$sib$ and $esc$ genes of Bacteriophage P22: A Dissertation

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sibB and esc GENES OF BACTERIOPHAGE P22

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ABSTRACT

The superinfection exclusion gene (sieB) of Salmonella phage P22 was mapped using phage deletion mutants. The DNA sequence in the region was re-examined in order to find an open reading frame consistent with the deletion mapping. Several discrepancies from the previously published sequence were discovered. The revised sequence revealed a single open reading frame of 242 codons with six likely translation initiation codons. On the basis of deletion and amber mutant phenotypes the second of these six sites was inferred to be the translation initiation site of the sieB gene. The sieB gene encodes a polypeptide with 192 amino acid residues with a calculated molecular weight of 22,442, which is in reasonable agreement with that estimated from polyacrylamide gels. The transcription start-site of sieB was identified by the use of an RNAase protection assay. The sieB promoter thus identified was inactivated by a two-base substitution in its -10 hexamer. The sieB gene of coliphage λ was also identified. The promoter for λ sieB was identified by homology to that of P22 sieB.

sieB aborts the lytic development of some phages. P22 itself is insensitive to the lethal effect of SieB because it harbours a determinant called esc. It was found that the sieB gene encodes two polypeptides—SieB, which is the exclusion protein, and Esc, which is a truncated version of SieB that inhibits its action. Superinfecting P22 synthesizes an antisense RNA, sas, that inhibits synthesis of SieB but allows continued synthesis of Esc, thus allowing P22 to by-pass SieB-mediated exclusion. This translational switch induced by sas RNA is essential to vegetatively developing P22; a mutation that prevents this
switch causes P22 to commit SieB-mediated suicide. It was also found that P22's Esc allows it to circumvent the SieB-mediated exclusion system of bacteriophage λ.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Signature page</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>iii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>Table of contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of figures</td>
<td>x</td>
</tr>
</tbody>
</table>

Chapter 1: Introduction

ABORTIVE INFECTIONS

- **sieB** genes of P22 and \( \lambda \)  
  page 3
- **rex** genes of \( \lambda \)  
  page 6
- **pit** genes of F-plasmid  
  page 9

GENE REGULATION by ANTI-SENSE RNA

- Plasmid DNA replication  
  page 11
- Regulation of transcription  
  page 13
  - Autoregulation by **crp**  
    page 13
  - **c4** repressors of P1 and P7  
    page 14
- Regulation of translation  
  page 17
  - **sar** of P22  
    page 17
  - **RNA-OUT** of IS10  
    page 19
  - **OOP** RNA of \( \lambda \)  
    page 21

Note  

Chapter 2: The Superinfection Exclusion (**sieB**)  
Genes of Bacteriophages P22 and \( \lambda \)

INTRODUCTION  

MATERIALS and METHODS

- Bacteria and Phage  
  page 35
- Plasmids  
  page 36
- RNAase protection  
  page 38
- DNA sequencing  
  page 40
Maxicells

RESULTS
- Deletion mapping of P22 sieB
- Sequence and expression of the P22 sieB gene
- Identification of the sieB promoter
- Identification of \( \lambda \) sieB

DISCUSSION
- P22 sieB
- \( \lambda \) sieB
- sieB promoters of P22 and \( \lambda \)

Chapter 3: A Switch in Translation Mediated by an Antisense RNA

INTRODUCTION

MATERIALS and METHODS

RESULTS
- esc as Antisense RNA
- esc as Protein
- Superinfecting P22 requires sieB antisense RNA or Esc protein to circumvent SieB-mediated exclusion
- A P22 mutant that commits suicide
- sieB/esc antisense RNA inhibits synthesis of SieB but not Esc
- The sieB antisense effector RNA, sas
- Esc, but not sas RNA, allows P22 to bypass \( \lambda \) SieB-mediated exclusion

DISCUSSION
- Molecular mechanism for esc
- sas RNA
- Relation to other exclusion systems
Chapter 4: Preliminary studies on transcriptional regulation of \textit{sieB} and \textit{esc} expression

INTRODUCTION 111

MATERIALS and METHODS 112

RESULTS and DISCUSSION
Does \(P_{\text{esc}}\) have promoter activity? 116
Is \(P_{\text{esc}}\) repressed by LexA? 118
Is \(P_{\text{esc}}\) repressed by IHF? 118
Is \(P_{\text{esc}}\) repressed by c2? 119

BIBLIOGRAPHY 123
LIST OF TABLES

SieB phenotypes of cells expressing different alleles of P22 or λ sieB 50

Esc phenotypes of P22 bearing different alleles of sieB/esc on cells expressing different alleles of P22 sieB 93

Esc phenotypes of P22 bearing different alleles of sieB/esc on cells expressing different alleles of λ sieB 95
LIST OF FIGURES

The pathway of pairing between RNAI and RNAII 23
Genetic structure of bacteriophages P1 and P7 in the vicinity of the c4 antisense RNA gene 25
Genetic structure of the bacteriophage P22 immI region 27
Genetic structure of bacteriophage λ PR operon in the vicinity of the cII gene 29
Deletion mapping of P22 sieB 52
Sequence of the sieB region of P22 54
Maxicell analysis of P22 and λ sieB expression 56
RNase protection to detect sieB mRNA 58
Map of the sieB region of λ 60
The sieB promoter region 62
Comparison of the hydrophilicity profiles of P22 and λ SieB 64
Fine structure mapping of P22 esc 96
P22 SieB and Esc are expressed from the same gene 98
Substitution mutations introduced into the untranslated region of sieB 100
sieB/esc antisense RNA inhibits synthesis of SieB but not Esc 102
RNase protection to detect sieB antisense RNA 104
Structures of plasmids expressing λ sieB 106
Molecular mechanism for P22 esc 108
Operon fusions between P22 sieB and lacZYA
CHAPTER 1

INTRODUCTION
P22 is a temperate *Salmonella* phage. Upon infecting a sensitive host, it follows one of two mutually exclusive developmental pathways. In the lytic mode, the injected phage DNA is replicated, recombined, encapsulated; and a crop of a hundred or so progeny phages is released by lysing the cell. In the lysogenic pathway, following early gene expression, the injected phage DNA is integrated into the host cell chromosome, and expression of most prophage genes is repressed (for reviews of P22 biology, see Susskind and Botstein, 1978; Poteete, 1988).

In the prophage state, P22 assumes territorial rights over its host cell. It elaborates two kinds of systems that prevent the lytic growth of superinfecting phages. An immunity system which operates on P22 itself and other closely related phages (by sequence), and a non-specific one which excludes a variety of phages. P22 and coliphage 21 are unable to grow in a P22 lysogen because transcription from their major promoters P_L and P_R is sensitive to repression by the prophage encoded c2 repressor; and the lysogen is said to be 'immune' to infection by P22 (Levine, 1957; Zinder, 1958; Botstein and Herskowitz, 1974; Poteete *et al.*, 1980). A P22 prophage expresses another transcription repressor, Mnt, which prevents expression of the *antirepressor* gene of superinfecting P22 (and, of course, of its own *ant* gene), whose function is to neutralize any c2 (or c2-like) repressor present in the cell (Bezdek and Amati, 1968; Gough, 1968; Botstein *et al.*, 1975; Levine *et al.*, 1975; Susskind and Botstein, 1975). The second system, which is comprised of three genes (which seem to function independently of one another), prevents the growth of P22
itself and other phages like L, MG178 and MG48. The sieA (for superinfection exclusion) gene interferes with the DNA injection process (Susskind et al., 1974a); the a1 gene alters the structure of the host phage receptor and prevents adsorption of some phages (Susskind and Botstein, 1978). Another gene, sieB, aborts the lytic development of superinfecting phages other than P22 (Susskind et al., 1974b). This last mechanism—abortive infection—of preventing the growth of superinfecting phages is quite widespread among lambdoid phages, and even among some low copy number plasmids (Duckworth et al., 1981). What follows is a survey of two other such superinfection exclusion systems; namely, the rex system of coliphage \( \lambda \) which prevents the growth of some T-even mutants, and the pif system of the F-plasmid which causes the abortive infection of T7 and related phages, but I review first what was known about sieB prior to this work.

**Abortive Infections**

sieB genes of P22 and \( \lambda \)

Susskind and co-workers, using prophage deletions, mapped the sieB gene of P22 to the PL operon (Susskind et al., 1974b). This observation, at the time, was paradoxical because the PL operon is repressed in a lysogen, but the sieB gene is evidently expressed. They proposed two explanations two resolve this paradox: the repressed level of sieB expression is sufficient to confer a SieB\(^+\) phenotype, or that sieB is transcribed independently of the other genes in the operon. As it turns out, the latter possibility is correct. Based on her sequencing studies, Franklin later proposed a rightward open reading frame between genes ral and 24 to be the sieB gene (Franklin, 1985).
Susskind and co-workers also examined the physiology of SieB-mediated exclusion (Susskind et al., 1974b). They found that when a SieB+ culture was infected with a sensitive phage like L, the culture stopped increasing in turbidity approx. 20 min after infection and showed slow partial lysis thereafter, and phage development was blocked. Although lysis of the superinfected culture was only partial, all of the cells were killed. In contrast, when a non-lysogen or a SieB- lysogen was infected with phage L, the turbidity of the culture increased till approx. 40 min after infection, but by 50 min the culture lysed completely. Using hybrid L phage bearing mutant alleles of P22 lysis genes—19, which encodes lysozyme and 13, which makes 'holes' in the cell membrane and allows lysozyme access to the peptidoglycan—they showed that the 'early partial lysis' observed upon infection of a SieB+ lysogen requires expression of these genes. The growth arrest of an L-infected SieB+ culture however, was not alleviated by mutations in genes 19 and 13; the turbidity of such cultures neither increased nor decreased 20 min after infection.

The pattern of DNA, RNA and protein synthesis upon infection of a SieB+ culture by L was also examined. It was observed that incorporation of appropriate radioactive precursors into SieB+ or SieB- infected cultures was indistinguishable for about 20 min after infection. But then there was a precipitous drop in precursor incorporation in the SieB+ lysogen, whereas incorporation of radioactive precursors continued for 20 min more (till the culture was lysed) in the SieB- culture. Cessation of DNA, RNA and protein synthesis occurred simultaneously, suggesting that inhibition of one does not cause inhibition of the others. To rule out the possibility that the drop in the incorporation of radioactive precursors was due to a defect in their transport into
the cell, a direct assay of protein synthesis was used—lysozyme activity in infected SieB+ and SieB- cultures was measured. It was found that lysozyme activity reached a plateau 20 min after infection of a SieB+ lysogen, but it continued to increase in a SieB- lysogen, suggesting that the drop in the uptake of radioactive precursors (at least 3H-phenylalanine) was not due to inhibition of precursor transport. As will become apparent, this phenomenon—normal phage development till midway through the latent phase, followed by a dramatic shut-off of macromolecular synthesis—is characteristic of abortive infections, suggesting that all these exclusion systems may function through a common mechanism. Another characteristic common to these abortive infections, is our lack of understanding of their underlying molecular mechanism.

The closely related coliphage λ also harbours a sieB gene. It interferes with the growth of the same Salmonella phages as does P22's sieB; as with P22 sieB, P22 is insensitive to λ SieB-mediated exclusion (Susskind and Botstein, 1980). In fact, it is not known whether any coliphage is sensitive to λ SieB-mediated exclusion. With the help of bio-sustituted λ (rex-) lysogens of Salmonella, Susskind and Botstein mapped the sieB gene to the PL operon, between genes cIIl and N; a position analogous to that of sieB on the P22 genome. Some of the bio-substituted prophages (bio255 and bio247) behaved anomalously though: they excluded P22 as well, suggesting that sequences that may be involved in negatively regulating sieB were deleted in these mutants. Alternatively, host sequences which were brought into proximity of the sieB gene by the substitutions may have resulted in its overexpression. Studies described later are consistent with the latter hypothesis. Based on the sequencing studies of Ineichen et al. (1981), Court and Oppenheim (1983) later
proposed a rightward open reading frame in this region as the $\lambda$ sieB gene. The physiological effects of $\lambda$ SieB-mediated exclusion have not been studied; but in light of the similar specificities of P22 and $\lambda$ sieB, it seems likely that the aberrations caused by $\lambda$ SieB will be similar to those caused by P22 SieB.

P22 is insensitive to all of the lethal effects of SieB (P22 and $\lambda$) described above. The reason for this, Susskind et al suggested, is that it harbours a determinant called esc, that allows it to escape SieB-mediated exclusion. Phage L is sensitive to SieB-mediated exclusion because it lacks the esc determinant. It was found that sieB and esc are tightly linked, but not identical; some point mutants are SieB- but Esc+. Using mixed infections of Esc+ P22 and Esc- L, they showed that the ability to escape SieB-mediated exclusion is dominant—Esc- phage L is insensitive to the lethal effects of SieB in the presence of Esc+ P22. This observation also suggests that P22 esc can function in trans. It was found, using the mixed infection protocol with different P22 mutants, that esc is negatively regulated by c2 repressor acting at OL/PL, indicating that esc is a part of the PL operon.

rex genes of $\lambda$

Escherichia coli lysogenic for $\lambda$ excludes T-even phages with mutations in their rII genes (Benzer, 1955). This effect is attributable to the products of two genes expressed by the prophage, rexA and rexB (Matz et al., 1982; Landsmann et al., 1982). The rex genes also exclude a variety of other phages including coliphage $\phi$80 (Toothman and Herskowitz, 1980a) and, when expressed in Salmonella, phages P22 and L (Susskind and Botstein, 1980). Other phages, including $\lambda$ are naturally insensitive to Rex-mediated exclusion,
but become susceptible when they harbour mutations in specific genes. These include T5 lr (Court and Oppenheim, 1983 and references therein) and λ red and ren mutants (Toothman and Herskowitz, 1980b).

The rexA and rexB genes, which encode proteins of approx. 30 kD and 16 kD respectively, map to the immunity region of λ, and are part of an operon that includes the λ repressor gene (Landsmann et al., 1982). In a λ prophage this operon is expressed from the PRM promoter, in the order: cl, rexA, rexB (reviewed in Ptashne, 1986). Another promoter, Plit, located at the 3' end of the rexA gene promotes the expression of rexB, but obviously not of rexA (Hayes and Szybalski, 1973). Pli_t is inactive in the prophage state, but is active in an induced lysogen; the products of the DNA replication genes (O and P) of λ and the E. coli dnaB and dnaG genes are required for transcription from Pli_t (Hayes and Szybalski, 1973). The precise function of transcription from Pli_t is unclear; recent studies have fueled the speculation that overexpression of rexB from Pli_t causes an imbalance in the ratio of RexA to RexB, thereby protecting an induced λ prophage from Rex-mediated exclusion (Parma et al., 1992).

The physiological aberrations caused by RexA and RexB are similar to those effected by P22 SieB. Following infection of Rex+ cells by sensitive mutants of T4 (for review, see Duckworth et al., 1981) or λ (Toothman and Herskowitz, 1980c), phage development proceeds normally for 10-20 min, but then there is a rapid and complete shut-off of all DNA, RNA and protein synthesis. Although a vast amount of effort has been made to understand Rex-mediated exclusion, the molecular mechanism underlying this phenomenon remains obscure. Recent studies have provided some encouragement. One study examined the effect of altering the ratio of RexA to RexB in uninfected
cells (Snyder and Williams, 1989). It was found that overexpressing rexA, in the presence of limiting amounts of RexB, resulted in many of the aberrations seen in phage-infected Rex+ cells, suggesting that the ‘trigger’ for Rex-mediated exclusion may be an alteration of the RexA to RexB ratio by some phage-encoded activity. The converse experiment—overexpression of rexB in the presence of limiting amounts of RexA—produced an interesting result. It was found that overexpression of rexB in a Rex+ λ lysogen conferred a Rex− phenotype (Parma et al., 1992), raising the possibility that RexA is the lethal effector of exclusion, and RexB protects the cell from RexA. Some investigators have proposed that membrane dysfunction may be involved in Rex-mediated exclusion because the defect caused by Rex can be alleviated by adding cations like Mg$^{2+}$ to the medium (reviewed in Duckworth et al., 1981). Germane to this proposal, are studies which indicate that RexB is an integral membrane protein (Parma et al., 1992) and that the T4 rII gene-products which confer resistance to Rex are associated with the cell membrane (Weintraub and Frankel, 1972).

It is not at all clear how the protective functions of T4 and λ exert their effect. Since λ red mutants which lack their recombination functions are sensitive to Rex, it seems possible that some recombination and/or replication intermediate is sensitive to Rex (or Rex is triggered by this). The map position of the other mutation, ren, which renders λ sensitive to Rex is consistent with this hypothesis; ren maps adjacent to the replication genes O and P (Toothman and Herskowitz, 1980b). Given the functional clustering of phage genes, it seems likely that ren is involved in DNA replication.
**pif genes of F-plasmid**

Female-specific T phages, like T7, are unable to develop in hosts containing the conjugative plasmid F, a phenomenon known as F-exclusion. This effect has been attributed to the *pifABC* (for phage inhibition by F) genes. However, the primary lethal effector seems to be PifA, a 70 kD protein encoded by the *pifA* gene (Rotman *et al.*, 1983), since PifA⁻ PifB⁻ cells are fully permissive, whereas the plating efficiency of T7 on a PifA⁺PifB⁺ host is reduced only 2-fold (Morrison and Malamy, 1971). It is thought that the *pifC* gene negatively regulates expression of itself and of *pifA* (Miller and Malamy, 1983). The role, if any, of *pifB* in F-exclusion is a mystery. In fact, even the map position of *pifB* is unclear, since some transposon insertions in the region where *pifB* was thought to be located do not confer a phenotype (Cram *et al.*, 1984).

