Control of gene expression in bacteriophage P22 by a small antisense RNA.

II. Characterization of mutants defective in repression

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Phage P22 produces antirepressor protein early after infection from a transcript initiated at the P_{ant} promoter. After the first few minutes of infection, transcription from P_{ant} is repressed by a protein encoded by the *arc* gene. Antirepressor is not produced late in infection, even though the antirepressor gene, *ant*, is transcribed from the late operon promoter P_{late} . We describe the isolation of P22 mutants that synthesize antirepressor from the P_{late} transcript. The mutations inactivate a promoter P_{sar} , which lies within the *ant* coding sequence and directs the synthesis of *sar* RNA, a small antisense regulatory RNA complementary to the *ant* ribosome binding site. Characterization of the P_{sar} down-mutants shows that transcription from P_{sar} interferes with synthesis of antirepressor from both the P_{late} and P_{ant} transcripts. Since *sar* RNA represses synthesis of antirepressor *in trans*, we propose that *sar* RNA base-pairs with *ant* mRNA to inhibit antirepressor synthesis at a post-transcriptional level. The role and importance of *sar* RNA in P22 biology are discussed.

[Key Words: Antisense RNA; antirepressor protein; repression-defective mutants; RNA–RNA pairing]

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In recent years, it has become clear that some biological regulatory mechanisms are mediated by interactions between complementary RNA molecules (reviewed by Green et al. 1986). For example, replication of plasmid ColE1 is controlled by pairing between RNA II, the precursor of the primer for DNA replication, and RNA I, a small regulatory RNA that is complementary to the 5' end of RNA II. This interaction, which is facilitated by a protein called Rop or Rom, interferes with the processing of RNA II to form the mature primer RNA (Tomizawa and Itoh 1981; Lacatena et al. 1984; Tomizawa and Som 1984). The expression of genes encoding proteins can also be controlled by the specific interaction of mRNAs and complementary (antisense) RNAs. Naturally occurring regulation by antisense RNAs has been described for several bacterial proteins, including Tn10 transposase (Simons and Kleckner 1983), the replication proteins of plasmids R1 (Light and Molin 1983) and pT181 (Kumar and Novick 1985), and the OmpF outer membrane protein of *Escherichia coli* (Mizuno et al. 1984).

In this paper, we present evidence that the synthesis of phage P22 antirepressor is regulated by a small antisense RNA called *sar* (for small antisense regulatory RNA). P22 antirepressor is a protein that inhibits the specific DNA-binding activity of P22 *c2* repressor, phage

λ *cI* repressor, and the analogous repressors of other, related phages (for review, see Susskind and Youderian 1983). These repressors, which are structurally and functionally similar but differ in DNA sequence specificity, turn off transcription of lytic phage genes during lysogeny. The antirepressor gene, *ant*, is expressed from a strong, rightward promoter called P_{ant} , which is regulated by two repressor proteins (see Fig. 1). The first gene in the *ant* operon, *arc*, encodes a repressor that binds an operator within P_{ant} and turns down transcription from P_{ant} during lytic growth of the phage. The adjacent gene, *mnt*, encodes a repressor that binds an operator at the start point of P_{ant} transcription and turns off P_{ant} during lysogeny. The *mnt* gene is transcribed leftward from P_{mnt} , a divergent promoter that overlaps P_{ant} .

For phages with the P_{ant} promoter and *ant* gene intact, Arc repressor is essential for lytic phage growth and Mnt repressor is essential for lysogeny. Without Arc repressor P_{ant} -promoted synthesis of antirepressor occurs at such a high rate that synthesis of other essential phage proteins is hampered (Susskind 1980). Without Mnt repressor, P22 cannot maintain stable lysogeny because even low levels of antirepressor inhibit *c2* repressor and cause prophage induction (Levine et al. 1975; Botstein et al. 1975). The Arc and Mnt repressors ensure that transcription from P_{ant} occurs only early after infection with P22

Mutations that allow synthesis of antirepressor from the P_{late} transcript were isolated by mutagenizing P22 $P_{\text{ant}} \downarrow$ RU411 9-*del*/TB phage and selecting mutants that form plaques on the pMS317-bearing host (see Materials and methods). Like the parental phage, most of the mutants cannot form plaques on hosts that produce P22 $c2$ repressor, indicating that they have not created a new ($c2$ -insensitive) promoter for transcription of *ant*. Eight mutants were chosen for further study; they are designated P_{sar} down-mutants, for reasons that are clarified below.

