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

I. Characterization in vitro of the P_{sar} promoter and the *sar* RNA transcript

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The characterization in vitro of a newly discovered promoter (P_{sar}) in the bacteriophage P22 *immI* region is described. P_{sar} is located within the *ant* gene and is directed toward the major *immI* promoter, P_{ant} . The entire intercistronic region between the P22 *arc* and *ant* genes (69 bp) is transcribed. The initiation and termination of *sar* (small antisense regulatory) RNA transcription are unusual. Frequent abortive initiation occurs in the presence of all four NTPs; RNA products 3–13 nucleotides in length are produced in about 15- to 25-fold larger numbers than full-length transcripts. Termination of *sar* RNA synthesis occurs after transcription of the first and second Ts of a TTTA sequence following a region of hyphenated dyad symmetry. The effects of convergent transcription between P_{ant} and P_{sar} were investigated on linear and supercoiled templates. Active transcription from P_{ant} interferes with full-length transcription from P_{sar} ; several factors that interfere with P_{ant} initiation (e.g., P_{ant} down-mutation, Mnt repressor protein, Arc repressor protein) result in indirect activation of *sar* RNA synthesis. The *sar* RNA pairs rapidly with *ant* mRNA to form a stable stoichiometric complex. The location and properties of P_{sar} suggest an important regulatory function for *sar* RNA as a negative effector of *ant* expression. The results of Wu et al. (this issue) support this suggestion.

[Key Words: RNA polymerase; transcription; abortive initiation; RNA–RNA pairing]

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Bacteriophage P22 is a temperate phage of *Salmonella typhimurium*. Most aspects of the control of the lysis-lysogeny decision and other pathways in development are similar to those in bacteriophage λ (for review, see Susskind and Youderian 1983). Thus, the *immC* region of P22 is similar in genetic organization to the immunity region of λ . Unique to P22 is a second immunity region, termed *immI*, which includes an antirepressor gene (*ant*) and its regulators (Fig. 1). Antirepressor is a 35-kD protein that inhibits various lambdoid repressors, including the P22 *c2* repressor. The *ant* gene is transcribed rightward from the P_{ant} promoter and lies within an operon containing the *arc* gene. Genetic and biochemical studies suggest that Arc protein binds at P_{ant} to repress the *ant* operon shortly after infection (Susskind 1980; Vershon et al. 1985). *Ant* gene expression is turned off in P22 lysogens by a second repressor, the product of the *mnt* gene. Mnt protein binds to an operator located at the start point of P_{ant} transcription (Sauer et al. 1983). Repression of P_{ant} by Mnt also results in activation of P_{mnu} , a leftward (divergent) promoter that overlaps P_{ant} (Vershon et al. 1987b).

The *ant* gene is also transcribed late during lytic infection as part of the P22 late operon, but Ant protein is not synthesized. Susskind and co-workers devised a genetic selection to obtain P22 mutants that can synthesize Ant late in infection. These mutations were found to lie in the extreme 5' end of the *ant* gene. At about the same time, experiments performed in vitro in the McClure laboratory showed that a small RNA was initiated from a promoter in this same region. Further analysis showed that the P22 mutations were in the –10 region of the promoter responsible for the synthesis of the small RNA. This RNA (*sar* RNA, for small antisense regulatory RNA) spans the entire intercistronic region between *arc* and *ant* in the antisense direction.

The collaborative experiments described in this report and in Wu et al. (this issue) examine the regulatory significance of the *sar* RNA and its promoter (P_{sar}). Two plausible functions for P_{sar} follow immediately from its location and orientation: (1) transcription from P_{sar} might interfere with convergent transcription of *ant*, an effect predicted to occur in *cis*; or (2) the synthesis of *sar* RNA might interfere with *ant* expression because *sar*

