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A Role for a Small Stable RNA in Modulating the Activity of DNA-Binding Proteins

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Summary

The 10Sa RNA, encoded by the E. coli *ssrA* gene, appears to modulate action of some DNA-binding proteins. When *ssrA* is inactivated, *lacZ* expression from the *lac* operon, as well as *galK* from a *gal* operon fused to a phage λ promoter, is reduced from that observed in bacteria wild-type for *ssrA*. These differences are not observed if the relevant repressor is inactive, suggesting that in the absence of 10Sa RNA binding of LacI and λ cI repressors is enhanced. Gel mobility shifts show that 10Sa RNA binds these repressors and that an excess of 10Sa RNA competes for binding of λ cI with a DNA fragment containing the O_R2 repressor-binding sequence. Similar observations were made in studies of the E. coli LexA repressor and phage P22 C1 transcription activator proteins. These results suggest that direct interaction with 10Sa RNA may explain this modulation of protein–DNA interactions.

Introduction

Classically, RNA molecules have been identified as ribosomal, transfer, and messenger RNAs according to their roles in the translation process. However, it is now recognized that RNA also functions in a variety of other roles, including, but not limited to, those of catalysis, regulation of gene expression, and secretion (for examples see Wightman et al., 1993; Darr et al., 1992; Altman et al., 1993; Takayama and Inouye, 1990; Brockdorff et al., 1992; Brunkow and Tilghman, 1991; Inouye and Delihas, 1988; Jensen et al., 1994). In Escherichia coli, small stable RNAs have been identified that are not recognizable as one of the three classical types of RNAs (reviewed by Inouye and Delihas, 1988). Although the functions of some of these RNAs have been identified, the functions of others remain a mystery.

We focus on one such RNA, the E. coli 10Sa RNA. Encoded by the *ssrA* gene, which lacks significant open reading frames, the 362 nt 10Sa RNA is derived from a 457 nt precursor that is processed at its 5′ and 3′ ends by RNases P and III, respectively (Subbarao and Apirion, 1989; Chauhan and Apirion, 1989; Komine et al., 1994). Homologs of this relatively abundant RNA, which is found at ~1000 copies per haploid genome in E. coli (Subbarao and Apirion, 1989), have been identified in such diverse bacteria as Mycobacterium tuberculosis (Tyagi and Kinger, 1992), Alcaligenes eutrophus (Brown et al., 1990), Vibrio cholerae (C. Huang, V. DiRita, and D. I. F., unpublished data), Bacillus subtilis, and Mycoplasma capricolum (Ushida et al., 1994), leading us to suspect that homologs may be present in most, if not all, bacteria.

Although E. coli *ssrA* mutants are viable, indicating that 10Sa RNA is not essential, subtle differences in the growth rate and physiological response of these mutants have been observed. E. coli with point, deletion, or insertion mutations in *ssrA* exhibit slow growth, delayed recovery from carbon starvation, and decreased motility in soft agar (Oh and Apirion, 1991; Komine et al., 1994). Although 10Sa RNA is not essential, the conservation of *ssrA* in a wide range of bacterial genera and the abundance of 10Sa RNA in E. coli suggest a physiologically important role for this RNA. This is not without precedent, since other functions with important physiological roles first identified in E. coli and later identified in other bacteria, such as integration host factor, HU, H-NS, (Drlica and Rouviere-Yaniv, 1987), RNase III (Court, 1993), and cyclic AMP–cyclic AMP receptor protein (Adhya and Garges, 1990), are not essential for viability.

Studies from both this laboratory and that of S. Gottesman have exploited an unusual *ssrA* mutation, *sipB391* (also known as Δ(CP4-57)), to identify additional phenotypes resulting from alterations in 10Sa RNA (Kirby et al., 1994; Retallack et al., 1994). First identified because of its effect on the growth of certain phages (Strauch et al., 1986), the *sipB391* mutation is an ~3–4 kb deletion created by the excision of a cryptic prophage adjacent to the sequences encoding the 3′ terminus of mature 10Sa RNA (Kirby et al., 1994). The deletion leaves the sequence encoding the mature form of 10Sa RNA essentially unaffected, but deletes the sequence encoding the 3′ processed portion of precursor 10Sa RNA. Kirby et al. (1994) observed that these mutants express a new protease activity that is also observed in E. coli carrying an insertionally inactivated *ssrA* gene, *ssrA::cat*. Using Northern blot analysis, we identified an RNA corresponding to 10Sa RNA in the *sipB391* mutant (Retallack et al., 1994), but failed to detect 10Sa RNA in the *ssrA::cat* mutant (D. M. R. and D. I. F., unpublished data).