The physiological defects seen in T7-infected F-containing cells are reminiscent of those seen in SieB⁺ and Rex⁺-infected cells. Early gene expression appears to be normal, but later stages of the infection are grossly abnormal. DNA and protein synthesis are severely affected (reviewed in Duckworth *et al.*, 1981). Recent studies indicate that late mRNA synthesis is also aberrant (Beck and Molineux, 1991).

Phage T3, which is related to T7 by sequence, is insensitive to F-exclusion because it has gene 1.2. T3 bearing null mutations in gene 1.2 are efficiently excluded by F-bearing *E. coli* (Molineux and Spence, 1984; Schmitt *et al.*, 1987). T7 also harbours a gene 1.2, which is approx. 40% identical to that of T3 (Schmitt *et al.*, 1987), but the differences are such that it can't protect T7 from the lethal effect of PifA; on the contrary, expression of T7 gene 1.2 is lethal to F plasmid-containing *E. coli* (Schmitt and Molineux, 1991). T7 gene
1.2 encodes a protein that inhibits the activity of dGTPase (Huber et al., 1988), a host enzyme with no known physiological function. Normal T7 and T3 growth is resistant to low levels of nalidixic acid, a DNA gyrase inhibitor, but the growth of T7 and T3 lacking gene product 1.2 is extremely sensitive to nalidixic acid, suggesting that gene product 1.2 interacts with gyrase (Molineux, 1991). Molineux speculated that this function of 1.2 may be relevant to F-exclusion because preliminary studies revealed that PifA interferes with DNA gyrase activity.

Several studies have led to the notion that the capsid protein, product of gene 10, of T3 and T7 triggers the lethal action of PifA. Missense mutations in gene 10 suppress the growth defect of T3, in F-containing cells, conferred by gene 1.2 mutations (Condreay and Molineux, 1989). T7 mutants that are no longer sensitive to F-exclusion harbour mutations in both genes 1.2 and 10 (Molineux et al., 1989). Moreover, expression of gene 10 of either phage is toxic to F-bearing E. coli (Schmitt and Molineux, 1991).

**Gene Regulation by Antisense RNA**

In prokaryotes, antisense RNAs regulate gene expression in a variety of ways. They affect the expression of target genes directly at the level of transcription termination, translation initiation and mRNA stability; and indirectly by regulating the replication of some plasmids (for reviews, see Simons and Kleckner, 1988; Takayama and Inouye, 1990). What follows is a survey of each of these modes of action. Although this section may seem incongruous with the preceding one, sieB links the two—as described later, expression of sieB appears to be regulated by an antisense RNA. This survey is not meant to be
encyclopaedic; rather, I have described those systems which have been the subject of intense study and those which I considered relevant to this thesis.

Plasmid DNA replication

Replication of the CoIE1 family of plasmids initiates by synthesis of a specific RNA molecule (RNAII) which serves as a primer for DNA synthesis 555 nucleotides (nt) downstream of its transcription initiation site (Itoh and Tomizawa, 1980). In general, only a small stretch of the newly synthesized RNA stays base-paired with the template. In the case of RNA II, as transcription approaches the site of origin of DNA synthesis, the newly synthesized RNA stays hybridized to the template. The RNA:DNA hybrid is then processed by RNaseH, thus providing DNA polymerase with a 3’ end for new strand synthesis. Because of base-pairing with the antisense RNA (RNAI), however, the conformation of RNAII is affected in such a way that it is no longer able to form a persistent RNA:DNA hybrid (Masukata and Tomizawa, 1986). As a result, RNAII cannot function efficiently as a primer for DNA synthesis. RNAI is a small molecule of approx. 108 nt, and is transcribed from the same region of the plasmid as is the primer RNAII, but in the opposite direction, such that RNAI can hybridize completely with its target RNAII (Tomizawa et al., 1981).

This inhibition of primer formation by antisense RNA is the molecular basis for the phenomenon of plasmid incompatibility (Tomizawa and Itoh, 1981). Two plasmids of the same 'incompatibility group' have antisense regulators with the same specificity, i.e., one can interfere with the replication of the other. Because of this mutual hindrance, eventually each cell has only one 'kind' of plasmid.
The secondary structures of the two RNA species have been deduced using a structure prediction program, and these have been refined by nuclease sensitivity and mutagenesis studies (Tamm and Polisky, 1983; Masukata and Tomizawa, 1986). The antisense RNA can be folded into a secondary structure that consists of three stem-loops separated by small regions of unpaired nucleotides. The stems are approximately thirty base-pairs in length and the loops consist of seven nucleotides. The target RNAII can also be folded into a number of stems and loops connected by regions of unpaired nucleotides. Unlike RNAI however, the target RNAII assumes different conformations depending on the presence or absence of RNAI. In terms of pairing between antisense and sense RNAs, it is the loop regions in RNAII and RNAI that are of critical importance. Comparisons between the antisense effectors of compatible plasmids show that major differences occur in the loops of the effector RNAs, although the overall structures of the antisense RNAs are very similar (Selzer et al., 1983). Furthermore, mutations which affect the specificity of antisense control usually map to the loop regions (Tomizawa and Itoh, 1981). Some of these loop mutations are such that they confer a phenotype when present in only one pairing partner, but not when a compensating mutation, which restores pairing, is present in the other pairing partner. In general, these kinds of 'compensatory mutations' in a sense/antisense RNA pair are adduced as definitive genetic evidence that base-pairing between two RNA species is important for the system in question.

The pathway of RNAI:RNAII base-pairing has been worked out in vitro, using nuclease sensitivity and gel electrophoresis assays (Tomizawa, 1984, 1985, 1986; see Figure 1). The first step in pairing between RNAI and RNAII,
one that is sufficient for antisense control, is a reversible interaction between the loops of the antisense RNAI and the complementary loops of the target RNAII—the kissing reaction. This is followed by a more stable interaction between the two molecules which involves pairing between the 5' tail of RNAI and a single-stranded 'bubble' of RNAII. This intermediate is so stable that it survives dilution into EDTA and 7 M urea. RNAI molecules which are unable to form this intermediate are only about a fourth as efficient as full-length molecules in copy number control. This initial pairing between the 5' end of RNAI and RNAII is then propagated over the entire RNAI molecule, resulting in complete pairing between RNAI and RNAII. This 'zippering reaction' is very slow—a significant fraction of the molecules are not completely paired even after 64 minutes—and is not required for antisense control.

The RNAI:RNAII interaction is further complicated by the involvement of a plasmid-encoded protein. The 'Rom' (for RNAI modulator) or 'Rop' (Repressor of primer) protein increases the efficiency of antisense control by increasing the rate of formation of the intermediate stable complex (Cesareni et al., 1982; Tomizawa and Som, 1984). It is thought that Rom functions by aligning the loops of RNAI and RNAII, thus facilitating base-pairing between the two molecules.

Regulation of transcription

There have been two cases described where antisense RNA is thought to regulate gene expression by causing premature transcription termination.

The expression of the E. coli catabolite receptor gene (crp, also referred to as cap) is negatively regulated by cAMP-CRP itself. This repression can be
attributed to the activation of a promoter, by cAMP-CRP, which directs synthesis of an RNA antisense with respect to crp RNA (Okamoto and Freundlich, 1986). Nuclease protection experiments have identified the transcription initiation site of the ‘transcription inhibitory complementary RNA’ (tic RNA) to be three nucleotides upstream of and on the strand opposite to that of the transcription initiation site of crp. Although the antisense effector and the target RNAs are non-overlapping, nucleotides two through fifteen of tic RNA are complementary to nucleotides two through eleven of crp mRNA. The notion that tic RNA causes premature transcription termination of crp mRNA is engendered by the observation that addition of tic RNA to an in vitro transcription system inhibited synthesis of crp mRNA, although, as yet, prematurely terminated crp mRNA has not been detected. Indirect evidence for the involvement of tic RNA in regulating expression of crp is provided by the observation that autoregulation is lost due to point mutations which debilitate the tic promoter.

The c4 repressors of bacteriophages P1 and P7 are thought to function in a manner analogous to that of tic RNA (Biere et al., 1992). The c4 gene is part of the immI operon of these phages, and regulates the expression of the antirepressor (ant) gene. To maintain lysogeny, expression of the ant genes must be repressed, and this is accomplished by c4 (Citron and Schuster, 1990). In all other cases of regulation by antisense RNA, the target and the effector are transcribed from two different, usually convergent, promoters; the c4 antisense effector RNA and the target ant mRNA are unusual in that both are part of the same operon and are transcribed from a common promoter, with c4 being proximal to the promoter (see Figure 2).
The c4 antisense RNA is a small 77 nt species that is processed from the larger precursor c4-ortx-ant RNA (Citron and Schuster, 1992). The host activity responsible for this processing is unknown, but the tRNA processing enzyme, RNase P, has been implicated because the sequence in the vicinity of the processing site in the c4 precursor RNA is similar to that found in tRNA precursors. The secondary structure of c4 has been deduced; it is, in essence, a two pronged fork. The two 'prongs' have nine and four nucleotide loops at their ends. Nucleotides present in the nine nucleotide loop are important for interaction between c4 and the target. One of the 'prongs' has a bulge of seven nucleotides at its base; nucleotides present in this bulge are also important for interaction with the target RNA. Critical genetic evidence for base-pairing between the 'loop' and 'bulge' of c4 and its target comes from a compensatory mutation—a mutation in c4 RNA which abolishes antisense control can be 'reverted' by a mutation in the target RNA which restores base-pairing (Citron and Schuster, 1990). Furthermore, c4 RNAs of P1 and P7 are closely related, but differ from each other only in these two regions. The heteroimmunity of P1 and P7 can be explained, therefore, by these differences in their c4 RNAs. As mentioned above, c4 negatively regulates expression of ant, but the target of c4 is far upstream of the translation initiation site of ant. How then does c4 exert its effect?

The expression of ant is coupled to a small, overlapping open reading frame (ortx) that lies upstream of the ant gene, and c4 functions by interfering with the translation of ortx (Biere et al., 1992). The Orfx gene-product does not appear to play a role in regulating ant expression since frameshift mutations which result in replacement of 10 of the 73 residues of Orfx have no effect.
Expression of cloned orfx does, however, cause cell filamentation, suggesting that it may have some other function. In any case, it was found that when translation of orfx was inhibited by amber mutations no ant mRNA could be detected in phage infected cells, at least as judged by Northern blots, indicating that mutations in orfx are polar on ant expression. Since this polarity could be reversed by a mutation in the transcription termination factor ρ, it was concluded that inhibiting translation of orfx affects synthesis of ant mRNA but not its stability. The c4 antisense RNA functions analogously to the amber mutations in orfx—it inhibits translation of orfx, as suggested by its effect on orfx-lacZ gene-fusion expression, most likely by base-pairing with the orfx ribosome-binding site. It has been proposed that preventing translation of orfx by c4 creates a structure in orfx-ant mRNA that mimics a ρ-dependent transcription terminator, thus blocking synthesis of ant mRNA.

Since the antisense effector RNA is synthesized prior to the target ant mRNA (c4 being promoter-proximal), it raises the question of how ant is expressed at all. Schuster and co-workers speculated that this may be achieved by alternative base-pairing between c4 and orfx RNAs. There are two, short regions (7 and 8 nucleotides) of complementarity between c4 and orfx, only one of which overlaps the Shine-Dalgarno sequence of orfx; the other is located such that base-pairing between this sequence and c4 is unlikely to have an effect on orfx translation. The proposed mechanism for ant expression in the presence of c4, entails a switch in base-pairing between c4 and orfx RNAs—from c4 pairing with the Shine-Dalgarno sequence of orfx to c4 pairing with the region far upstream of the initiation site of orfx. This begs the question though, of what causes this switch in base-pairing. A more economical
explanation may be that the unprocessed form of c4 can not exert its effect, perhaps, because it can not assume the appropriate structure. It was found that early during P1-infection full-length c4-orfx-ant mRNA can be detected, but at later time-points only RNA that terminates within orfx is detectable. It may be that ant is translated off of the precursor of c4 RNA, thereby solving the problem of ant expression in the presence of c4.

Regulation of translation

Antisense RNAs can affect the translation of target RNAs in at least two ways; they can prevent translation initiation by base-pairing with the ribosome-binding site and preventing access to ribosomes, or they can destabilize the target RNA by creating a site for a double-strand specific ribonuclease (RNaseIII, for example). I review below examples of each class.

sar of P22

Bacteriophage P22, unlike its relative λ, possesses a second immunity system, imml, which encodes an antirepressor gene ant, and its regulators. Ant is a 35 kD protein that obstructs the action of a number of phage repressors, including that of P22 itself. Ant and another gene, arc, are expressed rightward from a common promoter, Pant. Ant expression is tightly regulated since its over-expression is lethal to P22. Arc inhibits the expression of ant by binding to an operator near Pant. In a lysogen, ant expression is inhibited by another repressor, Mnt. The ant gene is additionally transcribed late in infection from another phage promoter, Plate, but Ant is not synthesized from the resulting RNA (reviewed in Susskind and Youderian, 1982). Susskind and co-workers
isolated P22 mutants that synthesize Ant late during infection. Surprisingly, some of these mutations caused silent changes in ant (Wu et al., 1987). In vitro studies revealed that a small RNA, antisense with respect to ant, is synthesized from this region of P22 (Liao et al., 1987). The mutations that were isolated were found to affect the promoter for this antisense RNA.

Sar (small antisense RNA) is a 68 or 69 nt species that spans the entire intercistronic region between arc and ant, and is transcribed in the direction opposite to that of arc and ant (see Figure 3). Gel-mobility shift assays have revealed that sar and ant RNA rapidly form a complex (Liao et al., 1987), but the kinetics of base-pairing between the two have not been studied. The mechanism of sar inhibition of ant expression is not known, but given that a part of sar is complementary to the Shine-Dalgarno region of ant, it seems likely that sar functions by base-pairing with the ant ribosome-binding site, thus preventing access to ribosomes. It is possible, however, that sar could function by destabilizing ant mRNA. Computer modeling, refined by nuclease protection and mutagenesis experiments, suggests that sar can be folded into a non-symmetric dumb-bell shaped secondary structure (Jacques and Susskind, 1991). In keeping with the theme of loop-to-loop interactions being important for antisense function, the region of sar that is complementary to the ribosome-binding site of ant is present in the 5' loop. Whether the target ant mRNA also folds into some structure is not known.

The following model has been proposed for the lack of Ant synthesis late during infection by P22 (Liao et al., 1987). Early during infection there is a burst of transcription from the strong ant promoter, resulting in elevated levels of Ant. In vitro studies revealed that high level transcription from P_{ant} inhibits
transcription from $P_{sar}$, leading to the notion that early during infection there is little or no $sar$ present. As Arc accumulates, however, expression from $P_{ant}$ is turned down, with a concomitant increase in transcription from $P_{sar}$. Late during infection, when there is little or no transcription from $P_{ant}$, sar efficiently prevents expression of Ant from the Plate transcript.

**RNA-OUT of IS10**

The insertion sequence IS10 encodes a single protein, transposase, that acts on the ends of the insertion sequence to promote transposition. IS10 also encodes an antisense RNA, called RNA-OUT, which inhibits synthesis of transposase (reviewed in Simons and Kleckner, 1988). As the number of IS10s increases in the cell, expression of transposase and hence the frequency of transposition per IS10 copy decreases, a phenomenon known as multi-copy inhibition. Multicopy inhibition is due to the action of RNA-OUT, and can be explained by the ability of the antisense effector to act in trans along with the ability of transposase to function preferentially in cis (Simons and Kleckner, 1983; Morisato et al., 1983). Consistent with the role of RNA-OUT in multi-copy inhibition, antisense RNA has little effect on transposase expression when only a single-copy of IS10 is present in the cell. This is because the concentration of RNA-OUT is too low. When the number of IS10 copies increases to as few as three however, significant inhibition of transposase expression can be seen.

RNA-OUT is a 69 nucleotide species that initiates in the 5’ end of the transposase gene. RNA-OUT can be folded into a single stem-loop structure; complementarity to the target transposase RNA (RNA-IN) begins in the loop domain and extends down one side of the stem for 35 nucleotides (Kittle et al.,
1989). Unlike sar, however, RNA-OUT buries its region of complementarity to the ribosome-binding site of RNA-IN in the stem-domain. Based on genetic and in vitro binding studies, the following model has been suggested for pairing between RNA-OUT and RNA-IN (Kittle et al., 1989). Pairing initiates, as in the CoIE1 family, by interactions between the loop domain of RNA-OUT and the complementary region of RNA-IN. A mutation in the loop domain of RNA-OUT reduces significantly the rate of hybridization in vitro and affects multicopy inhibition in vivo. This defect is remedied by a compensatory mutation in the target RNA. Base-pairing then propagates down one part of the stem domain of RNA-OUT, and is reminiscent of the 'strand invasion' reaction observed in homologous recombination—the new IN-OUT duplex is formed by displacing one strand of the RNA-OUT stem. In contrast to RNAI:RNAII pairing, hybridization between RNA-OUT and RNA-IN is rapid and is complete in two min, as judged by a gel mobility shift assay. The in vitro pairing reaction probably closely resembles the in vivo process because single base mutations which affect the rate of pairing in vitro also affect multi-copy inhibition.

