 22. Purified CII was used at concentrations (nM) specified at the top of each lane. Lanes labelled GT represent corresponding sequencing reactions generated from pEC622 DNA with ^{32}P kinased primer 22. The region protected by CII is shown at the left of the Figure.

Figure 2.2.2 (Bottom)

Summary of the CII DNase I footprinting data.

The *l*-strand data is from a DNase I protection experiment (not shown) carried out with 186 DNA PCR amplified using ^{32}P 5' labelled primer 99 and unlabelled primer 80 as described in Fig . Enhancements are shown by vertical lines with their size indicating relative strengths of the effect. Protections are indicated with asterisks. A dotted line or square indicates that assignment of the protection or enhancement at this position is done with a reduced degree of confidence. The two 7 bp perfect inverted repeats which correspond to the CII DNA binding determinants are indicated by arrows, as is the location of the 186*c*IV476 mutation. The stop codon of Apl and start codon of CII are indicated in bold. The filled dot represents the centre of the binding site and is located at sequence position 3135.


186+
cIV476
G T 0 42 21 4.2 0 42 21 4.2
3086
3152
apl gene
cII gene


TGCTTAATTCACTTTATGTGAATTGTAAGGATGCAACATGTTTGATTTTCAGGTTTCCAAACATCCCCACTA
ACGAATTAAGTGAAATACACTTAACATTCCTACGTTGTACAAACTAAAAGTCCAAAGGTTTGTAGGGGTGAT
3084
3155
T
cIV476
att
CP69
int
cI
pR
pL
apl
cII
fil
dhr
tR

sequence (Ian Dodd, personnal communication). Fig 2.3.1 lists these sites, highlighting their divergence from the pE inverted repeats, and specifies their exact location on the 186 genome. The potential site located at the start of the *B* gene, which encodes the late gene activator protein, was of interest as it might reflect an involvement of CII in the control of late gene expression. However, purified CII (at 420 nM) was unable to bind this site in a gel shift (data not shown). Although the other two putative CII DNA binding sequences were not tested it seems unlikely that CII would act at such sites as they also contain three sequence changes and map within coding regions.

2.3 DISCUSSION

2.3.1 Model of DNA binding by CII

CII appears to have an unusual mode of DNA binding. The sequence conservation, DNase I footprinting and mutational data strongly support CII recognition sequences that are arranged in inverted orientation, two turns of the DNA helix apart. The lack of dyad symmetry within the 7 base repeat sequence (ATGTTTG) suggests that each sequence is contacted by one CII monomer (although a multimer could in fact be involved in binding). Mutation at one of these sequences eliminated binding at both sequences. Thus the sequences appear to function as half sites for contact by a CII dimer. Equilibrium sedimentation experiments, carried out with purified CII in the presence of a 40 bp fluorescein labelled double stranded DNA fragment containing the CII binding site, showed that the CII species bound corresponded to a dimer (Keith Shearwin, personnal communication), not a tetramer as was suggested from solution association experiments (Neufing *et al.*, 1996) .

CII, being a likely HTH protein, should contact its recognition sequence via the major groove. These data thus suggest a model for CII binding in which CII monomers, as part of a dimer, contact major grooves two turns of the helix apart. This model is

Figure 2.3.1 (Top)

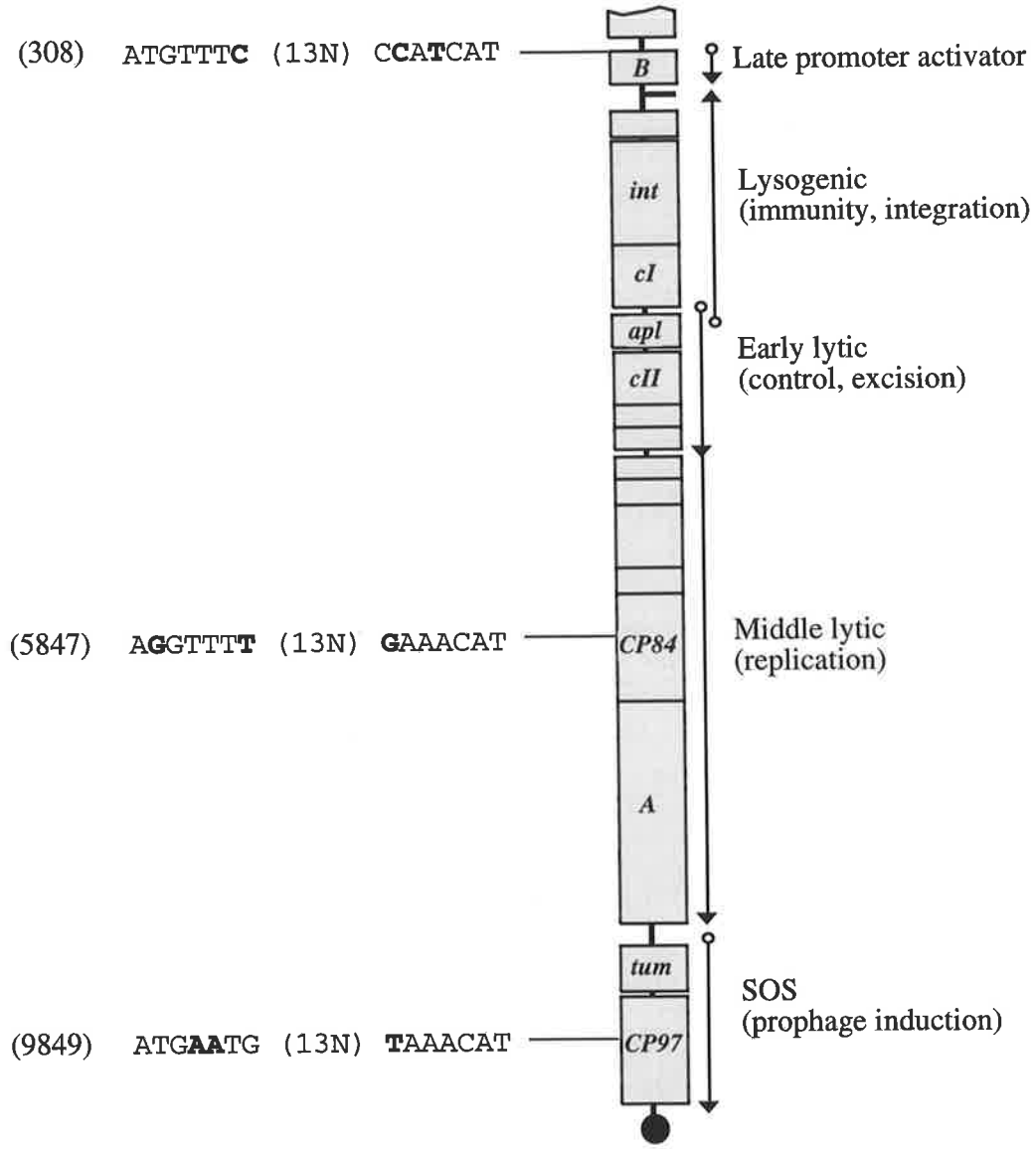
Location of 186 sequences with an 11 out of 14 bp match with the CII DNA binding sequence identified by DNase I footprinting

The CII binding site sequence corresponds to the repeat ATGTTTG(13N) CAAACAT (the *c*IV476 mutation is shown in bold). Bases that diverge from this sequence are shown in italics. The right Cos end is represented by a filled circle at the bottom of the figure. Sequence position 1 corresponds to the unique Pst I site (Kalionis *et al.*, 1986a).

Figure 2.3.2 (Bottom)

Sequence aligment of the CII binding site of 186 with the putative CII binding sequence at the start of orfb of ϕR67

Conserved bases are shown as vertical lines and inverted repeats are shaded. Putative -10 consensus sequences are underlined.


(308) ATGTTTC (13N) CCATCAT
B
Late promoter activator
int
cI
Lysogenic
(immunity, integration)
apl
cII
Early lytic
(control, excision)
(5847) AGGTTTT (13N) GAAACAT
CP84
Middle lytic
(replication)
A
tum
(9849) ATGAATG (13N) TAAACAT
CP97
SOS
(prophage induction)


Ec67 - ATGTTTGATTACCAAGTTTCCAAACAT
186 - ATGTTTGATTTTCAGGTTTCCAAACAT

supported by the strong enhancements of DNase I cleavage between the half sites. These enhancements are diagnostic of a distortion of the minor groove of the DNA, often produced when the minor groove lies on the outside of a DNA bend (Drew and Travers, 1985; Hochschild and Ptashne, 1986). In the model, this distorted minor groove would lie on the opposite face of the helix from the CII-contacted major grooves, consistent with its accessibilty to DNase and suggesting that CII bends the DNA around itself. This proposed mode of binding by 186 CII is quite different from lambda CII and its P22 equivalent, which bind to direct repeats one turn of the helix apart (Ho *et al.*, 1983; Ho *et al.*, 1992). To date I have only come across one HTH protein (the resolvase of transposon γδ, Tn*1000*) which, like 186 CII, binds to non-successive major grooves (Oram *et al.*, 1995). Whether there is a functional significance for HTH proteins to associate with major grooves two turns of the helix apart is not clear.

2.3.3 Identification of a putative CII DNA-binding sequence in ϕR67

Ec67 is a reverse transcriptase element (retron) found in *E. coli* (Lim, 1991) which has inserted into a viable prophage related to 186 (Hsu *et al.*, 1990). Sequence data infers that ϕR67 possesses a switch region similar to that of 186 (Dodd and Egan, 1996). The retrophage switch region is predicted to contain two face-to-face promoters p_I and p_A corresponding to the 186 promoters p_L and p_R. Whereas the p_L/p_R transcripts overlap by 62 bp, the p_I/p_A predicted transcripts only overlap 40 bp. p_I and p_A are flanked by *orf-i* and *orf-a*, which share 30% and 26 % amino acid identity with the *cI* and *apl* genes of 186 respectively. Most strikingly however *orf-b* of ϕR67 retains 69 % amino acid identity with the 186 *c*II gene (Dodd and Egan, 1996). Moreover, the inverted repeat sequences identified as the 186 CII DNA-binding determinants are exactly conserved between the two phages (See Fig 2.3.1). It will be of interest to determine whether the 186 CII protein can bind to the ϕR67 CII like DNA binding

determinants and conversely whether the ϕR67 CII protein can bind to the 186 CII binding site.

CHAPTER 3

CII activates a leftward promoter p_E upstream of p_L

3.1 INTRODUCTION

Although 186 is a non-lambdoid phage its CII protein binds to a site whose location is conserved amongst λ and the lambdoid phages HK022 and P22. Since these lambdoid CII analogues function by activating a promoter for establishment of lysogeny upstream of the lysogenic promoter, the hypothesis that 186 CII acts in a similar fashion was tested. This Chapter concerns itself with experiments carried out to test this hypothesis.

3.2 RESULTS

3.2.1 CII activates a leftward promoter p_E

To determine whether 186 CII could activate leftward transcription from the *Bsp*M I-*Sna*B I (2983-3238) fragment which contains the CII binding site (Fig 3.1), this region was fused to the *lacZ* gene of pMRR9 and subsequently placed at a single copy into the host chromosome, by the method of Simons *et al.* (1987), as described in Section 7.1.4 . When this strain, MC1061.5(λPN68), was host to the CII expression plasmid (pPN72), 1272 units of β-galactosidase activity were observed (Table 3.1), compared with 3 units in the absence of CII. This indicated that there exists, upstream of p_L, a promoter which is activated by CII. This promoter was denoted as the promoter of establishment, p_E.

3.2.2 The start site of the p_E transcript is mapped by primer extension

Primer extension analysis was used to map the start site of the p_E transcript. Total RNA was prepared from a culture of DH5α containing the CII expression plasmid

Figure 3.1

The *Bsp*M I to *Sna*B I restriction fragment used in reporter studies contains the CII DNA binding site.

Early lytic and lysogenic functions encoded within the *Xho* I to *Bgl* II region of 186 are shown at the top of the figure. The CII binding site (filled circle) is centered at position 3135 and is contained within the *Bsp*M I (2983) to *Sna*B I (3238) restriction fragment. This restriction fragment was cloned with the *Bsp*M I region adjacent to the *lacZ* reporter gene of pMRR9 such that leftward transcription could be measured.

Figure 3.1

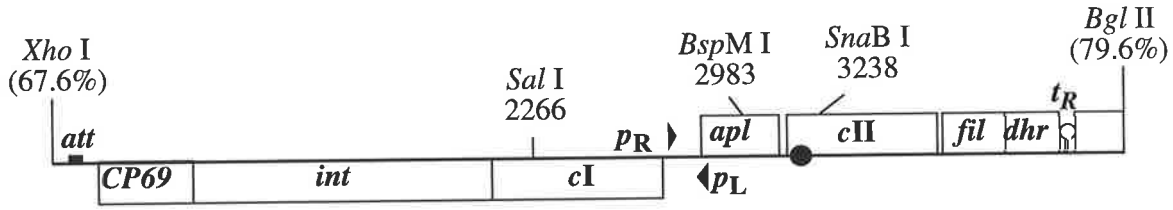


Table 3.1

pE is activated by CII and repressed by CI

CII[a]	CI[b]	pE-*lacZ* transcription[c]
-	-	3 ± 1
+	-	1272 ± 169
-	+	1 ± 1
+	+	283 ± 39

a + indicates the presence of the CII expression plasmid pPN72. - indicates the presence of a control plasmid (pPN178) carrying a mutant *c*II gene; the same value was obtained in the absence of the plasmid.

b + indicates the presence of the pET3aCI expression plasmid. - indicates the presence of the corresponding control plasmid carrying a mutant *c*I gene.

c MC1061.5(λPN68) Miller units. Background activity (0 units) was determined by measuring β-galactosidase activity of MC1061.5 strains lysogenised for λMRR9 and containing either pPN72 or pPN178 in the presence or absence of functional CI protein.

(pPN72) and a compatible plasmid clone of the *Sal* I-*Sna*B I (2266-3238) region of 186, which encompasses the p_E region (pPN79). These and control RNA preparations were subsequently reverse-transcribed using labelled primer 67 and electrophoresed on a sequencing gel. Consistent with the *lacZ* reporter studies, a 119 bp extension product was observed only in the presence of both pPN72 and pPN79 (Figure 3.2), confirming that CII functions as a transcriptional activator. The size of the extension product corresponded to p_E transcription initiating with a G at position 3086.

The start site relative to the CII footprint is recorded in Figure 3.2. A 4/6 match with the σ^{70} -10 consensus hexamer is located upstream of the start site, but no likely -35 consensus sequence is apparent, consistent with dependence of p_E on CII.