We found that our original *sipB391* mutant, as well as the *ssrA::cat* mutant, failed to support growth of some hybrid phages formed from E. coli phage λ and the related Salmonella typhimurium phage P22 (Strauch et al., 1986; Retallack et al., 1994). Based upon genetic and physiological studies, we proposed that 10Sa RNA modulates the binding of the P22 C1 protein to its specific DNA-binding site, P_{RE} (Retallack et al., 1993, 1994). The inhibition of P22 growth in the absence of functional 10Sa RNA was postulated to result from unusually avid binding of C1 to P_{RE}, which could block expression of genes encoding functions essential for lytic growth (Retallack et al., 1993, 1994). To explore further the interaction between 10Sa RNA and DNA-binding proteins, we have examined the effect of 10Sa RNA in vivo on the activities of three well-defined repressor proteins, λ cI as well as E. coli LacI and LexA, to their cognate operators and assessed binding of these

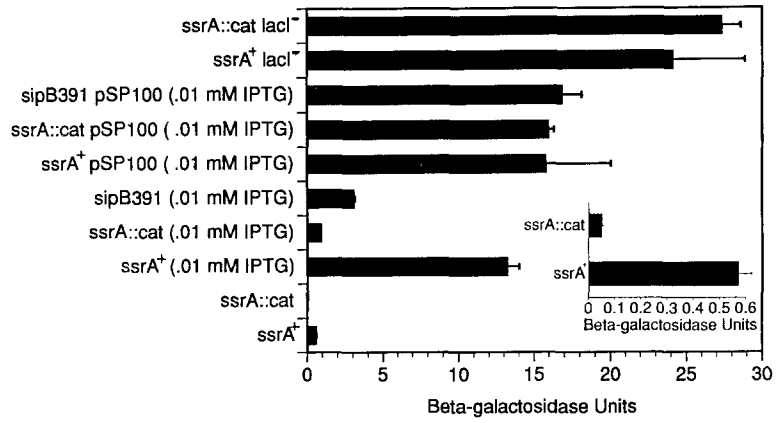

Figure 1. Effect of *ssrA::cat* and *sipB391* Mutations on LacI Activity as Measured by *lacZ* Expression

The relevant genotypes of the bacteria tested are shown on the Y axis, and *lacZ* expression is shown on the X axis. β-Galactosidase units are defined as Miller units divided by 1000. The inset graph is an enlargement of the portion of the graph describing the basal levels of expression (without IPTG induction). Each value represents the average of three experiments, and the error bars represent standard deviations.

proteins, along with the P22 C1 protein, to 10Sa RNA synthesized in vitro.

Results

Effect of *ssrA* Mutations on LacI Activity In Vivo

The E. coli *lac* operon, repressed by *lacI* gene product, is derepressed in the presence of compounds, such as the gratuitous inducer isopropyl-β-D-thiogalactopyranosidase (IPTG), that bind and inactivate LacI (Beckwith, 1987). To assess the role of 10Sa RNA in LacI binding to its operator DNA, we determined levels of the *lacZ* gene product, β-galactosidase, as a measure of *lac* operon expression. Enzyme levels were determined following induction with varying amounts of IPTG in congenic *ssrA*$^+$ and *ssrA::cat* strains. The level of *lacZ* expression was consistently 10-fold lower in the *ssrA::cat* strain at concentrations of IPTG ranging from 0 to 4 mM. Figure 1 shows the effect of 10Sa RNA on the expression of the *lac* operon as measured by β-galactosidase synthesis. We emphasize the following observations about the *ssrA::cat* strain. There is a consistent 10-fold lower basal, as well as induced (in the presence of 0.01 mM IPTG), level of *lac* operon expression in the *ssrA::cat* strain. The effect of the *ssrA::cat* mutation appears to be due to an interaction between LacI and 10Sa RNA, since wild-type levels of β-galactosidase were expressed in the *ssrA::cat* strain under two conditions: if the bacterium carried a plasmid, pSP100 (Retallack et al., 1994), expressing a wild-type *ssrA* gene or if the bacterium was also *lacI*$^-$ and thus did not express active LacI. The expression of *lacZ* in the *sipB391* mutant, which produces 10Sa RNA with an altered 3′ terminus at approximately wild-type levels (Retallack et al., 1994), is reduced only about 4-fold (Figure 1).