The mechanism of inhibition by RNA-OUT is fairly well understood, at least in vitro. Toe-printing studies, which measured the binding of 30S and 70S ribosomal subunits to the target RNA-IN, revealed that binding is significantly inhibited when RNA-IN is base-paired with the antisense regulator RNA-OUT, indicating that RNA-OUT functions by blocking translation initiation (Ma and Simons, 1990). The IN-OUT duplex, in vivo and in vitro, is susceptible to cleavage by RNaseIII, followed by degradation by sundry cellular exonucleases. But this destabilization is unlikely to be the primary cause of
inhibition of transposase expression, since antisense regulation functions efficiently in an RNaseII* host (Case et al., 1990).

**OOP RNA of λ.**

Like P22, upon infection, bacteriophage λ follows one of two developmental pathways—lytic or lysogenic. In lysogeny, the expression of lytic functions is prevented by cl repressor. The expression of cl is established from the PRE promoter, and is then maintained in the prophage at low levels by transcription from the PRM promoter. PRE functions only when activated by the cll protein; thus cll plays a pivotal role in the lysis-lysogeny decision (Ptashne, 1986). The expression of cll is regulated, in part, by a small antisense RNA, OOP. Studies by Krinke and Wulff (1987, 1990) indicate that OOP RNA functions by destabilizing its target RNA.

OOP RNA is transcribed from its own promoter in the direction opposite to that of the target cll RNA (see Figure 4). The promoter for OOP RNA overlaps the ribosome-binding site of a DNA replication gene, O, which is adjacent to cll. OOP RNA terminates at a strong transcription terminator within the cll gene, such that the 3' 55 nt of OOP (out of 77 nt) are complementary to the 3' 55 nt of the cll gene (reviewed in Wulff and Rosenberg, 1983).

OOP antisense RNA is unusual in the sense that it is complementary to the 3' end of its target gene, unlike all the other antisense RNAs described so far which are complementary to the 5' end of their target genes. Studies by Krinke and Wulff revealed that expression of cll, as measured by the expression of a cll-activated galactokinase gene, is inhibited approx. 100-fold when OOP is produced from a strong promoter in a multi-copy plasmid. Little or no inhibition
was seen in an RNaseIll- (a double-strand specific RNase) host, leading to the notion that OOP RNA destabilizes the target cll RNA by creating a site for RNaseIII cleavage (Krinke and Wulff, 1987). Primer extension experiments with RNA isolated from induced lysogens, in the presence of excess OOP RNA produced from a plasmid, helped identify a specific RNaseIII cleavage site, that lies ten nt beyond the cll termination codon, in the poly-cistronic cll-O mRNA. Nuclease protection experiments suggested that, following RNaseIII cleavage, cll mRNA is rapidly degraded, whereas O mRNA is not. Two minor OOP RNA dependent cleavage sites in cll-O mRNA were seen in an RNaseIll- host: one near the RNaseIII site and one further upstream beyond the region of complementarity of cll and OOP. In contrast to the situation in an RNaseIll+ host, cll mRNA upstream of these non-RNaseIll cleavage sites is stable, but now O mRNA is rendered unstable.

The biological role of OOP was revealed by experiments which tested the effect of OOP RNA on the burst size of an induced \( \lambda \) prophage (Krinke et al., 1991). It was found that a UV-induced \( \lambda \) prophage unable to make OOP RNA, because of a mutation in the OOP promoter, had a two-fold lower burst size as compared to that of wild-type \( \lambda \). However, upon infection no differences were seen between wild-type \( \lambda \) and the OOP- mutant with regard to frequency of lysogenisation, plaque morphology and one-step growth curves, suggesting that the main role of OOP RNA is in prophage induction.
**Figure 1:** The pathway of pairing between RNAI and RNAII (Eguchi *et al.*, 1991). Pairing initiates by base-pairing interactions between the loops of the two RNAs to form C**. This 'kissing' reaction facilitates complete pairing between RNAI and RNAII that starts at the 5' end of RNAI, and then propagates along the entire length of RNAI.
Figure 2: Genetic structure of the *imml* operon of bacteriophages P1 and P7 (Biere *et al.*, 1992). The *c4*, *orfX* and *ant* genes are transcribed from promoter 51 (p51). Wavy lines below indicate transcripts. The full-length and prematurely terminated *ant* RNAs are shown. The antisense RNA, *c4*, is also shown, and its site of interaction with *orfX/ant* mRNA is indicated by an arrow.
*imm1 operon*

- **RNA**: ant mRNA, c4 RNA processed

Diagram:
- **Op51**
- **c4**
- **orfX**
- **ant1/ant2**
- **kilA**

Legend:
- p
Figure 3: Genetic structure of the bacteriophage P22 imml region (Liao et al., 1987). A: Striped arrows show the pattern of transcription in this region. Arc and ant are transcribed from Pant; the antisense sar is transcribed from its own promoter Psar. Oarc and Omnt refer to the operators of Arc and Mnt repressors respectively. B: The DNA sequence between arc and ant is shown. The numbers indicate distance from the +1 site of sar. The -35 and -10 hexamers of Psar are underlined. The ribosome binding-site of ant is indicated with asterisks. The termination sites for sar are labelled Tsar.
ant arc ant

Hinc n, ,

"GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG

AACATACACDGCGCACAGTCAAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGATGGTCAGTCGCGACAGCAGTCAAGGAGACCTCTTTTTGAGT

CGTTAAATCTTCGCAAGCAATGATATAGCAATTTTATGAAAGCAGTTAGGAGACCTCTTTTTGAGTTACCTTTAACTTCGGGGTTGACGCGAT

GlyAlnTerm. 60 40 20 . . .

GGCGCGTAAGTTGAAAGCCCGAACAGTACGCGGGTACGCGGCTCCGGTGTCAATCTCTTGGAGAAGAAAACCAAAAGCAATGATATAGCAATTTTATGAAAGCAGTTAG
Figure 4: Genetic structure of the PR operon of bacteriophage λ in the vicinity of the cll gene (Krinke and Wulff, 1987). The orientation is reverse of the conventional prophage genetic map. Wavy arrows indicate transcripts. Transcription from PR results in expression of lytic genes, with the exception of cll which plays a role in the establishment of lysogeny. The OOP antisense RNA is transcribed from P₀ and terminates at t₀. cll protein activates transcription from PRE which results in expression of the cl repressor.
NOTE

Chapters 2 and 3 form the experimental core of this thesis; chapter 4 delineates preliminary studies on transcriptional regulation of \textit{sieB} and \textit{esc}. Chapter 2 has been published in the \textit{Journal of Bacteriology} (1993, 175: 4712-4718). Most of Chapter 3 has been published in \textit{Genes and Development} (1993, 7: 1498-1507). A. Poteete is a co-author on both papers. In the following chapters, plasmids with the prefix, 'pTP' were constructed by him. Some of the observations about the \textit{\lambda sieB} gene reported in Chapter 2 were originally made by A. Poteete (unpublished); I confirmed and extended his studies on \textit{\lambda sieB}. 
CHAPTER 2

The Superinfection Exclusion \((sieB)\) Genes of Bacteriophages P22 and \(\lambda\)
INTRODUCTION

Lambdoid prophages encode systems that prevent the lytic growth of superinfecting phages. Thus λ and P22 are unable to grow vegetatively in λ or P22 lysogens respectively because transcription from their major promoters (PL and PR) is sensitive to repression by cl (in the case of λ) or c2 (in the case of P22) repressors present in the lysogen (for reviews see Gussin et al., 1983; Poteete, 1988). P22 encodes an antirepressor which is capable of inducing any resident lambdoid phages by neutralizing the repressor already present in the cell. A P22 prophage itself is not induced by this antirepressor because it elaborates another repressor (Mnt) whose function is to prevent transcription of ant (which encodes the antirepressor) (for review see Susskind and Youderian, 1983).

P22 and λ also have mechanisms to prevent the growth of superinfecting phages that are insensitive to repression. Thus the λ rex genes prevent the growth of rII mutants of phage T4 (Benzer, 1955). P22 has at least two genes which prevent superinfection by other phages; sieA (for superinfection exclusion) interferes with the DNA injection process (Susskind et al., 1974a) and a1 causes conversion of the O-antigen of the Salmonella host, thus preventing adsorption of some phages (Susskind and Botstein, 1978 and references therein). Both λ and P22 possess another superinfection exclusion gene—sieB; its product aborts the lytic development of some superinfecting phages (Susskind et al., 1974b; Susskind and Botstein, 1980). Perhaps
surprisingly, these two genes have the same specificity in that they exclude the same *Salmonella* phages L, MG178 and MG40 (although *Salmonella* is not a normal host for λ).

The *sieB* gene of P22 was found to map in the P_L operon between genes *c3* and 24 (Semerjian *et al.*, 1989; Susskind *et al.*, 1974b). Sequencing studies by Franklin (1985) revealed a rightward open reading frame (orf) which was a likely candidate for *sieB*. Studies by Susskind and Botstein (1980) showed that λ too had a *sieB* gene; and using bio substituted λ phages, they showed that it mapped in an analogous position in the P_L operon of λ, between genes *cIII* and N. Sequencing studies by Ineichen *et al.* (1981) revealed a rightward reading frame in the region which was later proposed by Court and Oppenheim (1983) to be the λ *sieB* gene.

Data presented below serve to identify the two *sieB* genes, and their respective promoters. The λ *sieB* gene is identical to the one proposed by Court and Oppenheim; however the P22 *sieB* gene that we have identified differs substantially from the one proposed earlier (Franklin, 1985).
MATERIALS and METHODS

Bacteria

*Escherichia coli* W3110 *lacQ* (Brent and Ptashne, 1981) was used for the propagation of plasmids. Strain CSR603 (*recA1 uvrA6*; Sancar and Rupert, 1978) was used for maxicell analysis. *Salmonella typhimurium* LT2 strains MS1868 (*leuAam414 r*^+^) and MS1362 (*leuAam414 supD*^6^) were provided by M. Susskind. Strain CV112 (*polAts*) was obtained from A. Wright.

Phage

Phage L *cll-101* and P22 *immC* _esc^+/p22_ and P22 *sieA44 Ap2* were obtained from M. Susskind. P22 *sieA44 Ap2* is an oversized phage (it has a Tn1 insertion in the _a1_ gene), and hence is unable to form plaques on single infection (Weinstock _et al._, 1979). P22 *sieA44 a1-327* is a plaque-forming revertant of this phage, and its genome is approximately the same size as wild-type P22 (L. Hardy and A. Poteete, unpublished observations). Mutant alleles of *sieB* were introduced into P22 by crossing mutation-bearing plasmids with oversized kanamycin resistance-carrying P22 as described (Semerjian _et al._, 1989). For crossing deletions in *sieB* into P22, the oversized phage P22 *sieA44 m44 ral :: Kn467* (Semerjian _et al._, 1989) was employed. This oversized phage has a kanamycin resistance gene inserted at the HindIII site in _ral_. Point mutations in *sieB* were crossed into P22 *sieA44 a1 sieB::Kn567*. In this phage, the 732 bp between the HindIII site (in _ral_) and the EcoRV site (in _sieB_) have been substituted by 5.04 kb of sequences from Tn5, including the kanamycin resistance gene, thus making it oversized. It was constructed by crossing
pKR662 (see below) with P22 sieA44 a1-327 as described (Semerjian et al., 1989).

**Plasmids**

Plasmids pTP83, pTP425, and pAS474 have been described (Poteete, 1982; Semerjian et al., 1989). pAS474 was used to make nested deletions in the *ral-sieB* region as follows. pAS474 was opened at the HindIII site in *ral* and digested with BAL31 for various times, filled-in and religated in the presence of a HindIII linker or a 1100 bp kanamycin-resistance encoding BamHI (filled-in) DNA fragment with Sall sites very close to both ends (Semerjian et al., 1989). This series includes pTP520, pTP649, pTP650, pTP651, pTP652 and pTP652a. To provide flanking homology for plasmid by phage crosses, these deletions were recloned into pTP425 by ligating the HindIII-EcoRI deletion-bearing fragment from pTP520 and pTP651 with the HindIII-EcoRI backbone of pTP425; or the Sall (filled-in)-EcoRI deletion-bearing fragment from pTP649, pTP650, pTP652 and pTP652a with the HindIII (filled-in)-EcoRI backbone of pTP425. This sub-cloning step also ensures that all the deletions have a defined right end-point at the HindIII site in *ral*. The extent of deletions is indicated in Figure 1 and the deletion end-points in Figure 2. Plasmid pKR682 (which was used for maxi-cell analysis) was constructed by ligating a 969 bp HindIII-MluI *sieB* containing fragment from pAS474 (see Figure 1) with the large HindIII-PvuII ori containing fragment from pBR322. A derivative of pKR682 was constructed by replacing the HindIII-EcoRV fragment with a similar fragment bearing an amber mutation at codon 4 of the *sieB* gene.

Mutant alleles of P22 *sieB* were constructed in plasmids and then introduced into P22 by recombination. To this end, the 969 bp HindIII-MluI
fragment from pAS474 was cloned into the polylinker of M13mp19 and mutations were introduced as described (Kunkel et al., 1987). The mutagenic oligonucleotides (mismatched nucleotide(s) underlined) used were as follows:

\[
\begin{align*}
\text{PsieB } -35 & : 5' \text{ AGGGGTACCGCGAGAACC} \\
\text{PsieB } -10 & : 5' \text{ GTGAATAGCGTTGTTCTCGC} \\
\text{Ser 4 to am} & : 5' \text{ CTGCCACCACTAGTTGTCAT}
\end{align*}
\]

(PsieB -35 and PsieB -10 refer to the -35 and -10 hexamers of the promoter for the P22 sieB gene; Ser 4 refers to the codon of the sieB gene that was changed to an amber.). After confirming by DNA sequencing that the region had only the desired mutation, a HindIII-EcoRV fragment (see Figure 1) carrying the mutation was used to replace the wild-type HindIII-EcoRV fragment in pTP83. These pTP83 derivatives were used to introduce the mutations into P22 by homologous recombination. A pTP83 derivative was also used to construct the oversized phage P22 sieA44 a1 sieB::Kn567. This derivative, pKR662, was constructed by substituting the 732 bp HindIII-EcoRV (ral-sieB) segment with a 5.04 kb kanamycin resistance encoding Hpal fragment from Tn5 (isolated from pPB20 :: Tn5-13; Semerjian et al., 1989).

A sieB-containing segment of the \( \lambda \) cl-857 S-am7 chromosome extending from the Xhol site at 33,500 to the BgIII site at 38,100 (Sanger et al., 1982) was inserted between the Xhol and Pvull sites of pTP83. In the process of construction, the BgIII end was filled in with \( E. \ coli \) DNA polymerase I large fragment in the presence of dNTP's and the Pvull and filled-in BgIII ends were joined via an EcoRI linker; the resulting plasmid was designated pTP108. Plasmid pTP200 was constructed by digesting pTP30 (Berget et al., 1983) with Pvull, and recircularizing the large fragment by ligating in the presence of Ncol
linkers (5'-CCCATGGG-3'). Plasmid pTP442 was constructed by digesting pTP108 with AatII and XhoI, filling in the ends, and recircularizing the large fragment by ligating in the presence of XbaI linkers. Plasmid pTP462 was constructed by digesting pTP442 with EcoRI and Hpal, filling in the EcoRI ends, and recircularizing the large fragment by ligating in the presence of BglII linkers (CAGATCTG; this restores the EcoRI site at the junction). Plasmid pTP466 was constructed by joining the lacUV5 promoter-containing fragment of pTP200 generated by digesting with Ncol, filling in the ends, and digesting with PstI, to the origin-containing fragment of pTP462 generated by digesting with BamHI, filling in the ends, and digesting with PstI. Plasmid pTP482 was constructed by ligating the sieB-containing EcoRI fragment of pTP466 into the EcoRI site of pMC7 (the lacIQ-bearing version of the plasmids described in Calos, 1978).

In order to synthesize large amounts of riboprobe, a 426 bp Sall-Hpal fragment from pTP650 was placed in the polylinker region of pGEM-3Z (Promega). The orientation of the insert in the resulting plasmid is such that sieB antisense RNA can be made by digesting with EcoRI and transcribing with T7 RNA polymerase.

**RNase protection**

RNA for nuclease protection experiments was isolated as follows. A 30 mL culture of the appropriate P22 lysogen was grown to approximately 2x10^8 cells/mL. The pelleted cells were washed with 1 mL TE (10 mM Tris pH 7.6 and 1 mM EDTA) and suspended in 500 μL RNA lysis buffer (10 mM Tris pH 7.6, 1 mM EDTA and 0.2% SDS). An equal volume of phenol (equilibrated with water) was added and the mixture was shaken at room temperature for 5 min. The aqueous supernatant was extracted 2-3 times with chloroform and RNA
was precipitated by adding 1/10 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes ethanol. The pelleted RNA was dissolved in 200 μL diethyl pyrocarbonate (DEPC)-treated water and precipitated again as above. The precipitate was washed with 70% ethanol, dried and dissolved in 50 μL of DEPC-treated water. RNA was quantitated by measuring absorbance at 260nm, assuming 1 A$_{260}$ unit equals a concentration of 40 μg/mL of RNA. 

Riboprobe was synthesized as described by the supplier of plasmid pGEM-3Z (Promega).