As shown in Figure 2, three different mutations were found, each of which is a single base-pair substitution in the fourth codon of the *ant* gene. U3 and U5 change the

An explanation for these mutations is based on the results presented by Liao et al. (this issue). A small transcript, *sar* RNA, is synthesized in vitro from this region of the genome. The RNA is 68–69 bases in length and extends leftward across the intercistronic region between *arc* and *ant*, initiating at the base pair immediately to the left of the translational start codon for *ant* (see Fig. 2) and terminating heterogeneously at two base pairs at the end of *arc*. As shown in Figure 2, the promoter for *sar* RNA, P_{sar} , is embedded in the *ant* coding sequence. All three of our mutations are in the –10 hexamer of P_{sar} , and decrease its homology with the consensus –10 hexamer (5'-TATAAT-3'). Thus, with respect to P_{sar} , U7 changes the consensus T : A base pair at position –12 to A : T, whereas U3 and U5 change the consensus A : T base pair at position –11 to T : A and C : G, respectively. These findings suggest that the mutations decrease P_{sar} promoter activity. If so, this effect could result in overproduction of antirepressor, if synthesis of *sar* RNA interferes with *ant* gene expression.

To demonstrate that these mutations decrease P_{sar} promoter activity, we measured their effects on the kinetics of open complex formation *in vitro*. As described in Materials and methods, we used the abortive initiation assay to determine the rates of open complex formation at several RNA polymerase concentrations. The results using purified restriction fragments as templates are shown in Figure 3 in the form of TAU plots for P_{sar}^{+} and $P_{\text{sar}} \downarrow \text{U7}$, the mutant at position -12 . As summarized in Table 1, part A, this analysis indicates that P_{sar}^{+} is a relatively strong promoter, with high values for K_{B} , the apparent equilibrium constant for initial binding of free polymerase to the promoter, and k_{f} , the rate constant for isomerization of closed complexes to open complexes. Our measurement of overall promoter strength for P_{sar}^{+} (estimated by the apparent association rate constant, the product $K_{\text{B}} \times k_{\text{f}}$) is about sixfold lower than that predicted from the sequence of P_{sar} by the TARGSEARCH program of Mulligan et al. (1984). The $P_{\text{sar}} \downarrow \text{U7}$ mutation at position -12 causes a large decrease in the isomerization constant (30-fold) and a small decrease in the initial binding constant (fivefold). $P_{\text{sar}} \downarrow \text{U3}$ and U5 , the mutations at position -11 , have an even more deleterious effect on P_{sar} promoter strength. In fact, these two promoters are so weak that open complex formation did not proceed to completion under our standard assay conditions. We estimate that the product $K_{\text{B}} \times k_{\text{f}}$ for these mutants is about 500-fold lower than that for wild-type P_{sar} (Table 1). The absence of RNA polymerase occu-

Figure 2. Sequence of P_{sar} down-mutations. The top DNA strand includes the *ant* Shine-Dalgarno (1974) sequence, indicated by asterisks, and beginning of the *ant* coding sequence, as determined by DNA and protein sequencing (Sauer et al. 1983). The bottom DNA strand is shown only for the -35 and -10 hexamers of P_{sar} . The consensus sequences for these regions are 5'-TTGACA-3' and 5'-TATAAT-3', respectively (Hawley and McClure 1983). Below are shown the 5' end of the P_{sar} transcript and the sequence changes for three P_{sar} down-mutations. Among eight sequenced mutations, three were U7, four were U3, and one was U5.

Figure 3. TAU plots for P_{sar}^{+} and P_{sar}^{-} U7 DNA fragments. τ_{obs} is plotted versus the reciprocal RNA polymerase concentration for P_{sar}^{+} (A) and P_{sar}^{-} U7 (B). Template DNAs were *Hind*III fragments containing P_{sar} (from the *Hind*III site in *arc* to the first *Hind*III site in *ant*) purified from digests of pMS200 and pMS434. Abortive initiation reactions were performed at 37°C using standard assay conditions, and values of τ_{obs} were determined as described in Materials and methods. The template concentration was 1 nM; the RNA polymerase concentration was varied as indicated.

pancy at the U3 and U5 mutant promoters was confirmed by digesting DNA fragments containing P_{sar} with *Hinc*II (which cleaves in the -35 region; see Fig. 2) in the presence and absence of RNA polymerase. Complete protection by RNA polymerase was observed for the wild-type promoter, whereas the U3 and U5 mutant promoters were rapidly cleaved in the presence of RNA polymerase (data not shown).