Effect of 10Sa RNA on λ cI Activity In Vivo

The activity of λ cI in vivo was assessed using a chromosomally located fusion of a defective λ prophage to the E. coli *gal* operon, rendering *gal* operon expression dependent on transcription from the λ P_R promoter (Reyes et al., 1979; Dambly-Chaudiere et al., 1983). The essential components of this fusion are shown in Figure 2A. Elements from phage λ include the *N* gene, the operator–*cI*–promoter region, the tR1–Rho-dependent transcription terminator, and the *nutR* antitermination signal. Other elements include an IS2, with its strong Rho-dependent terminator (De Crombrugghe et al., 1973), followed by the fused *gal* operon. Because the IS2 blocks transcription from the

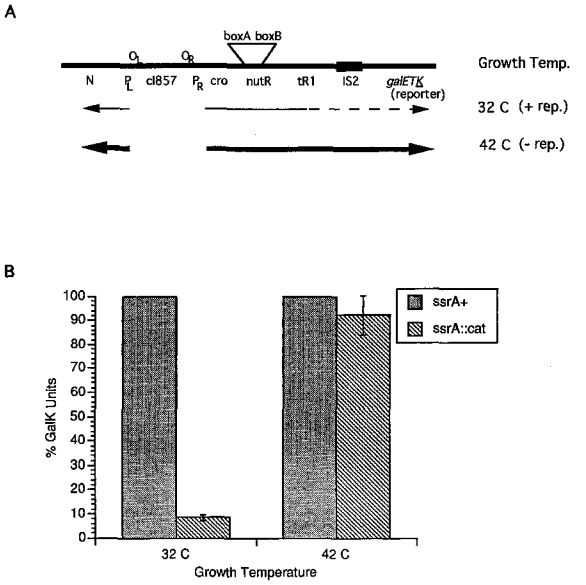

Figure 2. Effect of *ssrA::cat* on λ cI Action Measured with a λ–*gal* Fusion

(A) Shown at the top is a diagram of the λ–*gal* chromosomal fusion (not drawn to scale). Expression of the *gal* operon is driven from the λ P_R promoter. The *gal* promoter is rendered inactive by the *gal3* IS2 insertion (Reyes et al., 1979). The *nutR* signal in this fusion carries the *boxAcon* mutation (Friedman et al., 1990). The lines with arrowheads below the genetic map show the transcription from P_L and P_R, and the thickness of the lines indicates the differences in levels of expression from the phage promoters at high and low temperatures. At low temperature (32°C), the cI857ts repressor is active and binds to the operator sites, repressing transcription from the P_R and P_L promoters. At high temperature (42°C), the cI857ts repressor is inactive, allowing a higher level of transcription from P_R and P_L. See text for more details on the fusion.

(B) The graph illustrates the effect of *ssrA::cat* on *galK* expression from the λ P_R promoter at temperatures at which the cI857 repressor is active (32°C) or inactive (42°C). For purposes of comparison, the levels of galactokinase measured in the fusion strain carrying *ssrA*$^+$ (stippled bars) are set at 100%, with the levels in the congenic strain carrying the *ssrA::cat* allele (hatched bars) indicated as percentages of those levels. Each bar represents the average of at least five experiments, and error lines indicate standard deviations.

P-gal promoter (Adhya et al., 1974), expression of the *gal* operon depends on transcription from the λ P_R promoter. Additionally, the λ N antitermination protein, which modifies RNA polymerase at the *nut* sites, allows transcription to proceed through the IS2 and tR1 terminators into the *gal* operon. Therefore, the level of galactokinase, encoded by the *galK* gene, provides a measure of expression from the λ promoters. Since expression from P_R and P_L is repressed by cI, *galK* expression from the fusion is inversely related to λ cI activity; i.e., when λ cI is bound to one of its DNA targets, O_R, expression from P_R is repressed, resulting in low *galK* expression. Conversely, when λ cI is not bound to operators, there should be high levels of *galK* expression. The fusion carries the *cI857* mutation and thus expresses a temperature-sensitive *cI* gene product (Sussman and Jacob, 1962). Therefore, in this bacterium P_R and P_L are repressed at low (32°C) but not high (42°C) temperature.