3.2.3 Activated p_E increases leftward transcription past p_R

I next wished to determine whether the p_E transcript extends into the lysogenic operon beyond p_R. A *lacZ* fusion (pPN157) measuring leftward transcription from the *Sal* I to *Sna* BI (2266-3238) (Fig 3.1) restriction fragment of 186*c*I$^+$*apl* Δ11 DNA (Dodd *et al.*, 1993) was constructed to this end. In single copy and in the presence of inactive CII (from pPN178) this construct yielded 7 units of β-galactosidase activity. In the presence of CII (pPN72) lysogenic transcription increased to 430 units. Thus, it appears that although CII activated transcription from p_E is inhibited by p_R activity (compare 430 units to the intrinsic activity of p_E, 1272 units), transcription from p_E can proceed past p_R and into the lysogenic operon.

3.2.4 The p_E promoter is repressed by CI

The CI repressor of 186 has been shown to bind at p_R and p_B (the late lytic gene activator of 186) as well as at a site within the CI coding region and in the *apl*/*c*II intergenic region (Dodd and Egan, 1996). It is of interest to note that this last binding

Figure 3.2 (Top)

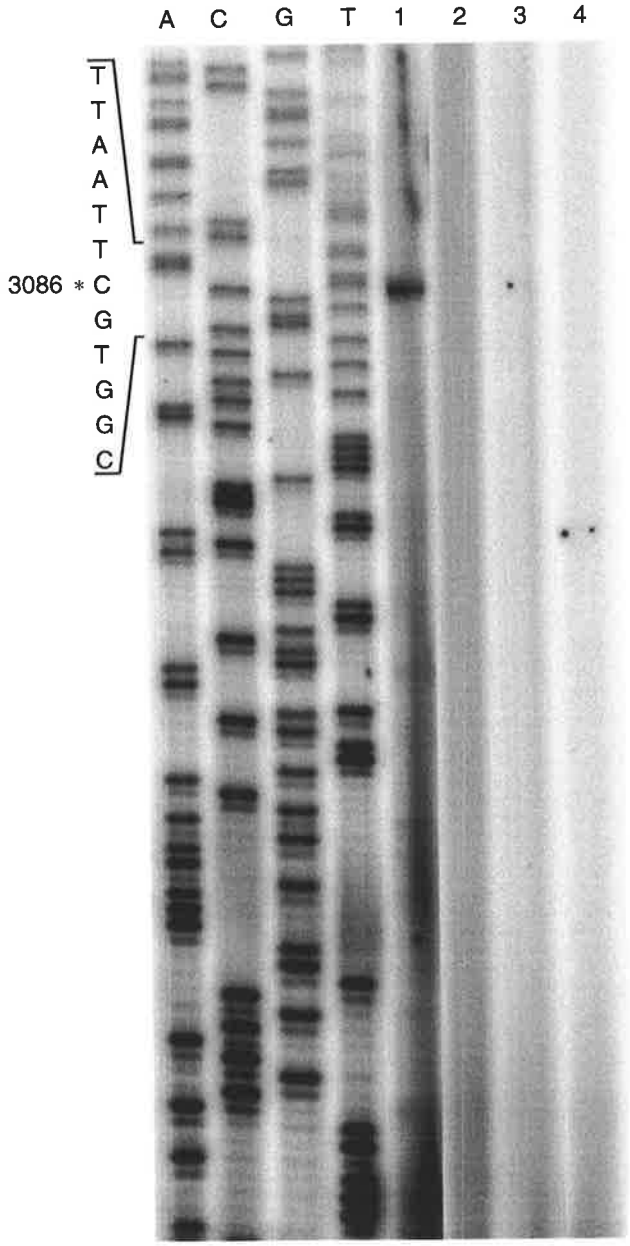
Locating the start site of the CII dependent p_E transcript by primer extension.

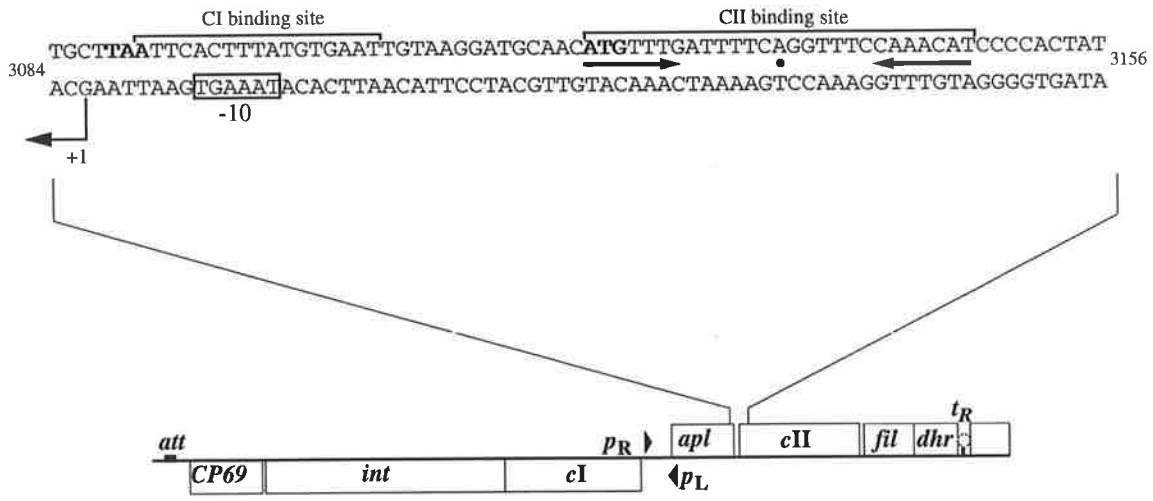
Lane 1 shows the primer 67 extension product obtained from a DH5α strain containing pPN79 in the presence of the CII expression plasmid pPN72. Lanes 2 and 3 show extension of RNA isolated from cultures carrying pPN79 with pACYC184 and pMRR9 with pPN72 respectively. Lane 4 shows primer extended in the absence of RNA. The ACGT sequencing reactions to the left were generated from pEC449 with kinased primer 67 as described in Materials and Methods. A portion of the sequence read from the gel and the base with which the extension product lines up are shown to the left of the Figure.

Figure 3.2 (Bottom)

Sequence of p_E.

Shown on the sequence is the relative location of the CII binding site(s) (arrows), the start of the p_E transcript (at position 3086), the putative -10 region of the promoter and the CI binding site, FR (Dodd and Egan, 1996).


A C G T 1 2 3 4
T
T
A
A
T
T
3086 * C
G
T
G
G
C


CI binding site
CII binding site
TGCTTAATTCACTTTATGTGAATTGTAAGGATGCAACATGTTTGATTTTCAGGTTTCCAAACATCCCCACTAT
3084
3156
ACGAATTAAGTGAAATACACTTAACATTCCTACGTTGTACAAACTAAAAGTCCAAAGGTTTGTAGGGGTGATA
-10
+1
att
CP69
int
cI
pR
pL
apl
cII
fil
dhr
tR

site coincides with the putative -10 region of p_E (Figure 3.2) (Dodd and Egan, 1996). This strongly suggested that p_E may be CI repressible. I tested this possibility by comparing β-galactosidase activity from the activated single copy p_E-*lacZ* transcriptional fusion (lPN72) in the presence of either the CI expression plasmid pET3aCI or the equivalent construct (pET3aCI^{FS}) which does not express functional CI protein. The uninduced pET3aCI plasmid produces sufficient CI protein to confer immunity to superinfection by a 186*c*I phage (I.Dodd, personal communication). This is presumably due to endogenous *E.coli* transcription at the T7 ϕ10 promoter. CI supplied from this vector repressed CII-activated p_E 4-fold and also repressed the slight basal activity of p_E (Table 3.1). This indicates that p_E is directly subject to negative feedback from the first protein product of the p_L transcript, whose transcription it presumably establishes.

3.3 DISCUSSION

3.3.1 Role of p_E in establishing lysogeny

In order to establish lysogeny the 186 must establish transcription of CI from the weak promoter p_L in the face of converging transcription from the much stronger promoter p_R. Work in this Chapter shows not only that CII is the transcriptional activator of a leftward promoter p_E which spans the *apl*/*c*II intergenic region upstream of the lysogenic promoter p_L but also that the p_E transcript extends into the lysogenic operon past the rightward transcribing lytic promoter. It is expected that expression of CI from the p_E transcript would lead to repression of p_R and relief of p_R's inhibition of p_L. The activity of p_L would then be sufficient to maintain CI production.

As the lysogenic operon of 186 not only encodes CI but also the Int protein, which is required to catalyse phage integration, the single p_E promoter is functionally equivalent to the two CII activated promoters of λ, p_{RE} and pI (Schmeissner *et al.*, 1980, 1981, Shimatake and Rosenberg, 1981). The p_{AQ} and third role of lambda CII (Hoopes and

McClure, 1985, Ho and Rosenberg, 1985) presumably does not exist in 186 as CII is unlikely to bind elsewhere in the known sequence of 186 (See Section 2.3.2). However, a role equivalent to the p_{AQ} transcript restricting expression of the late control protein Q could be accomplished by CI repression of transcription of the late activator gene *B* (Dibbens *et al.*, 1992).

3.3.2 The p_R-*cro*-p_{RE} and p_R-*apl*-p_E arrangements of lambda and 186

In both lambda and 186 the CII dependent promoter for establishment of lysogeny, p_{RE} (lambda) and p_E (186), spans the *cro*/*c*II (lambda) and *apl*/*c*II (186) intergenic regions respectively. This places both establishment promoters in a face-to-face arrangement with the lytic promoter. In lambda this allows interfering p_{RE} transcription to dampen p_R activity (Schmeissner *et al.*, 1980) even before CI represses p_R directly. Such a mechanism may also exist in 186 (see Chapter 5). In 186, due to the face-to-face arrangement of p_R and p_L, such a mechanism could have the additional advantage of reducing p_R inhibition of p_L, hence aiding the establishment of lysogenic transcription from p_L. These possibilities are investigated further in subsequent chapters.

As mentioned in Section1.2.1.3 , in λ, the placement of p_{RE} beyond *cro* has been speculated to play a role in the differential expression of antagonist proteins (Cro and CII) transcribed from a common lytic promoter, by production of an anti-sense *cro* RNA from p_{RE} which may reduce expression of Cro during the establishment of lysogeny (Spiegelmann *et al.*, 1972). I propose that, since the p_E transcript of 186 also has opposing polarity to the transcript originating from p_R, the RNA initiating from p_E has an anti-sense RNA function which may reduce Apl expression and thereby decrease Apl repression of p_L, and result in greater expression of CI. In addition, since Apl is an excisionase (Dodd *et al.*, 1993), the reduced level of Apl should foster

prophage integration. Experiments which address this question are presented in Chapter 5.

3.3.3 CI repression of p_E

The last phase of the establishment of lysogeny displays indirect and direct negative feedback on p_E. p_E activity is presumed to be reduced indirectly, due to CI repression of *c*II transcription from p_R, as well as directly by CI repression of p_E. The importance of such a double control mechanism over p_E is unclear. It is possible that too high a level of transcription from CII-activated p_E during the establishment of lysogeny could generate an elevated level of Int that inhibits prophage integration, as found in the closely related coliphage P2 (Yu and Haggård-Ljungquist, 1993).

CHAPTER 4

Lysogenic transcription in the face of p_R

4.1 INTRODUCTION

In 186, the activity of the lytic promoter far exceeds that of the lysogenic promoter (Dodd *et al.*, 1990). This bias is further exacerbated by the face-to-face arrangement of p_R and p_L which results in transcription from p_R interfering with p_L activity (Dodd *et al.*, 1990). As a consequence lysogeny cannot be established by transcription from p_L alone. As described in chapter 3 the phage solves this problem by establishing lysogenic transcription from another promoter p_E, itself arranged facing p_R. This means that we are now dealing with two sets of face-to-face promoters, p_L/p_R, unable to establish lysogeny and $(p_E+p_L)/p_R$, proficient in its establishment and I therefore set out to establish that the (p_E+p_L) promoter combination is more effective in establishing lysogenic transcription in the face of p_R than p_L alone.

4.2 RESULTS

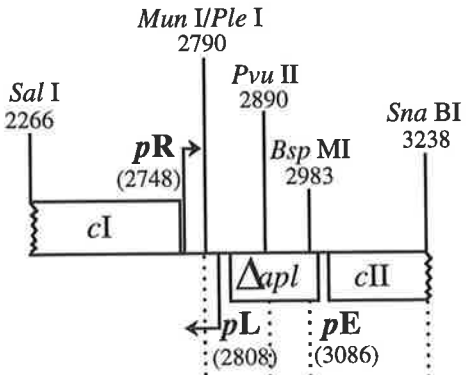
4.2.1 p_L activity is context dependent

One of the reasons why p_E but not p_L is able to establish the lysogenic transcript in the face of p_R may be due to the greater transcriptional activity of p_E compared to p_L. In order to compare the transcriptional activities of p_E and p_L two single copy transcriptional β-galactosidase fusions were initially constructed. The p_E fusion (pPN68) contains the *Bsp* MI to *Sna* BI (3238-2983) region of 186 such that *lacZ* transcription is under the control of the p_E promoter (Fig 4.1). The p_L fusion (pPN340) contains the *Ple* I to *Pvu* II (2790-2890) region obtained from 186 $cI^+apl\Delta11$ DNA (Dodd *et al,* 1993) such that p_L is driving *lacZ* expression. Both constructs were placed as a single copy into the bacterial chromosome to yield strains

Figure 4.1

Measure of individual and combined contributions of p_L and p_E (in the abscence of p_R) from different 186 restriction fragments

Restriction fragments shown to the left of the figure were cloned in front of the promoterless *lacZ* gene of pMRR9 and introduced at a single copy in the chromosome of MC1061.5. β-galactosidase activity obtained from these strains transformed with either the CII^+ expression plasmid pPN72 or its CII^- derivative (pPN178) are shown to the right of the figure. The units shown are the mean of units determined for 6 individual cultures of each strain on the same day. The errors are those determined for a 90 % confidence limit of the mean using Student's *t*-statistic (Wilks, 1948). The error between 4 independent single lysogens of the same clone was determined to be less than 10 %. The background activity (1 unit) has been substracted from the figures shown and was determined by measuring β-galactosidase activity of MC1061.5 strain lysogenised for pMRR9 and transformed either with pPN72 or pPN178



	+ pPN72 (CII$^+$)	+ pPN178 (CII$^-$)
MC1061.5(λPN68)	81 ± 5	86 ± 6
MC1061.5(λPN340)	1514 ± 69	3 ± 1
MC1061.5(λPN155)	1660 ± 56	117 ± 9

MC1061.5(λPN68) and MC1061.5(λPN340) respectively. Upon transformation of the CII expression plasmid (pPN72), or the control plasmid which expresses a truncated inactive CII (pPN178), into the p_E and p_L *lacZ* fusion strains β-galactosidase activities were measured. Results presented in Figure 4.1 show that p_E is 18 to 19 fold more active than p_L (compare 1514 obtained for CII activated p_E transcription to the p_L values of 81 and 86). This result is consistent with the observation that p_E is proficient in establishing lysogeny whereas p_L is not.