To detect sieB mRNA, approximately 50 μg of total RNA was hybridized to 2 μL of ribprobe (approximately 10$^6$ Cerenkov cpm) in 50 μL of hybridization buffer (10 mM Tris pH 7.2, 1 mM EDTA and 1 M NaCl; Berk, 1989). The RNA and riboprobe mixture was denatured at 85°C for 5 min and then hybridized at 65°C for 60 min. Excess riboprobe and unhybridized RNA were digested as described (Sambrook et al., 1989), with some modifications. The hybridization mixture was cooled to room temperature and 450 μL of RNase digestion buffer (9.0 mM Tris pH 7.5, 5 mM EDTA and 120 mM NaCl) containing 30μg/mL nuclease P1 and 10 μg/mL RNaseT1 was added. After incubation at 37°C for 60 min the digestion mixture was extracted by adding 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated by adding two volumes of ethanol to the aqueous phase. The pelleted RNA was suspended in 8 μL of TE (pH 7.6) and 8 μL of Stop solution (USB Sequenase). The protected riboprobe was denatured at 85°C for 5 min and fractionated on a 5% acrylamide: 8M urea gel. Urea was removed from the gel by soaking in cold 5% TCA for 20 min. The gel was then dried and placed on X-ray film (Fuji) at room temperature for 19 hr.
DNA sequencing

P22 DNA was sequenced as described (Rennell et al., 1991). To confirm the DNA sequence between ral and 24, BAL31 deletions generated to map *sieB* (see above) were cloned into M13mp18 and M13mp19 and then sequenced (using the Sequenase kit from USB) using the universal primer or special primers obtained from the University of Massachusetts DNA synthesis facility. For obtaining sequence of the *sieB* sense strand, Sall-MluI fragments from pTP649 and pTP652 and a HindIII-ClaI fragment from pTP651 were cloned into M13mp18. The sequence of the anti-sense strand was obtained by cloning the Sall-MluI and HindIII-ClaI fragments from pTP649 and pTP651 respectively into M13mp19.

Maxi-cells

Maxi-cell analysis was performed essentially as described (Silhavy et al., 1984), except that *E. coli* strain CSR603 was used. Labelled proteins were fractionated on a Tricine-sodium dodecyl sulfate 10% polyacrylamide gel (Schagger and Jagow, 1987).
RESULTS

Deletion Mapping of P22 *sieB*

To map the *sieB* gene precisely, P22 mutants with deletions in the *ral-sieB* region were constructed. These phages carry deletions of varying lengths with a common left end-point at the HindIII site in *ral* (see Figure 1). Deletion end-points were identified by sequencing phage DNA; these are indicated in Figure 2.

The SieB phenotype of deletion-bearing P22 *sieA44* prophages was determined by their ability to exclude phage L. In the absence of *sieA*, the only known P22 gene-product that excludes L is SieB (Susskind *et al.*, 1974b). As indicated in Figure 1, lysogens of P22 *sieA44*, P22 *sieA44 Δ649* and P22 *sieA44 Δ650* plate L with the same low efficiency (10^{-2} to 10^{-3} relative to its efficiency of plaque formation on a non-lysogen). A L/P22 hybrid phage, that carries a P22 determinant *esc* (for escape) that allows it to overcome SieB-mediated exclusion has an efficiency of plating of approx. one. Lysogens of P22 *sieA44 Δ520*, P22 *sieA44 Δ651*, P22 *sieA44 Δ652* and P22 *sieA44 Δ652a* fail to exclude L however; L plates as well on these lysogens as it does on a non-lysogen. Combined with previous studies (Susskind *et al.*, 1974b; Semerjian *et al.*, 1989), these results indicate that sequences to the left of nucleotide 169 are not essential for *sieB* function, but a critical *sieB* determinant lies between 169 bp and 288 bp, in the numbering shown in Figure 2.

Sequence and Expression of the P22 *sieB* Gene

The sequence of the *ral-sieB* region was re-examined in an attempt to identify a *sieB* open reading frame consistent with the mapping experiments
described above. A number of discrepancies from the previously published sequence (Franklin, 1985) were discovered. The corrections are as follows: there are two A's after position 278 (instead of 3), there is no A after position 317, there is no T after 454, there is a T after 473, there are only 2 A's after 837 (as opposed to 3) and a single A after 840 (instead of 2), and finally there is a T after residue 922. The net effect of these revisions is to substantially alter the location of the putative sieB open reading frame; the correction at position 922 causes a frame-shift in the 3' end of gene 24. The revised sequence shown in Figure 2 revealed a candidate sieB open reading frame with the potential to encode a polypeptide of 242 amino acids.

This open reading frame has six likely initiation ATGs, and these are underlined in Figure 2. P22 sieA44 Δ650 lacks the N-terminal 23 codons of this orf but is still SieB+; therefore one can exclude the first ATG as the sieB initiation codon. An amber mutation was introduced four codons after the second initiation codon, and its SieB phenotype was tested. As indicated in Table I, Salmonella bearing this mutant allele of sieB plates L as efficiently as does a P22 prophage with a deletion of the sieB region. This observation suggests that the sieB orf initiates at the second ATG (also see below). Whether the amber is suppressible in single copy could not be tested because the supD strain when lysogenised by this phage became resistant to phage infection—the control esc+ P22 (which is insensitive to SieB-mediated exclusion) failed to grow on this strain (data not shown). The same supD strain bearing the sieB amber gene on a multicopy plasmid (pKR682a) however, excludes phage L with the same efficiency as does wild-type sieB (see Table I). The same
plasmid in a \textit{sup}^{0} strain however, is completely innocuous to the growth of phage L.

The open reading frame thus identified (see Figure 2) as the \textit{sieB} gene is read rightward in the conventional P22 prophage genetic map, and has the potential to encode a polypeptide of 192 amino acids with a calculated molecular weight of 22,442.

Maxicells were used to analyse expression of proteins from this region of P22. To this end, a 969 bp HindIII-Mul wild-type or amber-carrying \textit{sieB} (see Figure 1) fragment was subcloned between the HindIII and Pvull sites of pBR322. These plasmids were analysed using maxicells, and the results are presented in Figure 3. As shown in lane 3, two polypeptides with apparent molecular weights of approx. 20,000 and 18,000 are synthesized from the wild-type \textit{sieB} gene. We identify the larger protein as SieB because the N-terminal amber mutation at codon 4 (Ser4Am) confers a SieB\textsuperscript{-} phenotype and prevents expression of this protein, but not the smaller, which is labelled SieB\textsubscript{inh} (Figure 3, lane 4). The smaller polypeptide is due to internal initiation in the \textit{sieB orf} (manuscript in preparation).

\textbf{Identification of the \textit{sieB} promoter}

The start-site of \textit{sieB} transcription was identified using RNase protection. A uniformly labelled \textit{sieB}-antisense riboprobe (Figure 1) was digested with nucleases P1 and T1 in the presence of RNA isolated from a SieB\textsuperscript{+} or a SieB\textsuperscript{-} lysogen. The wild-type \textit{sieB} mRNA yields a protected fragment of approximately 350 nucleotides (Figure 4, lane 1). A prophage which has the whole \textit{ral-sieB} region deleted fails to give any protected fragment (Figure 4,
An examination of the upstream DNA sequence in this region reveals a candidate $\sigma^{70}$ promoter (see Figure 2).

To unequivocally identify the $sieB$ promoter, mutations were made in the putative -10 and -35 sequences, and these are indicated in Figure 2. The -10 hexamer was mutated at two sites; the highly conserved T residues at -7 and -12 were mutated to G, and the conserved T at position ‘-35’ (which in this case is actually at -37) was mutated to a C. The mutation-bearing $sieA44 a1$ prophages were tested for their ability to plate phage L. As indicated in Table I, the prophage with a mutation at the -35 position excludes phage L just as well as wild-type. The -10 mutant however, plates phage L with an efficiency as high as does a prophage which has its $sieB$ gene deleted.

An inference from these results is that the mutation at position -35 has little or no effect on transcription from the $sieB$ promoter, but the double mutation at -10 and -7 is a severe ‘down’. This is borne out by the RNase protection experiment shown in Figure 4. RNA isolated from mutant lysogens was subjected to nuclease protection as before. As can be seen in Figure 4, $sieB$ mRNA is readily detectable in the -35 mutant (lane 3), but not in the -10 mutant (lane 4).

**Identification of $\lambda$ $sieB$**

Based on genetic and sequencing studies by Susskind and Botstein (1980) and Ineichen et al. (1981) respectively, Court and Oppenheim (1983) speculated that a rightward open reading frame between $cIII$ and $N$ is the $sieB$ gene of $\lambda$. Data presented in Table I indicate that this is indeed the case. When the said open reading frame is expressed in *Salmonella*, it excludes phage L, but not wild type P22. The relevant genetic structure of the $P_L$ operon of $\lambda$ is
illustrated in Figure 5. A *sieB* containing fragment from the BamHI site at 34,499 (Sanger *et al.*, 1982) to the Hpal site at 35,261 (228 bp past the *sieB* termination codon) was used. The putative *sieB* open reading frame was fused at the initiating ATG using the BamHI site at 34,499 to the controllable *lacUV5* promoter; an in-frame initiation codon was provided by an Ncol linker used in the construction. In addition this plasmid—pTP482—bears the *lacI*^Q^ allele. As shown in Table I, due to partial derepression of the *lacUV5* promoter in the presence of the inducer IPTG, L plates with a very low efficiency on plasmid-bearing cells; however, the control esc^+^ P22 plates with a high efficiency. In the absence of IPTG both phages plate with a high efficiency. Thus, expression of the open reading frame is necessary and sufficient to confer a SieB^+^ phenotype on wild-type *Salmonella*.

Another plasmid, pTP466, expresses λ *sieB* from the *lacUV5* promoter, but, unlike pTP482, it does not express the Lac repressor. As indicated in Table I, cells bearing pTP466 exclude L and P22. The simplest interpretation of these results is that overexpression of λ *sieB* (in the absence of Lac repressor as opposed to partial derepression by IPTG) leads to exclusion of L and P22. That physiological levels of λ SieB are needed for the correct specificity of exclusion is further illustrated by the result with plasmid pTP462. As shown in Figure 5, this plasmid bears the *ea10-ral-sieB* (and a part of *N*) region from λ; consequently, λ *sieB* is expressed, most likely, from its own promoter. As indicated in Table I, cells bearing plasmid pTP462 exclude phage L but not P22.

An examination of the sequence of λ *sieB* revealed two potential internal initiation sites (not shown). To test whether these are functional, maxicell
analysis was performed using plasmid pTP462. The result is shown in Figure 3. As can be seen in lane 4, only one polypeptide is detected; its apparent molecular weight—20,000—is in good agreement with the calculated molecular weight—20,982—of λ SieB.
DISCUSSION

**P22 sieB**

Several lines of evidence support the notion that the orf identified as *sieB* is indeed the *sieB* gene discovered by Susskind and co-workers (1974b). Deletion mutants of P22 which lack parts of this orf are SieB-. At least one amber mutation in this orf confers a suppressible SieB- phenotype. The said open reading frame encodes a polypeptide with a calculated molecular weight (MW) of 22,442 which is in reasonable agreement with the apparent MW of approx. 20,000 observed on SDS-PAGE.

It transpires that the orf identified here as *sieB* encodes two polypeptides—a large one ("SieB" in Figure 3, lane 3) which we have identified as SieB, and a truncated version of this protein ("SieBinh" in Figure 3). The shorter protein is a product of internal initiation (at the fifth ATG in Figure 2) in *sieB*, and in fact is an inhibitor of SieB (manuscript in preparation). It is clear that the larger protein is responsible for conferring a SieB+ phenotype because an amber mutation in its fourth codon confers a SieB- phenotype but still directs synthesis of the shorter protein, at least in maxi-cells and presumably in P22 lysogens. The formal possibility that the shorter polypeptide contributes to SieB-mediated exclusion in some way is not ruled out by the data presented in this paper.

**λ sieB**

Ineichen *et al.* (1981) identified a rightward open reading frame (*git*) in the P_L operon of λ. Based on the deletion mapping experiments of Susskind and Botstein (1980), Court and Oppenheim (1983) speculated that *git* is *sieB*. 
Data presented above confirm this idea. When git/sieB is placed under control of lacI and P_lac, it excludes phage L (but not P22) in an IPTG-dependent manner. Data shown in Table I indicate that expression of λ sieB from the lacUV5 promoter, in the absence of any Lac repressor, causes exclusion of L and P22. This observation reveals the basis of the 'super-sieB' phenotype described by Susskind and Botstein (1980). They observed that some λ bio-substituted prophages excluded L and P22; in the light of the result with pTP466, this is most likely due to overexpression of λ sieB by these prophages.

The λ sieB orf encodes a polypeptide of molecular weight 20,982 which is in good agreement with the apparent MW of 20,000 estimated from SDS-PAGE.

There is no appreciable homology between the sieB genes of the two phages, although the two genes exclude the same Salmonella phages. As shown in Figure 7 however, they have remarkably similar hydrophilicity profiles. Both proteins appear to have a hydrophilic N-terminus followed by two hydrophobic regions that are of sufficient length to be transmembrane domains. These hydrophobic regions are separated by a small stretch of hydrophilic amino acid residues. In both proteins the second hydrophobic domain is followed by a region rich in hydrophilic residues. Finally, both proteins have a hydrophilic C-terminus which is relatively rich in charged amino acids. Whether this similarity reflects a common exclusion mechanism remains to be determined. The idea that SieB may be a membrane protein is consistent with the physiological studies of Susskind et al. (1974b) which suggested that damage to the cell membrane may be involved in SieB-mediated exclusion. A comparison of the deduced sequences of λ SieB with λ RexA and RexB (which exclude some rif mutants of T4) revealed two regions of partial homology.
between RexB and \( \lambda \) SieB; they share the sequences GLLLLS and VFVFAL (except that the RexB sequence differs from the latter in that it has an I instead of F at the second position). There is no comparable homology between RexB and P22 SieB.

\textbf{sieB promoters of P22 and \( \lambda \)}

The transcription start-site of P22 sieB was mapped using RNase protection. Point mutations served to confirm that the sequence upstream of this site is indeed the sieB promoter. The double mutation in the -10 hexamer conferred a SieB\(^{-} \) phenotype; moreover, sieB mRNA was undetectable in mutant lysogens. The mutation at -35 had no apparent effect on SieB phenotype or mRNA levels. This lack of effect is almost certainly due to the context of the sieB promoter since a similar mutation in another P22 promoter (Pant) had a severe effect (Moyle \textit{et al.}, 1990).

As shown in Figure 6, the sequence in \( \lambda \) of the region upstream of its sieB gene is identical to that in P22. We suspect therefore, that \( \lambda \) uses the same sequence as a promoter for its sieB as the one used by P22. Another feature in the sequence around \( P_{\text{SieB}} \) stands out. In P22 and \( \lambda \) there is a ten nucleotide direct repeat around \( P_{\text{SieB}} \) (indicated in bold in Figure 6)—one overlapping the putative -35 of \( P_{\text{SieB}} \) and one after +1. This leads us to speculate that this sequence may be involved in regulating sieB expression.