We have previously observed that when the fusion has the *boxAcon* mutation in the *boxA* component of the λ *nut* region, N action is enhanced (Friedman et al., 1990); there is a low level of *gal* expression in the presence of cI, a level of expression not observed with the fusion carrying a wild-type *nut* region (D. M. R. and D. I. F., unpublished data). This indicated that repression was incomplete and suggested that the fusion with the *boxAcon* mutation could provide a sensitive means to assess the biological role of 10Sa RNA in λ cI action. We reasoned that in the absence of 10Sa RNA, increased activity of λ cI would reduce this low level of *gal* expression. The level of expression from the fused *gal* operon could be quantified by measuring levels of galactokinase. Moreover, because of the *cI857ts* mutation, by choosing appropriate temperatures for the assay, we could assess expression of *galK* from the fusion in both the presence and absence of active repressor.

The results of two experiments, shown in Figure 2B, suggest that the presence of 10Sa RNA influences the activity of λ cI protein in vivo. First, we find that at 32°C, a temperature at which the cI857 repressor is active, *galK* expression in the *ssrA*$^+$ strain (K5316) is 10-fold higher than in the congenic *ssrA::cat* strain (K7823). Second, at 42°C, a temperature at which the cI857 repressor is inactive, expression from both the *ssrA*$^+$ and *ssrA::cat* derivatives are essentially the same. Thus, the effect of 10Sa RNA on expression of *galK* is observed in the presence, but not in the absence, of repressor. Provided that temperature does not influence the action of 10Sa RNA (and we have no evidence suggesting that temperature has an effect on 10Sa RNA action), these experiments indicate that λ cI action is more effective in the absence of 10Sa RNA.

Interactions between 10Sa RNA and the λ cI and LacI Repressors In Vitro

One possible explanation for these in vivo observations is that a physical interaction with 10Sa RNA reduces the effective binding of some proteins to their specific DNA targets. To assess the possibility of a direct interaction, we tested the binding of purified λ repressor, λ cI, and lactose repressor, LacI, proteins to 10Sa RNAs synthesized in vitro using gel mobility shifts. The sequences of preprocessed and mature forms of 10Sa RNA prepared in vitro were based on the work of Chauhan and Apirion (1989) (Figure 3). Computer analysis revealed no similarity between 10Sa RNA sequence and that of the LacI- or λ cI–binding sites (data not shown). Therefore, any RNA–protein interactions detected would be with sequences other than those resembling the natural binding sites of the repressors.

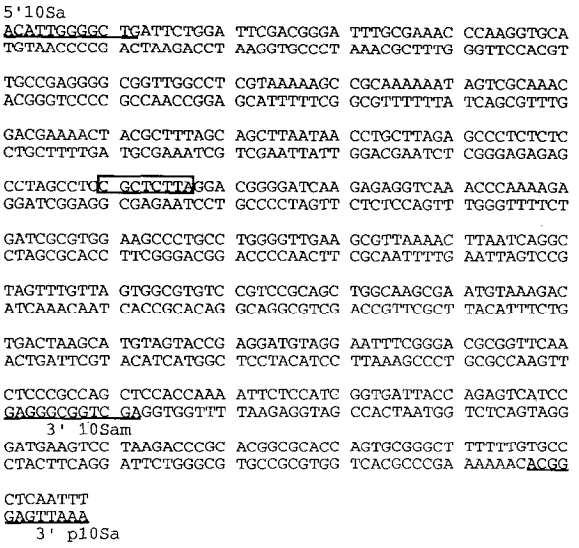

Figure 3. Sequence of the *ssrA* Gene Indicating the 5′ and 3′ Ends of the Mature and Preprocessed RNAs Used in the Gel Retardation Studies

Data published by the Apirion laboratory provided this sequence information (Chauhan and Apirion, 1989). Underlining indicates sequences of oligonucleotides used as polymerase chain reaction primers. A HindIII restriction site was engineered onto the 5′ end of the 5′ 10Sa primer, and a BamHI restriction site was engineered onto the 5′ end of the 3′ mature 10Sa (10Sam) and 3′ preprocessed 10Sa (p10Sa) primers to facilitate cloning into pGEM-3Z. The boxed area outlines a sequence identical to the phage λ *boxA* sequence. The significance of this *boxA* sequence for 10Sa RNA function, if any, is unknown.