I next investigated whether transcription from p_E and p_L is synergistic, additive or whether these two promoters interfere with one another. To address this question the *Mun* I to *Sna* BI region from 186 *c*I$^+$*apl*Δ11 DNA was cloned in front of the promoterless *lacZ* gene of pMRR9 such that *lacZ* expression is under the control of p_L and p_E (pPN155). In single copy in the presence of CII this construct yielded 1660 Miller units whereas, in the absence of active CII, 117 units were obtained, suggesting that the activities of p_E and p_L are approximately additive. This conclusion however, is cast into doubt as the value for p_L obtained from λPN155 (117+/-9) is 27 to 31% greater than the p_L values measured for the λPN68 construct (81+/-5 and 86+/-6). Therefore there was concern that the particular context of the promoter could effect the activity measured. In order to reduce the error factor introduced by measuring constructs that differ in context, all future constructs contained the same DNA fragment of 186, namely the *Sal* I to *Sna* BI (2266-3238) region. This fragment ends well within the *c*I coding region and thus measures the level of *c*I trancription. As the essence of establishing lysogeny is the establishment of *c*I transcription such constructs allow a direct appreciation of the processes involved in establishing lysogeny. The generation of these constructs required p_R, p_L and p_E to be inactivated by mutation. As p_E in the absence of active CII only yields 3 Miller units, a mutation inactivating p_E was not introduced. However, inactivating mutations needed to be introduced into p_R and p_L.

4.2.2 Construction of p_R^- and p_L^- mutants

A p_L mutation consisting of 3 base pair changes, shown in Figure 4.2 was introduced in the -10 region of the promoter. To determine that this mutation reduces p_L transcription, the *Sna* BI to *Ple* I fragment of 186 *c*I$^+$*apl*Δ11 DNA containing the mutation was cloned in front of the promoterless *lacZ* gene of pMRR9 such that *lacZ* expression is under the control of the lysogenic promoters (pPN467). At a single copy, strain MC1061.5(λPN467) yielded 3+/-1 β-galactosidase units whereas the comparable construct containing the wild type p_L promoter (λPN155) yielded 117+/-9 units, suggesting that the mutation dramatically reduces p_L activity.

The p_R mutation used in this work had previously been generated in this laboratory by introducing four base pair changes in the -35 region of the promoter (Reed, 1994) (Fig 4.2). Single copy reporter studies (in the presence of p_L) indicate that this p_R mutation reduces p_R activity to 1 β-galactosidase unit (Rachel Schubert, personal communication)

4.2.3 p_E is 13 fold more active than p_L

In order to reconfirm the transcriptional activities of the p_L and p_E promoters on the same DNA fragment two new single copy transcriptional β-galactosidase fusions were constructed. λPN610 (Fig 4.3) was used to measure p_E transcription - it carried the *Sal* I-*Sna*B I (2266-3238) fragment of 186 *c*I$^+$*apl*Δ11 DNA (Dodd *et al,* 1993) in which both p_R and p_L have been inactivated by mutation. λPN538 is a clone of the same restriction fragment of 186 *c*I$^+$*apl*Δ11 DNA but only p_R is mutated and hence, in the absence of CII, transcription originating from p_L can be measured. As recorded in Figure 4.3, p_L (λPN538 + pPN178) generated 115 units and p_E (λPN610 + pPN72) 1447 units. This indicates that, when the activities of p_E and p_L are compared on the same sized fragment, p_E is 13 fold more active than p_L.

Figure 4.2

Mutations introduced into the p_R (Reed, 1994) and p_L promoters

Base pairs mutated are represented in bold on the wild type sequence. Specific base substitutions and restriction sites formed are represented above the sequences. The predicted -35 and -10 hexamers are underlined.

Xho I

G GCTC

pR CTA**TTTACT**ATCTCTCAATTGGGAGATATATTTTGGCTAAACCCA

-35 -10

Bss HII

GC C

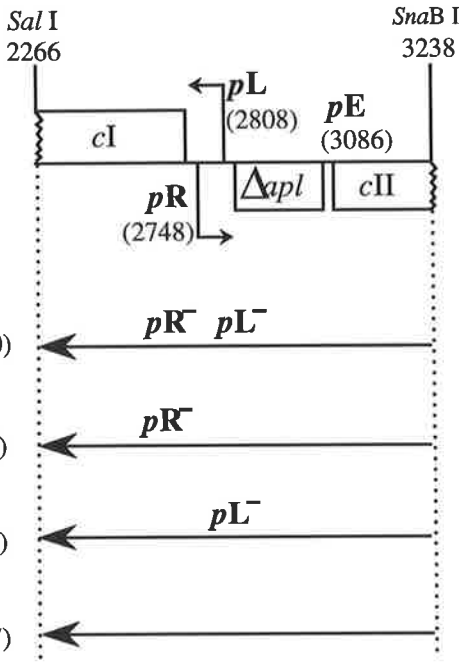
pL ATTGCGATTTCAGAAGCCATATTG**CTAGAT**TCCCTATTAGCCAAA

-35 -10

Figure 4.3

Measure of lysogenic transcription in the face of p_R

Fragments shown to the left of the figure were cloned in front of the promoterless *lacZ* gene of pMRR9 and introduced at a single copy in the chromosome of MC1061.5. β-galactosidase activity obtained from these strains transformed with either the CII^+ expression plasmid pPN72 or its CII^- derivative (pPN178) are shown to the right of the figure. The units shown are the mean of units determined for 6 individual cultures of each strain on the same day. The errors are those determined for a 90 % confidence limit of the mean using Student's *t*-statistic (Wilks, 1948). The error between 4 independent single lysogens of the same clone was determined to be less than 10 %. The background activity (1 unit) has been substracted from the figures shown and was determined by measuring β-galactosidase activity of MC1061.5 strain lysogenised for pMRR9 and transformed either with pPN72 or pPN178.



	+ pPN72 (CII$^+$)	+ pPN178 (CII$^-$)
MC1061.5(λPN610) *p*R$^-$ *p*L$^-$	1447 ± 34	2 ± 1
MC1061.5(λPN538) *p*R$^-$	1625 ± 41	115 ± 15
MC1061.5(λPN612) *p*L$^-$	696 ± 67	2 ± 1
MC1061.5(λPN157)	444 ± 51	8 ± 0.3

4.2.4 The activities of p_E and p_L are additive

I now wished to reconfirm that transcription from p_E and p_L, on the *Sal* I to *Sna* BI fragment of 186 *cI*$^+$*apl*Δ11, was additive. To this end, β-galactosidase activity of the λPN538 strain supplied with CII was determined. Expression of p_L and p_E from the same DNA fragment yielded 1625 +/- 41 Miller units, close (104 %) to the sum of the individual measurements of p_E and p_L (1562 +/- 49). Thus, the transcriptional activities of p_E and p_L in the absence of p_R appear to be additive.

4.2.5 Transcription from p_E is inhibited less by p_R than is transcription from p_L

The challenge faced by p_L is strong opposing transcription from p_R and the question next investigated was whether transcription from p_L and p_E is equally sensitive to converging transcription from p_R.

In order to address this objective I constructed single copy *lacZ* fusions of the *Sal* I *Sna*BI fragment carrying either p_R, p_E and an inactivated p_L (λPN612) or all three promoters (λPN157), such that lysogenic transcription could be measured. In the presence of the CII expression plasmid pPN78, λPN612 yielded 696 Miller units (Fig 4.3). Comparison of this figure with the value of 1447 miller units obtained when p_E activity was measured in the presence of mutated p_R and p_L (λPN610 + pPN78), shows that p_R inhibits p_E 2 fold. In contrast, p_R was found to reduce p_L activity 14 fold on comparable constructs (compare λPN538 with λPN157 activities in the absence of CII). This shows that transcription from p_L is more sensitive than is transcription from p_E to the opposing transcription from p_R.

4.2.6 In the face of p_R transcription the combined activities of p_E and p_L are more inhibited than is the activity of p_E alone

Given that the activities of p_E and p_L are additive I therefore expected that the combined activities of p_E and p_L would be even less inhibited by p_R transcription than p_E activity alone. Surprisingly the reverse was true, with the presence of p_L apparently antagonising the situation. Thus the combined activity of p_L and p_E in the presence of an active p_R (444 units - Fig 4.3) was 36 % less than that found for p_E alone in the presence of an active p_R (696 units). It would appear then that the presence of p_L is inhibitory to the establishment of lysogeny.

4.3 DISCUSSION

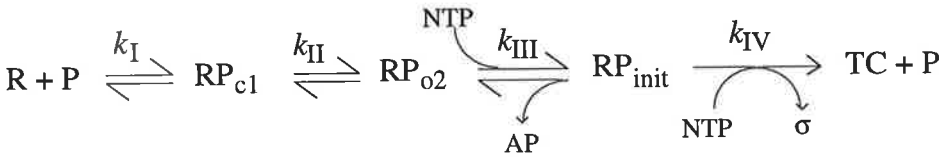
Establishment of lysogeny requires transcription of CI in the face of converging p_R transcription. Transcription from p_L alone is not sufficient to establish CI transcription (see Sections 1.3.3.1 and 1.3.3.2). p_E however, is 13 fold more active than p_L, is less inhibited than is p_L by p_R transcription and proficiently establishes lysogeny. In fact p_E+p_L is 55 fold better than p_L alone at transcribing CI in the face of converging transcription from p_R. It remains to be discussed why p_E performs better than p_L in the face of p_R transcription.

4.3.1 Why is p_E activity less inhibited than is p_L by converging p_R transcription ?

Results described in sections 4.2.3. and 4.2.5. indicate that p_E and p_L not only differ in their relative transcriptional activity, but are also differentially inhibited by p_R trancription, with transcription from p_L inhibited 14x and transcription from p_E inhibited 2x by transcription from p_R. The differential inhibition of p_E and p_L by transcription from p_R may be due to a difference in the intrinsic p_E/p_L promoter parameters which predisposes p_L to be more sensitive to p_R inhibition than p_E.

Alternatively, proximity to p_R may be detrimental to transcription from converging promoters. To differentiate between these two possibilities, it would be instructive to evaluate lysogenic transcription, in the presence and absence of CII, from a reporter construct in which the native positions of p_E and p_L have been reversed. If p_E activity is more inhibited when it is located 60 bp rather than 338 bp downstream of p_R and p_L activity is less inhibited when it is positioned 338 bp rather than 60 bp downstream of p_R, this would suggest that the extent of p_R inhibition is dependent on the relative spacing between converging promoters (Section 4.3.1.2). Alternatively, if transcription from p_E and p_L is inhibited to the same extent regardless of proximity to p_R, this suggests that p_R transcription interferes differently with transcription initiation at p_E than at p_L (Section 4.3.1.3). To allow a better appreciation of these outcomes a brief summary of transcription initiation is given.

Transcription initiation can be described by the following equation (Record *et al.*, 1996)



First RNA polymerase (R) associates with its promoter (P) to form an initial closed complex (RP_{c1}), in which the promoter region remains entirely double helical, with a characteristic constant k_I. A 10 to 15 bp region of DNA near the start of transcription is subsequently unwound to form the final open complex (RP_{o2}) which can be described by the rate constant k_{II}. Nucleoside triphosphates (NTP) complementary to the template strand of DNA at the open start site bind to form a series of initiation ternary complexes (RP_{init}). The RP_{init} may either progress by binding to the next nucleotide or reverse by removing the last nucleotide added. Alternatively the complete nascent chain may be released (aborted product, AP) and the complex reverts back to the RP_{o2} form in a process called abortive initiation. Upon addition of 7 to 12 nucleotides, the σ

subunit is released and the polymerase clears the promoter to form a processive ternary complex (TC) (Record *et al.*, 1996). Experimentally the most widely measured parameters are the binding constant (K_B) which measures the affinity with which RNA polymerase associates with its promoter and the rate constant (k_2) which corresponds to the rate of RP_{init} formation. Theoretically, p_R could interfere with any of these stages of transcription initiation and elongation at p_E and p_L. Moreover, because transcripts also initiate from p_R, any stage of p_R initiation and/or elongation has the potential to interfere with p_E and p_L transcription. The following discussion considers how the association of polymerase at p_R, the formation of open complex at p_R and the elongating complexes originating from p_R may differentially interfere with p_E and p_L transcription.

4.3.1.1 Polymerases at p_R, p_L and p_E are not expected to interfere with each other at the level of K_B

Association of the holoenzyme with its promoter has been shown to protect a region spanning from -55 to +20 relative to the transcription start site by DNase I footprinting (von Hippel et al., 1985; McClure, 1985, Record et al., 1996). As the +1 sites of p_R and p_L are located 60 bp apart, polymerases at p_R and p_L are not expected to compete for their respective binding site. Similarly as the +1 sites of p_R and p_E are located 338 bp apart, these two promoters are also not expected to interfere with each other's activity at the level of k_I.

4.3.1.2 Open complex formation at p_R is more likely to interfere with initiation at p_L than at p_E

During isomerisation the DNA spanning the -10 to +2 region of the promoter is unwound to its single stranded form (Schickor *et al.*, 1990; Suh *et al.*, 1993), resulting in the DNA adjacent to the polymerase becoming transiently overwound (Schwartz,

1988 as quoted in Su and McClure, 1994). It is conceivable that such a transient topological change at p_R could potentially interfere with initiation some 60 bp downstream at p_L, yet not effect p_E located 338 bp downstream of p_R.

Abortive initiation assays carried out on the face-to-face promoters of transposon element IS10, whose transcripts overlap by 36 bp (p_{IN} and p_{OUT}), showed that the K_B and k_2 for p_{IN} and p_{OUT} were the same regardless of whether both or just one promoter was present on the DNA fragment studied (Simons *et al,* 1983). In other words, opening of the DNA helix by nearly one turn at one promoter did not effect the binding constant nor the rate of RP_{init} formation of the facing promoter. However, Simons *et al* carried out their kinetic studies using linear DNA templates. Transient torsional strain, caused by unwinding of one turn of the DNA helix, can be easily dissipated from a linear template by rotation about the DNA ends. This situation differs from that in the chromosome, where DNA ends are anchored (Liu and Wang, 1987) and where transient topological changes have been observed to diffuse rather more slowly (Rahmouni and Wells, 1992). Therefore it seems possible that abortive initiation experiments, such as those carried out by Simons *et al* . (1983) but using supercoiled minicircle templates (Choy and Sankar, 1993), may show that isomerisation at one promoter can influence initiation of transcription at a facing promoter located in close proximity.

4.3.1.3. p_R elongating complexes may interfere with transcription from p_L and p_E differently

The above experiment may equally well show that isomerisation at p_R is not a contributing factor in explaining why transcription from p_E and p_L is inhibited differently. Such an outcome would not only suggest that the elongating complex from p_R is the module which interferes with transcription from facing promoters but also that elongating p_R complexes inhibit transcription from p_L and p_E differently.