DNA supercoiling significantly increased the strength of all three P_{sar} down-mutants. The results of the TAU plot analyses on supercoiled templates are listed in Table 1, part B. Overall promoter strength ($K_B \times k_f$) of wild-type P_{sar} was reduced about 50% on the supercoiled template compared to the linear template. In contrast, DNA supercoiling increased the activity of the U7 mutant promoter about 40-fold. The two mutants at position -11 , which were immeasurably weak on DNA fragments, were stimulated by supercoiling to an even greater extent.

It is difficult to predict accurately the effects of the P_{sar} down-mutations on promoter strength in vivo from their behavior in vitro, because the superhelical tension of DNA in the cell is not known precisely. As isolated, plasmid DNAs are negatively supercoiled to an extent of about six turns/100 bp ($\sigma = -0.06$). The equivalent torsional tension in vivo has been estimated to be about half as great (Pettijohn and Pfenninger 1980). For an intermediate superhelical density ($\sigma = -0.03$) the theory developed by Davidson (1972) was used to predict that initiation from the P_{sar} down-mutant promoters would be reduced at least 90% from the level predicted for P_{sar}^{+} .

Overproduction of antirepressor by P_{sar} down-mutants

To determine the effects of the P_{sar} mutations on *ant* gene expression in vivo, we assayed the rate of antirepressor synthesis in cells infected with isogenic P_{sar}^{+}

and P_{sar}^{-} phages. The experiment shown in Figure 4 examines synthesis of antirepressor from the P_{late} transcript. In this case, all of the infecting phages carry the P_{ant} $\downarrow$ RU411 deletion, and proteins were pulse-labeled 32 min after infection, when the late operon is maximally expressed. The left half of Figure 4 shows that the P_{sar} down-mutants produce considerable amounts of antirepressor, whereas no detectable antirepressor is produced by the isogenic P_{sar}^{+} control phage. The right half of Figure 4 shows that otherwise isogenic P_{sar}^{-} phages with two amber mutations in gene 23 synthesize reduced levels of antirepressor and other phage late proteins. Since p23 is specifically required for efficient ex-

Table 1. Effect of mutations and DNA supercoiling on P_{sar} promoter strength in vitro

Promoter	K_B (M^{-1})	k_f (sec^{-1})	$K_B \times k_f$ (relative)
A. Linear templates			
P_{sar}^{+}	4.9×10^7	0.11	100
P_{sar}^{-} U7 ($-12, T \rightarrow A$)	1.1×10^7	0.003	0.6
P_{sar}^{-} U3 ($-11, A \rightarrow T$)	N.D.	N.D.	≤ 0.2
P_{sar}^{-} U5 ($-11, A \rightarrow C$)	N.D.	N.D.	≤ 0.2
B. Supercoiled templates			
P_{sar}^{+}	1.5×10^7	0.19	53
P_{sar}^{-} U7 ($-12, T \rightarrow A$)	4.0×10^7	0.032	24
P_{sar}^{-} U3 ($-11, A \rightarrow T$)	7.9×10^7	0.012	18
P_{sar}^{-} U5 ($-11, A \rightarrow C$)	9.1×10^7	0.007	12

K_B and k_f values were determined as described in Materials and methods from the slopes and intercepts of TAU plots such as those shown in Fig. 3. For the comparisons discussed in the text, apparent association rate constants ($K_B \times k_f$) are shown relative to that for P_{sar}^{+} on a linear template. Supercoiled templates were plasmids pMS200 and 432-434. Linear templates were *Hind*III fragments purified from digests of these plasmids (see legend to Fig. 3).

Figure 4. P_{sar} down-mutants produce antirepressor from the P_{late} transcript. Proteins pulse-labeled with [35 S]methionine 32–34 min after infection of DB7000 (sup^0) cells were analyzed by SDS-polyacrylamide gel electrophoresis. All infecting phages carry the P_{ant} $\downarrow$ RU411 deletion and $c1-7$ clear-plaque mutation, and differ in P_{sar} and gene 23 genotypes as indicated. (23^-) 23-*am*H335 23-*am*H79; (Uninf) uninfected cells in this and subsequent figures. Three P22 late proteins are designated by the corresponding gene numbers.

pression of the late operon, we infer that the antirepressor protein made by $P_{sar} \downarrow$ 23^+ phages is produced from the P_{late} transcript. We conclude that synthesis of the P_{sar} transcript interferes with synthesis of antirepressor from the P_{late} transcript.

