A switch in translation mediated by an antisense RNA

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Antisense RNAs regulate expression of target genes in a variety of ways—transcription termination, translation initiation, and mRNA stability. We describe a case in which the target gene encodes two polypeptides, and antisense RNA causes a switch in its translation by selectively inhibiting synthesis of one of the polypeptides. Bacteriophage P22 is a temperate *Salmonella* phage; in the prophage state it expresses only a handful of its genes. One of these genes, *sieB*, aborts the lytic development of some phages. P22 itself is insensitive to the lethal effect of SieB because it harbors a determinant called *esc*. We show 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 bypass 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. Finally, we show that P22's Esc allows it to circumvent the SieB-mediated exclusion system of bacteriophage λ .

[Key Words: Superinfection exclusion; antisense RNA; translational switch]

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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. 1974). In a SieB⁺ host, vegetative development of sensitive phages proceeds normally for ~20 min, 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 harbors 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 Fig. 1). Below, we present the likely molecular mechanism that P22 utilizes to escape SieB-mediated exclusion.

In a previous study we identified the *sieB* gene and its promoter (Ranade and Poteete 1993). The *sieB* gene, which encodes a polypeptide of 192 amino acid residues, is embedded in the *p_L* 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 an inhibitor of the exclusion protein SieB. The inhibitor, Esc, is a truncated version of SieB itself; it lacks the amino-terminal 25 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 that 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

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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. Finally, we present evidence indicating that P22's Esc can disarm the *sieB* exclusion system of bacteriophage λ .

Results

esc as antisense RNA

Preliminary experiments designed to detect post-transcriptional regulation of the major leftward operon (p_L) of bacteriophage P22 revealed that the p_L transcript is processed in the vicinity of the *sieB* gene. This processing was investigated further using an RNase protection assay, and the results are presented in Figure 1.

Wild-type *Salmonella* was infected with virulent P22, and RNA was isolated 10, 20, and 30 min after infection. The riboprobes used in the experiment are shown in Figure 1A; two of the riboprobes (Hpa and Nde) have a common 5' end that is 75 bp upstream of the transcription start site of *sieB/esc*. They have different 3' ends; the Hpa probe extends to the *HpaI* site and has ~426 nucleotides of complementarity with the p_L transcript. The

Nde probe has ~175 nucleotides of complementarity and extends to the *NdeI* site in *sieB/esc*. The third probe (Xmn) has a different 5' end; it extends from the *HindIII* site in *ral* for 426 nucleotides to the *XmnI* site in *sieB/esc*. As can be seen in Figure 1B, the Hpa probe is fully protected by RNA extracted 10 min postinfection (where observed, the difference in size between the fully protected and the untreated probes is the result of the presence of vector sequences in the probe); between 10 and 20 min after infection, however, there is a decrease in the full-length probe with a concomitant increase in a 105-nucleotide protected fragment. This pattern is mirrored in the experiment with the Nde probe, suggesting that the 5' end of the 105-nucleotide fragment is to the left of the *NdeI* site. A 105-nucleotide protected riboprobe is obtained even when one uses a probe with a different 5' end (Xmn probe). Taken together, these results indicate that the p_L transcript (in this region) is processed into a 105-nucleotide RNA species, and this processed fragment lies within the boundaries of the Nde probe. In addition to the 105-nucleotide 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 Fig. 1B) are partially processed forms of the p_L transcript; their likely provenance is diagrammed in Figure 1A. These two partially processed RNAs also allow one to identify the most likely ends of the 105-nu-

Figure 1. 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 p_L transcript. (300 and 280) The singly processed forms of p_L RNA and (105) 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) Autoradiograph of a 6% polyacrylamide/8M 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) Untreated riboprobe; (U) 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-nucleotide fragments discussed in the text are marked at right. Markers (M) are *HinfI* fragments of pBR322, filled in by the Klenow fragment of *E. coli* DNA polymerase I in the presence of [α - 32 P]dATP. The numbers at left indicate the length of each fragment (in nucleotides).

Table 1. *Esc* phenotypes of P22 bearing different alleles of *sieB/esc* on cells expressing different alleles of *sieB*

<i>sieB/esc</i> allele in P22	<i>sieB</i> allele in host ^a		
	wild type	substitution 2	am115
Wild type	+	+	+
Substitution 1	+	+	+
am4	+	+	+
am14	+	+	+
am22	+	+	+
am29	+	–	+
am115	+	–	+
P _{sieB-10}	+	–	+
Substitution 1 am115	–	–	+
Substitution 2 am115	–	–	+

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 phase in LB + ampicillin, and 0.1 ml of culture was used to make lawns on LB-ampicillin plates. Tenfold 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.