Figure 1. Transcriptional patterns within the P22 immunity I region. (A) The map shows the region cloned in pMS200, in which the P22 insert is flanked by *EcoRI* sites from the vector plasmid. The locations of the *mnt*, *arc*, and *ant* genes are indicated by bars. Arrows show the pattern of transcription originating at *P_{mnt}*, *P_{ant}*, and *P_{sar}*. *O_{arc}* and *O_{mnt}* represent operator sites to which Arc and Mnt proteins bind. The locations of several restriction endonuclease cleavage sites are also shown. (B) The DNA sequence of the *P_{sar}* region is shown (Sauer et al. 1983). The -10 and -35 hexamers of *P_{sar}* are underlined. The numbers correspond to the distance in base pairs from the *P_{sar}* transcription start point at +1. Arrows below the sequence indicate a region of dyad symmetry, which may result in stem-and-loop structures in the RNA involved in termination of transcription; the dotted portions of these arrows indicate potential G:U base pairs in the stem structure for leftward transcripts. The termination sites for the small antisense RNA are labeled *T_{sar}*. The translational codons that correspond to *arc* termination and *ant* initiation are indicated above the sequence. The Shine-Dalgarno sequence for *ant* translation is indicated with asterisks.

RNA is complementary to the *ant* ribosome binding site region, an effect predicted to occur in *trans*. Our results do not support the first suggestion. In fact, when convergent transcription was examined in vitro, we found that transcription from *P_{ant}* interfered with *sar* RNA synthesis. The in vivo and in vitro properties of *P_{sar}* and its transcript strongly support a model for *trans* inhibition of *ant* expression by RNA-RNA pairing.

Results

sar RNA is transcribed from the region between *arc* and *ant*

We first noticed the *sar* RNA on gels that were used to separate RNA products initiated at the *P_{ant}* and *P_{mnt}* promoters. The results of initial attempts to locate *P_{sar}* by transcribing templates cleaved with various restriction enzymes were difficult to interpret because, as shown below, transcription from *P_{ant}* interferes with transcription from *P_{sar}*. Ultimately, we searched the DNA sequence of the *ant* operon using the TARGESEARCH program described by Mulligan et al. (1984) and located three potential promoters. The site of greatest homology with the consensus promoter sequence (homology score = 57) turned out to be the one responsible for *sar* transcription as described below.

By sequencing the 5' and 3' termini of *sar* RNA as described in Materials and methods, we established that *sar* RNA is transcribed from the intercistronic region be-

tween the *arc* and *ant* genes (Fig. 1). Partial digestions of [γ -³²P]GTP-labeled *sar* RNA with ribonucleases T₁ and U₂ showed that the RNA initiates at the base pair immediately to the left of the *ant* translational start codon ATG. By labeling the 3' ends of *P_{sar}* transcripts [³²P]pCp using T4 RNA ligase, the RNA was shown to terminate heterogeneously at positions +68 and +69 adjacent to and within, respectively, the termination codon of *arc* (Fig. 1). The addition of *Escherichia coli* Rho factor did not significantly affect the overall termination efficiency at this site or the relative amounts of the two RNA species (data not shown). In Figure 1 we have designated this Rho-independent termination site as *T_{sar}*. Thus, the *sar* RNA is transcribed from the entire intercistronic region between the *arc* and *ant* genes, in the antisense direction.

RNA polymerase at *P_{sar}* makes abortive and full-length transcripts

The RNA products synthesized from a linear template carrying *P_{sar}* in the presence of all four NTPs included not only full-length transcripts (68 and 69 nucleotides), but also many abortive products 3–13 residues in length (Fig. 2). This characteristic pattern of abortive products was observed on both linear and supercoiled templates, and at UTP concentrations varying from 50 μ M to 200 μ M (data not shown). Since these experiments were performed in the presence of heparin, the abortive synthesis corresponds only to dissociation of the RNA product

Figure 2. RNA product distribution from P_{sar} transcription. The number of RNA chains synthesized per template is shown as a bar at the corresponding product length. The standard reaction conditions were 40 mM TrisCl (pH 8), 100 mM KCl, 10 mM MgCl₂, 100 μ g/ml bovine serum albumin, 1 mM dithiothreitol. The DNA template concentration was 5 nM; the RNA polymerase concentration was 50 nM. [γ -³²P]GTP-labeled transcripts were synthesized in vitro from the 832-bp *Eco*RI fragment cleaved with *Fnu*DII (at position +75 from the P_{sar} start site). Transcription was limited to a single round of synthesis (see Materials and methods). The number of RNA chains per template was calculated from the amount of 5'-terminal GTP in each product as described in Materials and methods.

from ternary complexes rather than dissociation of RNA polymerase from the promoter (i.e., premature termination). The expected dinucleotide, pppGpU, and RNA products from 14 to 67 nucleotides in length were not detected. The total number of abortive products was about 15–25 times the number of full-length transcripts. The efficiency of termination of full-length transcripts was about 50% at position +68, and more than 90% of the remaining RNA chains terminated at position +69. The overall termination efficiency at T_{sar} , calculated from the yield of readthrough transcripts that extended to the end of the linear template, was >95%.