The experiment presented in Figure 5 examines the effect of the P_{sar} down-mutations on synthesis of antirepressor from the P_{ant} transcript. To prevent repression of P_{ant} by the Arc and Mnt repressors, the infecting phages carry an amber mutation in *arc* and Kn6, a substitution that deletes nearly all of *mnt* (see Fig. 1). The phages also carry $P_{ant} \downarrow$ RU1204 (–10, T : A $\rightarrow$ G : C), a promoter down-mutation that decreases P_{ant} activity about five-fold in vivo (Youdarian et al. 1982). This mutation is included so that the rate of antirepressor synthesis is not too high; with *mnt* $^-$ P_{ant}^+ *arc* $^-$ phage, most of the protein pulse-labeled during infection is antirepressor (Graña et al. 1985), and any further increase due to the P_{sar} down-mutations would be difficult to detect.

The left half of Figure 5 shows that in sup^0 cells pulse-labeled 20 min after infection, the rate of antirepressor synthesis is about twofold higher for Kn6 $P_{ant} \downarrow$ RU1204 *arc-am* $P_{sar} \downarrow$ phages than for the isogenic P_{sar}^+ control. At this early time, antirepressor is synthesized primarily from the P_{ant} transcript, since little or no antirepressor is

produced by phages with the $P_{ant} \downarrow$ RU411 deletion (Fig. 5, right half). We conclude that synthesis of the P_{sar} transcript also interferes with synthesis of antirepressor from the P_{ant} transcript, although the effect on P_{ant} -promoted synthesis is less than the effect on P_{late} -promoted synthesis.

P_{sar} produces a trans-acting repressor of antirepressor synthesis

The experiment presented in Figure 6 shows that the wild-type *sar* region cloned on a plasmid acts *in trans* to repress synthesis of antirepressor by $P_{sar} \downarrow$ phage. A small fragment containing the *sar* region (255 bp, from the *Hind*III site in *arc* to the first *Hind*III site in *ant*; see Fig. 1) was cloned in a derivative of pBR322. A sup^0 host carrying this plasmid, or an isogenic $P_{sar} \downarrow$ plasmid, was infected with P22 Kn6 $P_{ant} \downarrow$ RU1204 *arc-am* $P_{sar} \downarrow$ U7 phage. The kinetics of antirepressor synthesis were assayed by pulse-labeling at various times after infection. The left half of Figure 6 shows that antirepressor is not produced in the presence of the P_{sar}^+ plasmid. Transcription from P_{sar} on the plasmid is required for this effect, since the same phage synthesizes high levels of antirepressor in the presence of the $P_{sar} \downarrow$ plasmid (right half of Fig. 6); the kinetics of antirepressor synthesis in the host with the $P_{sar} \downarrow$ plasmid are virtually identical to the kinetics observed when the host carries no plasmid or the vector plasmid with no P22 insert (data not shown). We

Figure 5. P_{sar} down-mutations increase synthesis of antirepressor from the P_{ant} transcript. DB7000 (sup^0) cells were infected with $c1-7$ phages with the indicated P_{ant} and P_{sar} genotypes. $P_{ant} \downarrow$ RU1204 phages also carry Kn6 and *arc-am*H1605. Proteins were pulse-labeled 20–22 min after infection.

conclude that P_{sar} directs the synthesis of a diffusible product that inhibits synthesis of antirepressor.

Discussion

By selecting P22 mutants that synthesize antirepressor from the P_{late} transcript, we obtained three different mutations in P_{sar} , a promoter within the *ant* coding sequence. The results of transcription experiments in vitro (Liao et al. 1987) show that P_{sar} directs the synthesis of a small antisense RNA from the intercistronic region upstream of *ant*. All three mutations decrease the homology of P_{sar} with the consensus promoter sequence and decrease P_{sar} promoter strength, as measured by an abortive initiation assay in vitro (Fig. 3; Table 1). When compared with isogenic P_{sar}^+ strains, the P_{sar} down-mutants produce considerably higher levels of antirepressor from the P_{late} transcript and slightly higher levels from the P_{ant} transcript in vivo (Fig. 4 and 5). These findings suggest that transcription from P_{sar} interferes with the synthesis of antirepressor.