^aA minus sign indicates that the phage plates 100- to 1000-fold less efficiently on a particular host as compared with its plating efficiency on *Salmonella* expressing the am115 allele of *sieB/esc*; a plus sign indicates that the plating efficiency is ~1.

cleotide fragment: The 5' end of this RNA is at or very near the *sieB* initiation codon, and the 3' end is ~70 nucleotides to the left of the *sieB/esc* transcription initiation site. This processed fragment of the *p_L* transcript is referred to, from now on, as *sas* (*sieB* antisense) RNA.

Studies by Susskind and co-workers (1974) showed that *esc*—the determinant in P22 that allows it to bypass SieB-mediated exclusion—is part of the *p_L* operon and is tightly linked to *sieB*. Our deletion mapping studies revealed that a critical *esc* determinant maps almost precisely to the region encompassed by *sas* RNA (data not shown). Taken together, these observations suggest that *esc* is *sas* RNA. Given the small size of this RNA and the fact that it has the potential to base-pair with the ribosome-binding site of *sieB*, it seemed plausible that *sas* RNA might be involved in negatively regulating *sieB* expression. If *sieB* antisense RNA, synthesized by super-infecting P22, acting on the untranslated region of *sieB* mRNA, were responsible for allowing P22 to grow in a SieB⁺ cell, one might expect that mutations in the antisense effector that abolish base-pairing with the target *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 *sieB*. This mutation—substitution 1 (substitution 1 in Fig. 3B, below)—replaces almost the entire untranslated region of *sieB* mRNA (24 of 31 nucleotides). The substitution was crossed into virulent P22, and its *Esc* phenotype was tested. As indicated in Table 1, substitution 1-bearing P22 had no phenotype; P22 *sub1* plates as well as does wild-type P22 on cells expressing wild-type *sieB*. This result, prima facie, would rule out base-pairing be-

tween *sas* RNA and the untranslated region of *sieB* mRNA as a necessary part of the mechanism by which P22 bypasses SieB-mediated exclusion. As is shown 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*.

esc as protein

When expression of proteins was analyzed, using maxicells from the *sieB* region of P22, we discovered that two proteins are expressed from this region (see Fig. 2B, lane 3). On the basis of the phenotype of an amber mutation (am4 in Fig. 2A), we inferred that the larger polypeptide is expressed from the *sieB* gene (Ranade and Poteete 1993). An examination of the sequence of the *sieB* gene revealed four in-frame initiation codons downstream of the *sieB* initiation codon (indicated as 2, 3, Esc ATG, and 5 in Fig. 2A). To test the possibility that the smaller polypeptide is the result of initiation at one of these ATGs, amber mutations were introduced between consecutive ATGs; an amber mutation was introduced

Figure 2. (A) The *sieB/esc* gene is shown with the amber mutations (am) discussed in the text. The deduced initiation codons of *sieB* and *esc* are indicated as SieB ATG and Esc ATG, respectively. (B) Autoradiograph of ³⁵S-labeled proteins expressed in maxicells and separated by SDS-PAGE (10%) (tricine system; Schagger and Jagow 1987). (Lane 1) No plasmid; (lane 2) pBR322 with its tetracycline resistance-conferring gene deleted. Lanes 3–7 are derivatives of pKR682 expressing different alleles of *sieB/esc*. (Lane 3) Wild type; (lane 4) am4; (lane 5) am14; (lane 6) am22; (lane 7) am29. (Lane 8) am115. Arrows indicate positions of β-lactamase, SieB, and Esc. The numbers at left indicate molecular mass markers (Pharmacia) (in kilodaltons).

toward the carboxyl terminus of *sieB* as well. Derivatives of pKR682 bearing these amber alleles were constructed, and expression of proteins from these plasmids was analyzed using maxicells. (Plasmid pKR682 is a derivative of pBR322 bearing the *HindIII*–*MluI* *ral*–*sieB* region of P22; see Fig. 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 as a result of translation reinitiation by ribosomes terminating at these ambers. Two conclusions can be drawn from these results: First, *SieB* and the smaller polypeptide (*Esc*) are synthesized from the same open reading frame; and 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 [e.g., p1 and p2 proteins of transposon Tn5 (Isberg et al. 1982; Johnson et al. 1982) S₁₀₇ and S₁₀₅ proteins of bacteriophage λ (Young 1992)], we guessed that *Esc* may be an inhibitor of *SieB*-mediated exclusion. 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, one would predict that superinfecting P22 bearing amber mutations in *sieB* upstream of the *esc* initiation codon (am4, am14, and am22; see Fig. 2A) would be able to bypass *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 host expressing wild-type *sieB* 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 that 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 bypass *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