Transcription from P_{ant} interferes with transcription from P_{sar}

The effect of transcriptional interference resulting from the convergent orientation of P_{sar} and P_{ant} is examined in the experiments of Figure 3. When a supercoiled DNA template containing both P_{sar} and wild-type P_{ant} was transcribed, no full-length *sar* transcripts were synthesized (Fig. 3A, lane 2). However, repression of P_{ant} by Arc protein (lane 3) or Mnt protein (lane 4) resulted in the synthesis of full-length P_{sar} transcripts. *sar* RNA was also produced in the absence of Arc and Mnt from an otherwise identical template in which P_{ant} promoter strength was severely reduced by a mutation $P_{ant} \downarrow$ RE167 (Fig. 3A, lane 5). The P_{ant} transcript was not observed on these supercoiled templates because of readthrough from the insert into vector DNA.

The effect of P_{ant} transcription on P_{sar} transcription was examined in detail using an 832-bp *Eco*RI fragment containing both P_{sar} and P_{ant} (see Fig. 1). This DNA fragment was purified and cleaved in four separate reactions with restriction endonucleases *Ava*II, *Hha*I, *Hinc*II, and *Eco*RV. The products of in vitro transcription of these digested templates and of the intact *Eco*RI fragment are shown in Figure 3B. *sar* RNA was observed only in lanes 2 and 3, where the template had been digested with *Ava*II and *Hha*I, respectively. In both of these cases, the restriction cleavage site lies between the convergently oriented P_{ant} and P_{sar} promoters. The absence of *sar* RNA synthesis in lane 4 follows from the fact that *Hinc*II cleavage in the –35 region of P_{sar} destroys the promoter. The absence of *sar* RNA synthesis in lane 1 (intact *Eco*RI fragment) and in lane 5 (*Eco*RV-cleaved template) shows

Figure 3. The effects of convergent transcription on the activity of the P_{sar} promoter. Transcripts synthesized in vitro on various DNA templates were separated on 5% acrylamide–7 M urea gels as described in Materials and methods. The standard reaction conditions of Figure 2 were used. The DNA concentration in the reactions was 3 nM, the enzyme concentration was 50 nM, the UTP concentration was 20 μ M, and [α -³²P]UTP was added to a specific activity of 1250 cpm/pmol. (A) Transcription of supercoiled DNA templates: (lane 1) pMS99 (control without P22 DNA insert); (lane 2) pMS200 (P_{ant} wild-type); (lane 3) pMS200, 1 μ M Arc protein added; (lane 4) pMS200, 280 nM Mnt protein added; (lane 5) pMS206 ($P_{ant} \downarrow$ RE167). The locations of pBR322 RNAI and P_{sar} transcripts are shown on the right. The longer RNAs at the top of the gel were not analyzed. (B) Transcription of the 832-bp *Eco*RI fragment cleaved with various restriction enzymes. (Lane 1) uncut; (lane 2) cleaved with *Ava*II; (lane 3) *Hha*I; (lane 4) *Hinc*II; (lane 5) *Eco*RV. Numbers on the right indicate the expected lengths of the run-off transcripts from P_{ant} (see Fig. 1A). The bands labeled a and b initiate at P_{ant} and are discussed in the text.

that transcription from P_{ant} on the same template molecule is sufficient to interfere with *sar* RNA synthesis. In those cases where we observed interference with *sar* RNA synthesis (lanes 1 and 5), we also found that the distribution and yield of abortive products from P_{sar} described above were similar to those observed in reactions in which interference was not occurring (data not shown).