Various possible mechanisms for this interference may be envisioned. One possibility is that leftward transcription from P_{sar} directly interferes with rightward transcription of *ant* on the same DNA molecule. However, transcriptional interference in vitro works in the opposite way: when P_{ant} and P_{sar} are on the same DNA template, transcription from P_{ant} interferes with transcription from P_{sar} (Liao et al., this issue). Furthermore, whereas transcriptional interference is a *cis*-acting phenomenon, the experiment shown in Figure 6 demonstrates that *sar* repression works well *in trans*.

We do not believe that the *trans*-acting *sar* repressor is a protein. If the P_{sar} transcript produced in vivo is the same as the Rho-independent, full-length transcript produced in vitro, then *sar* RNA has no AUG or GUG codons for translation initiation. The codon UUG, which initiates translation of a few genes in *E. coli* (Aiba et al. 1984; Gren 1984; Hediger 1985), occurs near the 5' end of *sar* RNA (see Fig. 2). We argue that this UUG codon does not initiate a polypeptide because it is not preceded by a Shine-Dalgarno sequence, is not followed by an in-frame termination codon before the 3' end of *sar* RNA, and can be changed to UCG by mutation without affecting the efficiency of *sar* repression (M.M. Susskind unpubl.). As discussed below, it is inviting to speculate that P_{sar} transcripts may be extended in vivo, at least some of the time, and serve as mRNAs for synthesis of Mnt repressor. However, Mnt repressor is not responsible for any of the effects shown here, since the *mnt* gene is deleted in all experiments. An extended *sar* RNA would contain a short, leftward open reading frame corresponding to the end of the *arc* gene (base pairs 483–521 in Sauer et al. 1983); however, this reading frame cannot be responsible for *sar* repression, since it would encode a polypeptide of only 12 amino acids, it is not preceded by a Shine-Dalgarno sequence, and part of it is deleted on the Sar^+ plasmid used in the experiment shown in Figure 6. These considerations lead to the conclusion that *sar* repression is not mediated by a protein.

Figure 6. P_{sar} produces a *trans*-acting repressor of antirepressor synthesis. Derivatives of MS1868 (*sup*⁰) carrying plasmid pMS390 (P_{sar}^+) or pMS391 ($P_{sar} \downarrow$ U7) were infected with Kn6 $P_{ant} \downarrow$ RU1204 *arc-amH1605* $P_{sar} \downarrow$ U7 *c1-7* phage. Proteins were pulse-labeled for 2 min at various times after infection as indicated.

We propose that the *trans*-acting *sar* repressor is the *sar* RNA itself, and that it interferes with antirepressor synthesis by base-pairing with *ant* mRNA. Since *sar* RNA is complementary to the region immediately upstream of the *ant* coding sequence, including the Shine-Dalgarno sequence, base-pairing between *ant* mRNA and *sar* RNA would be expected to interfere with initiation of translation of antirepressor. In addition, pairing of *sar* RNA with *ant* mRNA might destabilize the mRNA, either because mRNA is more susceptible to degradation when it is not undergoing translation, or because it is attacked by a double strand-specific RNase. Destabilization of mRNA by antisense RNA is suggested by results reported for other systems, in which the abundance of an mRNA has been shown to decrease in the presence of antisense RNA (Som and Tomizawa 1983; Mizuno et al. 1984). Further experiments are needed to establish the exact mechanism of inhibition of gene expression by antisense RNA.

The efficiency of repression by *sar* RNA varies widely in the experiments shown here; *sar* reduces the rate of synthesis of antirepressor about 20-fold in Figure 4 and 80-fold in Figure 6, but only about twofold in Figure 5. These differences are presumably due to variations in the amounts of *sar* RNA and *ant* mRNA produced in each experiment. Thus, the amount of P_{ant} transcript produced by the $P_{ant} \downarrow$ RU1204 phages in Figure 5 is apparently greater than the amount of P_{late} transcript produced by the 23⁺ phages in Figure 4, as judged by the amounts of antirepressor produced by the P_{sar} down-mutants in each experiment. Furthermore, the level of *sar*