The gel mobility studies indicate that λ cI binds to both mature and preprocessed 10Sa RNAs. As shown in Figures 4A and 4B, radioactively labeled 10Sa RNAs are shifted in the presence of λ cI. Western blot analysis confirmed that the protein associated with the shifted bands was λ cI (data not shown). Although a 200-fold molar excess of transfer RNA (tRNA) does not compete with 10Sa RNA for λ cI binding, a 10-fold excess of unlabeled 10Sa RNAs competes with labeled 10Sa RNA.

LacI, like λ cI, binds to both forms of 10Sa RNA, as shown by the shifted bands in Figures 4C and 4D. A 200-fold molar excess of tRNA does not compete with 10Sa RNA for LacI binding, while a 10-fold excess of unlabeled 10Sa RNA does compete. The nature of LacI binding to 10Sa RNA must differ from that to *lac* operator DNA, since binding to the preprocessed form of 10Sa RNA is not affected by the addition of millimolar amounts of IPTG to the shift reaction mixture, concentrations significantly higher than those necessary to derepress the *lac* operon (data not shown).

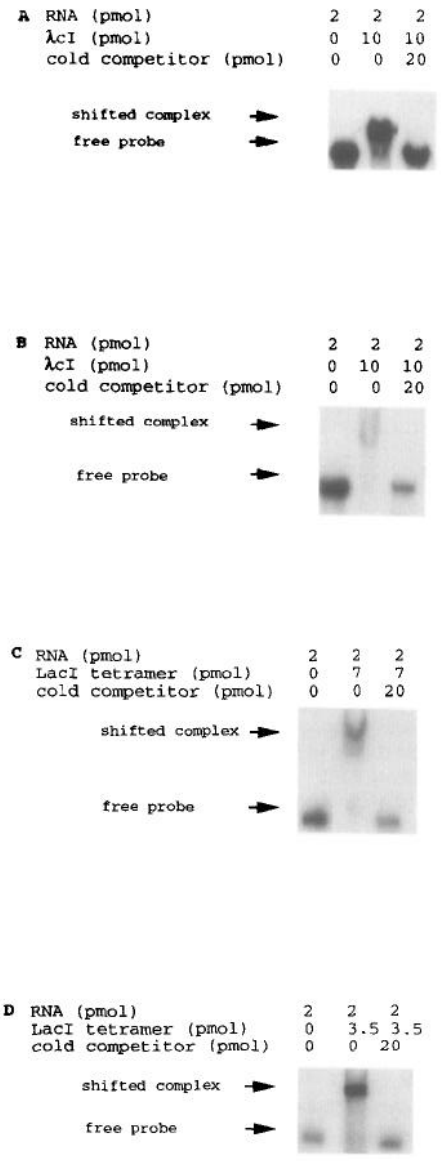

Figure 4. Mobility Shift Analysis of the Association of λ cI and LacI Repressors with 10Sa RNA

The amounts of protein and RNA added are listed above each lane. Nonspecific tRNA competitor (400 pmol) was added to each reaction. Arrows indicate positions of the free probe and the shifted protein–RNA complex. Shown are binding of λ cI to mature 10Sa RNA (A), binding of λ cI to preprocessed 10Sa RNA (B), binding of LacI to mature 10Sa RNA (C), and binding of LacI to preprocessed 10Sa RNA (D).

Specificity for 10Sa RNA

The observation that a 200-fold molar excess of tRNA failed to compete with 10Sa RNA for binding of either λ cI or LacI suggested that binding of the proteins could be an unusual property of 10Sa RNA. We assessed the ability of another RNA to bind these two proteins. An ~360 nt RNA synthesized using a portion of the *tetR* gene of pBR322 as the template was tested in the gel mobility shift assay. Unlike 10Sa RNA, the *tetR* RNA failed to show a shift in mobility following incubation with either λ cI or LacI (data not shown).