Because mutations that would (1) prevent *sieB* antisense RNA from interacting with its sense partner (substitution 1), or (2) 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 *p_L* operon (*sieB* antisense RNA) or *Esc* protein, but not both, to bypass *SieB*-mediated exclusion. To test this possibility, a P22 strain bearing substitution 1 in combination with the *sieB/escam115* allele was constructed, and its ability to grow on a *SieB*⁺ host was tested. The results are presented in Table 1. As can be seen, P22 *sub1 sieB/escam115* plates inefficiently on cells expressing wild-type *sieB*. Neither mutation alone has any effect on the plating behavior of P22. On the control *SieB*[−] host (expressing the *sieB/escam115* allele), 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 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 experiment should also yield the same result, that is, 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 9 nucleotides (2–11) 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; data not shown). The plating behavior of P22 bearing various amber alleles of *sieB/esc* was

Figure 3. (A) The *sieB/esc* gene is shown, and the antisense *p_L* transcript is indicated as a broken arrow below. (Sub1 and Sub2) Substitutions 1 and 2, respectively. (B) The untranslated sequence of P22 *sieB* is shown; (+1) The transcription start site of *sieB*; the *sieB* initiation codon is underlined. Shown below the wild-type sequence are the changes present in the two substitutions.

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 behavior of P22 bearing another mutation, *P_{sieB-10}* was also tested. This mutation alters the –10 hexamer of the *sieB* promoter, thus preventing synthesis of *sieB* mRNA (Ranade and Poteete 1993). P22 *P_{sieB-10}* plates efficiently on cells expressing wild-type *sieB*, suggesting that the *P_{sieB-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 hypothesis is 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 for a description of how this was obtained) 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 harboring 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 attributed solely to *SieB* made by the infecting phage because P22 bearing substi-

tution 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 relevant structures are indicated in Figure 4A. These plasmids are derivatives of pKR682, which carries the *sieB/esc* region of P22 (but not *p_L*, from which the antisense RNA is normally expressed). In addition, but at a different location, these plasmids have different fragments of the *sieB/esc* region, which when transcribed from the heterologous *lacUV5* promoter, produce different lengths of *sieB/esc* antisense RNAs (only these additional sequences are indicated as arrows under the genetic map in Fig. 4A). All three plasmids would direct synthesis of antisense RNA initiated at the *EcoRV* site in *sieB/esc*. Plasmid pKR687 would make a ~732-nucleotide antisense RNA up to the *HindIII* site in *ral*; plasmid pKR688 would direct synthesis of a ~528-nucleotide antisense RNA, and plasmid pKR689 would make a ~448-nucleotide antisense RNA. To ensure that antisense RNAs 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 (Fig. 4A), and expression of proteins from these plasmids was analyzed using maxicells (Fig. 4B). As can be seen in lane 2, in the absence of any *sieB/esc* antisense RNA, both *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 (Fig. 4B, lane 1). Plasmid pKR687 confers a *SieB[–]* phenotype and, in maxicells, directs synthesis of *Esc* but not *SieB* (Fig. 4B, lane 3), suggesting that this antisense RNA causes a switch in the translation of *sieB/esc* RNA, from directing synthesis of both *SieB* and *Esc* to *Esc* alone. Plasmids pKR688 and pKR689 confer a *SieB⁺* phenotype and, in maxicells, direct synthesis of *SieB* and *Esc* (Fig. 4B, lanes 4 and 5, respectively). The result with pKR688 suggests that sequences in the *p_L* transcript, which lie beyond the region of complementarity with *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 (c.f. lanes 2 and 5). Plasmid pKR688 seems to overproduce *SieB* and *Esc*; we do not understand the basis for this.

Esc, but not sas RNA, allows P22 to bypass SieB-mediated exclusion by bacteriophage λ

Susskind and co-workers showed that coliphage λ too

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has a *sieB* gene, and this excludes the same *Salmonella* phages as P22's *sieB*, although there is little or no homology between the two genes. They also showed that P22 is insensitive to λ SieB-mediated exclusion (Susskind and Botstein 1980). Below, we present evidence to suggest that Esc, but not *sas* RNA, allows P22 to circumvent λ SieB-mediated exclusion.