One of the sequence corrections mentioned above (see Results) causes a frame-shift in the 3' end of gene 24 resulting in an increase in the length of gene 24 protein by four amino acid residues; its new calculated molecular weight is 10,969. The revised deduced amino acid sequence of the C-terminus of gene 24 protein (from residue 86 to 101) reads: LYAAGHRKSKQITAR.
Table 1
SieB phenotypes of cells expressing different alleles of P22 sieB or λ sieB

<table>
<thead>
<tr>
<th>Prophage or plasmid</th>
<th>Excludes phage</th>
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<tr>
<td></td>
<td>L</td>
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<tr>
<td>P22 (SieB+)</td>
<td>+</td>
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<tr>
<td>P22 PsieB-35</td>
<td>+</td>
</tr>
<tr>
<td>P22 PsieB-10</td>
<td>-</td>
</tr>
<tr>
<td>pKR682 (SieB+)</td>
<td>+</td>
</tr>
<tr>
<td>pKR682a Ser4am in sup0</td>
<td>-</td>
</tr>
<tr>
<td>pKR682a Ser4am in supD</td>
<td>+</td>
</tr>
<tr>
<td>pTP462 (λ sieB)</td>
<td>+</td>
</tr>
<tr>
<td>pTP462 (lacIQ P\text{lacUV5-λ sieB}) -IPTG</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+IPTG</td>
</tr>
<tr>
<td>pTP466 (P\text{lacUV5-λ sieB})c</td>
<td>+</td>
</tr>
</tbody>
</table>

aSalmonella strain MS1868 (sup0) was lysogenized with the appropriate P22 sieA44 a1 phage or transformed with the appropriate plasmid; where indicated, the supD strain (MS1362) was used. Lysogens or plasmid-bearing cells were grown to late-log in LB or LB supplemented with the appropriate antibiotic and 0.1 mL of culture was used to make lawns on LB or LB+antibiotic plates. Where indicated, IPTG was added to the bacterial lawn at a final concentration of
approx. 1 mM. Ten-fold dilutions of P22 \textit{vir3} and L \textit{cll-101} were spotted on such lawns. After the spots had dried, plates were incubated overnight at 30°C. 

b A '+' indicates that the phage plates 100-1000 fold less efficiently on the particular strain as compared to its plating efficiency on a prophage with a deletion in the \textit{ral-sieB} region (the plating efficiency with respect to a non-lysogen is the same, although the plaque size is larger on a non-lysogen); a '-' indicates that the efficiency of plating is close to 1.

cThis plasmid, unlike pTP482, does not bear the \textit{lacI^Q} allele.
**Figure 1.** Deletion mapping of the P22 sieB gene. Open boxes below the map represent sequences deleted from the phage. A '+' indicates that the deletion mutant excludes phage L as well as does a wild-type (SieA-) P22 prophage and '-' indicates that L plates as well on the deletion mutant as it does on a non-lysogen. The solid line indicates the riboprobe used in the nuclease protection experiments.
SieB phenotype

Δ 649  +
Δ 650  +
Δ 520  -
Δ 651  -
Δ 652  -
Δ 652a -
Figure 2. Sequence of the P22 genome in the vicinity of the $sieB$ gene. It was published first in (Franklin, 1985); nucleotides 1-104 were re-examined in (Semerjian et al., 1989) and 104-973 were revised in this study (see text). Deletion end-points are numbered and indicated by Δ; the underlined nucleotide is the last nucleotide that is absent in the deletion mutant. The six initiation codons (ATG) discussed in the text are underlined. The deduced amino acid sequence of SieB is shown; the serine whose codon was mutated to amber is shown in bold and underlined. The -35 and -10 hexamers of the $sieB$ promoter and the transcription start-site are indicated in bold; the $P_{sieB}$ -35 and -10 mutations are indicated immediately above the hexamers. Complementary nucleotides of the likely 5' and 3' ends of sas RNA (see Chapter 3) are boxed.
Figure 3. Autoradiogram of $^{35}$S-labelled proteins expressed in maxicells and separated by sodium dodecyl sulphate-polyacrylamide (10%) gel electrophoresis (tricine system). Lanes: 1, no plasmid; 2, pBR322 with its tetracycline resistance-conferring gene deleted; 3, pKR682—a P22 $sieB$ expressing plasmid; 4, a derivative of pKR682 that has an amber allele (Ser4am) of $sieB$; 5, pTP462—a plasmid expressing $\lambda$ $sieB$. Arrowheads indicate position of $\beta$-lactamase, P22 and $\lambda$ SieB and 'SieBinh' (SieB inhibitor). The numbers on right indicate molecular weight markers (Pharmacia) in kilodaltons.
Figure 4. RNase protection experiment to detect P22 sieB mRNA. Lanes: 1, RNA isolated from a SieB+ lysogen; arrow indicates position of the approx. 350 nucleotide protected riboprobe; 2, RNA isolated from a P22 lysogen that has a deletion in the ral-sieB region; 3, RNA isolated from a P_{sieB}-35 lysogen; 4, RNA isolated from a P_{sieB}-10 lysogen; 5, untreated riboprobe; 6, markers—HinfI fragments of pBR322, filled-in by the Klenow fragment of *E. coli* DNA polymerase I in the presence of α-32P-labelled-dATP. The numbers on the side indicate the length of each fragment in nucleotides. Note that lanes 5 and 6 are taken from a shorter exposure of the same gel.
Figure 5. Map of the \( \lambda \) genome in the vicinity of the \( sieB \) gene. Sequences present in the \( sieB \)-expressing plasmids constructed for these studies are indicated by solid lines below; large bold arrow represents the \( lacUV5 \) promoter.
Figure 6. The *sieB* promoter region. The DNA sequence around the transcription start-site of P22 *sieB* is shown at the top; the -35 and -10 hexamers of the P22 *sieB* promoter are underlined; the homologous sequence in λ upstream of its *sieB* gene is shown below. The likely λ *sieB* promoter is underlined as well. The ten nucleotide direct-repeat referred to in the text is shown in bold.
P22

TTCTCGTGTACCCCTACAGCGAGAAATCGGATAAACTCTATTCACCCCTACAGAGGAGTAAAAAGAGAA

TTCTCGTGTACCCCTACAGCGAGAAATCGGATAAACTATTACAAACCCCTACAGTTTGATGAGTATAGAA

λ
Figure 7. Hydrophilicity profiles of P22 (panel A) and λ SieB (panel B).
Hydrophilicity was analysed using the MacVector protein analysis program, using the algorithm of Kyte and Doolittle (1982). A window size of 20 residues was used.
A. P22 SieB

Hydropphilicity

B. λ SieB

Hydropphilicity
CHAPTER 3

A Switch in Translation Mediated by an Antisense RNA
INTRODUCTION

In prokaryotes, antisense RNAs have been shown to regulate gene expression in a variety of ways. In some cases antisense RNAs regulate expression of the target gene by base-pairing with its ribosome-binding site and preventing translation initiation; in other cases they have been shown to cause premature transcription termination by hybridizing with the target RNA and forming a structure that mimics a transcription terminator. In another case, the antisense RNA has been shown to destabilize the target mRNA by creating a site for RNase III cleavage (for reviews, see Simons and Kleckner, 1988; Takayama and Inouye, 1990). On this theme of regulation by antisense RNA, we present a variation. We describe a case in which the target gene encodes two polypeptides, and the antisense effector RNA causes a switch in phenotype by selectively inhibiting synthesis of one of them.

Bacteriophage P22 is a temperate Salmonella phage that closely resembles coliphage λ in its genetic structure and the regulation of expression of its genes (for a review of P22 biology, see Poteete, 1988). Like other temperate phages, P22 expresses only a handful of its genes in the prophage state; their function, at least in part, is to prevent the growth of superinfecting phages. One of these genes, sieB, aborts the lytic development of some superinfecting phages (Susskind et al., 1974b). In a SieB+ host, vegetative development of sensitive phages proceeds normally for about twenty minutes, but then there is an abrupt cessation of all—phage and host—macromolecular synthesis; as a result release of progeny phage is prevented and the host cell
dies. Susskind and co-workers showed that P22 itself is not susceptible to SieB-mediated exclusion because it harbours a determinant esc (for escape from SieB-mediated exclusion); they showed further that this P22 function can act in trans and is negatively regulated by the prophage repressor acting at the major leftward promoter of P22 (see Figure 1). We present below the likely molecular mechanism that P22 utilises to escape SieB-mediated exclusion.

In a previous study we identified the sieB gene and its promoter (Ranade and Poteete, submitted). The sieB gene, which encodes a polypeptide of 192 amino acid residues, is embedded in the PL operon; unlike other genes in this operon, which are read leftward and repressed in the prophage state, sieB is read rightward and expressed in a lysogen. We now show that the sieB gene encodes another polypeptide (referred to as Esc), which is, in fact, an inhibitor of the exclusion protein—SieB. The inhibitor, Esc, is a truncated version of SieB itself; it lacks the N-terminal twenty-five amino acid residues, and is synthesized from an internal initiation codon in sieB. We present genetic evidence to show that superinfecting P22 synthesizes an antisense RNA which causes ribosomes to switch from synthesizing both the exclusion and inhibitor proteins (SieB and Esc respectively) to synthesizing only Esc, thereby allowing P22 to escape SieB-mediated exclusion. There is redundancy in the system because functional amounts of Esc can be made either by the prophage or the superinfecting phage. We show further that, in the presence of SieB, this switch in translation is essential for the lytic development of P22; a mutation that likely prevents this switch is lethal to vegetatively growing P22. We show, as a lagniappe, that P22 uses the same mechanism to disarm the sieB exclusion system of bacteriophage λ.
MATERIALS and METHODS

Bacteria

Escherichia coli W3110 lacQLB (Brent and Ptashne, 1981) was used for the propagation of plasmids; except for plasmids pKR687, pKR688 and pKR689, which were propagated in strain HB101 (Sambrook et al., 1989). Strain CSR603recA uvrA6 (Sancar and Rupert, 1978) was used for maxicell analysis. Salmonella typhimurium LT2 strains MS1868 (leuAam414 rm+) and DB53 (cysA1348 hisC527 fels) were provided by M. Susskind.

Plasmids

Plasmid pTP546 was constructed by cutting pAS474 (Semerjian et al., 1989) at the unique HindIII site followed by digestion with BAL31. The ends were filled-in by the Klenow fragment of E. coli DNA polymerase I in the presence of all four deoxynucleotides, and ligated in the presence of HindIII linkers. Plasmids pKR687, pKR688 and pKR689 were constructed as follows. The EcoRI-HindIII fragment from pKR682 (Ranade and Poteete, submitted) was replaced by a tant bearing EcoRI-HindIII fragment from pTP550 (Semerjian et al., 1989); the resulting plasmid was called pKR683. The large ori containing PstI-EcoRV fragments from plasmids pAS474, pTP546 and pTP520 (Ranade and Poteete, submitted) were ligated with the P_lacUV5-bearing PstI-PvuII fragment from pTP30 (Berget et al., 1983); the resulting plasmids were called pKR684, pKR685 and pKR686 respectively. These plasmids were cut with HindIII, filled-in, and digested with PstI. The resulting 'sieB/esc antisense-expressing' small fragments were ligated to the large ori containing PstI-AfIII (filled-in) fragment from pKR683. To minimize intra-molecular recombination,
the new plasmids were propagated in strain HB101. The recombinant plasmids are called pKR687, pKR688 and pKR689 respectively.

Mutant alleles of \textit{sieB/esc} were constructed using site-directed mutagenesis as described (Kunkel \textit{et al.}, 1987; Ranade and Poteete, submitted). Construction of the \textit{am4} and \textit{P_{sieB-10}} alleles of \textit{sieB/esc} has been described (Ranade and Poteete, submitted). The mutagenic oligonucleotides (mismatched nucleotides(s) underlined) used to construct the other mutations were as follows:

\begin{verbatim}
am14: 5' CATCCTTGTAGGAAAAACG
am22: 5' GCA TGAA ACTGTTTAAG
am29: 5' CAATAGATCTAGATGATTAG
am115: 5' CGAATGCCAAQTACGCTCTTTC
substitution1: 5' GTTCATCGGCGAAAGAGAAAAATGAGAGACATCCCG
          TGAATAGAGTTTATCCG 3'
substitution 2: 5' CTCTTTTACTCTGACATCCCCGGAATAGAGTTTATCC
sorfam 33: 5' ATAGAGTTTACCCGATTTC
\end{verbatim}

('\textit{am}' refers to the amber mutation that was introduced into the \textit{sieB/esc} gene, and the number that follows refers to that codon of \textit{sieB/esc} that was mutated to amber; \textit{sorf} refers to the short open reading frame in the P\textsubscript{L} operon that is discussed in the text and \textit{am33} indicates that the thirty-third codon in this orf was mutated to amber; substitutions 1 and 2 refer to the mutations that were introduced into the untranslated region of \textit{sieB/esc}). For crossing mutations into P22 (see below), a HindIII-EcoRV fragment, from an M13 clone carrying the mutation, was used to replace the wild-type HindIII-EcoRV fragment from pTP83 (Poteete, 1982). For maxicell analysis, derivatives of pKR682 were constructed
that carried the amber alleles of *sieB/esc*. This was accomplished by replacing the wild-type HindIII-EcoRV fragment from pKR682 with the mutation-bearing HindIII-EcoRV fragment from the corresponding M13 clone. Substitution 1 and substitution 2 were combined with am115 as follows. Mutation-bearing HindIII-Hpal fragments were used to replace the corresponding wild-type fragment from a derivative of pKR682 carrying the am115 allele of *sieB/esc*. HindIII-EcoRV fragments (bearing both mutations) from such derivatives of pKR682 were used to replace the corresponding fragment from pTP83 as outlined above.

Out-of-frame deletions in *sieB/esc* were constructed as follows. 433 bp and 601 bp HindIII-Xmnl and HindIII-Hpal fragments respectively, from plasmid pKR682 were ligated with the large ori-containing HindIII-EcoRV fragment from pTP83. The resulting plasmids, in effect, have the Xmnl-EcoRV or Hpal-EcoRV regions in *sieB/esc* deleted (see Figure 1). These deletions were crossed into P22 by homologous recombination as described below.

In order to synthesize large amounts of riboprobe, 426 bp Sall-Hpal and 732 bp HindIII-EcoRV fragments from pTP650 (Ranade and Poteete, submitted) and pKR682 respectively were cloned into the polylinker of pGEM-4Z (Promega). The orientation of the inserts in the resulting plasmids is such that riboprobes complementary to PL RNA can be made by digesting with the appropriate restriction endonuclease and transcribing with T7 RNA polymerase.

**Phage**

In some of the experiments, L was used as the control Esc" phage (Susskind *et al.*, 1974). Deletion-bearing virulent P22 were constructed by crossing P22 *sieA44* bearing deletions in *sieB/esc* with P22 *vir3*. DNA was
extracted from stocks of virulent progeny phage and tested for the presence of deletions with restriction endonucleases and gel electrophoresis. Mutant alleles of \textit{sieB/esc} were introduced into virulent P22 by crossing mutation-bearing plasmids with P22 \textit{vir3 $\Delta$474}. This phage was constructed by crossing P22 $\Delta$474 (Semerjian \textit{et al.}, 1989) with P22 \textit{vir3}, selecting for virulence and screening for the deletion. P22 \textit{vir3 $\Delta$474} has a large deletion in the \textit{kil-ral} region; since the phage lacks gene 17, it is unable to grow on a fels lysogen (Susskind and Botstein, 1978). \textit{Salmonella} strain MS1868 was transformed with mutant derivatives of plasmid pTP83, and plasmid by phage crosses (using P22 \textit{vir3 $\Delta$474}) were performed as described (Semerjian \textit{et al.}, 1989). The lysates from the crosses were plated on \textit{Salmonella} strain DB53 (a fels lysogen) to select for recombinants that had picked up gene 17 from the plasmid. A large proportion of these also pick up mutations in \textit{sieB/esc}. Virulent progeny were identified by their ability to form plaques on a P22 \textit{sieA44 a1 sieB::Kn567} lysogen (Ranade and Poteete, submitted). Virulent P22 carrying mutations in \textit{sieB/esc} were identified by DNA sequencing, as described (Rennell \textit{et al.}, 1991). P22 \textit{vir3 sorfam33 sieB/escam115} was obtained essentially as described above except that P22 \textit{vir3 $\Delta$474 sieB/escam115} was used instead of P22 \textit{vir3 $\Delta$474}. P22 \textit{vir3 $\Delta$474 sieB/escam115} was obtained by crossing P22 \textit{vir3 $\Delta$474} with a derivative of pTP83 bearing the am115 allele of \textit{sieB/esc}. Recombinant phage were identified by their inability to form plaques on a fels-lysogen and on cells expressing $\lambda$ \textit{sieB} from the \textit{lacUV5} promoter. P22 \textit{vir3 sub2} was constructed as follows. A derivative of pTP83 bearing \textit{substitution 2 sieB} was constructed and transformed into \textit{Salmonella} strain MS1868. A plasmid by phage cross was
performed. When the lysate from such a cross was plated on DB53 (to select for recombinants that had picked up gene 17) few progeny phage were recovered, and the few tested did not carry substitution 2. Consequently, the lysate was then plated on DB53 that constitutively expressed the SieB-inhibitor—Esc—by virtue of having a derivative of plasmid pKR682 that expressed an amber allele of sieB (am22). A number of virulent P22 were picked and their plating behaviour on cells that do or do not express Esc was tested. Some of these plated with high efficiency only on cells that expressed Esc, and some plated with high efficiency on both. We inferred that the former class had substitution 2; whereas the latter was wild-type.

**Maxicells and RNase protection**

Maxicell analysis was performed as described (Silhavy *et al.*, 1984). RNase protection was performed essentially as described (Ranade and Poteete, submitted), except that 10-15 μg of RNA was used; RNase A was used at a final concentration of 40 μg/mL (instead of nuclease P1), and proteinase K digestion (final concentration 300 μg/mL, in the presence of 3% SDS) was used to degrade ribonucleases. Riboprobes were synthesized as described by the supplier of plasmid pGEM-4Z (Promega). Following synthesis, the riboprobes were purified by fractionating on a 5% acrylamide:8 M urea gel. Riboprobes were eluted from the gel as described (Sambrook *et al.*, 1989). RNA was isolated using the 'lysozyme freeze-thaw' method as described (Belfort *et al.*, 1990). Phage infections to determine the status of P_L RNA were performed as follows. Cells were grown in LB medium at 37°C to a density of 2×10^8 cells/mL. The cells were then infected with P22 vir3 at a multiplicity of infection of 5.
Phage were allowed to adsorb at room temperature for 5 min, and the infected cultures were aerated at 37°C. Approximately 10, 20 and 30 min after infection, 30 mL of culture was withdrawn and treated as described (Belfort et al., 1990). Just before infection, 30 mL of culture was withdrawn as the uninfected control.
RESULTS

esc as Antisense RNA

Studies by Susskind and co-workers had shown that P22 is not susceptible to the lethal effects of sieB because it harbours a determinant called esc (for escape from superinfection exclusion). They showed that sieB and esc are tightly linked, but not identical—point mutations in sieB are phenotypically Esc+. To map esc precisely, we crossed deletions that had been used to map sieB (Ranade and Poteete, submitted) into a virulent background and tested the ability of the resulting recombinant phages to form plaques on a SieB+ host. As shown in Figure 1, these deletions have a common left end-point (at the HindIII site in ral) and extend rightward into the sieB gene.