Transcriptional inhibition by elongating complexes may be broken down into two components: elongating complexes may either interfere with opposing elongating polymerases or alternatively with initiation of transcription at the opposing promoter (Elledge and Davis, 1989). In the former case, the probability of one polymerase inhibiting progress of the converging other polymerase would be expected to be the same for both elongating polymerases assuming that the two complexes share the same characteristics. It is more likely that an elongating polymerase interferes with initiation at different promoter types to different extents.

Simplistically, weak promoters may be limited in the formation of K_B and/or k_2 (Ellinger *et al.*, 1994; Knaus *et al.*, 1994; Record *et al.*, 1996). One would suspect that a polymerase initiating from a promoter which is rate limited in K_B but not k_2 is less susceptible to being dislodged by a converging elongating RNA polymerase compared to a polymerase initiating from a promoter which is limited in K_B as well as k_2. p_E may represent an example of the former case whereas p_L may be limited in K_B as well as k_2. As a consequence p_E may be less inhibited by p_R complexes than p_L. A comparison of the various rate constants of p_E and p_L on linear as well as supercoiled templates may yield some answers to this question.

4.3.2 Why is transcription from p_L strongly inhibited by rightward transcription from p_R but not by leftward transcription from p_E ?

While polymerases from p_R and p_E both extend through the p_L promoter, albeit from different directions, p_R transcription inhibits p_L activity (14 fold) yet p_L activity is assumed to be unaffected by transcription from p_E since the activites of p_L and p_E are approximately additive. Why does transcription from p_R and p_E effect p_L transcription so differently ?

4.3.2.1 Is it because transcription from p_R and p_L is in opposing directions whereas transcription from p_E and p_L is in the same direction ?

Since polymerases originating from p_R and p_E must both elongate through the p_L region it might be expected that transcription from p_R and p_E would both interfere with initiation at p_L. To determine whether a second promoter is more detrimental to p_L transcription when it transcribes in an opposing direction rather than in the same direction as p_L it would be instructive to evaluate p_L transcription from the following two constructs. The first construct contains p_L oriented such that it now faces p_E rather than p_R transcription. The second construct is identical to the first but contains the p_R-mutation. If p_L activity is inhibited by a second promoter which transcribes in an opposing direction to p_L but not by one which transcribes in the same direction, p_L transcription in the first construct in the absence of CII is expected to be greater than p_L transcription already measured for MC1061.5(λPN157) (also in the absence of CII). Similarly, p_L activity measured from the second construct in the presence of CII is expected to be less than that measured for MC1061.5(λPN538) (in the presence of CII).

If these experiments determine that p_L transcription is inhibited when opposed to p_R or p_E this does not necessarily imply that convergent transcription is the sole inhibitory factor. Transcription elongation requires the movement of RNA polymerase around the DNA. If RNA polymerase is unable to rotate around the DNA, perhaps due to frictional drag, the tracking process is expected to generate local regions of overwound DNA ahead and underwound DNA behind the polymerase (Liu and Wang, 1987). Since Apl is translated from the p_R transcript but not from p_E, we might expect elongation from p_R to perturb local DNA topology more than elongation from p_E because of the additional frictional torque caused by Apl translation . p_L activity may be inhibited by such a large change in superhelical density. To determine whether Apl

translation effects p_L activity, evaluation of p_L transcription from a construct where Apl is translated from the p_E transcript may be useful.

4.3.2.2 Is it because p_R is in closer proximity to p_L than p_E ?

The relative proximity of p_R and p_E to p_L may also present an explanation as to why p_L is inhibited by p_R but is not adversely effected by p_E transcription. As mentioned in section 4.3.1.2 open complex formation at p_R could potentially be inhibitory to p_L transcription. Moreover, when p_R and p_L are in close proximity, transcription from these two promoters may form supercoiled domains which are particularly detrimental to p_L activity (Liu and Wang, 1987; Rahmouni and Well, 1992). Result of an experiment described in the afore mentioned section in which p_R and p_L are located 338 bp apart would have already determined whether or not the inhibitory effect of p_R transcription on p_L transcription can decrease over distance.

4.3.2.3 Is it due to the greater transcriptional activity of p_R compared to p_E ?

If neither the relative orientation nor the distance separating transcriptional units can fully explain the opposing effects that p_R and p_E transcription have on p_L transcription, then perhaps the difference in the relative activities of p_R and p_E may be important. Although the transcriptional activity of p_R in the absence of p_L has not been measured to date, β-galactosidase measurements presented in the next chapter indicate that p_R is at least 1.6 fold more active than p_E. To determine whether p_L inhibition is due to the greater transcriptional activity of p_R compared to p_E, it would be instructive to measure p_L transcription in the presence of CII in a construct where p_E is placed in the native p_R position. the complementary experiment in which p_E is substituted with p_R may also be useful.

CHAPTER 5

Apl and CII act in concert to repress transcription from p_R

5.1 INTRODUCTION

In coliphage Lambda the face-to-face arrangement of p_R and p_{RE} allows interfering p_{RE} transcription to dampen p_R activity even before CI represses p_R directly (Schmeissner *et al.*, 1980). Since Lambda and 186 have conserved the arrangement of their p_R-*cro*-p_{RE} and p_R-*apl*-p_E regions, I wished to determine whether p_E transcription was able to inhibit p_R transcription similarly in 186.

In 186 the lytically expressed Apl protein has been found to bind in the p_R/p_L region and repress transcription from both p_R and p_L (Dodd *et al.*, 1993), with p_R being repressed more efficiently than p_L (Reed *et al.*, 1997). As a second aim in this chapter it was therefore of interest to also determine whether, during the establishment of lysogeny, Apl could act in concert with p_E to repress p_R transcription.

5.2 RESULTS

5.2.1 p_E (in the presence of p_L) inhibits transcription from p_R 1.5 fold

In order to determine whether p_E inhibits transcription from p_R an attempt was made to clone the *Sna* BI to *Sal* I (2266-2983) region of 186 encoding the p_L^- mutation such that p_R controls *lacZ* expression of pMRR9. It was expected that an evaluation of such a construct in the presence and absence of CII would determine whether transcription from p_E inhibits p_R transcription. However, despite several attempts I was unsuccessful in obtaining such a clone.

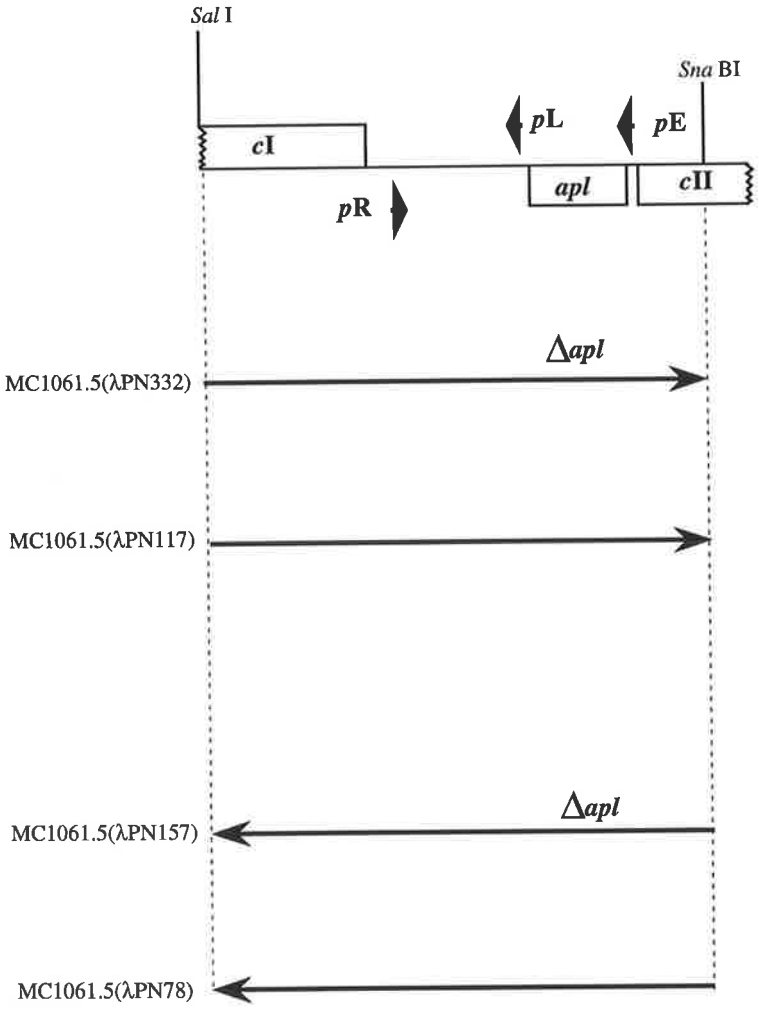
Dodd *et al.* (1990) had previously attempted to measure p_R activity in the absence of p_L in pKO2T but had also failed to clone such a construct. I was equally unsuccessful in cloning the *Sal* I-*Ple* I (2266-2790) fragment, which encodes p_R but not p_L, into pMRR9 or into a derivative of pMRR9 which contains the λt_{R1} terminator from pKO2T positioned between the polycloning site and the *lacZ* gene. Nor were clones obtained when the ligations were transformed into a CI expressing strain. The *Sal* I-*Ple* I (2266-2790) fragment was eventually cloned into a derivative of pMRR9T in which additional terminators from the ω fragment of pHP45ω-Km^r (Fellay *et al*, 1987) had been cloned in front of the plasmid origin of replication. However when attempts were made to transfer this construct (pPN367) at a single copy into the host chromosome it gave rise to unstable lysogens. Single lysogen colonies which appeared blue on X-Gal agar plates repeatedly gave rise to a population of approximately 50 % white and 50 % blue colonies. Despite this unsatisfactory precedent the *Sal* I-*Sna* BI (2266-2983) fragment of 186 carrying p_L^- was cloned into pMRR9Tω such that *lacZ* is under p_R control. However, lysogens of the construct (λPN470) gave approximately 10 fold lower β-galactosidase units than identical constructs carrying wild type p_L (λPN462), suggesting that p_R activity was increased by the presence of p_L. As this seemed unlikely, λPN470 was deemed unsuitable for further reporter studies.

It is not clear why it is so difficult to measure p_R activity in the absence of p_L in λRS45 derived lysogens. It is intriguing that p_R in the absence of p_L appears to be tolerated in lysogens when lysogenic transcription is measured and p_R transcription is directed towards the four tandem repeats of the *E. coli* $rrnBt_1$ transcriptional terminator (ie. λPN612), yet is not tolerated when it is directed towards the single repeat of λt_{R1} between the polycloning site and the *lacZ* gene. It may be of interest to determine whether p_R activity, in the absence of p_L, can be measured in lysogens which contain four tandem repeats of $rrnBt_1$ downstream of the *lac* operon .

Figure 5.1

Apl alters the flow of lytic and lysogenic transcription during the establishment of lysogeny

Fragments shown to the left of the figure were cloned in front of the promoterless *lacZ* gene of pMRR9 and introduced at a single copy in the chromosome of MC1061.5. β-galactosidase activity obtained from these strains transformed with either the CII^+ expression plasmid pPN72 or its CII^- derivative (pPN178) are shown to the right of the figure. The units shown are the mean of units determined for 6 individual cultures of each strain on the same day. The errors are those determined for a 90 % confidence limit of the mean using Student's *t*-statistic (Wilks, 1948). The error between 4 independent single lysogens of the same clone was determined to be less than 10 %. The background activity (1 unit) has been substracted from the figures shown and was determined by measuring β-galactosidase activity of MC1061.5 strain lysogenised for pMRR9 and transformed either with pPN72 or pPN178



	Miller units	
	+ pPN72 (CII^+)	+ pPN178 (CII^-)
MC1061.5(λPN332)	1474 ± 96	2275 ± 75
MC1061.5(λPN117)	760 ± 46	1278 ± 78
MC1061.5(λPN157)	444 ± 51	8 ± 0.3
MC1061.5(λPN78)	517 ± 43	6 ± 1

Since multiple attempts to measure p_R transcription in the absence of p_L failed the effect of p_E on p_R was determined in the presence of p_L. pPN332 was constructed to this end and subsequently transfered at a single copy into the host chromosome (Fig 5.1). MC1061.5(λPN332) in the absence of active CII yielded 2275 β-galactosidase units. When the same lambda lysogen was measured in the presence of active CII supplied from pPN78, 1474 β-galactosidase units where obtained (Fig 5.1). I concluded that p_E transcription, in the presence of p_L, reduces transcription from p_R 1.5 fold.

5.2.2 CII and Apl act in concert to decrease p_R transcription

To determine whether Apl could act in concert with active p_E and decrease p_R transcription even further, a *lacZ* promoter fusion monitoring lytic transcription from the *Sal* I to *Sna*B I region of 186$^+$ DNA was constructed and placed at a single copy into the bacterial chromosome to yield strain MC1061.5(λPN117) (Fig 5.1). β-galactosidase activity was subsequently determined for strains MC1061.5(λPN117) transformed with either pPN72 or pPN178 and compared with units obtained for the Δ*apl* strain MC1061.5(λPN332) also containing either pPN72 or pPN178. Results (Fig 5.1) indicate that CII and Apl act in concert to reduce p_R transcription. Apl reduces p_R by 44 % (from 2275 to 1278) and active p_E a further 40 % (from 1278 to 760) for an overall reduction of 67 % when Apl and CII act in concert.

5.2.3 The presence of Apl slightly increases transcription of the lysogenic operon (beyond p_R) during establishment

Since repression of p_R by the combined activities of p_E and Apl would be predicted to decrease p_R inhibition of p_L transcription by virtue of the face-to-face arrangement of p_R and p_L, and since Apl also represses p_L transcription, it was of interest to determined how Apl ultimately effects lysogenic transcription during the establishment

of lysogeny. To address this question, a single copy transcriptional *lacZ* fusions of the *Sal* I to *Sna*B I region of *apl*Δ11 DNA, MC1061.5(λPN157) was constructed such that lysogenic transcription could be monitored. Upon transformation of either pPN72 or pPN178 into this strain β-galactosidase activity was measured and compared to the activity obtained previously for strains MC1061.5(λPN78) transformed with either pPN72 or pPN178. Figure 5.1 shows a representative set of results obtained. Strains MC1061.5(λPN78) (*apl*$^+$) and MC1061.5(λPN157) (Δ*apl*) yielded 517 and 444 Miller units respectively in the presence of the CII expression plasmid. These figures suggest that the establishment of lysogeny benefits from the presence of Apl. Such a conclusion was tentative as the errors fail to indicate a significant difference between these two figures; however it must be stressed that this trend was observed consistently. To date five independent β-galactosidase assays, each time done at least in quadruplicate, have been performed on MC1061.5(λPN78) (*apl*$^+$) and MC1061.5(λPN157) (Δ*apl*). Although the absolute β-galactosidase values obtained showed daily variation, the relative values for these two constructs on any one day remained relatively. In fact when the mean of the units obtained each time for the *apl*$^+$ strain is deducted from the corresponding mean of the Δ*apl* strain, both in the presence of CII, numbers of 73, 84, 105, 115 and 73 are obtained. The mean of this difference at a 90 % confidence limit is 90 +/- 17. Since these confidence limits do not include zero it would seem that at the 90 % confidence limit the *apl*$^+$ and Δ*apl* strains are significantly different. This suggests that the presence of Apl is beneficial to the establishment of lysogeny.