RNA may be higher in Figure 4 than in Figure 5, as predicted by the observation that strong, rightward transcription of *ant* interferes with synthesis of *sar* RNA from the same DNA template (Liao et al., this issue). Consistent with this idea, *sar* repression is highly effective in the experiment shown in Figure 6, where *sar* RNA is provided *in trans* by a plasmid that has no promoter opposing P_{sar} . Although the plasmid is a ColE1 derivative, efficient repression by the plasmid is not due to Rop protein (see introductory section), since all but the carboxy-terminal end of the *rop* gene is deleted. In short, although direct measurements are needed to assess the effects of varying the absolute and relative concentrations of the complementary RNAs, these parameters probably account for variations in the efficiency of *sar* repression.

The control of antirepressor synthesis during the P22 life cycle is obviously complex, involving three repressors (Mnt protein, Arc protein, and *sar* RNA) that not only regulate antirepressor but also, in many cases, are known or suspected to regulate themselves and each other. Without dwelling on all possible complications, we can summarize our current picture of the regulation of antirepressor by P22 wild type as follows. Initially after infection, none of the repressors is present, and the P_{ant} operon is actively transcribed, resulting in synthesis of Arc protein and antirepressor. By about 10 min after infection of unirradiated cells, the rate of synthesis of antirepressor begins to decline and is rapidly turned off. The phenotype of P22 *arc*⁻ mutants shows that Arc protein is the principal effector of this early turnoff (Susskind 1980), although the phenotype of P_{sar} down-mutants indicates that *sar* RNA also plays a role in determining the level of early, P_{ant} -promoted synthesis of antirepressor. The phenomenon of transcriptional interference suggests that *sar* RNA may not be synthesized efficiently until after Arc protein turns down transcription from P_{ant} . If so, then part of the role of Arc protein would be to stimulate synthesis of *sar* RNA, which would help to complete and maintain the early shutoff of antirepressor synthesis. During the late stage of infection, when *ant* is transcribed as part of the late operon, *sar* RNA efficiently blocks synthesis of antirepressor from the P_{late} transcript. In cells undergoing establishment of lysogeny, P22 eventually synthesizes Mnt repressor, which turns off P_{ant} and turns on P_{mnt} . How Mnt repression becomes established is not clear, since the P_{ant}/P_{mnt} region is initially bound by Arc protein, which *in vitro* represses both P_{ant} and P_{mnt} (S.-M. Liao, unpubl.). One possibility, considered in the accompanying paper, is that P_{sar} transcripts occasionally read through the *sar* transcription terminator and extend to the *mnt* gene; such extended P_{sar} transcripts could serve as mRNAs for synthesis of the first Mnt repressor molecules, which then stimulate efficient transcription of *mnt* from P_{mnt} . At present, however, we have no evidence that P_{sar} promotes synthesis of Mnt repressor.

Even though there are two other repressors of *ant* gene expression, *sar* RNA plays an important role in P22 biology. We have constructed recombinants with any one

of the P_{sar} ↓ alleles and no other mutation in the P22 genome. These *mnt*⁺ *arc*⁺ P_{sar} ↓ phages form clear plaques; like *arc*⁻ mutants, they are defective in establishment of lysogeny. Thus, all three regulators of *ant* gene expression are essential for lysogeny by P22 *ant*⁺ phage. Since *mnt*⁻ mutants do not form clear plaques, the requirement for *sar* is not simply to stimulate establishment of Mnt repression; direct control of antirepressor synthesis by *sar* (as well as by Arc repressor) is necessary to prevent antirepressor from interfering with establishment of *c2* repression.

Materials and methods

Bacteria

S. typhimurium strains used in this study are described by Susskind (1980) and Graña et al. (1985). They include DB7000 (*leuA-am414 sup*⁰), MS1363 (*leuA-am414 supE*), MS1367, a derivative of MS1363 carrying a P22 *c2*⁺ prophage deleted for *immI*, MS1868 (*leuA-am414 r⁻m⁺ sup*⁰), MS1883 (*leuA-am414 r⁻m⁺ supE*), and MS1655, a derivative of MS1363 that carries plasmid pKM101.