P_{ant} transcripts were relatively unaffected by P_{sar} activity. Transcripts were not observed for the template cleaved with *Avall*, which cuts near the start site for P_{ant} (Fig. 3B, lane 2); the other templates produced run-off P_{ant} transcripts that vary in size, as predicted from the known locations of the restriction sites (Fig. 1). The only evidence for termination of P_{ant} transcripts within *sar* is the presence of bands labeled a and b in Figure 3B. Both of these RNAs originate from P_{ant} because they were labeled by [γ - ^{32} P]ATP (data not shown), while the 5' end of *sar* RNA is labeled by [γ - ^{32} P]GTP. The 3' termini were not determined, but the following conclusions can be drawn based on gel mobilities. First, band a was slightly longer than the run-off transcript from the *HhaI*-cleaved template. Termination of this RNA probably occurred near the T_{sar} site and was observed even when P_{sar} was inactivated by *HincII* cleavage. Second, band b, which was found when the intact *EcoRI* fragment or *EcoRV*-cleaved template was transcribed, corresponded in length to termination at approximately +20 with respect to the P_{sar} initiation site. This product was not found when P_{sar} was inactivated by *HincII* cleavage. Thus, band b is the only candidate for P_{sar} -mediated interference with P_{ant} transcription.

The inhibition of *sar* RNA synthesis by P_{ant} transcription was also shown to be dependent on P_{ant} promoter strength by using increasing concentrations of Arc repressor to inhibit P_{ant} transcription initiation progressively. Arc protein binds to its operator within P_{ant} with a relatively low affinity (Vershon et al. 1987a). Half-maximal repression of P_{ant} occurs at an Arc concentration of 2×10^{-7} M. As shown in Figure 4, it is evident that repression of P_{ant} by Arc results in coordinate stimulation of full-length transcription from P_{sar} . This stimulation is due entirely to the indirect effect of a reduction of P_{ant} transcription initiation by Arc because Arc protein at these concentrations has no direct effect *sar* RNA synthesis when DNA fragments containing the P_{sar} promoter are used (data not shown). Repression of P_{ant} and coordinate activation of *sar* transcription were also seen in the presence of Mnt protein (data not shown). Because of high binding affinity of Mnt for its operator, the midpoint of the coordinate switch in P_{ant} and P_{sar} transcription occurred at much lower Mnt concentrations (10–20 nM).

sar RNA binds to *ant* mRNA

Pairing of *ant* mRNA and *sar* RNA was examined in the experiment shown in Figure 5. The synthesis of *sar* RNA and *ant* mRNA in separate reactions is shown in lanes 1 and 2, respectively, of Figure 5A. Lane 3 in Figure 5A

Figure 4. The effect of Arc repressor on the synthesis of RNA from P_{ant} and P_{sar} . The relative numbers of P_{ant} run-off transcripts (●) and full-length *sar* RNAs (■) are plotted versus the concentration of Arc protein. Transcripts synthesized in vitro from the 832-bp *EcoRI* fragment cleaved with *EcoRV* were analyzed as described in Materials and methods. The standard reaction conditions of Fig. 2 were used except that 20 μ M UTP (sp. act. 1250 cpm/pmol) was used. The template concentration was 5 nM; the RNA polymerase concentration was 50 nM. After visualization by autoradiography, the transcript bands were cut out, and the radioactivity was measured in a scintillation counter. The number of RNA chains per template molecule was calculated from the amount of UMP incorporated into each transcript and the UMP composition of those RNAs. We also observed that the distribution and yield of abortive products from P_{sar} were approximately equal at all of the Arc concentrations used (data not shown).

shows the results of transcription when both *sar* RNA and *ant* mRNA were synthesized in the same reaction solution. We observed rapid formation of a complex between the two RNAs under standard transcription conditions. The complex migrated more slowly than *ant* mRNA on native TBE gels (Fig. 5A, lane 3). The portion of the gel containing the putative complex was cut out; the RNA was then denatured in urea, and the RNA complex was resolved on denaturing gels into two species which migrated to positions corresponding to the separate *ant* and *sar* RNAs (Fig. 5B, lane 3). Measurements of the radioactivity in the resolved bands yielded an average stoichiometric ratio for *sar/ant* of 0.9. We do not as yet know the precise location, extent, or stability of RNA–RNA duplex structure in the paired complex separated on native gels. These characteristics will be important in the future for understanding the mechanism of *sar* inhibition of *ant* expression.