P22 deletion mutants Δ649 and Δ650 are impervious to SieB-mediated exclusion—they plate as well on a SieB+ lysogen as on a P22 lysogen that has a large deletion in its sieB gene. On the other hand, Δ520 and deletions larger than Δ520 are extremely sensitive to SieB—they plate 100-1000 fold less efficiently on a SieB+ cell as compared to their plating efficiency on a SieB- host. Combined with previous studies (Susskind et al., 1974b; Semerjian et al., 1989), these results indicate that sequences to the left of the deletion end-point of Δ650 are not essential for P22 to grow on a SieB+ cell, but a critical esc determinant lies in the 120 nt between the right end-points of deletions Δ650 and Δ520.

Having mapped crudely the left end-point of esc, we attempted to identify its right end-point. Two out-of-frame deletions in the sieB gene were constructed, crossed into virulent P22, and their Esc phenotypes were tested.
As shown in Figure 1, both deletions have a common right end-point at the EcoRV site in sieB. The large deletion extends leftward for 300 bp to the XmnI site and the short deletion extends for 130 bp to the Hpal site. As indicated in Figure 1, P22 bearing either deletion plates as efficiently on a SieB+ cell as does wild-type P22, suggesting that sequences to the right of the XmnI site are dispensable for Esc function.

The 360 bp region between the end-point of \(\Delta649\) and the XmnI site has a small open reading frame (referred to as sort) of 71 codons reading leftward in the conventional prophage genetic map (indicated as a box just below the genetic map shown in Figure 1). In the light of earlier results (Susskind et al., 1974b), suggesting that the esc gene was part of the leftward reading PL operon, sort could be esc, although \(\Delta650\) deletes the C-terminal seventeen codons of this orf but is still Esc+. A formal possibility is that a truncated polypeptide synthesized by this deletion mutant retains function. To test this possibility, codon 33 of this open reading frame was mutated to an amber codon. Virulent P22 bearing this mutation shows no phenotype—it plates as efficiently on a \(sup^0\) SieB+ cell as does wild-type P22. This result suggests that expression of sort is not necessary for P22 to by-pass SieB-mediated exclusion.

Taken together these results would appear to point to an antisense RNA being responsible for by-passing SieB-mediated exclusion. Some antisense RNAs regulate gene expression by hybridizing to the ribosome-binding site on target mRNAs, thus preventing translation initiation. The critical esc determinant maps almost precisely to the untranslated region of sieB—\(\Delta650\) is Esc+ and the PL transcript synthesized by this mutant would retain the ability to base-pair with the entire sieB untranslated region; \(\Delta520\), on the other hand, lacks the region
that would base-pair with the untranslated region of \textit{sieB} mRNA, and is Esc\(^{-}\). If \textit{sieB} antisense RNA, synthesized by superinfecting P22, acting on the untranslated region of \textit{sieB} mRNA, were indeed responsible for allowing P22 to grow in a SieB\(^{+}\) cell, then one might expect that mutations in the antisense effector that abolish base-pairing with the target \textit{sieB} mRNA, would render P22 susceptible to SieB-mediated exclusion. To test this possibility a large deletion/substitution was introduced in the untranslated region of \textit{sieB}. This mutation—substitution 1 (see Figure 3B)—replaces almost the entire untranslated region of \textit{sieB} mRNA (24 out of 31 nucleotides). The substitution was crossed into virulent P22 and its Esc phenotype tested. As indicated in Table 1, substitution 1-bearing P22 had no phenotype—P22 \textit{substitution 1} plates as well as does wild-type P22 on a SieB\(^{+}\) cell. This result, prima facie, would rule out base-pairing between antisense RNA and the untranslated region of \textit{sieB} mRNA as a necessary part of the mechanism by which P22 bypasses SieB-mediated exclusion. As we show below however, this result reflects redundancy in the devices that P22 elaborates to circumvent SieB-mediated exclusion—knocking out the antisense effector reveals the action of the inhibitor, Esc.

\textbf{esc as Protein}

When expression of proteins was analysed, using maxicells, from the \textit{sieB} region of P22, we discovered that two proteins are expressed from this region (see Figure 2B, lane 3). Based on the phenotype of an amber mutation (am4 in Figure 2A and 2B), we inferred that the larger polypeptide is expressed from the \textit{sieB} gene (Ranade and Poteete, submitted). An examination of the
sequence of the *sieB* gene revealed four in-frame initiation codons downstream of the *sieB* initiation codon (numbered two through five in Figure 2A). To test the possibility that the smaller polypeptide is due to initiation at one of these ATG's, amber mutations were introduced between consecutive ATG's; an amber mutation was introduced toward the C-terminus of *sieB* as well. Derivatives of pKR682 bearing these amber alleles were constructed, and expression of proteins from these plasmids was analysed using maxicells. (Plasmid pKR682 is a derivative of pBR322 bearing the HindIII-MluI rat-*sieB* region of P22; see Figure 1.) All five amber mutations confer a SieB− phenotype (data not shown), and as shown in Figure 2C prevent synthesis of the larger—SieB—polypeptide. Only am29 and am115 prevent synthesis of the smaller—Esc—polypeptide; am14 and am22, on the other hand, result in slight overproduction of Esc, perhaps due to translation reinitiation by ribosomes terminating at these amber codons. Two conclusions can be drawn from these results: first, SieB and the smaller polypeptide (Esc) are synthesized from the same open reading frame. Second, Esc is initiated, most likely, from the fourth ATG in the *sieB* gene.

By analogy with other systems where a truncated protein inhibits the function of the full-length polypeptide (for example, p1 and p2 proteins of transposon Tn5[Isberg *et al.*, and Johnson *et al.*, 1982]; S105 and S107 proteins of bacteriophage λ [Young, 1992]), we guessed that Esc may be an inhibitor of SieB-mediated exclusion. Indeed when a plasmid expressing the am22 allele of *sieB*, which abolishes synthesis of SieB but not Esc, was introduced into a SieB+ lysogen, it converted the cell to a SieB− phenotype (data not shown). We interpreted this result to mean that Esc expressed from
the plasmid inhibited the action of endogenous full-length SieB. If Esc were responsible for neutralizing endogenous SieB in a P22-superinfected cell, then one would predict that superinfecting P22 bearing amber mutations in sieB upstream of the esc initiation codon (am4, am14 and am22; see Figure 2A) would be able to by-pass SieB-mediated exclusion. P22 bearing amber mutations within sequences encoding both sieB and esc (am29 and am115) however, would be sensitive to SieB. To test this prediction, all five amber mutations were crossed into virulent P22, and the ability of P22 bearing these amber mutations to grow on a SieB+ cell was tested. As shown in Table 1, not one of the amber mutations has an effect on P22's ability to grow on a SieB+ cell; P22 strains bearing amber mutations which prevent synthesis of the inhibitor, Esc—am29 and am115—plate with the same high efficiency as do P22 strains with amber mutations that do not diminish synthesis of Esc—am4, am14 and am22. These results would appear to rule out a role for Esc in P22's ability to by-pass SieB-mediated exclusion. However, as pointed out earlier, this reflects redundancy in the devices that P22 uses to circumvent SieB-mediated exclusion—knocking out the inhibitor, Esc, reveals the action of sieB antisense RNA.

Superinfecting P22 requires sieB antisense RNA or Esc protein to circumvent SieB-mediated exclusion

Since mutations that would: i) prevent sieB antisense RNA from interacting with its sense partner (substitution 1), ii) prevent synthesis of a small polypeptide encoded by the P_L operon (sorfam33), or iii) prevent synthesis of the SieB-inhibitor—Esc (sieB/esc am29 and am115), were found not to confer
an Esc^− phenotype, it seemed possible that superinfecting P22 requires either a function encoded by the PL operon (sieB antisense RNA or the small polypeptide encoded by sorf), or Esc protein, but not both, to bypass SieB-mediated exclusion. To test this possibility, P22 strains bearing substitution 1 or sorfam33 in combination with the sieB/escam115 allele were constructed, and their ability to grow on a SieB^+ host was tested. The results are presented in Table 1. As can be seen, P22 sorfam33 sieB/escam115 plates as efficiently on a SieB^+ host as does wild-type P22. P22 sub1 sieB/escam115, however, plates inefficiently. None of the mutations alone has any effect on the plating behaviour of P22 (Figures 1, 2 and Table1). On the control SieB^− host however, the sub1 sieB/escam115 double mutant and wild-type P22 plate with the same high efficiency. These results strongly indicate that a P22 strain unable to synthesize its own Esc relies on sieB antisense RNA (and not a small polypeptide encoded by the PL operon) to bypass SieB-mediated exclusion.

In the experiment described above the antisense effector of the infecting phage was wrecked by mutation, thus revealing the role of Esc. We reasoned that the converse should also be true, i.e., destroying, by mutation, the ability of the endogenous target sieB mRNA to interact with the wild-type antisense effector should reveal superinfecting P22's dependence on Esc for bypassing SieB-mediated exclusion. We could not use substitution 1 to test this because it confers a SieB^− phenotype (data not shown). Consequently, another mutation was introduced in the untranslated region of sieB. This mutation—substitution 2 in Figure 3B—replaces nine nucleotides (two through eleven) in the sieB untranslated region with unrelated sequence. A derivative of pKR682 was constructed carrying this mutation, and the SieB phenotype of cells bearing this
derivative was tested. *Salmonella* expressing *sub2 sieB* is phenotypically
SieB⁺; it excludes an Esc⁻ phage, but not wild-type P22 (Table 1 and data not shown). The plating behaviour of P22 bearing various amber alleles of *sieB/esc* was tested on cells expressing *sub2 sieB*, and the results are presented in Table 1. As expected, P22 strains bearing amber mutations upstream of the *esc* initiation codon—am4, am14 and am22—plate as efficiently as does wild-type P22. P22 bearing mutations in *esc*—am29 and am115—however, plate inefficiently. The plating behaviour of P22 bearing another mutation, *PsieB*-10, was also tested. This mutation alters the -10 hexamer of the *sieB* promoter, thus preventing synthesis of *sieB* mRNA (Ranade and Poteete, submitted). P22 *PsieB*-10 plates efficiently on cells expressing wild-type *sieB*, suggesting that the *PsieB*-10 mutation has no effect on the antisense effector. On cells expressing *sub2 sieB* however, the mutant plates with low efficiency. This latter result indicates that Esc is synthesized from the same transcript as SieB.

One might expect that mutations that restore the ability of the antisense effector RNA to base-pair with the target *sub2 sieB* mRNA, should restore the ability of P22 *escam115* to plate efficiently on cells expressing *sub2 sieB*. To test this possibility, substitution 2 was combined with *sieB/escam115* on a plasmid and then introduced into P22 by homologous recombination. As expected, this phage plates inefficiently on cells expressing wild-type *sieB*, but it also grows poorly on cells expressing *sub2 sieB* (see Table 1). P22 *sieB/escam115* bearing the larger substitution 1 also plates inefficiently on both cell-types. On a SieB⁻ cell however, both phages plate with high efficiency. These results suggest that substitution 2 wrecks the ability of the
sense/antisense pair to interact with each other even when it is borne by both effector and target RNAs. This could be due to a number of reasons, and these are discussed below.

A P22 Mutant that Commits Suicide

Attempts to cross substitution 2 alone (in the absence of am115 in sieB/esc) into P22 failed; few progeny phage were recovered from the cross by plating on wild-type Salmonella, and among these the few tested were non-recombinants (data not shown). In the light of results presented above—the inability of P22 sub2 sieB/escam115 to grow on cells expressing sub2sieB—it seemed possible that substitution 2 by itself is lethal to P22, by preventing the phage from shutting off synthesis of its own SieB. We reasoned that substitution 2-carrying P22 might be prevented from committing SieB-induced suicide if the host were constitutive for the SieB inhibitor—Esc. This is indeed the case. Substitution 2-carrying P22 (see Materials and Methods) was tested for its plating efficiency on cells that do or do not express Esc. P22 sub2 plates 100-fold more efficiently on Salmonella that expresses the SieB-inhibitor, Esc, than on cells that are deficient in Esc. (The host was constitutive for Esc by virtue of harbouring a derivative of pKR682 that carried the sieB/escam22 allele, and the control Esc- cells carried another derivative of pKR682 bearing the sieB/escam115 allele.) The lethality of substitution 2 is due solely to SieB made by the infecting phage because P22 bearing substitution 2 in combination with the sieB/escam115 allele plates with high efficiency on Esc- and Esc+ cells. This result suggests that wild-type P22 does not ‘SieB-exclude’ itself during lytic growth because antisense RNA inhibits synthesis of SieB.
**sieB/esc Antisense RNA inhibits synthesis of SieB but not Esc**

To test the effect of antisense RNA on the synthesis of SieB and Esc, plasmids expressing different lengths of **sieB/esc** antisense RNA were constructed; their structures are indicated in Figure 4A. These plasmids are derivatives of pBR322 bearing the **sieB/esc** region of P22 (but not PL, from which the antisense RNA is normally expressed). In addition, but at a different location, they have different fragments of the **sieB/esc** region, which when transcribed from the heterologous lacUV5 promoter produce different lengths of **sieB/esc** antisense RNAs. All three plasmids would direct synthesis of antisense RNA initiated at the EcoRV site in **sieB/esc**. Plasmid pKR687 would make a ~732 nt antisense RNA up to the HindIII site in ral. Plasmid pKR688 would direct synthesis of a ~528 nt antisense RNA that would terminate between the deletion end-points Δ650 and Δ520 (see Figure 1). Plasmid pKR689 would make a ~448 nt antisense RNA up to the end-point of Δ520. To ensure that antisense RNA of the expected sizes are synthesized, a strong transcription terminator $t_{ant}$ was placed after these fragments in all three plasmids.

The SieB phenotypes of these plasmids were determined (Figure 4A), and expression of proteins from these plasmids was analysed using maxi-cells (Figure 4B). As can be seen in lane 2, in the absence of any **sieB/esc** antisense RNA, SieB and Esc are synthesized. The control plasmid—pBR322 with its tetracycline resistance conferring gene deleted—directs synthesis of neither SieB nor Esc, as expected (Figure 4B, lane 1). Plasmid pKR687 confers a SieB⁻ phenotype, and in maxicells directs synthesis of Esc but not SieB (Figure
suggesting that this antisense RNA causes a switch in the translation of \textit{sieB/esc} RNA—from directing synthesis of both SieB and Esc to Esc alone. Plasmids pKR688 and pKR689 confer a SieB\textsuperscript{+} phenotype, and in maxicells direct synthesis of SieB and Esc (Figure 4B, lanes 4 and 5 respectively). The result with pKR688 suggests that sequences in the \textit{PL} transcript which lie beyond the region of complementarity with \textit{sieB} mRNA are required for the antisense RNA to function. Interestingly, expressing antisense RNA complementary to Esc has little or no effect on its synthesis (compare lanes 2 and 5). Plasmid pKR688 seems to overproduce SieB and Esc; we do not understand the basis for this.

**The \textit{sieB} Antisense Effector RNA—\textit{sas}**

Antisense RNAs that inhibit translation of target mRNAs are, in general, small molecules. In the case of \textit{sieB/esc} however, studies by Susskind and coworkers indicated that \textit{esc} is under negative control from \textit{OL/PL}, suggesting that the \textit{sieB} antisense RNA would be initiated at \textit{PL}, thus making it a relatively large species. It seemed possible that a small processed form of the \textit{PL} transcript might be the antisense effector. Such an effector would have to lie in the region identified as being important by the deletion mutants described before (\textit{Δ650} and \textit{Δ520}, see Figure 1). We looked for such a molecule in phage-infected cells using an RNase protection assay, and the results are presented in Figure 5. Wild-type \textit{Salmonella} was infected with virulent P22 and RNA was isolated 10, 20 and 30 minutes after infection. The riboprobes used in the experiment are shown in Figure 5A; two of the riboprobes ('Hpa' and 'Nde') have a common 5' end which is 75 bp upstream of the transcription start-site of
The 'Hpa' probe extends to the Hpal site and has ~426 nt of complementarity with the PL transcript. The 'Nde' probe has ~175 nt of complementarity and extends to the Ndel site in sieB/esc. The third probe ('Xmn') has a different 5' end; it extends from the HindIII site in ral for 426 nt to the Xmnl site in sieB/esc. As can be seen in Figure 5B, the Hpa probe is fully protected by RNA extracted 10 min post-infection (where observed, the difference in size between the fully protected and the untreated probes is due to the presence of vector sequences in the probe); between 10 and 20 min after infection though, there is a decrease in the full-length probe with a concomitant increase in a 105 nt protected fragment. This pattern is mirrored in the experiment with the Nde probe, suggesting that the 5' end of the 105 nt fragment is to the left of the Ndel site. A 105 nt protected riboprobe is obtained even when one uses a probe with a different 5' end (Xmn probe). Taken together, these results indicate that the PL transcript (in this region) is processed into a 105 nt RNA species and this processed fragment lies within the boundaries of the Nde probe. In addition to the 105 nt fragment, the Xmn probe yields three other protected species. The largest of these is the full-length probe. We suspect that the other two (280 and 300 in Figure 5B) are partially processed forms of the PL transcript; their likely provenance is diagrammed in Figure 5A. These two partially processed RNAs also allow one to identify the most likely ends of the 105 nt fragment—the 5' end of this RNA is at or very near the sieB initiation codon and the 3' end is ~70 nt to the left of the sieB/esc transcription initiation site. We note that the sequence encompassed by this 105 nt RNA is precisely the region of importance identified by deletion mapping (Figure 1) and substitution mutations (Figure 3 and Table 1). We
conclude that the \textit{sieB} antisense effector RNA is, most likely, a 105 nt processed form of the PL transcript, referred to, from now on, as \textit{sas (sieB antisense)} RNA.