5.3 DISCUSSION

5.3.1 p_E and Apl act in concert to reduce lytic transcription during the establishment of lysogeny

p_E apparently fulfills two roles in establishment. It serves as the alternative promoter to transcribe the *c*I gene and it also inhibits transcription from p_R 1.5 fold. Apl expressed

from the single copy construct further represses p_R transcription. In fact, Apl represses p_R 1.8 fold (Fig 5.1). Interestingly this value corresponds well with the burst size, an indirect measure of p_R activity, of the *apl*Δ11 phage which was determined to be 1.7 times greater than that of wild type phage (Dodd *et al.*, 1993). Co-expression of Apl and CII results in a 3 fold repression of p_R transcription beyond p_L which implies that p_E and Apl act in concert to reduce p_R expression. A reduction of lytic trancription during the establishment of lysogeny may be a means whereby the phage ensures that in the CI deficient cellular environment, which exists at the onset of establishment, expression of lytic functions is already partially controlled before CI represses p_R directly.

5.3.2 Apl slightly improves lysogenic transcription during the establishment of lysogeny

β-galactosidase assays measuring lysogenic transcription suggest that CII dependent leftward transcription is improved slightly by the presence of Apl. This trend was not detected when the frequency of lysogeny of wild type and *apl*Δ11 phage was measured (Dodd *et al.* 1993). Dodd *et al* (1993) measured the frequency of lysogeny independently three times and obtained figures of 7.3, 6.2, 6.8 for 186 wild type phage and figures of 7.0, 4.9, 3.8 for *apl*Δ11 phage. The errors fail to indicate a significant difference between the frequency of lysogeny of the wild type and *apl*Δ11 phages (6.8 SD 0.5 and 5.2 SD 1.8 respectively). Perhaps the error margins could be narrowed by repeating this experiment additional times.

It seems at first contradictory that Apl repression of p_R and p_L slightly improves lysogenic transcription during establishment. However this observation may be explained by postulating that by repressing p_R and p_L, Apl acts to decrease the frequency at which p_R/p_L interfering complexes occur (See 4.2.6) therefore allowing p_E to more easily transcrib beyond the p_L/p_R region.

5.3.3 An anti-*apl* type mechanism similar to that proposed for *cro* in the case of Lambda (Spiegelmann *et al.*, 1972) does not exist in 186

If p_E were to express an anti sense *apl* mRNA, effective in hybridizing to the sense *apl* message and thus reducing cellular Apl concentrations, then Apl repression of p_R would be expected to be less efficient in the presence than in the absence of CII. Results from rightward reporter assays presented in this chapter (Fig 5.1) in fact show if anything the reverse with Apl reducing p_R transcription 1.8 fold in the absence of CII (compare 2275 with 1278) and 1.9 fold in the presence of CII (compare 1474 with 760). This implies that the cellular Apl concentration has not been significantly affected by transcription from p_E. The figures indeed suggest that Apl repression of p_R is improved very slightly when CII is co-expressed. The complementary situation is also observed with CII inhibiting p_R trancription 1.5 fold in the absence of Apl (compare 2275 and 1474) and 1.7 fold in the presence of Apl (compare 1278 to 760). Although the increase in inhibition is not very significant it would however seem that p_E repression of p_R is slightly improved by the additional reduction in p_R activity due to Apl repression and vice versa. That is, Apl and active p_E act in concert to reduce p_R transcription and overall act in concert to establish lysogeny.

CHAPTER 6

Concluding Remarks

6.1 THE CLOSELY RELATED PHAGES 186 AND P2 ESTABLISH LYSOGENY DIFFERENTLY

6.1.1 P2 does not possess a CII-like function yet is able to establish lysogeny at similar frequencies than 186

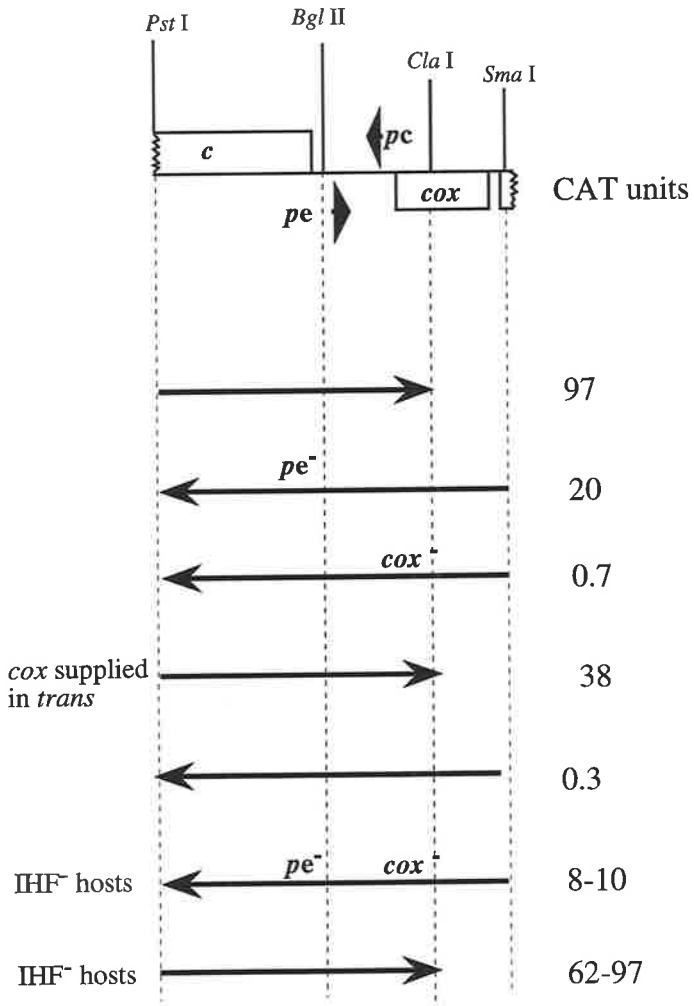
Coliphage 186 and P2 are highly homologous, as evidenced by heteroduplex mapping (Younghusband and Inman, 1974), their ability to form intervarietal hybrids (Hocking and Egan, 1982) and sequence data (Kalionis *et al.*, 1986a; Dibbens and Egan, 1992; Liu and Haggård-Ljungquist, 1994; Qing and Egan, 1995a, 1995b). Although there is little DNA sequence homology in the lysis/lysogeny region, both phages share the same overlapping face-to-face arrangement of lytic and lysogenic promoters (Fig 1.4). P2 and 186 also establish lysogeny at fairly similar frequencies with between 5 and 15 % of P2 infections giving rise to lysogens at multiplicities of infection (moi) ranging from 0.2 to 12.1 (Bertani, 1957; Bertani, 1962; Bertani and Bertani, 1971) and 24 % of 186 infections giving rise to lysogens at a moi of 0.07 (Dodd, 1994). Unlike 186 however, P2 does not possess a *c*II gene equivalent (Linderoth *et al.*, 1992) and so it is unclear how P2 is able to establish lysogeny at comparable levels to 186. Saha *et al* (1990) claim that the outcome of a P2 infection depends on the relative activities of the P2 lytic and lysogenic promoters. However, the relative ratio of lysogenic to lytic transcription in the P2 switch is 0.8 % (0.3 / 38) (Fig 6.1), given that P2 has a fol of 5 to 15 % this suggests that the relationship between the oucome of an infection and the relative level of lytic and lysogenic transcription is not a direct one.

Figure 6.1

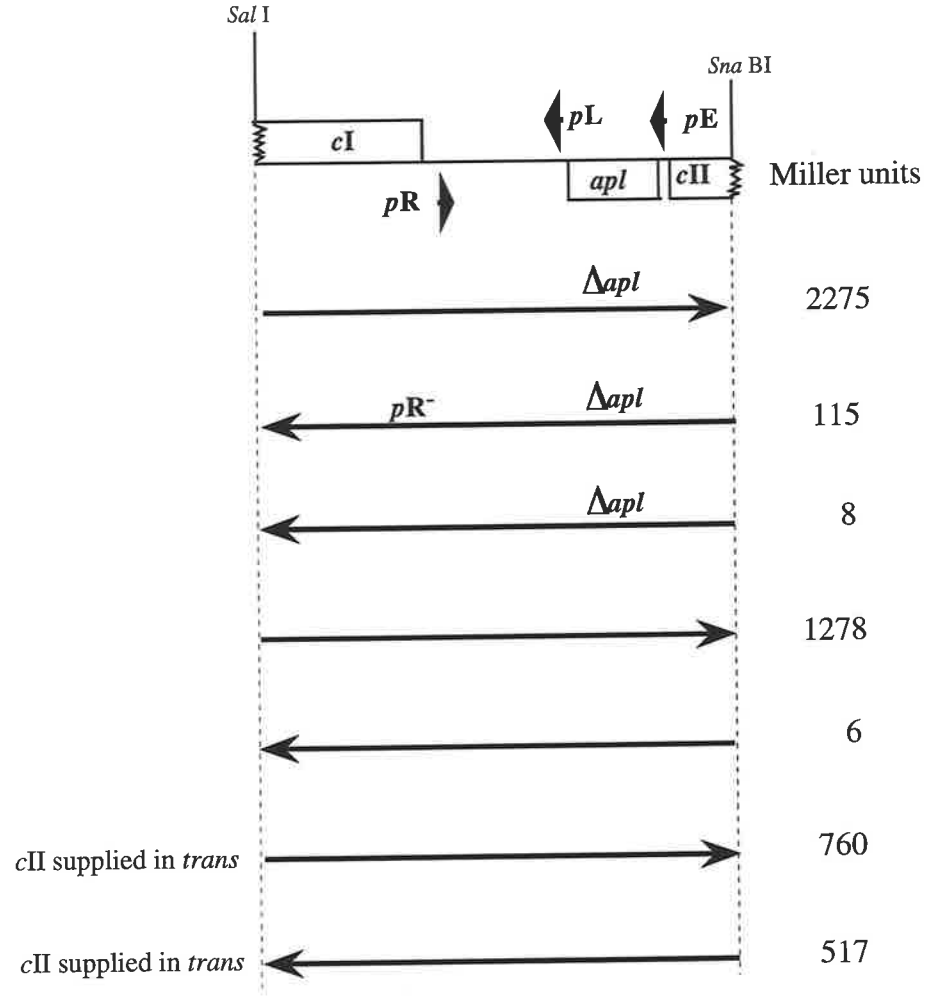
Transcriptional activities of the P2 and 186 switch regions

P2 reporter studies, reproduced from Saha *et al.* (1987) and Saha *et al.* (1990), are represented in the left half of the Figure. P2 transcription is expressed in CAT units and was measured from multicopy constructs without the inclusion of a copy number control. Relevant 186 reporter contructs measured in this study are listed to the right of the figure. 186 contructs denoted were placed at a single copy into the host chromosome and β-galactosidase units obtained for each of these constructs is shown.

Coliphage P2



Coliphage 186



The ratios of lysogenic to lytic transcription are similar in P2 and in 186 in the absence of CII (compare 6 / 1278 = 0.5 % for 186 with 0.3 / 38 = 0.8 % for P2) however, the same ratio for 186 in the presence of CII increases to 68 % (517 / 760). Unlike 186, P2 does not possess a CII function. Instead Cox and IHF have been shown to influence the P2 lytic/lysogenic decision (Section 1.3.2). Can these two proteins sufficiently alter the flow of lytic and lysogenic transcription in P2 to substitute for CII ?

The absence of Cox has been shown to favour a lysogenic outcome (Section 1.3.2). However, Cox repression of the P2 switch promoters is little different to repression of the 186 switch promoters by Apl. When Cox is overexpressed *in trans* and Apl is expressed from the same construct, P2 and 186 lytic transcription is decreased by 61 % and 44 %, and lysogenic transcription is decreased by 58 % and 25 %, respectively. Although the absence of Cox increases lysogenic transcription it does not effectively change the ratio of lysogenic to lytic transcription (0.7 / 96.5 = 0.7 %). therefore the absence of Cox would not seem to substitute for the presence of CII.

IHF appears absolutely essential for the lysogenic response in P2 yet, like Cox, only marginally effects the activities of the lytic and lysogenic promoters (Section 1.3.2). The ratio of lysogenic to lytic transcription mentioned in the above paragraph was determined in the presence of endogenous IHF (and absence of cox) and therefore represents the most favourable conditions for a P2 lysogenic outcome. Yet clearly, lysogenic transcription of P2 resembles that of 186 in the absence of CII more closely than it does that of 186 in the presence of CII. As neither Cox nor IHF are able to substitute for CII at the level of transcription how can one explain that 186 and P2 establish lysogeny at fairly similar levels ?

One possibility which may explain why 186 requires a CII function but P2 does not relates to the overlap between the lytic and lysogenic transcripts. Although the switch

regions of 186 and P2 are not significantly homologous in sequence, they are arranged identically, the notable exception being the spacing which separates the lytic and lysogenic transcripts. These transcripts overlap by 60 bases in 186 but by only approximately 30 bases in P2. We do not know how this different spacing may effect transcription from converging promoters. The closer spacing between the P2 lytic and lysogenic promoters may perhaps not allow two RNA polymerases to occupy the lytic and lysogenic promoters at the same time. This implies that the promoter which bind polymerase first has a direct advantage over the opposing promoter because it can repress opposing transcription and at the same time initiate its own transcript. The P2 lytic lysogenic decision may therefore be determined by the promoter which is the first to bind polymerase immediately after infection.

Alternatively, P2 might achieve a similar frequency of lysogeny than 186 without a CII function because C is much more efficient at repressing p_e than CI is at repressing p_R. This implies that only a few C transcripts initiated from p_C are required to extend beyond p_e to drive P2 lysogenic. In addition, small changes in p_C transcription caused by IHF and Cox would be expected to dramatically effect the frequency of lysogeny.

6.2 THE ESSENTIALLY UNRELATED PHAGES 186 AND λ EMPLOY SIMILAR STRATEGIES TO ESTABLISH LYSOGENY

6.2.1 DNA binding by the CII functions of 186 and λ

Coliphage 186 is essentially unrelated to the well studied phage Lambda. Specifically the Lambda and 186 CII proteins share no significant amino acid homology outside of the HTH motif. The two proteins have very different modes of binding to DNA. 186 CII binds to a 7 bp inverted repeat sequence separated by two turns of the helix with binding half sites centered at the -38 and -58 positions of p_E. In constrast λ CII binds

to 4 bp direct repeats separated by one turn of the helix with binding half sites centered at -27.5 and -37.5 relative to the start of transcription.