The relevant region of the λ genome is shown in Figure 5A. The structures of plasmids bearing the *sieB* gene are shown below. Plasmid pTP462 has the *ea10*–*ral*–*sieB* (and a part of *N* as well) region of λ ; consequently in this plasmid, λ *sieB* is expressed, most likely, from its own promoter. The region indicated by bold lines is homologous to that of P22; the untranslated regions of the *sieB* genes of the two phages are homologous but not identical (see Fig. 3B and 5B). Plasmid pTP482 has the *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 Fig. 5B). The plating behavior 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 mutation in *esc* (*sieB/esc*am115) or the *sieB/esc* promoter mutation ($P_{sieB-10}$), 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 6 we present a molecular mechanism to explain P22's ability to circumvent SieB-mediated exclusion. In

Figure 5. (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.

Table 2. *Esc* phenotypes of P22 bearing different alleles of *sieB/esc* on cells expressing λ sieB

<i>sieB/esc</i> allele in P22	<i>sieB</i> allele in host ^a	
	pTP462 ($P_{\text{sieB}}-\lambda$ sieB)	pTP482 ($P_{\text{lac}}-\lambda$ sieB)
Wild type	+	+
am22	+	+
am115	—	—
$P_{\text{sieB-10}}$	—	—

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.

^aA minus sign indicates that the phage plates 100- to 1000-fold less efficiently on a particular host as compared with its plating efficiency on cells bearing the am115 allele of P22 *sieB*. A plus sign indicates that the plating efficiency is ~ 1 .

a lysogen, the *sieB/esc* genes are expressed from a weak promoter just upstream of the *sieB* initiation codon; the strong leftward promoter p_L is repressed by the action of *c2* repressor. The cell is phenotypically *SieB*⁺ at this ratio of *SieB* (the exclusion protein) to *Esc* (the inhibitor protein); the prophage does not exclude itself, 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, p_L and p_R , and low-level transcription from the *sieB/esc* promoter as well. The region of the p_L transcript, which is antisense with respect to *sieB/esc* mRNA, is processed into a 105-nucleotide *sieB* antisense effector RNA, *sas*. *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 functional amounts of *Esc* can be made by the prophage or the superinfecting phage.

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* (Table 1), 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 Fig. 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* (*sub1 sieB/esc*am115 and *sub2 sieB/esc*am115; 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, superinfecting P22 depends on its own *Esc* to bypass *SieB*-mediated exclusion (P22 *sieB/esc*am29 and *am115*; $P_{\text{sieB-10}}$; Table 1). The switch induced by *sas* RNA is essential to vegeta-

tively developing P22; substitution 2, which renders *sieB* mRNA unresponsive to its antisense partner, causes P22 to *SieB*-exclude itself upon infection of a nonlysogen. When *sieB* is inactivated by an amber mutation (am115), however, substitution 2 is innocuous. Finally, artificial antisense RNAs inhibit the synthesis of *SieB* but not *Esc* (Fig. 4).

sas RNA

The status of the effector that causes the translational switch as an RNA molecule seems reasonably secure. (1) 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. (2) The p_L transcript is processed into a small 105-nucleotide 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 50–110 nucleotides in length (Simons and Kleckner 1988).

Figure 6. 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.

(3) There is no likely translation initiation site in *sas* RNA. Moreover, an amber mutation in the only substantive leftward open reading frame in this region of the p_L operon has no phenotype (data not shown).

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 that 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 attributable to at least two reasons. First, substitution 2 is a relatively large mutation (9 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 p_L 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 (data not shown). It is not clear whether the processing of p_L is absolutely essential for it to be an effective antisense RNA. The unprocessed form of p_L may be unable to interfere with the expression of *sieB* or *esc*, perhaps because it cannot fold into the appropriate structure. Another point that deserves to be mentioned is that the region of p_L RNA 5' to *sas* RNA is rapidly degraded; one can see only two protected species with the Hpa probe: full-length probe and *sas* RNA (see Fig. 1B). This observation suggests how P22 can synthesize Esc in the presence of the p_L transcript (because the p_L transcript is also antisense to *esc* mRNA)—the region of the p_L RNA that can base-pair with *esc* mRNA is rapidly degraded. 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, RNaseP, has been implicated (Citron and Schuster 1992).