Another point that deserves to be mentioned is that the region of PL RNA 5’ to the 105 nt \textit{sas} RNA is rapidly degraded; one can see only two protected species with the Hpa probe—full-length probe and the 105 nt fragment. This observation suggests how P22 can synthesize Esc in the presence of the PL transcript (because, of course, the PL transcript is antisense to \textit{esc} mRNA too)—the region of the PL transcript that can base-pair with \textit{esc} mRNA is rapidly degraded.

\textbf{Esc, but not sas RNA, allows P22 to bypass SieB-mediated exclusion by bacteriophage \(\lambda\)}

Susskind and co-workers showed that coliphage \(\lambda\) too has a \textit{sieB} gene and this excludes the same \textit{Salmonella} phages as P22’s \textit{sieB}, although there is little or no homology between the two genes. They also showed that P22 is insensitive to \(\lambda\) SieB-mediated exclusion (Susskind and Botstein, 1980). We present below evidence to suggest that Esc, but not \textit{sas} RNA, allows P22 to circumvent \(\lambda\) SieB-mediated exclusion.

The relevant region of the \(\lambda\) genome is shown in Figure 6A. The structures of plasmids bearing the \textit{sieB} gene are shown below. Plasmid pTP462 has the \textit{ea10-ral-sieB} (and a part of \textit{N} as well) region of \(\lambda\); consequently in this plasmid, \(\lambda\) \textit{sieB} is expressed, most likely, from its own promoter. The region indicated in bold is homologous to that of P22; the untranslated regions of the \textit{sieB} genes of the two phages are homologous, but not identical (see Figures 3B and 6B). Plasmid pTP482 has the \textit{sieB} gene
under control of the heterologous *lacUV5* promoter; the fusion is such that the entire untranslated region of *sieB* is replaced by unrelated sequence from the vector (see Figure 6B). The plating behaviour of some of the P22 mutants already described was tested on cells bearing these plasmids. The results are indicated in Table 2. As can be seen, wild-type P22 plates efficiently on *Salmonella* bearing either pTP462 or pTP482. P22 bearing an amber in *esc* (*sieB/escam115*) or the *sieB/esc* promoter mutation (PsieB-10) however, plates at least 100-fold less efficiently on cells expressing λ *sieB* from its own promoter (pTP462) or from the *lacUV5* promoter (pTP482). P22 bearing an amber mutation upstream of the *esc* initiation codon (am22), however, plates efficiently on both cell types. These results suggest that P22’s Esc is able to neutralize the action of λ SieB, but P22 sas RNA is unable to prevent neutralize of λ SieB.
under control of the heterologous *lacUV5* promoter; the fusion is such that the entire untranslated region of *sieB* is replaced by unrelated sequence from the vector (see Figure 6B). The plating behaviour of some of the P22 mutants already described was tested on cells bearing these plasmids. The results are indicated in Table 2. As can be seen, wild-type P22 plates efficiently on *Salmonella* bearing either pTP462 or pTP482. P22 bearing an amber in *esc* (sieB/escam115) or the *sieB/esc* promoter mutation (PsieB-10) however, plates at least 100-fold less efficiently on cells expressing *λ* *sieB* from its own promoter (pTP462) or from the *lacUV5* promoter (pTP482). P22 bearing an amber mutation upstream of the *esc* initiation codon (am22), however, plates efficiently on both cell types. These results suggest that P22's Esc is able to neutralize the action of *λ* SieB, but P22 sas RNA is unable to prevent synthesis of *λ* SieB.
DISCUSSION

In Figure 7 we present a molecular mechanism to explain P22's ability to circumvent SieB-mediated exclusion. In a lysogen, the sieB/esc genes are expressed from a weak promoter just upstream of the sieB initiation codon; the strong leftward promoter PL is repressed by the action of c2 repressor. The 'SieB-booby-trap' is set at this ratio of SieB (the exclusion protein) to Esc (the inhibitor protein); the trap does not go off in a lysogen, presumably because the trigger that activates the lethal action of SieB is present only during the lytic phase. Upon superinfection by virulent P22, the situation changes dramatically. There is a burst of transcription from the two major promoters of the superinfecting phage, PL and PR, and low level transcription from the sieB/esc promoter as well. The region of the PL transcript, which is antisense with respect to sieB/esc mRNA is processed into a 105 nt sieB antisense effector RNA, sas; moreover, the region of the PL transcript that can base-pair with esc mRNA is rapidly degraded. Sas RNA then binds sieB/esc mRNA (synthesized from the prophage and superinfecting phage) and causes a switch in translation—from directing synthesis of both SieB and Esc, sieB/esc mRNA now directs synthesis of only Esc. In essence, superinfecting P22 circumvents SieB-mediated exclusion by altering the ratio of SieB to Esc. This mechanism has redundancy built into it because sas RNA alone (by hijacking the endogenous sieB/esc mRNA) or Esc alone (perhaps by competing for a limiting substrate) can neutralize the action of SieB.

Several lines of evidence support this model. Amber mutations in sieB/esc have no effect on the plating efficiency of P22 on cells expressing wild-
type sieB (Figure 2), presumably because these phages are proficient at making sas RNA. Similarly a mutation that would hinder base-pairing between sas RNA and the target sieB mRNA (substitution 1 in Figure 3) has no effect on P22's ability to grow in a SieB+ host because the phage can still make Esc. When superinfecting P22 can make neither sas RNA nor Esc (Δ520 and larger deletions, Figure 1; sub1 sieB/escam115 and sub2 sieB/escam115, Table 1), it is no longer able to grow in a host expressing wild-type sieB. Alternatively, if the target sieB mRNA is rendered unresponsive to the translational switch induced by sas RNA, then superinfecting P22 depends on its own Esc to bypass SieB-mediated exclusion (P22 sieB/escam29 and am115; PsieB-10, Table 1). The switch induced by sas RNA is essential to vegetatively developing P22—substitution 2, which renders sieB mRNA unresponsive to its antisense partner, causes P22 to 'SieB-exclude' itself upon infection of a non-lysogen. When sieB is inactivated by an amber mutation (am115) however, substitution 2 is innocuous. Lastly, artificial antisense RNAs inhibit the synthesis of SieB but not Esc (Figure 4).

sas RNA

The status of the effector that causes the translational switch as an RNA molecule seems reasonably secure: i) Mutations that render P22 susceptible to the lethal effect of SieB (in the absence of Esc) map to the untranslated region of sieB which is where many antisense RNAs act to regulate gene expression. ii) The only substantial leftward open reading frame in the region can be inactivated by mutation (by deleting the C-terminal third or by introducing an amber in the N-terminal third) with no effect on P22's ability to
develop in a SieB+ host, even when the phage cannot synthesize its own Esc. We note that this does not rule out an accessory role for this leftward orf in P22’s ability to by-pass SieB; it could, for instance, enhance the rate of base-pairing between the antisense and target RNAs. This small polypeptide would have to be synthesized early in infection because its mRNA is processed into sas RNA.

iii) The PL transcript is processed into a small 105 nt molecule, and this molecule encompasses the region identified, by mutational studies, as being important for P22’s ability to bypass SieB. The small size of sas is in harmony with other reported antisense RNA effectors, which are 50 to 110 nt in length.

The genetic approach to showing definitively that base-pairing interactions between two RNA molecules are important for the system in question is to isolate mutations in one which are compensated by mutations, which restore base-pairing, in the other. We have not yet shown this to be true of sas and sieB RNAs. Even when substitution 2 is present in both sieB mRNA and sas RNA, the sense and antisense RNAs are unable to interact with each other, making P22 susceptible to SieB-mediated exclusion. This could be due to at least two reasons. First, substitution 2 is a relatively large mutation (nine nucleotides are changed), and this could affect the structure of the target and effector RNAs. Second, substitution 2 is very near the processing site in the PL RNA, and it may affect the rate of processing or abolish processing completely. We note that most compensating mutations (those that restore sense and antisense pairing) that have been described are single base changes (Simons and Kleckner, 1988).

Several interesting questions about sas RNA and its action remain unanswered. We imagine that sas RNA acts by occluding the ribosome-binding
site of *sieB* mRNA, but it is also possible that it acts by destabilizing this part of *sieB/esc* RNA. Such an effect has been documented in the case of *OOP* antisense RNA and the *hok/sok* plasmid maintenance system (Krinke and Wulff, 1990; Gerdes *et al.*, 1992). Preliminary evidence, however, suggests that the stability of *sieB* mRNA is unaffected by *sas* RNA (unpublished observations). It is not clear whether processing of P₇ is absolutely essential for it to be an effective antisense RNA. Also, the host enzymatic activity that is responsible for processing is unknown. The c₄ antisense RNA of bacteriophage P1 is also processed from a larger precursor; in that system, the tRNA processing activity—RNAaseP—has been implicated (Citron and Schuster, 1992).

**Relation to other exclusion systems**

We have presented genetic evidence that indicates that P22's Esc can circumvent the SieB-mediated exclusion system of λ (Table 2). Although the untranslated regions of the *sieB* genes of the two phages are closely related, evidently *sas* RNA, acting alone, is not sufficient to allow P22 to by-pass λ SieB-mediated exclusion (Table 2). In contrast, *sas* RNA and Esc appear to make individually sufficient contributions to P22's ability to circumvent its own SieB (Figures 1 and 2). We can envisage at least two reasons for this difference. (1) It may be that *sas* RNA can base-pair with λ *sieB* mRNA but can't cause a translational switch, perhaps because λ *sieB* does not encode an Esc-like inhibitor. According to this hypothesis, to disarm SieB, preventing its synthesis is not enough—SieB molecules already present in the cell must be neutralized, and this is accomplished by Esc. (2) It is possible that blocking SieB synthesizing is sufficient, but P22 *sas* RNA might not bind λ *sieB* mRNA.
because of the seventeen base mismatch in the overlap regions of the two RNAs. If this is true, then it suggests why P22 has evolved Esc—it enables superinfecting P22 to by-pass any exclusion system that functions through the same host macromolecule(s) as does SieB. If the first possibility is correct, then it raises the question of how λ is able to by-pass its own SieB-mediated exclusion system. The answer may be that λ is naturally insensitive to SieB-mediated exclusion (perhaps it lacks the ability to trigger SieB). Indeed, a mutant of λ with a large deletion in its sieB gene (and therefore its putative esc gene and sas RNA) plates efficiently on E. coli expressing plasmid-borne λ sieB (unpublished observations).

The SieB/Esc system of exclusion that we have described resembles the RexA/RexB exclusion system elaborated by phage λ (for review, see Court and Oppenheim, 1983). This system is composed of two genes, rexA and rexB, and their gene products abort the growth of certain mutants of phage T4; Rex+ λ lysogens of Salmonella also exclude P22. Parma et al. (1992) showed that when rexB is overexpressed in a Rex+ λ lysogen, it confers a Rex- phenotype, a result reminiscent of the effect of Esc on SieB-mediated exclusion. In that system however, the biological relevance of this observation is not clear. They speculated that overexpression of rexB may be the mechanism by which an induced λ prophage does not 'Rex-exclude' itself.
Table 1

Esc phenotypes of P22 bearing different alleles of *sieB/esc* on cells expressing different alleles of *sieB*.

<table>
<thead>
<tr>
<th><em>sieB/esc</em> allele in P22</th>
<th><em>sieB</em> allele in host</th>
<th>wild-type</th>
<th>substitution 2</th>
<th>am115</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sorfam33</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>substitution 1</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>am4</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>am14</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>am22</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>am29</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>am115</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PsieB-10</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>substitution 1 am115</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>substitution 2 am115</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>sorfam33 am115</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
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</table>

*Salmonella* strain MS1868 was transformed with plasmid pKR682 bearing the wild-type allele of *sieB*, or with derivatives of pKR682 bearing the substitution 2 or am115 alleles of *sieB*. Plasmid-bearing cells were grown to late-log in LB+Ampicillin and 0.1 mL of culture was used to make lawns on LB-Ampicillin plates. Ten-fold serial dilutions of P22vir3 bearing the
indicated alleles of *sieB/esc* were spotted on such lawns, and the plates were incubated overnight at 30°C. A '-' indicates that the phage plates 100-1000 fold less efficiently on a particular host as compared to its plating efficiency on *Salmonella* expressing the am115 allele of *sieB/esc*; a '+' indicates that the plating efficiency is close to one.
Table 2

Esc phenotypes of P22 bearing different alleles of sieB/esc on cells expressing λ sieB

<table>
<thead>
<tr>
<th>sieB/esc allele in P22</th>
<th>sieB allele in hostb</th>
<th>pTP462 (P_{sieB-λsieB})</th>
<th>pTP482 (P_{lac-λsieB})</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>am22</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>am115</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PsieB-10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a This experiment was performed essentially as described in Table 1, except that IPTG (at a final concentration of 1 mM) was added to lawns of cells bearing plasmid pTP482. b A '-' indicates that the phage plates 100-1000 fold less efficiently on a particular host as compared to its plating efficiency on cells bearing the am115 allele of P22 sieB. A '+' indicates that the plating efficiency is close to 1.
Figure 1. A map of the P22 genome in the vicinity of the sieB/esc gene is shown. Open boxes below the map represent sequences deleted from the phage. 'Sorfam33' indicates the mutation introduced into sorf. A '-' indicates that the deletion mutant plates 100-1000 fold less efficiently on a SieB+ lysogen as compared to its plating efficiency on a P22 lysogen with a large deletion in its sieB gene. A '+' indicates that the plating efficiency is approximately one.
Figure 2. A: The \textit{sieB/esc} gene is shown with the amber mutations ('am') discussed in the text. The deduced initiation codons of \textit{sieB} and \textit{esc} are indicated as 'SieB ATG' and 'Esc ATG' respectively. B: Autoradiogram of $^{35}$S-labelled proteins expressed in maxicells and separated by sodium dodecyl sulphate-polyacrylamide (10%) gel electrophoresis (tricine system; Schagger and Jagow, 1987). Lanes: 1, no plasmid; 2, pBR322 with its tetracycline resistance-conferring gene deleted; 3-7, derivatives of pKR682 expressing different alleles of \textit{sieB/esc}. 3, wild-type; 4, am4; 5, am14; 6, am22; 7, am29; 8, am115. Arrows indicate positions of $\beta$-lactamase, SieB and Esc. The numbers on left indicate molecular weight markers (Pharmacia) in kilodaltons.
**Figure 3:** A: The *sieB/esc* gene is shown and the antisense P_L transcript is indicated as a broken arrow below. Sub1 and sub 2 refer to substitutions 1 and 2 respectively. B: The untranslated sequence of P22 *sieB* is shown; '+1' refers to the transcription start-site of *sieB*; the *sieB* initiation codon is underlined. Below the wild-type sequence are shown the changes present in the two substitutions.
A

\[ P_{\text{sieB}} \rightarrow \text{SieB} \quad \text{ATG} \quad \text{Esc} \quad \text{ATG} \quad \text{sieB (esc)} \]

Sub 2

Sub 1

\[ P_{\text{L transcript}} \]

B

Wild-type

\[ +1 \quad \text{ACCCCUACAGAGUAAAAAGAGAAUCGCGAUG} \]

Substitution 1

\[ \text{GGGGAUGUCUCUAAUUUUCUCUU} \]

Substitution 2

\[ \text{GGGGAUGUC} \]
Figure 4: A: Plasmids used to analyse the effect of antisense RNA on expression of sieB and esc. The HindIII-Mlu region present in the parent plasmid pKR682 is shown as a solid line below. Arrows indicate the extent of antisense RNAs that would be synthesised from the three plasmids. 'Placuv5' refers to the lacUV5 promoter, and t_{ant} indicates a transcription terminator. The SieB-phenotype conferred by these plasmids is also shown. A '+' indicates that cells bearing that plasmid plate an Esc^+ phage (L) 100-1000 fold less efficiently as compared to its plating efficiency on cells expressing an amber allele (am115) of sieB. A '-' indicates that the plating efficiency is approx. 1. B: Autoradiogram of 35S-labelled proteins expressed in maxicells and separated by SDS-PAGE. Lanes: 1, pBR322 with its tetracycline resistance-conferring gene deleted; 2, pKR682; 3, pKR687; 4, pKR688; and 5, pKR689. The positions of β-lactamase, SieB and Esc are indicated by arrows.
A

\[ \text{SieB phenotype} \]

<table>
<thead>
<tr>
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<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

- Bla
- SieB
- Esc
**Figure 5:** RNase protection experiment to detect sas RNA. A: The riboprobes used in the experiment are indicated as solid lines. Arrowheads under the Xmn probe indicate the likely processing sites in the PL transcript. '300' and '280' are the singly processed forms of PL RNA and '105' is the antisense effector RNA, sas; the numbers refer to the number of nucleotides in each species, inferred from the gel shown in B. Broken lines indicate that the RNA species is longer than that detected by the Xmn probe. B: Autoradiogram of a 6% polyacrylamide:8 M urea gel used to separate riboprobes protected from nucleases by RNA extracted from phage-infected cells. Xmn, Hpa and Nde refer to the three riboprobes shown in A. 'P' is untreated riboprobe; 'U' is RNA extracted from uninfected cells. The numbers 10, 20 and 30 indicate that RNA was extracted 10, 20 and 30 min after phage infection. The '300', '280' and '105' nt fragments discussed in the text are marked on the right. Markers (M) are Hinfl fragments of pBR322, filled-in by the Klenow fragment of *E. coli* DNA polymerase I in the presence of α-32P-labelled dATP. The numbers on the left indicate the length of each fragment in nucleotides.
A.