Oligomerisation studies with purified 186 CII protein have indicated that although CII associates to a tetramer in solution it is a CII dimer which binds to DNA (Keith Shearwin, personal communication). Lambda CII like 186 CII associates to a tetramer in solution and although the oligomerisation status of λ CII on DNA remains to be determined it has been implied that λ CII binds to DNA as a tetramer.

The exact nature of interactions between λ CII and RNA polymerase required for activated transcription still remains to be understood (1.2.1.3). As yet no work has been done to address this question in 186. However, since λ CII and 186 CII bind to different regions of their promoters relative to the start of transcription is seems possible that they interact with different regions of RNA polymerase.

6.2.2 Strategies used by λ and 186 CII in establishing lysogeny

Despite fundamental differences between the two proteins and despite the fact that lambda and 186 CII are associated with switch regions in which the relative arrangement of lytic and lysogenic promoters is not conserved, both phages employ very similar basic strategies to establish lysogeny. Lambda and 186 CII mediate a transcriptional switch from a lytic type state to the lysogenic maintenance state. In both phages, CII achieves this by activating an alternative promoter of the lysogenic operon located downstream of the lysogeny maintenance promoter. Production of CI from the p_E and p_{RE} transcripts thus leads to repression of p_R, and maintenance transcription of CI.

The CII protein of Lambda and 186 not only downregulate lytic trancription by expressing CI but also control lytic transcription before the appearance of CI protein.

The face-to-face arrangement of p_R/p_{RE} and p_R/p_E allows p_E and p_{RE} activity to interfere with transcription from p_R thus downregulating expression of lytic genes. Both phages also use independent strategies to downregulate lytic transcription even further, in 186 for example Apl acts in concert with CII to repress p_R activity, whereas in Lambda CII directly represses lytic transcription by activating the p_{AQ} promoter which produces an anti-sense *Q* RNA and decreases expression of the late gene activator Q (Hoopes and McClure, 1985; Ho and Rosenberg, 1985). Presumably such mechanisms are important in dampening lytic gene expression early on in establishment before CI itself is able to repress lytic transcription.

As well as maintenance transcription of CI and repression of lytic transcription, lysogeny also requires the phage genome to be integrated into the host chromosome. The CII proteins of Lambda and 186 both activate expression of integrase thus achieving to co-ordinate the transcriptional switch with the recombinational switch. However, whereas in Lambda CII activates p_I to produce Int, in 186 p_E co-transcibes *int* and *c*I and is therefore functionally equivalent to λp_{RE} and λp_I.

The final phase of the establishment of lysogeny in both Lambda and 186 is characterised by negative feedback on the CII activated promoters. Since *c*II is encoded on the lytic transcript which is repressed in the course of establishment expression of CII and therefore transcription of the CII activated promoters eventually ceases. In addition to this mechanism in 186, CI directly negatively feedsback on p_E.

Unlike Lambda which only possesses one pair of converging promoters, p_R/p_{RE}, 186 possesses two pairs of converging promoters p_R/p_L and p_R/p_E. In 186, it was thought that during establishment of lysogeny this arrangement would allow p_E inhibition of p_R transcription in turn to reduce p_R inhibiton of p_L and hence aid the establishment of lysogenic trancription from p_L even prior to the production of CI protein. However, results from transcriptional reporter studies described in Chapters 4 and 5 do not

support this hypothesis. Quite to the contrary, p_L activity is found to slightly inhibit p_E transcription in the presence of p_R. It is presumed that converging transcription from p_R and p_L results in the formation of complexes which block elongation of a small proportion of p_E transcripts beyond p_R. In 186 the face-to-face arrangement of p_R and p_L is thus not advantageous to the establishment of lysogeny.

In Lambda the placement of p_{RE} beyond *cro* has been predicted to be important in producing an antisense *cro* RNA from p_{RE} which might reduce expression of the *cro* gene thereby aiding the establishment of lysogeny (Spiegelman *et al.*, 1972). Although p_{RE} and p_E are positionally analogous in Lambda and 186, expression of Apl from the p_R trancript favours lysogenic transcription and therefore an antisense role for the p_E transcript on Apl expression is counter intuitive. Presumably Apl repression of p_L decreases the frequency with which interfering p_R/p_L complexes form and thereby allows p_E to more easily transcribe through the p_R/p_L region.

6.2.3 Control of 186 CII

CII expression in Lambda is tightly controlled (1.2.1.3). CII transcription is regulated by t_{R1} and CI. Post transcriptionally CII mRNA levels are controlled by RNase III cleavage of the *cII/OOP* RNA hybrid. Translation is regulated by IHF and finally *hflA*, FtsH, CIII and removal of the first two N terminal amino acids of CII regulate CII protein turnover. Since Lambda and 186 CII perform analogous functions it is very likely that CII expression in 186 is also tightly regulated. At present we known nothing about the regulation of 186 CII at the post transcriptional, translational or post translational levels. For 186 CII to be truly the master regulator of the lysis/lysogeny switch its cellular concentration needs to vary in response to the physiology of its host. Perhaps the most pressing question in this respect is to determine whether 186 CII, like its Lambda counterpart, is targeted by a specific host protease. Preliminary experiments would suggest that unlike λ CII, 186 CII is not proteolysed by the *hflA*

nor the *hflB* complexes (Egan and Portelli, personal communication). It will obviously be of great interest to identify the 186 CII host interactions which allow this unusual phage to probe and respond to its cellular environment.

CHAPTER 7

Materials and procedures

7.1 MATERIALS

7.1.1 Bacteria

MC1061.5 F- (*ara*D139 Δ(*ara-leu*)7696 Δ(*lac*)χ74 *gal*U *gal*K *hsd*R2 ($r_K^-m_K^+$) *mcr*B1 *rps*L (Str^R)(Δ*lac* χ*74*, *recA*$^+$) (Koop *et al.*, 1987) was used as host for *lacZ* reporter plasmids and phage.

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) (Bethesda Research Laboratories) used for routine cloning and for propagation of plasmids used in large scale DNA preparations and for double stranded sequencing.

7.1.2 Bacteriophage

The 186 strains used were 186$^+$ (Jacob and Wollman, 1956), unless otherwise stated.

*c*IV476: 186 clear plaque mutant containing a T to A change at position 3126 (Lamont *et al.*, 1993).

λRS45 (Simons *et al.*, 1987) is a λ phage vector used to transfer transcriptional reporter fusions made in pMRR9, at a single copy, into the host chromosome by the method reported in Simons *et al.* (1987).

7.1.3 Plasmids

pACYC184: contains a p15A replicon and the genes encoding tetracycline and chloramphenicol resistance and was used for general cloning (Chang & Cohen, 1978).

pBluescript: contains a ColE1origin of replication, encodes β-lactamase and was used for general cloning, double-stranded sequencing and site-directed mutagenesis (Stratagene).

pEC449: Is the *Xho* I-*Bgl* II (629-4244) fragment of 186+ inserted into the *Sal* I-*Bam* HI sites of pACYC184 (Dibbens and Egan, 1992).

pEC622: contains the 3.6 kb *Xho* I-*Bgl* II (629-4244) fragment of 186*c*I^{+}*apl*Δ11 (Dodd *et al.*, 1993) inserted into the *Sma* I-*Bam*H I backbone of pUC18 (Yanisch-Perron *et al.*, 1985).

pET3aCI^{FS}: pET3aCI (Shearwin and Egan, 1996) was linearized with *Mun* I, treated with Klenow 3' to 5' exonuclease activity and subsequently butt ligated. The resulting construct contains a frameshift in the *c*I gene and did not confer immunity to 186 infection, consistent with the loss of CI activity (I. Dodd, personal communication).

pJC251: contains the *Sna*B I-*Bsp*M I (3238-2983) region of 186^{+} from pEC449 (Dibbens and Egan, 1990) inserted into the *Sma* I site of pBluescript(SK)$^{+}$ (Stratagene) such that the endfilled *Bsp*M I site is closer to the vector *Sac* I site.

pLH3: The CII expression vector pLH3 is pET3a (Studier *et al.*, 1990) carrying the *c*II gene under control of the T7 ϕ10 promoter and ribosome binding site. Plasmid pLH3 was constructed by cloning the 596 bp *Nde* I-*Sau*3A I (3120-3716) fragment of pLH2 into the large *Nde* I-*Bam*H I fragment of pET3a. pLH2 was constructed from mEC3 (Dodd *et al.*, 1990) by creating an *Nde* I site in the predicted translation initiation site of the *c*II gene (Kalionis *et al.*, 1986a) by site-directed oligonucleotide mutagenesis using primer 23. The ability of pLH3 to synthesise functional CII was tested by complementation as follows. The 186 *c*II deficient phage

(*c*II473) formed clear plaques when the strain HMS174(λDE3) pLysS containing pET3a was used as indicator, but turbid plaques when the indicator contained pLH3. The CII coding region of pLH3 was sequenced and proved identical to that published (Kalionis *et al*, 1986a; Richardson *et al.*, 1989).

pMRR3: contains the *Xho* I-*Bgl* II (629-4244) fragment from 186+ inserted into the *Sal* I-*Bam* HI sites of pUC19 (Dodd *et al.*, 1993).

pMRR7: p_R^- derivative of pMRR3 created by site directed mutagenesis with primer 39 (Reed, 1994).

pMRR9: is a derivative of the *lacZ* promoter assay plasmid pRS415 (Simons *et al.*, 1987) containing translation stop codons from pKO2 (de Boer, 1984) and the pUC19 polycloning site (Yanisch-Perron *et al.*, 1985). The 184 bp *Sma* I-*Eco*R I fragment of pKO2 containing adjacent translation stop codons in all 3 reading frames was blunted and inserted into the *Sma* I site of pRS415 such that the *Sma* I site of the pKO2 fragment is closer to the *Eco*R I site of pRS415. The *Bam*H I and *Sal* I sites were then destroyed by digestion, end-filling and religation. The *Eco*R I-*Hind* III polycloning sequence of pUC19 was then inserted at the *Eco*RI-*Hind* III sites (the *Hind* III site is in the pKO2 fragment) to give pMRR9.

pMRR9T: contains the lambda t_{R1} terminator inserted in front of the *lacZ* gene of pMRR9 (Reed, 1994).

pMRR9Tω: contains the Klenow treated *Hind* III fragment of pHP45ω-Km^r (Fellay *et al.*, 1987) which contains the ω fragment cloned into the Klenow treated unique *Eag* I site of pMRR9T. The construct used in this study contained the insert cloned such that the *Sma* I site of the ω fragment was located closest to the *lac* operon.

Due to the insertion of the ω fragment, the *Sma* I and *Sal* I sites in the pMRR9Tω polylinker are no longer unique.

pPN68 : was obtained by cloning the small *Bam* HI - *Eco* RI fragment of pJC251 into the corresponding sites of pMRR9. The construct contains the *Sna* BI - *Bsp* MI (3238-2983) region of 186 oriented such that the *Bsp* MI site is closer to the *lacZ* gene.

pPN72: is the *Bgl* II-*Eco*R V fragment of pLH3 (containing the T7 promoter and ribosome binding site and the *c*II gene) cloned into the large *Nru* I to *Bam*H I fragment of pACYC184 (Chang and Cohen, 1978). Expression of CII in the absence of T7 polymerase is presumably from the tetracycline promoter of pACYC184. pPN72 produces functional CII as tested by complementation.

pPN78: is the *Sal* I to *Sna* BI fragment from pEC449 cloned into pMRR9 which had been digested with *Sma* I and *Sal* I such that lysogenic transcription is measured.

pPN79: contains the *Sna* BI-*Sal* I (3238-2266) region of 186^{+} from pEC449 (Dibbens and Egan, 1990) cloned into the large *Sma* I - *Sal* I fragment of pMRR9 (*Sal* I end near *lacZ*).

pPN115: is the Klenow treated *Mun* I to *Sna* BI (2790-3238) region of pEC449 inserted into the *Sma* I site of pMRR9 such that lysogenic transcription can be measured.

pPN117: is the *Sal* I to *Sna* BI fragment from pEC449 inserted into pMRR9 which had been digested with *Hind* III, treated with Klenow and subsequently digested with *Sal* I. This construct measures lytic transcription.

pPN157: has the *Sna* BI-*Sal* I (3238-2266) region of 186*c*I$^+$*apl*Δ11 (Dodd *et al.*, 1993) inserted into the *Sma* I and *Sal* I sites of pMRR9 such that lysogenic transcription is monitored.

pPN178: is pPN72 with the unique *Pst* I site at position 3560 (70 bp from the 3' end of the *c*II gene) destroyed by treatment of cut ends with the 3' to 5' exonuclease activity of Klenow and butt ligation. The 4 bp deletion destroyed CII activity as tested by complementation.

pPN228: Used as template in megaprimer mutagenesis. pPN228 contains the *Sal* I-*Sna* BI region from pEC622 cloned into the Klenow treated *Hind* III site and the *Sal* I site of pMRR9T.

pPN332: is the *Sal* I to *Sna* BI fragment from pEC622 cloned into pMRR9, which had been digested with *Hind* III treated with Klenow and subsequently digested with *Sal* I, such that lytic transcription is monitored.

pPN340 : contains the Klenow treated *Ple* I - *Pvu* II (2790-2890) region of 186*c*I$^+$*apl*Δ11 (Dodd *et al.*, 1993) cloned into the *Sma* I site of pMRR9 such that *lacZ* expression is under the control of p_L transcription.

pPN367: is pMRR9Tω with the Klenow treated *Sal* I-*Ple* I (2266-2790) fragment of pEC622 cloned into the Klenow treated *Bam* HI site of pMRR9Tω such that *lacZ* expression is under p_R control. The p_R region of this clone was sequenced and was found not to contain any mutations.

pPN450: contains the *Sal* I to *Hae* III fragment of 186 encoding the p_L^- mutation inserted into the *Sma* I site of pBS(KS)$^+$ such that p_R transcription opposes

p_{Lac} transcription. The complete construction details of this clone are given in Section 7.2.5.

pPN462: is the Klenow treated *Sal* I-*Sna* BI (2266-2983) fragment of pEC622 cloned into the Klenow treated *Hind* III site of pMRR9Tω. *lacZ* expression of this construct is under the control of p_R.

pPN467: is the *Eco* RI to *Ple* I fragment of pPN477, which contains the p_L^- and *apl*Δ11 changes (as well as the overlooked mutation in the CII binding site), cloned into the *Eco* RI and *Sma* I sites of pMRR9 such that lysogenic transcription can be measured.

pPN470: contains the small *Kpn* I to *Hind* III fragment from pPN447 cloned into the indentical sites of pMRR9Tω.

pPN471: was constructed by cloning the *Eco* RI to *Sal* I fragment of pPN477 into corresponding sites of pMRR9 such that lysogenic operon activity is measured. This clone was found to contain a single base pair change in the CII binding site. This mutation was propagated from pPN477 where it had been overlooked during the sequencing of the insert of this clone.