Competition between 10Sa RNA and a λ cI-Binding Site

We next determined whether 10Sa RNA is able to compete with a site on DNA known to bind a specific protein. Using gel mobility shift analysis, we determined the effect of a 4-fold molar excess of preprocessed 10Sa RNA on λ cI binding to a 25 bp DNA fragment containing the λ operator site, O_R2. The amount of λ cI bound to O_R2 is reduced ~3-fold in the presence of preprocessed 10Sa RNA, as shown by the decrease in the intensity of the shifted band (Figure 5A). The addition of an ~25-fold molar excess of E. coli tRNA did not reduce λ cI binding to O_R2. Although two sets of bands can be seen on the gel, only the set at the higher position in the gel represents the specific interaction between λ cI and the O_R2 site. This interaction is specific, since a similar band was not observed when λ cI was mixed with a variant of the 25 bp operator fragment having four base pair changes known to eliminate λ cI binding (Harrison, 1992), O_R2M, while the lower band was still detected (Figure 5B). The observation that the higher band was supershifted in the presence of an antiserum raised against λ cI (Figure 5B) confirms that λ cI was complexed with the DNA in the higher shifted band.

Effect of 10Sa RNA on LexA Repressor Activity

To assess the range of 10Sa RNA action, we examined its effect on the activity of a third repressor, the E. coli LexA protein. The LexA repressor controls the expression of a regulon whose gene products are involved in protecting the cell from DNA damage (Little and Mount, 1982). Cleavage of LexA leads to the expression of these products, an event called the SOS response. The *sulA* gene, whose product interferes with cell division, is a member of this regulon (Huisman and D'Ari, 1981). Because expression of *sulA* is repressed by LexA, a *sulA–lacZ* fusion in a bacterium deleted for the chromosomal *lacZ* gene could be used to measure LexA activity. The level of β-galactosidase expressed is inversely related to the activity of LexA.

Mitomycin C was used to create DNA damage necessary to induce an SOS response. A consistently lower level of β-galactosidase expression from the *sulA–lacZ* fusion was observed in the *ssrA::cat* bacterium over time, as compared with the *ssrA* wild-type control. An ~4-fold difference was observed following a 60 min induction with mitomycin C (Figure 6).

Gel mobility shift analyses, like those performed with λ cI and E. coli LacI proteins, showed that LexA protein could bind to both mature and preprocessed 10Sa RNAs. Figure 7 shows binding of LexA to preprocessed 10Sa RNA. LexA binding to 10Sa RNA was found to be specific, since a 200-fold excess of tRNA did not compete for binding of LexA, while a 10-fold excess of unlabeled 10Sa RNA did compete.

Interactions between 10Sa RNA and the P22 C1 Protein In Vitro

As discussed above, our previous studies suggesting that P22 C1 protein binds more avidly to its DNA-binding site, P_{RE}, in E. coli derivatives with *ssrA* mutations (Retallack

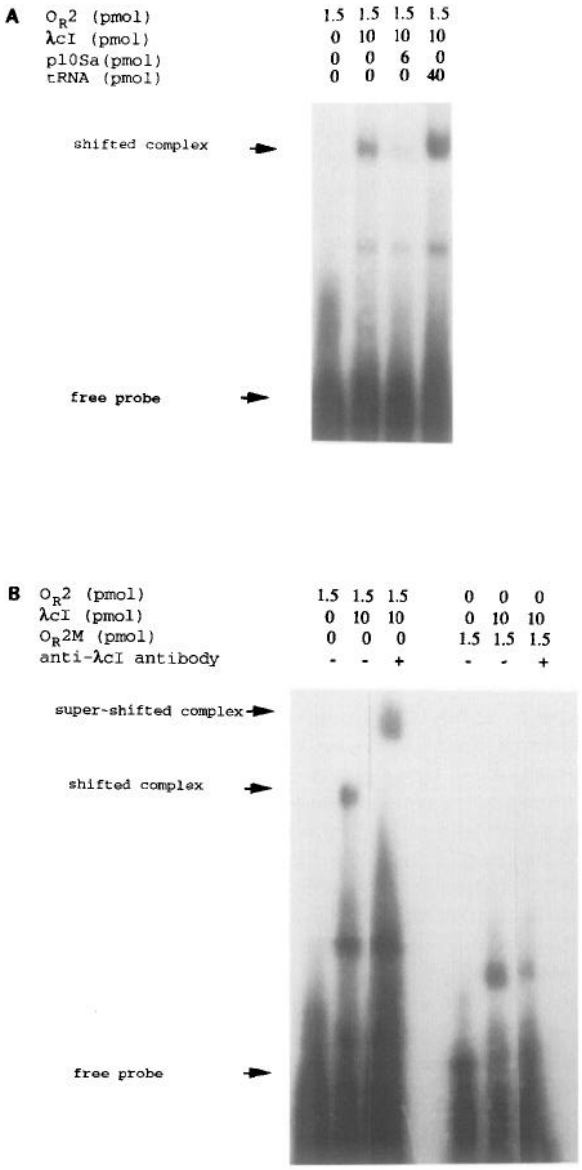

Figure 5. Competition of Preprocessed 10Sa RNA with λ O_R2 DNA for λ cI Binding

The amounts of protein, labeled DNA, and unlabeled RNA used in each reaction are listed above the figure. Addition of anti-λ cI antiserum is indicated by a plus. Arrows indicate positions of the free probe, the shifted protein–DNA complex, and the supershifted protein–DNA antibody complex.