Discussion

We have found that a bacteriophage P22 promoter (P_{sar}) directs the synthesis of a small antisense RNA (68–69 nucleotides long) from the intercistronic region between the *arc* and *ant* genes. Transcription from P_{sar} is directed towards P_{ant} , the major *immI* promoter. Active tran-

Figure 5. Complex formation between *ant* mRNA and *sar* RNA. (A) *sar* RNA and *ant* RNA were synthesized from different templates. (Lane 1) *Hind*III DNA fragment containing only the *sar* gene; (lane 2) the 832-bp *Eco*RI fragment cut with *Hinc*II; (lane 3) the above two DNA templates. The transcription reaction was performed at 37°C for 5 min under standard reaction conditions (see Materials and methods). After addition of rifampicin, the reaction mixtures were applied directly to a 5% acrylamide TBE gel. The locations of *sar* and *ant* RNAs are indicated between the panels as S and A, respectively. The putative complex is indicated in C. (B) Segments of gel shown in A corresponding to S in lane 1, A in lane 2, and C in lane 3 were cut out, soaked in 8 M urea–TBE buffer for 2 hr, and placed in boiling water 15 min. The products were analyzed by electrophoresis in a 5% acrylamide 7 M urea–TBE gel.

scription from P_{ant} inhibits full-length transcription from P_{sar} , but does not affect the synthesis of abortive products from P_{sar} . The location and transcriptional properties of P_{sar} suggest two possible in vivo functions for this promoter: (1) direct negative control of *ant* expression; and (2) transcription of *mnt* during establishment of lysogeny.

Even in the presence of all four ribonucleoside triphosphates, about 95% of the RNA molecules initiated at the P_{sar} promoter are abortive transcripts 3–13 nucleotides in length. This pattern of abortive products released from P_{sar} differs from the pattern of abortive products from the *lac*L8UV5 and Tn5 promoters (Munson and Reznikoff 1981). The tendency of certain promoter–polymerase complexes to release abortive products is not currently understood. It is noteworthy that the results of Figure 2 show that RNA polymerase can initiate at the P_{sar} start site after releasing abortive products 13 nucleotides in length without dissociating from the DNA. We believe that all of the oligonucleotide products result from the release of RNA chains rather than pausing because the molar yield of full-length *sar* RNA was equal to the template concentration. The presence or absence of the sigma subunit on

the enzyme during this process is an intriguing and important issue. On the one hand, it is difficult to imagine that the enzyme could reinitiate at the +1 position without the presence of sigma subunit. On the other hand, sigma has been shown to dissociate at 8 or 9 nucleotides on a poly(dAT) template (Hansen and McClure 1980). We believe it is likely that sigma subunit is still present in the abortively initiating complexes at P_{sar} ; however, we also recognize that the precise point of sigma release has not yet been established for any promoter.

Our in vitro transcription results demonstrate that transcription from P_{ant} interferes with full-length transcription from P_{sar} , even though promoter strength assays suggest that P_{sar} is more active than P_{ant} in vitro (Wu et al. 1987, and unpublished results). One explanation for this finding is based on the observation that P_{sar} produces a large molar excess of abortive products. It is possible that when RNA polymerase is cycling at the P_{sar} promoter and releasing abortive products, RNA polymerase transcribing rightward from P_{ant} reaches the P_{sar} region and disrupts the RNA polymerase– P_{sar} complex. Thus, productive initiation from P_{sar} would be severely disrupted by convergent transcription complexes originating at P_{ant} , while synthesis of abortive P_{sar} products would be essentially unaffected. It is only when P_{ant} initiation frequency is reduced that the complexes cycling at P_{sar} have the time required to negotiate a complete exit from the promoter to make a full-length RNA product.

There are several reports of the effects of convergent transcription in vivo. Transcription from the *his* promoter led to a fivefold decrease in the synthesis of the *rfb* gene product in a fusion between the two opposing operons (Levinthal and Nikaido 1969). When the *lac* and *trp* operons were fused in convergent orientation, no interference of gene expression was found (Miller et al. 1970). Studies on convergent transcription between the *E. coli trp* and λP_L promoters suggested that the interference due to convergent transcription was symmetrical, and that the inhibition of one promoter by the other reflected the unequal strengths of the two promoters (Ward and Murray 1979). An example of naturally occurring convergent transcription that resulted in interference was reported by Schmeissner et al. (1980), who found that cII activation of λP_E resulted in a 50% decrease in λP_R transcription.