pPN477: contains the *Sna* BI to *Bsp* MI region of 186 generated by PCR which has been inserted into pPN450 digested with *Pst* I subsequently treated with Klenow and then digested with *Bsp* MI. pPN477 contains mutation in the CII binding site which had been overlooked when this clone was first sequenced.

pPN538: contains p_R^- (Reed, 1994) and 186*c*I$^+$*apl*Δ11(Dodd *et al.*, 1993) mutations on the *Sal* I to *Sna*B I fragment of 186 cloned into pMRR9 such that lysogenic transcription is measured. Two DNA fragments, the *Sna*B I to *Ple* I

fragment of 186 containing p_E generated from pEC622 and alkaline phosphatase treated at the *Sna*B I terminus and the *Sal* I to *Ple* I digested PCR fragment generated with primers 55 and 97 from a pMRR7 template and alkaline phosphatase treated at the *Sal* I terminus were ligated. The products of this ligation were themselves ligated to a *Sma* I and *Sal* I digested pMRR9 backbone fragment. Putative clones were selected by PCR and checked for the presence of the *Xho* I and *Pvu* II sites corresponding to the p_R- and *apl*Δ11 mutations. The insert of the clone finally used was completely sequenced in one direction and was found not to contain any PCR generated mutations.

pPN540: was constructed in two stages. First two PCR DNA fragments the first generated with primers 39 and T7 from a pPN477 template and subsequently digested with *Bam*H I, treated with alkaline phosphatase before being digested with *Ple* I and the second generated with primers 55 and 97 from a pMRR7 template (Reed, 1994) and digested with *Sal* I, alkaline phosphatase treated followed by digestion with *Ple* I were ligated. The product of this ligation was then ligated a second time to pMRR9 which had been cut with *Bam*H I and *Sal* I. Putative clones were selected by PCR and checked for the presence of the p_L- and p_R- mutations by digestion with *Xho* I and *Bss*H II respectively. The insert of two pPN540 clones was entirely sequenced in one direction. Both clones were found to contain a single base pair change in the CII binding site. This mutation had been propagated from pEC477 where it had been overlooked in the sequence generated from the insert of this clone.

pPN581 : Contains the blunted *Afl* III- *Eco*R V fragment of 186 *apl*Δ11 cloned into the blunted *Eco*R I site of pACYC184 such that transcription from p_R opposes transcription from p_{Chlor}. The insert of pPN581 was generated by PCR with primers 78 and 22 from the pEC622 template. The *cI/apl* intergenic region which also encompasses p_R was sequenced and did not contain any PCR generated mutations.

pPN583 : Is pPN581 with the p_R- mutation. The pPN583 insert was generated with primers 78 and 22 from pPN538 template.

pPN610: contains the p_R- (Reed, 1994), p_L- (this work) and the *apl*Δ11 (Dodd *et al.*, 1993) mutations on the *Sal* I to *Sna*B I fragment of 186 cloned into pMRR9 such that lysogenic transcription from p_E is measured. pPN610 is derived from pPN540. pPN540 was found to contain a PCR generated mutation in the CII binding site. Therefore, the *Sna* BI-*Ear* I region of pPN540 was replaced with Wild Type sequence in pPN610. This was achieved first by ligation of the *Kpn* I-*Ear* I fragment of pJC251 with the *Sal* I-*Ear* I fragment of 186 sequence derived from pPN540. (To promote formation of the right product, both fragments were treated with alkaline phosphatase after initial digestion with either *Kpn* I or *Sal* I). The product of this reaction was subsequently cloned into the*Sal* I-*Kpn* I sites of pMRR9 to yield pPN610.

pPN612: is pPN471 in which the *Kpn* I to *Ear* I fragment, which was found to contain a mutation in the CII binding site, was replaced with wild type 186 DNA from pJC251. This was achieved by ligating the *Kpn* I-*Ear* I 256 bp fragment from pJC251 with the *Sal* I-*Ear* I 186 DNA fragment of pPN471 and subsequently ligating the products of this reaction with *Kpn* I-*Sal* I cut pMRR9. (So as to optimize formation of the wanted product in the first ligation, the appropriate constructs were first cut with *Kpn* I or *Sal* I respectively and treated with alkaline phosphatase before digestion with *Ear* I).

7.1.4 Chromosomal single copy *lacZ* fusions

MC1061.5(λMRR9) is strain MC1061.5 lysogenized with a pMRR9 recombinant derivative of λRS45 (Simons et al., 1987). The other pMRR9 derived reporter constructs described above were transfered at a single copy in the host chromosome of

MC1061.5 in the same way and gave rise to strains MC1061.5(λPNxxx), were xxx is the number corresponding to the parent multicopy construct.

7.1.5 Oligonucleotide primer sequences

22-CCAGCTTCGCCATGTTG- (3205-3189)

23-TCAAACATATGGCATCCTTAC- (3108-3128)

34-ACATCCACGTTGCTCCATCCTAAAGAATCT- (2660-2689)

39-GATAAAACCTACTCGAGATCTCTCAATTGGG- (2701-2731) Creating a *Xho* I site (underlined) in the p_R -35 region.

55-CACGGATCCAACCGCCAGCC- (2080-2099) Creates a *Bam* HI site (underlined) at the 5'end of the *c*I gene.

56-TTAACTGCGCGTCGCCGC- anneals between the polycloning site and the *lacZ* gene of pMRR9.

57-TGCCAGGAATTGGGGATC- anneals on the non-*lacZ* side of pMRR9 polycloning site.

67-GCACAATCCGTAAAGGCTGCAAGAAAGCAG- (2966-2996)

77-AATACGACTCACTATAG- T7 specific primer

80-CGGGATCCACCTCAAATCAGA- (3641-3621)

97-GAAGCCATATTGCGCGCTTCCCTATTAGCC- (2832-2861) Creates a *Bss* HII site (underlined) in the p_L -10 region.

99-ACTCGTCACCGGTGCTTA- (3089-3072)

USP-GTAAAACGACGGCCAGT- Universal sequencing primer.

RSP-CACACAGGAAACAGCTATGGACCATG- M13 Reverse sequencing primer.

7.1.6 Enzymes

Calf intestinal phosphatase: Boehringer Mannheim.

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

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.

pfu DNA polymerase : Stratagene (La Jolla)

Restriction Endonucleases: New England Biolabs, Toyobo, Bethesda Research Laboratories or Boehringer Mannheim.

AMV reverse transcriptase: 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 Polynucleotide kinase: Boehringer Mannheim.

T4 DNA polymerase : BRESATEC.

Taq DNA polymerase: BRESATEC.

pfu DNA polymerase : Stratagene.

7.1.7 Chemicals

7.1.7.1 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).

7.1.7.2 General 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.

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.

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.

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

Cesium chloride (CsCl): Bethesda Research Labs.

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

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

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

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.

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

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.

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.

Magnesium chloride: Ajax.

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.

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 fluoride (PMSF): Sigma Chemical Co.

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

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

rATP: Sigma Chemical Co.

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.

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

Urea: Sigma Chemical Co.

Xylene cyanol: Sigma Chemical Co.

7.1.7.3 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.

LB medium (LB): 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0.

TB medium (TB): 1% Bacto-tryptone, 0.5% NaCl, pH 7.0.

YENB medium (YENB): 0.75% Bacto yeast extract, 0.8% Bacto Nutrient Broth, pH7.0.

Antibiotic supplements to L and T 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}.

7.1.7.4 Solid media

L plates: 1.5% Bacto-agar was added to L broth.

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.

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. When selecting for putative clones by insertional inactivation of the *lacZ* gene, transformants were spread on LB plates containing the appropriate antibiotics and supplemented with X-Gal (0.02 mg ml^{-1}) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.02 mg ml^{-1}). Putative promoter fusions in pMRR9 were selected on the appropriate LB plate supplemented with 0.02 mg ml^{-1} X-Gal.

7.1.7.5 General buffers

10 x agarose loading buffer: 0.4% bromophenol blue, 0.2% xylene cyanol, 10 mM EDTA, 50% glycerol.

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.

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.

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.

TE: 100 mM Tris-HCl, pH 8.0, 0.1 mM EDTA was used for general storage of DNA.

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

7.1.7.6 DNA markers

DNA size markers were all purchased from BRESATEC (Australia) and 250 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.

7.2 PROCEDURES

7.2.1 Phage and bacterial procedures

7.2.1.1 Storage of bacterial and phage stocks

Bacterial stocks for short term storage (less than one month) 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 186 and λ phage stocks were mixed with a few drops of chloroform and stored at 4°C. Phage stocks for longer term storage were kept at - 80°C after addition of glycerol to a final concentration of 20%.

7.2.1.2 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 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.

7.2.1.3 Lambda phage stocks

Low titre stocks (10^8-10^{10} pfu ml^{-1}) of λRS45 derivatives (Simons et al., 1987) were prepared by liquid infection. An overnight of an appropriate *malt*$^+$ strain (usually MC1061.5) was pelleted and resuspended at half its original volume in 10 mM Mg SO4. 50 µl of this suspension was either inoculated with 10^5-10^6 phage or 1 single plaque and incubated at 37 °C for 5'. Upon addition of 2 ml of LB containing 10mM $MgSO_4$ the solution was incubated at 37°Cwith aeration for 4-6 hours or until lysis was visible. 500 ml of chloroform was subsequently added, the tube vortexed and placed on ice for 10'. Following centrifugation (4000 g), the supernatant was

transferred to a new tube and several drops of chloroform added. Stocks were usually stored at 4°C.

7.2.1.4 Phage assays

λ phage were assayed for plaque forming units (pfu) by incubating 0.1 ml of the phage diluted in PSB with 0.2 ml of stationary phase indicator bacteria in half a volume of 10 mM $MgSO_4$ for 5' at 37°C and then adding 3 ml molten (0.7%) soft agar overlay to the mixture before and pouring onto warmed (37°C) 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}).

7.2.1.5 Construction of chromosomal copy lacZ fusions

Single-copy chromosomal fusions of p_E *lacZ* and pMRR9 were obtained by *in vivo* homologous recombination with λRS45 followed by lysogenisation (Simons *et al.*, 1987). λRS45 and pMRR9 share portions of the amino terminus of both the ampicillin gene and the *lacZ* gene thus allowing the promoter insert to be recombined into the phage. Strain MC1061.5 transformed with pPN68 or pPN157 was used as host for growth of the λRS45 phage vector according to standard methods (Silhavy *et al.*, 1984). The phage stocks obtained were plated on MC1061.5 and single recombinant plaques were scored on the basis of colour in presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and purified once by replating. Independent blue lysogens isolated from the centre of four recombinant plaques were purified by restreaking. β-galactosidase activity of these lysogens was determined as described in Section 7.2.6. Lysogens giving the lowest Miller units were assumed to be single lysogens. Double lysogens were sometimes obtained giving twice the Miller units;

these were discarded. Two single lysogens derived from each of two plasmid clones of the same construct were assayed (four lysogens in total) on two different days, in order to improve accuracy and reproducibility of the assay.
As lysogens derived from the pPN68 plasmid gave low β-galactosidase activity, which made discrimination between single and double lysogens difficult, the CII expression plasmid was transformed into these strains before single copy status was determined.

7.2.1.6 Transformation by $CaCl_2$

For routine cloning events where high efficiency transformation was not required, competent cells prepared by CaCl2 were 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 1-24 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} = 0.4-0.6. The culture was chilled on ice for 10 min and the cells harvested by centrifugation (5 min, 5000 rpm, 4°C, JA10). The cells were then resuspended in 50 ml cold CaCl2 solution (60 mM $CaCl_2$, 15% glycerol) and left on ice for a further 10 min. The cells were again harvested and resuspended in 10 ml cold $CaCl_2$ solution. 500 µl aliquots were transferred to pre-chilled tubes snap frozen on a dry ice ethanol bath and stored at -75°C until use.

Transformation was carried out by mixing 0.1 ml competent cells with the appropriate plasmid or ligation mixture (in a volume not exceeding 20 µl) in chilled sterile eppendorf tubes and incubation on ice for 15-20 min. The cells were heat shocked by incubation at 45°C for 3 min and placed on ice for 2-5 min. L broth (1 ml) was added

to each tube and the tubes incubated at the appropriate temperature for 30 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. Plasmid transformation was also achieved directly by incubation of 0.1 ml competent cells with the appropriate DNA for 5' on ice and spreading this mixture on an appropriate selection plate prewarmed to 37 °C.

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, 40-80 µl of 20 mg ml^{-1} X-gal was spread onto the plates and allowed to dry before plating the transformation mix.

7.2.1.7 Transformation by electroporation

For routine cloning events were a higher degree of competency was required, electrocompetent cells were used. As salts are known to reduce the efficiency of electrotransformation and cause arcing in the electroporation cuvette (Beguinot *et al.*, 1986), bacteria to be electroporated were grown in YENB. Preparation of frozen electrocompetent cells and electroporation procedure was carried out as described by Sharma and Schimke (1996).

7.2.2 DNA manipulations

7.2.2.1 Large scale isolation of plasmid DNA

Large scale preparations of plasmid DNA were obtained by alkaline extraction followed by sedimentation on a CsCl gradient. A 200-500 ml saturated culture was

pelleted (5K 10' 4°C) and resuspended in 4 ml of lysozyme solution (15% glucose, 50mM Tris-Cl pH8.0, 5mM EDTA, 4mgml-1 lysozyme). Following incubation at room temperature for 10', 8 ml of 0.2M NaOH, 1% SDS was added, the solution was mixed gently and placed on ice for 10'. The pH was neutralized by addition of 5 ml of 3M NaAc pH4.6 and the solution placed on ice for a further 10' before cell debris was pelleted by centrifugation (16K 30' 4°C). The supernatant was extracted with 1 ml phenol and 1 ml of chloroform. Nucleic acids were precipitated with addition of 5 ml isopropanol (10' Room temperature) and pelleted by centrifugation (10K 40' 4°C). (For starting cultures of 100 ml above volumes were halfed).

The nucleic acid pellet was well drained and resuspended in 1.4 ml of water. 1.5 g of CsCl and 120µl of 10mgml^{-1} EtBr were dissolved in the solution before it was transfered to a 2.2ml quickseal polyallomer tube. After sealing the tube, DNA was banded in a Beckmann TL-100 at 80K for 12hrs at 20°C.

If DNA was required the same day, the dried nucleic acid pellet was resuspended in 720 µl of water and 1.26 g of CsCl was dissolved in this solution. Following addition of 120 ml of 10 mgml-1 EtBr, 500µl of this DNA solution was carefully layered under 1.4 ml of a 65% CsCl solution in a 2.2ml quickseal polyallomer tube. The DNA was banded by centrifugation at 100K for 3 hrs at 20°C.