Plasmids

All plasmids have the replicon and β-lactamase (*bla*) gene from pBR322. General procedures used for the construction, selection, and purification of plasmids have been described (Youderian et al. 1982). pMS317 (Fig. 1) was constructed in three steps from pPB13 (Berget et al. 1983), a derivative of pBR322 in which the *EcoRI*–*PvuII* (*tetA*) region is replaced by the P_{lac} UV5 promoter and P22 gene 9. First, the *TaqI*–*Clal* fragment of pPB13 containing the 5' end of gene 9 was inserted into the *Clal* site of pBR322. The resulting plasmid, pMS296, has the *TaqI* end of the insert (which is not converted to a *Clal* site) closer to the *EcoRI* site of pBR322. Next, the *PstI*–*Clal* fragment of pMS296, containing the 5' end of *bla* and the 5' end of gene 9, was inserted in place of the analogous fragment of pPB13, generating pMS297. Finally, pMS317 was generated by cleaving pMS297 at its unique *EcoRI* site and inserting an *EcoRI* fragment bearing the λ *cl* repressor gene and P_R promoter, in the orientation that places gene 9 under P_R control. The λ fragment extends from the *Clal* site in *rexA* to a *HaeIII* site in *cro*, and was obtained from pKB252 (Backman et al. 1976), in which the *HaeIII* site has been converted to an *EcoRI* site. The *cl* fragment was obtained by cleaving pKB252 with *Clal*, filling in the *Clal* ends, attaching 6-bp *EcoRI* linkers, and cleaving with *EcoRI*.

pMS316 was constructed by ligating the *EcoRI*–*HindIII* backbone of pBR322 with two fragments of P22 DNA: one extending from the second *HindIII* site in *ant* to the *TaqI* site past T_{ant} , and the other extending from the *BamHI* site past gene 9 to an *EcoRI* site past *int*. Ligation of the *TaqI* and *BamHI* ends, which had been filled in with Klenow fragment, regenerates a *BamHI* site and creates the deletion 9-*del*TB, which was subsequently crossed onto phage (see below).

pMS284 (Youderian et al. 1983) carries a substitution that replaces the *mnt* gene with the kanamycin-resistance (*kan*) gene from transposon Tn903. The *kan* substitution, which is flanked by *PstI* sites, was inverted by cleaving pMS284 with *PstI* and religating, generating pMS361. The inverted substitution, Kn6, was subsequently crossed onto phage (see below).

To construct pMS390 and pMS391, the *HindIII* fragment containing the *sar* region from P_{sar}^{+} and P_{sar} ↓ U7 phages, respectively, was filled in with Klenow fragment and inserted into the filled-in *BamHI* site of pMS99 (Youderian et al. 1982). Both

plasmids have the insert in the same orientation, with *sar* and *bla* transcribed in opposite directions.

Plasmids used for transcription experiments were pMS200 (Youderian et al. 1982) and $P_{sar} \downarrow$ derivatives of pMS200 (pMS432-434), constructed by replacing the *HindIII*–*HpaI* fragment containing P_{sar} .

Phage

Procedures for propagation of ϕ -phages are described by Youderian and Susskind (1980). Crosses between plasmids and phages were performed as described by Youderian et al. (1983), but without ultraviolet (UV) treatment. To cross the *9-delTB* deletion onto P22, a derivative of MS1883 carrying pMS316 was infected with the defective phage particles produced by induction of a lysogen of P22 Ap22, which has an insertion of transposon Tn1 in gene 9 (Weinstock et al. 1979). Progeny of the cross were plated on MS1363 plus p9 (gift of P. Berget). Since the Tn1 insertion in the parental phage makes the P22 genome too large to package in a single particle, the plaque-forming progeny of the cross are recombinants that have the *9-delTB* deletion in place of the Ap22 insertion. Similarly, the Kn6 substitution was recombined onto P22 by crossing plasmid pMS361 with P22 Ap521 defective particles, which have Tn1 inserted to the left of *mnt*. The *9-delTB* and Kn6 mutations were then combined with other P22 markers by standard phage crosses.

To obtain P_{sar} down-mutants, P22 *sieA44* $P_{ant} \downarrow$ RU411 *9-delTB* phage were irradiated with UV and passaged for a single growth cycle in UV-irradiated MS1655. Mutants were selected by plating the resulting lysates on host MS1883 carrying pMS317. The frequency of mutants was about 10^{-5} in mutagenized stocks, and undetectable ($<10^{-8}$) in the starting stock.