Relationship to other exclusion systems

We have presented genetic evidence indicating 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, *sas* RNA acting alone evidently is not sufficient to allow P22 to bypass 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 (Table 1). 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 cannot 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 synthesis is sufficient, but P22 *sas* RNA might not bind λ *sieB* mRNA because of the 17-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 bypass any exclusion system that functions through the same host macromolecule (or molecules) as does SieB. If the first possibility is correct, then it raises the question of how λ is able to bypass 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). A mutant of λ with a large deletion in its *sieB* gene (and therefore its putative *esc* gene and *sas* RNA) plates efficiently on *Escherichia coli* expressing plasmid-borne λ *sieB* (K. Ranade and A. Poteete, unpubl.).

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.

Materials and methods

Bacteria

E. coli W3110 *lacI^QL8* (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 CSR603 *recA1 uvrA6* (Sancar and Rupert 1978) was used for maxicell analysis. *Salmonella typhimurium* LT2 strains MS1868 (*leuAam414 r⁻m⁺*) and DB53 (*cysA1348 hisC527 fels*) were provided by M. Susskind (University of Southern California, Los Angeles).

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 1993) was replaced by a *t_{ant}*-bearing *EcoRI*–*HindIII* fragment from pTP550 (Semerjian et al. 1989); the resulting plasmid was called pKR683. The large *ori*-containing *PstI*–*EcoRV* frag-

ments from plasmids pAS474, pTP546, and pTP520 (Ranade and Poteete 1993) 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*-*AflIII* (filled-in) fragment from pKR683. To minimize intramolecular recombination, the new plasmids were propagated in strain HB101. The recombinant plasmids are called pKR687, pKR688, and pKR689, respectively.

Mutant alleles of *sieB/esc* were constructed using site-directed mutagenesis as described (Kunkel et al. 1987; Ranade and Poteete 1993). Construction of the *am4* and *P_{sieB-10}* alleles of *sieB/esc* has been described (Ranade and Poteete 1993). The mutagenic oligonucleotides (mismatched nucleotides underlined) used to construct the other mutations were as follows: *am14*, 5'-CATTCCTTGCTAGAAAAACG; *am22*, 5'-GCATATGAATCTACTGTTTAAG; *am29*, 5'-CAATACGATCTAGATGATTAG; *am115*, 5'-CGAATGCCAACTACGCTCTTTC; substitution 1, 5'-GTTTCATCGGCGAAAGAGAAAAATGAGAGACATCCCCTGAATAGAGTTTATCCG-3'; and substitution 2, 5'-CTCTTTTACTCTGACATCCCCTGAAATAGAGTTTATCC. (am refers to the amber mutation that was introduced into the *sieB/esc* gene, and the number that follows refers to that codon of *sieB/esc* that was mutated to amber; substitutions 1 and 2 refer to the mutations that were introduced into the untranslated region of *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. Substitutions 1 and 2 were combined with *am115* as follows. Mutation-bearing *HindIII*-*HpaI* 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.

To synthesize large amounts of riboprobe, 426-bp *SalI*-*HpaI* and 732-bp *HindIII*-*EcoRV* fragments from pTP650 (Ranade and Poteete 1993) 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 p_L 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). Mutant alleles of *sieB/esc* were introduced into virulent P22 by crossing mutation-bearing plasmids with P22 *vir3Δ474*. This phage was constructed by crossing P22 *Δ474* (Semerjian et al. 1989) with P22 *vir3*, selecting for virulence, and screening for the deletion. P22 *vir3Δ474* has a large deletion in the *kil-ral* region, because the phage lacks gene 17, it is unable to grow on a *fels* lysogen (Susskind and Botstein 1978). *Salmonella* strain MS1868 was transformed with mutant derivatives of plasmid pTP83, and plasmid by phage crosses (using P22 *vir3Δ474*) were performed as described (Semerjian et al. 1989). The lysates from the crosses were plated on *Salmo-*

nella 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 *sieB/esc*. Virulent progeny were identified by their ability to form plaques on a P22 *sieA44 a1 sieB::Kn567* lysogen (Ranade and Poteete 1993). Virulent P22 carrying mutations in *sieB/esc* were identified by DNA sequencing, as described (Rennell et al. 1991). P22 *vir3 sub2* was constructed as follows. A derivative of pTP83 bearing *substitution 2 sieB* was constructed and transformed into *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 behavior 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 1993), except that 10–15 μ g of RNA was used; RNase A was used at a final concentration of 40 μ g/ml (instead of nuclease P1), and proteinase K digestion (final concentration, 300 μ g/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/8M 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 previously (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 m.o.i. 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.

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