(A) Comparison of the competition of preprocessed 10Sa (p10Sa) RNA or control tRNA with O_R2 DNA.

(B) Sequence specificity of λ cI–λ O_R2 interaction. The left three lanes show supershift with antiserum raised against λ cI. The right three lanes show the lack of a shift when the DNA fragment carries a mutant O_R2, O_R2M, which contains four base pair mutations that are known to decrease binding to λ cI (Harrison, 1992). The wild-type O_R2 sequence, 5′-TATCTAACACCGTGCGTGTTGACTA, is changed to the mutant O_R2M sequence, 5′-TATCT**T**ACA**T**CGTGC**A**TGT**A**GACTA (altered bases shown in bold).

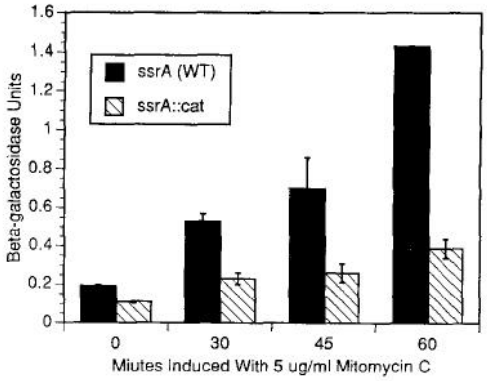

Figure 6. Effect of *ssrA::cat* on LexA Activity

Shown is the level of β-galactosidase expressed from a *sulA::lacZ* fusion in bacteria carrying Δ*lacZ* and either *ssrA*$^+$ or *ssrA::cat*. Because LexA represses *sulA* expression, its activity is inversely related to the level of β-galactosidase expressed. Time of treatment with mitomycin C (5 μg/ml) is shown on the X axis. β-Galactosidase activity is shown on the Y axis. β-Galactosidase units are defined as Miller units divided by 1000. The closed bars represent activity in an *ssrA*$^+$ bacterium, and the hatched bars represent activity in an *ssrA::cat* bacterium. Each value represents the average of three experiments. The standard deviations are indicated by the error bars.

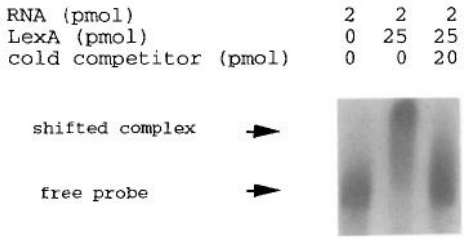

Figure 7. Mobility Shift Analysis of LexA Binding to Preprocessed 10Sa RNA

The amounts of protein and RNA used are indicated above each lane. Arrows point to the positions of free probe and the shifted protein–RNA complex.

et al., 1993; 1994) led to the studies reported in this paper. We therefore used the gel mobility assay to determine whether P22 C1 protein binds to 10Sa RNA. As in the case of the repressor proteins, P22 C1 was able to bind both forms of 10Sa RNA, even in the presence of a 200-fold molar excess of tRNA (data not shown).

Using gel mobility shift analysis, we determined whether the binding of P22 C1 to its P_{RE} DNA-binding site is affected by a 20-fold molar excess of preprocessed 10Sa RNA. The amount of P22 C1 bound to the labeled DNA fragment containing P_{RE} is reduced ~3-fold in the presence of preprocessed 10Sa RNA, as shown by the decrease in the intensity of the shifted band (Figure 8). However, the addition of a 20-fold molar excess of tRNA did not reduce the binding of P22 C1 to the DNA fragment containing P_{RE}.