In the absence of Rho factor, *sar* RNA terminates at +68 and +69. There is no evidence for nontemplated oligo(A) addition such as that reported for the *loop* RNA termination site (Rosenberg et al. 1975; Smith and Hedgcock 1975). The efficiency of the *sar* RNA termination at this site is >95% under our in vitro transcription conditions. The DNA sequence of the *sar* region near the termination site shares certain features with the consensus structure of other Rho-independent terminators (von Hippel et al. 1984; Platt 1986). The transcribed DNA contains a relatively G-C-rich potential stem-loop structure centered about 20 bp upstream of the termination sites. However, there are only three, instead of

four or more, consecutive T residues at T_{sar} following the dyad symmetric region; moreover, the transcript terminates at the first or second of these Ts. One might expect some inefficient termination of P_{ant} transcription on the opposite DNA strand, since it also carries the dyad symmetric sequences, but the stretch of several consecutive T residues is absent. A possible candidate for an inefficiently terminated transcript of this sort was band a RNA, which originated from P_{ant} (see Fig. 3B).

The regulatory function of *sar* RNA as an inhibitor of antirepressor synthesis from both P_{ant} and P_{late} transcripts is strongly supported by the genetic analysis described in Wu et al. (this issue). The mechanism of this inhibition by direct RNA–RNA complex formation is supported by the stoichiometry and speed of the in vitro pairing reaction described in this paper. We cannot rule out a direct transcriptional effect of P_{sar} on P_{ant} ; however, the predominant effect of opposing transcription in vitro is that transcription from P_{ant} interferes with *sar* RNA synthesis.

The indirect activation of full-length *sar* transcription by Arc observed in vitro (see Fig. 4) suggests that Arc and *sar* RNA might act in concert to effect the turn-off of *ant* expression early in infection. We speculate that the following temporal sequence might occur in vivo. Strong transcription from P_{ant} immediately after infection would interfere with *sar* RNA synthesis and result in rapid synthesis of antirepressor and Arc. As the concentration of Arc increased, P_{ant} activity would be gradually reduced. As a consequence, *sar* RNA would increase in amount and would complex the remaining *ant* mRNA to complete the turn-off of *ant* expression.

A judgment on the possible contribution of P_{sar} to *mnt* expression during establishment of lysogeny is not possible at this time. In favor of such a role is the finding that P_{sar} is about 30 times stronger than P_{mnt} . However, the frequent abortive initiation from P_{sar} and, more important, the efficient termination at T_{sar} observed in vitro combine to reduce dramatically (>95%) the number of transcription complexes that could reach the *mnt* region. Moreover, host termination factors or phage antitermination factors could significantly affect transcription from P_{sar} in vivo. On balance we consider the possibility of P_{sar} 's contribution to early *mnt* expression an open question.

Materials and methods

DNA templates and enzymes

Plasmids pMS200 (P_{ant}^+) and pMS206 ($P_{ant} \downarrow$ RE167) have the 818-bp *Sau3A1* fragment containing P_{ant} and P_{sar} inserted into the *Bam*HI site of pMS99 which is flanked by *Eco*RI sites (Yoderian et al. 1982). The 832-bp *Eco*RI fragment was purified from a 5% acrylamide gel following *Eco*RI digestion of pMS200. A *Hind*III fragment containing wild-type P_{sar} was also inserted into the *Bam*HI site of pMS99, designated as pMS390 in Wu et al. (this issue). The 283-bp DNA fragments were separated from the plasmid backbone DNA using 7% PEG fractionation (Lis and Schleif 1975). The purified fragments were precipitated with ethanol and dialyzed versus 0.01 M Tris, 0.10 mM EDTA. DNA concentrations were calculated using $E_{260} = 6.5 \text{ mm}^{-1}$

cm^{-1} DNA phosphorus. *E. coli* RNA polymerase was purified according to Burgess and Jendrisak (1975) and Lowe et al. (1979). Restriction endonucleases (New England Biolabs and Bethesda Research Laboratories), T4 RNA ligase, and ribonucleases T1 and U2 (P-L Biochemicals) were purchased from the indicated suppliers. Arc and Mnt proteins were gifts from A. Vershon and R. Sauer (MIT).