Processed forms of P<sub>L</sub> RNA

B.

Processed forms of P<sub>L</sub> RNA
Figure 6: A: Map of the λ genome in the vicinity of the sieB gene. Sequences present in the sieB-expressing plasmids constructed for these studies are shown below. B: The sequence of the untranslated region of λ sieB is indicated, and the sequence of the untranslated region in the lac fusion plasmid is shown below.
A

\begin{align*}
\text{Ea 10} & \quad ral & \quad \text{sieB} & \quad N \\
\text{Xhol} & & \text{BamH} & \text{HpaI} \\
\end{align*}

B

\begin{align*}
\text{Wild-type} & \quad +^1 \quad \text{ACCCCUACAGUUUGAUGAGUAUGAAUAGAAUUGUGAGCGGAUAACAAUUUCACACAGGAACAGCCAUCUG} \\
\text{lacUV5} & \quad \text{AAUUGUGAGCGGAUAACAUUUUCACACAGGAACAGCCAUCUG} \\
\end{align*}
**Figure 7:** Molecular mechanism to explain P22's ability to bypass SieB-mediated exclusion. A. In a lysogen, both SieB and Esc are synthesized from a transcript initiated at P_{sieB}; P_L is repressed by the phage repressor, c2. B. In an infected lysogen, P_{sieB} and P_L of the superinfecting phage are transcribed. The P_L transcript is processed into sas RNA which then acts on the target sieB/esc mRNA to cause a translational switch, thus allowing synthesis of only Esc. Broken arrows indicate transcripts.
A. Uninfected lysogen

B. Infected lysogen
CHAPTER 4

Preliminary Studies on Transcriptional Regulation of \textit{sieB} and \textit{esc} Expression
INTRODUCTION

The preceding chapter described the regulation of expression of sieB and esc at the level of translation, but it left open the question of regulation at the level of transcription. This chapter describes preliminary experiments designed to answer this question. As will be apparent from the results described below, there is no compelling reason to think that sieB or esc are subject to transcriptional regulation.

As discussed in Chapter 3, the sieB gene has four potential internal translation initiation sites, and esc appears to initiate at the third of these sites. However, if a protein initiated most likely at the fourth ATG is expressed from the lacUV5 promoter on a multicopy plasmid (pTP529, see Figure 1) in a SieB+ lysogen, then the lysogen becomes only weakly SieB+; phage L plates 10-20 fold more efficiently on pTP529-bearing lysogens than on the control SieB+ lysogens. This result suggested that a protein initiated at the fourth ATG in sieB may also have some Esc activity. Semerjian et al. (1989) noted a σ70 promoter-like sequence just upstream of this ATG, raising the possibility that alternative transcription initiation at the sieB promoter or at this hypothetical promoter may play a role in regulating exclusion activity in the cell. One specific hypothesis was that if, for some reason, SieB were overexpressed in a lysogen, then it may be harmful to the cell. Somehow, high levels of SieB would induce transcription from this hypothetical promoter leading to synthesis of 'Esc' which would then neutralize excess SieB present in the cell.
MATERIALS and METHODS

Bacteria and Phage

*E. coli* strain NK5031 (*lacΔM5265 naf sulII*) was used for all the β galactosidase assays, unless otherwise stated. Strains DE192 (*lexA51 Δ(lac-pro)X111 rpsL31 sulA211*), DM2251 (*Δ(lac-pro)X111 rpsL31 sulA211*), and DRS361 (*himA ΔSmaI::Tn 10 Kan himD Δ3::CamR lacU169 Δtrp(EA)Z*) were obtained from M. Marinus. Strains RS6184 (*ara Δ(lac-pro) thi glyA::Tn5*) and RS6181 (*ara Δ(lac-pro) thi glyA::Tn5 rcn105*) were obtained from D. Wulff. Phage λ RS45 (*bla' lacZSc imm21 indk*) was obtained from M. Marinus, and is described by Simons *et al* (1987).

Operon fusions were constructed on plasmids and then introduced into λ RS45 by homologous recombination. Dilutions of phage lysates from such crosses were spotted on a lawn of NK5031 containing the indicator X-Gal (200 μg/mL). Lysogens of NK5031 were isolated by streaking out from the middle of a blue plaque, and they were purified by repeated streaking on LB plates spread with X-Gal till only blue colonies were observed. Recombinant λ phage bearing the operon fusions were obtained from these lysogens by inducing them with mitomycin C.

Plasmids

Plasmids pTP10, pTP520, pTP650, pTP651 and pTP652a have been described (Chapter 2; Poteete and Roberts, 1981). Plasmid pTP529 has the Ndel-ClaI 'esc'-bearing fragment downstream of the *lacUV5* promoter (A. Poteete, personal communication). The resulting plasmid is expected to make a version of Esc that initiates at the fourth ATG in *sieB*. Operon fusions between
**sieB** and **lacZ** were constructed as follows; their relevant structures are shown in Figure 1. DNA fragments carrying both the **sieB** and the putative **esc** promoters (P_{sieB} and P_{esc} respectively), P_{esc} alone and neither promoter were cloned into a 'promoter-probe' vector. This vector, pNK1415, is a pBR322 derivative carrying a promoter-less **lac** operon (referred to as pRS415 in Simons *et al.*, 1987). In this plasmid, just upstream of the translation initiation region of **lacZ** is a polylinker region into which fragments of interest can be cloned. Because there are four tandem transcription terminators just upstream of the polylinker, background expression of **lacZ** from plasmid promoters is very low. Plasmid pTP650 was cut with Sall, filled-in, EcoRI linkers were ligated and the plasmid was then cut with EcoRI and EcoRV. The resulting EcoRI-EcoRV P_{sieB}, P_{esc}-bearing fragment was ligated with an EcoRI-BamHI (filled-in) fragment of pNK1415 to yield the recombinant plasmid pKR653. The negative control, pKR656, lacking both promoters, was constructed similarly except that plasmid pTP652a was used as the source. Plasmids pKR654 and pKR655 bearing P_{esc} alone were constructed by digesting plasmids pTP520 and pTP651 with HindIII, filling-in, ligating on EcoRI linkers and digesting with EcoRI and EcoRV. Plasmid pKR654 has an intact P_{esc}; plasmid pKR655, however, substitutes the first two nucleotides of the -35 hexamer of P_{esc} with linker sequence. Plasmid pTP514 carries a gene fusion between **sieB** and **lacZ** (A. Poteete, personal communication). In this plasmid, the **sieB** gene is fused in-frame at the Hpal site (via a BamHI linker that maintains the **sieB** reading frame) to the tenth codon in **lacZ**. This fusion was introduced into the promoter-probe vector as follows. Plasmid pTP514 was cut with HindIII, filled-in, EcoRI linkers were ligated, followed by digestion with EcoRI and EcoRV. The resulting **sieB-
lacZ-bearing fragment was ligated with the large ori-containing EcoRI-EcoRV fragment from pNK1415 to yield plasmid pKR663.

**RNase protection and in vitro transcription**

RNase protection was done as described in Chapter 2. In vitro transcriptions were performed as suggested by the supplier of *E. coli* RNA polymerase (Boehringer Mannheim). Approx. 500 ng of template and 2-10 units of RNA polymerase were used in the transcription reactions. All transcription reactions were carried out in the presence of 1 unit/μL of RNAsin (Promega). For linear templates, the transcription reaction was incubated at 37°C for 30 min in the presence of 20 μCi of α-32P-labelled rCTP and 1 mM each of rGTP, UTP and ATP. Transcription reactions with supercoiled templates were performed similarly except that unlabelled rXTPs were used at a final concentration of 2 mM and the amount of template was increased to approx. 2 μg. After incubation at 37°C for 60 min DNAase I was added to a final concentration of 25 μg/μL and incubation was continued for 15 min more. After phenol and chloroform extraction, RNase protection was done using the supernatant. The in vitro synthesized labelled RNA and the protected riboprobe were run on 8% and 5% sequencing gels respectively along with labelled HindIII fragments of pBR322. Linear templates for in vitro transcription were obtained by digesting plasmids pTP650 with Sall and Hpal, and pTP520 and pTP651 with HindIII and Hpal. The resulting 425, 305 and 265 bp DNA fragments respectively, were BAC gel-purified prior to using them in the in vitro transcription reaction. The supercoiled templates used were plasmids pKR653 and pKR654.
β galactosidase assays

β galactosidase (β gal) assays were performed as described by Miller (1972).
RESULTS and DISCUSSION

Does P_{esc} have promoter activity?

The promoter-like sequence in P22 noted by Semerjian et al (1989) and, for comparison, the consensus $\sigma^{70}$ promoter are given below:

<table>
<thead>
<tr>
<th></th>
<th>P22</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATGACA......17 bp.....TATGCT</td>
<td>TTGACA......17 bp.....TATAAT</td>
</tr>
</tbody>
</table>

Experiments described below were designed to test whether this P22 sequence has any promoter activity, and the answer seems to be that it does not. Two kinds of experiments were performed: indirect ones in which fusions to lacZ were used, and direct assays, like RNase protection and in vitro transcription to detect RNA initiated at this hypothetical promoter. The results of the lacZ fusion and RNase protection experiments are summarized in Figure 1.

Operon fusions

As shown in Figure 1, when both P_{SieB} and P_{esc} are present, high levels of $\beta$ gal are synthesized, both in multicopy and single-copy. When P_{SieB} is deleted $\beta$ gal activity is reduced approx. two-fold when the fusion is present in multi-copy, whereas in single-copy, activity is reduced approx. ten-fold. As expected, the control shows little or no $\beta$ gal expression. These results would appear to suggest that P_{esc} may be a weak promoter.

RNase protection

Transcripts initiated at P_{esc}, however, could not be detected with an RNase protection assay. The riboprobe used to detect sieB mRNA in Chapter 2
was used in these experiments (see Figure 1). When RNA extracted from cells bearing plasmids pKR653 or pKR654 was used, an RNA species approx. 100 nt shorter than the expected $P_{esc}$ mRNA was detected (indicated as 'short' in Figure 1). The inferred 5' end of 'short' RNA is such that it cannot direct synthesis of Esc. This 'short' RNA can not be simply a processed form of $sieB$ mRNA because pKR654 lacks the $sieB$ promoter, but still yields 'short' RNA. It seems likely that the $\beta$ gal activity observed in the absence of $P_{sieB}$ is due to this 'short' RNA.

The sequence in the vicinity of the 5' end of 'short' RNA resembles an RNaseIII processing site, suggesting that RNA initiated at $P_{esc}$ may be undetectable because it is rapidly processed into the short RNA. When RNA was extracted from an RNaseIII- strain bearing plasmids pKR653 or pKR654, RNA initiated at $P_{esc}$ could not be detected, but the short RNA was readily detectable, indicating that its presence is not due to RNaseIII processing of transcripts initiated at $P_{esc}$. RNaseIII may be involved in processing $sieB$ mRNA because a $sieB$-lacZ gene fusion has three-fold higher $\beta$ gal activity (613 units) in the RNaseIII- strain as compared to that in the RNaseIII+ strain (216 units). However, no short RNA could be detected in either strain bearing the gene fusion plasmid pKR663.

Attempts to detect RNA initiated at $P_{esc}$ in lysogens and phage-infected cells were also unsuccessful, although $sieB$ mRNA (as judged by the size of the protected riboprobe) could be reliably detected in both cases. 'Esc' RNA with a 5' end that would be similar, if not identical, to RNA initiated at $P_{esc}$ could be easily detected when expressed from the heterologous lacUV5 promoter in
cells bearing plasmid pTP529, suggesting that esc mRNA should be detectable if it were actually synthesized.

The lack of promoter activity in a sequence that closely resembles the consensus σ70 promoter may be explained if it were repressed by some trans-acting factor(s). The next three sections describe experiments to test this idea.

**Is Pesc repressed by LexA?**

The sequence flanking the -35 hexamer of Pesc suggests a binding site for LexA, a transcriptional repressor of genes that comprise the SOS regulon (Walker, 1984). The sequence in P22 and, for comparison the consensus LexA binding-site are given below:

\[
P22: \text{TcCTGcAaggAatgACAgTt} \\
LexA: \text{taCTGTatata_a_ACAGta}
\]

(The most conserved nucleotides in the LexA binding site and those in the P22 sequence that match the consensus LexA binding site are capitalised.)

To test the effect of LexA on Pesc, LexA+ and LexA− strains were lysogenised with λ bearing P\text{SieB}, Pesc or Pesc lacZ fusions and β gal activity in these lysogens was measured. The fusion bearing both P\text{SieB} and Pesc yielded 295 units of β gal in the LexA+ strain and 181 units in the LexA− strain. The fusion bearing Pesc alone had 45 and 50 units of β gal in the LexA+ and LexA− strains respectively. These results indicate that LexA does not repress transcription from Pesc.

**Is Pesc repressed by IHF?**

The sequence in the vicinity of the -35 hexamer of Pesc resembles closely the binding site of another DNA-binding protein called IHF. Depending on where IHF binds, it can either activate or repress a promoter. Since the
binding-site for IHF flanks the binding site for RNA polymerase, it seemed likely that IHF might repress transcription from $P_{esc}$. IHF+ (NK5031) and IHF- (DRS361) strains were transformed with the $lacZ$ fusion plasmids, and $\beta$ gal activity was measured. Plasmid pKR653 bearing both $P_{sieB}$ and $P_{esc}$ yielded 3816 and 7326 units of $\beta$ gal in the IHF+ and IHF- strains respectively, and plasmid pKR654 bearing $P_{esc}$ alone gave 802 and 1102 units of $\beta$ gal in the corresponding strains. These results indicate that IHF is not involved in regulating expression from $P_{esc}$.

Keeping in mind the theme of 'redundancy' developed in Chapter 3, it is conceivable that in the absence of LexA, IHF represses transcription from $P_{esc}$ and vice versa. The experiment of testing for $P_{esc}$ activity in an IHF-, LexA- double mutant has not been done.

**Is $P_{esc}$ repressed by c2?**

Semerjian *et al.* noted that the sequence flanking the -35 hexamer of $P_{esc}$ is reminiscent of a binding site for the c2 repressor of P22. Whereas bona fide repressor binding sites have four base pairs between symmetrically arrayed half-sites, the sequence in $P_{esc}$ has eight base-pairs, suggesting that if it were to bind P22 repressor, it would do so only weakly. Whether P22 repressor has any effect on expression from $P_{sieB}$ or $P_{esc}$ was tested using the $lacZ$ fusion-bearing $\lambda$ phages. $\lambda$ lysogens were transformed with plasmid pTP10, a plasmid that overexpresses P22 repressor. It was found that $\beta$ gal expression from the $lacZ$ fusion that has both $P_{sieB}$ and $P_{esc}$ is repressed approx. three-fold by P22 repressor (1103 units in the absence of repressor and 345 units when repressor is overexpressed). $\beta$ gal synthesis from the fusion that bears only $P_{esc}$ is insensitive to repression, however (168 and 148 units in
the presence and absence of repressor). These observations suggest that repressor binding within the \(sieB\) gene interferes with expression from \(P_{sieB}\). Whether this repression has any physiological role remains to be determined.

**In vitro transcription**

The lack of transcription from \(P_{esc}\) in IHF\(^-\) and LexA\(^-\) strains could be due to the action of an as yet undefined transcriptional repressor. If this were true, then one might expect \(P_{esc}\) to be active in vitro. To test this prediction, in vitro transcription experiments were performed with linear or supercoiled templates bearing both \(P_{sieB}\) and \(P_{esc}\), \(P_{esc}\) alone and neither promoter. Transcripts initiated at \(P_{esc}\) could not be detected from linear or supercoiled templates. However, transcripts initiated at \(P_{sieB}\), as judged by the size of the protected riboprobe, could be detected readily from linear and supercoiled templates.

At present, it is not clear why \(P_{esc}\) does not have promoter activity in vivo and in vitro. One reason may be that the sequence in the vicinity of \(P_{esc}\) has intrinsic curvature. In light of a study by Rojo *et al.* (1991), who showed that 'bent' DNA could substitute for the action of the p4 repressor of phage \(\phi 29\), it seems possible that curvature around \(P_{esc}\) prevents it from functioning as a \(\sigma^{70}\) promoter.
**Figure 1**: Genetic structure of P22 in the vicinity of the *sieB* gene. A number of restriction enzyme cleavage sites that are mentioned in the text are also shown. The *sieB* promoter, the putative *esc* promoter and the transcription initiation site of 'short' RNA are indicated as P_{sieB}, P_{esc} and P_{short} respectively. Shown below the genetic map are partial structures of the *lac* operon fusion plasmids; in all four plasmids (and the corresponding λ clones) the *lac* operon is fused at the EcoRV (R5) site in *sieB*. β-gal activity from each fusion when present in multi-copy (MC) or single-copy (SC) is shown. Activities are normalised to the pKR653 fusion values which were 4167 Miller units (MC) and 1111 Miller units (SC). RNA detected in an RNase protection experiment are shown under 'Transcripts'. RNase protection was done exactly as described in Chapter 2. A '+' indicates that the transcript was readily detectable, and a '-' indicates that the transcript was undetectable. The relevant structure of plasmid pTP529 is shown; it has the Ndel-ClaI fragment fused to the *lacUV5* promoter. The bold arrow represents PlacUV5.
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