The DNA band obtained after centrifugation was removed and purified from EtBr and CsCl by isopropanol extraction. The DNA solution was extracted with an equal volume of saturated isopropanol (isopropanol saturated with 5M NaCl, 10mM Tris pH8.0, 1mM EDTA pH8.0) at least 3 times or until all visible traces of colour were removed and subsequently precipitated by addition of 2 volumes of water and 6 volumes of 95% ethanol, incubation at -20°C for 20' and centrifugation 12K 15' 4°C. The DNA obtained was pelleted twice from 70% ethanol before being used.

7.2.2.2 Small scale isolation of plasmid DNA

DNA was routinely isolated from 1.5 ml cultures by alkaline lysis (Birnboim and Doly, 1979). The culture was pelleted at 12K 30", resuspended in 100µl of GET buffer (50mM glucose, 10mM EDTA pH8.0, 25 mMTris-HCl pH8.0). Following addition of 200 µl 0.2M NaOH, 1% SDS and 5' incubation on ice, the mixture was renatured with 150 µl 3 M potassium 5M acetate and placed on ice for 5'. Cell debris was removed by centrifugation 12K 5' 4°C. The supernatant was treated with 2 µl of 10mgml-1 RNase and incubated at 37°C for 20'. Following phenol/chloroform extraction, nucleic acids were precipitated by addition of 2 volumes of 95% ethanol incubation on ice 5' and centrifugation (12K 5' room temperature). Plasmid isolations from 50 ml cultures were carried out by scaling up the above protocol accordingly.

7.2.2.3 Ethanol precipitation

Generally, 1/10th volume of NaAc pH5.2 and two volumes of ethanol were added to the DNA solution. Following vortexing, the DNA was precipitated either at -20°C for 5' or overnight, but generally for 15'. The DNA was then collected by centrifugation (12K, 15' 4°C Eppendorf centrifuge or 16K, 20' 4°C JA20 rotor). The supernatant was removed and the pellet sometimes resuspended in 70% ethanol (v/v) and centrifuged as previously or rinsed in 70% ethanol (v/v) and dried in vacuo for 10'. The DNA was finally resuspended in TE or water and stored at 4°C or -20°C.

7.2.2.4 Phenol Chloroform extraction

DNA solutions were vortexed with a half volume of TE-equilibrated phenol and chloroform unless specified otherwise and centrifuged (12K 5' room temperature or 5K 10' 20°C, JA20 rotor for 50 ml oakridge tubes). The aqueous phase was then removed without disturbing the interface and ethanol precipitated.

7.2.2.5 Restriction enzyme digestions

Digestions were carried out in NEB buffers according to the suppliers specifications. As restriction enzymes are diluted in buffers that contain glycerol, the digest volume was adjusted so that the amount of enzyme added did not exceed one tenth of the total volume. Digestion times varied from one hour to overnight. Digests were checked by gel electrophoresis.

7.2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in horizontal minigels (1%, 2% or 3% w/v agarose in 1xTAE, stored at 65°C). Agarose loading buffer was added at 1x concentration to the DNA sample and electrophoresis carried out in 1xTAE buffer at 150mA.

7.2.2.7 Polishing DNA ends

*7.2.2.7.1 With the Klenow fragment of E. coli DNA polymerase I.*The Klenow fragment was used to endfill and chew back 5' and 3' overhangs respectively thus allowing such fragments to be joined to blunted DNA ends. DNA to be cloned was treated with 1 unit of Klenow fragment in a reaction containing 10mMTris-HCl pH8.0, 10mM MgCl2, 25μM dNTPs (added from a stock containing 0.25 mM of each of the four dNTPs in 5 mM Tris-HCl pH8.0, imM EDTA). The reaction was incubated at 37°C for 15' and terminated by heating at 70°C for 15' or by the addition of EDTA to a final concentration of 0.1 mM or by phenol/chloroform extraction.

As the Klenow fragment has been shown to have a terminal transferase activity, in order to increase the ligation efficiency of blunt ended products, either T4 polymerase or pfu was used.

7.2.2.7.2 With bacteriophage T4 polymerase. Blunting reactions with T4 polymerase were carried out in 33mM Tris-acetate (pH8.0), 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, 0.1 mgml-1 BSA, with 2mM of each of four dNTPs and 1 unit of T4 polymerase and incubation at 37°C for 5'. The enzyme was inactivated by heating to 70°C for 5'.

7.2.2.7.3 With pfu. Restriction digests to be treated with pfu were first ethanol precipitated. Reactions were ususally carried out in a 10 µl volume conatining 1x pfu reaction buffer (20 mM Tris-HCl pH8.0, 2mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 10µgml^{-1} nuclease-free BSA) 2 mM of each of four dNTP and 0.5 units of pfu polymerase. Following incubation at 68°C for 5', the enzyme was inactivated by phenol chloroform extraction.

7.2.2.8 Reactions with alkaline phosphatase

To reduce the frequency of religated vector obtained from ligations of vectors with compatible ends, the 5' phosphate of the linear vector was removed with calf intestinal phosphatase (CIP). 1 x CIP buffer (50 mM Tris-HCl pH8.5, 0.1 mM EDTA pH8.5) was added to the digest and upon addition of 1 unit of CIP the reaction was allowed to proceed for 1hr at 37°C. The enzyme was either inactivated by phenol/chloroform extraction or by addition of EDTA to 20mM final concentration and heating to 65°C for 10'. CIP reactions were deemed efficient when the number of colonies obtained after transformation of the same concentration of cut vector DNA with or without the addition of ligase were the same.

7.2.2.9 Purification of DNA fragment from agarose

DNA fragments were isolated from agarose gel slices with the Bresaclean kit (BRESATEC) according to the manufacturers specifications.

7.2.2.10 DNA ligations

DNA fragments with compatible 5' and 3' overhangs were ligated at a 1 : 3 molar ratio of vector : insert respectively, in a 10-20 µl reaction containing 66 mM Tris-HCl pH7.6, 6.6 mM MgCl2, 10 mM DTT, 0.06 mM ATP and 1 unit of ligase. Reactions were incubated between 2-12 hrs at 15°C. (Blunt ended DNA fragments were ligated at a 1 : 6 molar ratio of vector : insert under the same conditions).

Three factor ligations were usually performed in two stages. The first reaction involved ligation of the two smaller DNA fragments with one compatible terminus at a 1 : 1 molar ratio. The products of this reaction were subsequently ligated with the appropriately digested vector backbone. Sometimes in order to maximise formation of the correct product in the first ligation, DNA fragments used were treated with alkaline phosphatase after digestion with the first restriction enzyme (in these cases to allow the second ligation reaction to occur the vector DNA was not treated with CIP).

7.2.2.12 PCR from a single colony

PCR was used to screen putative pMRR9 recombinant colonies for the presence of the correct sized insert and to generate DNA fragments for the DNase I footprint analysis. PCR reactions of 5 µl used to screen colonies contained 25 ng each of primers 56 and 57, 2.5 mM $MgCl_2$, 200 µM NTP, 15 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl pH8.8, 0.15 % Triton X-100, 200 µg ml^{-1} BSA and 0.1 units of Taq polymerase. Colonies were picked and partially resuspended in the PCR mix. Following the addition of 30 µl of liquid paraffin, the PCR reaction was hot started (94 ^{o}C for 2 min) and cycled thirty times at 94^{o}C for 5 s, 50^{o}C for 5 s and 74^{o}C for 45 s.(Alternatively products were analysed by restriction digestion of mini-DNA-preparations)

7.2.2.13 Sequencing reactions

Dideoxy sequencing reactions used as size markers in the DNase I footprinting and the primer extension were performed with ^{32}P labelled primer 22 and 67, respectively,

according to the Sequenase Version 2.0 kit protocol (USB). 1 µl of the crude kinasing reaction was used for this purpose. Sequencing reactions for other purposes were carried out by direct incorporation of (α-^{32}P)-dATP as described in the Sequenase Version 2.0 kit protocol (USB).

7.2.3 DNase I footprinting

Double-stranded DNA fragments with one strand ^{32}P labelled were generated by PCR, as described earlier, in a final volume of 10 µl. The template consisting of either 1 ng pEC622 DNA or 1 µl of a 100 µl eluate of a fresh *c*IV476 plaque, was amplified with unlabelled primers 34 or 80 used in conjunction with 5 µl of the primer 22 or the primer 99 kinasing reactions (as descibed in the General DNA Manipulations section), respectively. The PCR fragment was subsequently gel purified and the footprinting reaction carried out with purified CII protein as described in Dodd *et al.* (1993), with the exception that the DNase I reaction was stopped by extraction with 50 µl of phenol and ethanol precipitated once only.

7.2.4 Primer extension analysis

Primer extension analysis was performed as described by Dibbens and Egan (1992), with the following two exceptions. Firstly, RNA was isolated from the appropriate strains as described by Ausubel *et al.* (1989). Secondly, the RNA was resuspended in 2 ml of diethyl pyrocarbonate (DEPC) treated water and further purified on a CsCl gradient by dissolving 1 g ml^{-1} of solid CsCl into the RNA solution, carefully layering the mixture onto 1.2 ml of a 5.7 M CsCl, 0.1 M EDTA cushion and centrifuging in a Beckman SW 50L rotor at 35,000 rpm for 12 hr at 25^{o}C. After centrifugation, the supernatant was removed with a pasteur pipette and the clear RNA pellet was dissolved in DEPC treated water and ethanol precipitated. 10 µg of this RNA was dried down

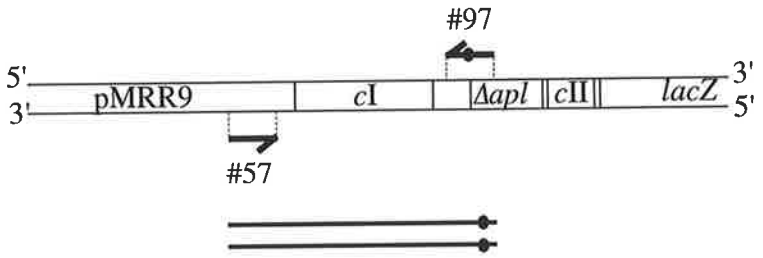
with 5 ng of gel purified primer 67 and the extension extension reaction performed and analysed as described by Dibbens *et al.* (1992).

7.2.5 Mutagenesis with mega primer

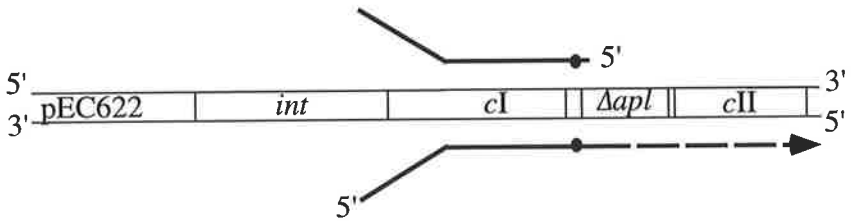
The p_L^- mutation was introduced by the Megaprimer method, using parallel templates as described by M. Upender et al, (1995) and is illustrated in Fig 1. The megaprimer was generated with primers 57 and 97 from a pPN228 template in a 5 µl reaction containing 1x Taq reaction buffer, 1 ng of template DNA, 25 ng of each primer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0,1 U Taq. Following incubation at 94°C for 2' the reaction was cycled 29 times (at 94°C for 5", 50°C for 5", 74°C for 45") before being returned to 4°C. The megaprimer was subsequently gel purified with bresaclean and its concentration estimated by running an aliquot on a 1% 1xTAE agarose gel and comparing the intensity of the ethidium bromide staining band with DNA markers of known concentration. The second round of PCR was performed in a final volume of 40 µl containing 16 ng of megaprimer, 16 ng of the pEC622 template, 1x Taq reaction buffer, 200 µM dNTPs,2.5 mM $MgCl_2$, and 1 U Taq. After the reaction had cycled 10 times at (94°C for 1', 55°C for 2', 72°Cfor 5'), 40 µl of a mix containing 1x Taq reaction buffer, 200 µM dNTPs, 2.5 mM $MgCl_2$, 100 ng primer #22 and # 57 and 1 U Taq was added beneath the parafin layer of the previous PCR mix. The combined PCR mixes were cycled 29 times at (94°C for 1', 55°C for 2', 72°C for 2') before being returned to 4°C. The length of the final PCR product obtained corresponded to the expected size. Following precipitation of nucleic acids with ethanol, the PCR product was digested with *Hae* III and *Sal* I, and the DNA ends were polished with Klenow. The DNA fragment was gel purified with Bresaclean before being ligated with *Sma* I digested pBluescript(KS)$^+$. The direction of the insert in the resulting clone pPN450 was such that p_R transcription opposes transcription from p_{Lac}.

Figure 7.1
Strategy used to introduce the p_L^- mutation by Megaprimer PCR site directed mutagenesis

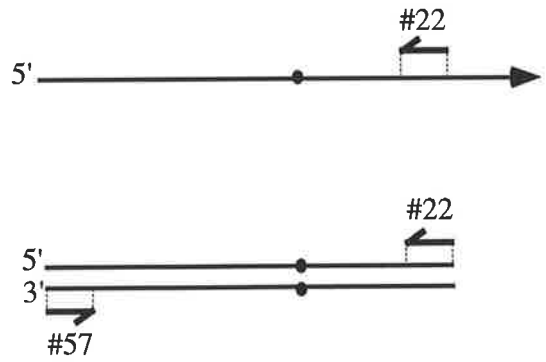
(1) Generation of a PCR megaprimer with the mutagenic primer #97 and primer #57 from a pPN228 template. (Filled circles represent base changes intoduced in the -10 region of pL with primer #97)



(2a) The megaprimer is used in a second round of PCR in which megaprimer is first extended from a pEC622 template. As the leftward end of the megaprimer and the pEC622 template diverge in sequence, formation of an extension product from the 3' end of the megaprimer which is homologous in sequence to a portion of the pEC622 template is favoured. (The shaded area shown below is the region homologous in sequence to the pPN228 template used to generate the megaprimer).



(2b) Primers #22 and #57 are now added to the PCR reaction. Further cycling amplifies the shown PCR product.



7.2.6 β-galactosidase assay

β-galactosidase activity was determined by the O-nitrophenyl-beta-D-galactopyranoside (ONPG) method described by Miller (1992), with the exception that cultures were grown in LB (absorbance contributions of LB were substracted from the OD_{600} and OD_{420} readings of the samples) and permeabilized with 30 µl of chloroform.

APPENDIX

Work presented in Chapters 2 and 3 has been published in:

Neufing, P.J., Shearwin, K.E., Camerotto, J., and Egan, J.B. (1996) The CII protein of bacteriophage 186 establishes lysogeny by activating a promoter upstream of the lysogenic promoter. *Mol. Microbiol.* **21:** 751-761.

A reproduction of this paper is included here.

Neufing, P.J., Shearwin, K.E., Camerotto, J., and Egan, J.B., (1996) The CII protein of bacteriophage 186 establishes lysogeny by activating a promoter upstream of the lysogenic promoter.
Molecular Microbiology, v. 21 (4), pp. 751-761.

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