In vitro RNA synthesis

The in vitro RNA synthesis reactions were performed in standard reaction buffer: 40 mM TrisCl (pH 8), 100 mM KCl, 10 mM MgCl_2 , 100 $\mu\text{g/ml}$ bovine serum albumin, 1 mM dithiothreitol. The enzyme and substrate concentrations were: 50 nM RNA polymerase, 200 μM ATP, GTP, and CTP, 20 μM [α - ^{32}P]UTP with a specific activity of 1250 cpm per pmole. DNA concentrations are indicated in the figure legends. All four ribonucleoside triphosphates (ICN), DNA, and Arc or Mnt protein (where present) were preincubated at 37°C for 10 min. The transcription reactions were initiated by addition of RNA polymerase to a final volume of 75 μl . After 5 min reaction at 37°C, heparin was added to a final concentration of 50 $\mu\text{g/ml}$. After an additional 5 min incubation, the reaction was stopped by addition of 25 μl 0.1 M EDTA. Samples were then extracted with neutralized phenol, precipitated with ethanol, and analyzed on a 25-cm 5% polyacrylamide, 7 M urea, TBE gel. Gel electrophoresis was performed at 200 V until the bromophenol blue dye was 3 cm from the bottom; the gel was then autoradiographed using Kodak XAR-5 film. For one round of transcription shown in Figure 2, the concentration of UTP was 200 μM and [γ - ^{32}P]GTP was 100 μM (at a final specific activity of 5000 cpm/pmole). DNA and enzyme were preincubated for 10 min at 37°C, the nucleotides and heparin (to 20 $\mu\text{g/ml}$) were then added to initiate the reaction. After 2, 4, 7, and 10 min at 37°C, the reaction was stopped with EDTA. Samples were analyzed on 20% acrylamide–7 M urea–TBE gel as described above. The individual RNA products ranging in length from 3 to 69 nucleotides were identified on the autoradiogram. The corresponding portions of the gel were cut out and counted in a scintillation counter (Cerenkov effect). The radioactivity in each sample and the specific radioactivity of the [γ - ^{32}P]GTP were used to calculate the number of RNA products per template molecule.

Determination of 5' and 3' termini of *sar* RNA

In vitro synthesis was performed as described above in the presence of [γ - ^{32}P]GTP. Indeed, no labeling was observed with [γ - ^{32}P]ATP. The RNA was separated from triphosphates and (a large amount of) oligonucleotides using a P4 column (1.5 ml). The labeled RNA was precipitated with ethanol and dissolved in T1 buffer (20 mM sodium citrate, pH 5.5, and 7 M urea) containing 20 $\mu\text{g/ml}$ tRNA. Various amounts of RNase T1 (P-L Biochemicals) were added to separate tubes. Partial digestions were carried out at 55°C. The digests were run on 20% acrylamide, 7 M urea, TBE gels, along with a partial digest (using pH 9.0, HCO_3^- , 90°C, 15 min) to provide length standards. An autoradiogram of the gel yielded a clear pattern of cleavages from +1 to +26, except that cleavage after +4 or +5 was markedly reduced. Additional digestion experiments with RNase U2 confirmed the location of the 5' terminus shown in Figure 1. The 3' ends were labeled using [$5'$ - ^{32}P]pCp and T4 RNA ligase (P-L Biochemicals). Preliminary partial digestion experiments and reactions using chain-terminating triphosphates (3'-O-methyl NTPs) suggested that the 3' termini were near +70. We also suspected that there were (at least) two termination sites. To determine these sites unambiguously, the 3'-labeled mixture

was digested with RNase T1 to completion. The products were run on 20% acrylamide, 7 M urea, TBE gels. The resulting autoradiograms showed only two labeled oligonucleotides corresponding to lengths 8 and 9 (with pCp ends). These oligonucleotides were extracted from the gel and purified by passage over QAE Sephadex and elution with 2.5 M triethylamine bicarbonate. The purified 3' terminal oligonucleotides were digested partially and completely with pancreatic RNase A and RNase U2. Electrophoretic and chromatographic comparison of the digested products with known standards showed that the 3' termini were at positions 68 and 69. The most important products supporting this conclusion were: (1) complete digestion with RNase A yielded nearest neighbor transfer of *pCp only to Up*; and (2) complete digestion with RNase U2 yielded CpUpU*pCp and CpUp*Cp. The identity of the U2 products was also confirmed by *pCp labeling of authentic CpUpU and CpU. Direct determination of the 3' terminal structures was required because the electrophoretic mobilities of the oligonucleotide products depended somewhat on base composition.

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