P_{sar} down-mutations were recombined into other genetic backgrounds as follows. The original mutants (*sieA44* $P_{ant} \downarrow$ RU411 $P_{sar} \downarrow$ *9-delTB*) were crossed with P22 *sieA44 mnt-ts1 arc-am* H1605 (Susskind 1980). $P_{ant} \downarrow$ RU411 9^+ recombinants were selected by plating on DB7000 at 37°C without exogenous p9. (The *arc-am* parental phage cannot grow in *sup*⁰ cells because of lethal overproduction of antirepressor.) These recombinants were tested for presence of the P_{sar} down-mutation by test crosses with P22 *sieA mnt-ts* $P_{ant} \downarrow$ RU1283 *arc-am* (Youderian et al. 1982). This P_{ant} down-mutant forms tiny plaques on MS1367 (marginal Ant⁺ phenotype), whereas *sieA mnt-ts* $P_{ant} \downarrow$ RU1283 *arc-am* $P_{sar} \downarrow$ recombinants generated in the test cross form large, clear plaques.

Kn6 $P_{ant} \downarrow$ RU1204 *arc-am* $P_{sar} \downarrow$ strains were constructed by crossing $P_{ant} \downarrow$ RU411 $P_{sar} \downarrow$ phages with P22 Kn6 $P_{ant} \downarrow$ RU1204 *arc-am*. Progeny were plated on MS1367, which is nonpermissive for $P_{ant} \downarrow$ RU411. Thus, the progeny that plate under this condition must have the Kn6 $P_{ant} \downarrow$ RU1204 *arc-am* mutations (since they are allelic with $P_{ant} \downarrow$ RU411); whereas the P_{sar}^+ parental type forms turbid plaques, recombinants that also have a P_{sar} down-mutation form clear plaques.

The clear-plaque mutation *c1-7* was used to ensure lytic development upon infection in protein labeling experiments. To cross in *c1-7* without segregation of markers in *imm1*, a *c1-7* strain with a deletion of *imm1* and gene 9 (Ap68tpfr72; Youderian and Susskind 1980) was used. *c1-7* derivatives of Kn6 $P_{ant} \downarrow$ RU1204 *arc-am* $P_{sar} \downarrow$ phages, which already form clear plaques, were identified by complementation tests for lysogeny. P22 Ap68tpfr72 *c1-7* 23-*am* H335 23-*am* H79 was used to cross the *c1* and 23-*am* mutations into $P_{ant} \downarrow$ RU411 P_{sar} strains. The presence of each 23-*am* mutation in recombinants was confirmed by test crosses with 23-*am* single mutants.

Assay of antirepressor synthesis in vivo

Procedures for infection and labeling of unirradiated cells, rapid preparation of lysates, SDS-polyacrylamide gel electrophoresis, and gel autoradiography were done as described by Graña et al. (1985). In the experiment shown in Figure 6, ampicillin (100 µg/ml) was added to the medium.

Abortive initiation assay

The properties of the abortive initiation assay in the study of RNA initiation have been described (McClure et al. 1978). Standard reaction buffer was 40 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM MgCl₂, 100 µg/ml bovine serum albumin, and 1 mM dithiothreitol. The substrates for the reaction with P_{sar} were 200 µM GpU (Sigma) and 50 µM UTP (ICN) with [α -³²P]UTP (Amersham) added to a specific activity of 200 cpm/pmol. DNA and RNA polymerase concentrations used are indicated in the legend to Figure 3. Lag reactions were initiated by addition of RNA polymerase after 10 min preincubation of DNA and nucleotides at 37°C. Samples were taken at appropriate times and spotted on Whatman 3MM paper prespotted with 0.1 M EDTA. The product (GpUpU) was separated from labeled UTP by ascending chromatography in WASP solvent (water/saturated ammonium sulfate/isopropanol, 18 : 80 : 2, by volume). The average time required for open complex formation (τ_{obs}) was calculated using a computer program (Mulligan et al. 1985). Based on the two-step model for open complex formation (McClure 1985), τ_{obs} is related to the RNA polymerase concentration $[R]$ as follows:

$$\tau_{obs} = 1/k_f + 1/K_B k_f [R]$$

The parameters for the two steps in open complex formation were quantified using TAU plots (τ_{obs} plotted versus $[R]^{-1}$): k_f is the reciprocal of the intercept and K_B is the ratio of intercept/slope. The values of K_B listed in Table 1 were corrected for the fraction of active holoenzyme (0.60) used in these experiments.

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