Specificity of 10Sa RNA for DNA-Binding Proteins

The fact that bovine serum albumin (BSA) (up to 1 μg) did not bind to 10Sa RNA (data not shown) suggests that 10Sa RNA may have some preference for DNA-binding proteins. To examine this question further, we tested whether another protein that does not bind to DNA, the E. coli PII protein, which is involved in nitrogen assimilation (Kamb-

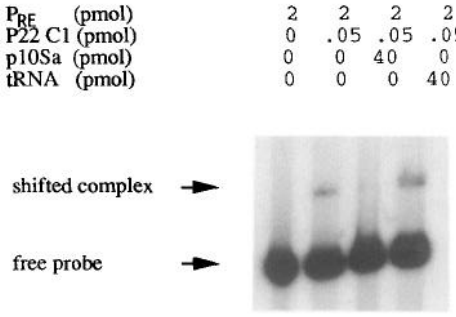

Figure 8. Competition of Preprocessed 10Sa RNA with P22 P_{RE} DNA for P22 C1 Binding

The amounts of protein, labeled DNA, and unlabeled RNA used in each reaction are listed above the figure. Arrows point to positions of the free probe and the shifted protein–DNA complex.

erov et al., 1994), binds to 10Sa RNA. We found that even 3 μg of PII fails to bind 10Sa RNA, as measured by a mobility shift assay (data not shown). Since to date we have examined only a small number of proteins, we can only speculate as to the nature of proteins that are capable of binding to 10Sa RNA.

Discussion

Both the in vivo physiological studies and the in vitro RNA-binding studies reported here are consistent with a model in which the association of some DNA-binding proteins with 10Sa RNA changes the nature of the interaction of the proteins with their DNA targets. First, in vivo studies of at least four proteins, λ cI, LacI, and LexA reported in this communication and P22 C1 reported in previous communications (Strauch et al., 1986; Retallack et al., 1993, 1994), suggest that binding of each of these proteins to its cognate target is enhanced in the absence of 10Sa RNA. Second, the DNA-binding proteins E. coli LacI, LexA, λ cI, and P22 C1 bind to 10Sa RNA in vitro, while PII and BSA, which are not DNA-binding proteins, do not bind to 10Sa RNA. Third, gel mobility shift experiments suggest that 10Sa RNA can compete successfully with λ cI protein for binding to the O_R2 operator DNA site and with P22 C1 protein for binding to its P_{RE} DNA site.

Our gel mobility shift experiments indicate that the P22 P_{RE} site has ~30-fold higher affinity for the P22 C1 protein than the λ O_R2 site has for λ cI protein. This is consistent with our observation that it takes substantially more preprocessed 10Sa RNA to compete successfully with the binding of P22 C1 to its DNA site than it takes to compete with binding of λ cI to bind to its DNA site.

We have initiated studies on the interactions of 10Sa RNA with other DNA-binding proteins. Preliminary results show that two well-characterized DNA-binding proteins are unable to bind specifically to 10Sa RNA in vitro. E. coli integration host factor, which binds to the minor groove of DNA primarily via two pairs of β-sheet arms (Yang and Nash, 1989) and TFIIIA, a Xenopus laevis zinc finger protein that binds specifically to both DNA and RNA (Pieler and Theunissen, 1993), are both unable to bind 10Sa RNA in the presence of nonspecific tRNA competitor (D. M. R., J. Withey, and D. I. F., unpublished data).

There are examples of molecules that share some of the attributes we have outlined for 10Sa RNA. First, the P22 Ant protein removes repressors from their operators (Susskind and Botstein, 1975). Second, the eukaryotic Mot1 protein (also known as ADI) removes TATA-binding protein from its binding site in an ATP-dependent reaction (Auble et al., 1994). Third, the X. laevis transcription factor TFIIIA, a specific DNA-binding protein, activates transcription of the 5S RNA gene and also binds to the 5S RNA product of that gene. TFIIIA binds to both DNA and RNA through zinc fingers, although the nature of the binding to the two molecules is different. It has been suggested that the partitioning of TFIIIA between its DNA target and 5S RNA might be a means of feedback regulation (reviewed by Pieler and Theunissen, 1993). Fourth, the *agr* regulatory locus of Staphylococcus aureus, which regulates expression of toxins, is thought to operate through a multifunctional RNA that has been postulated to interact with regulatory proteins (Novick et al., 1993). Fifth, RNAs can be engineered to bind a wide array of proteins, provided there is a powerful selection mechanism (Tuerk and Gold, 1990). Sixth, chaperones, proteins that interact with a wide variety of polypeptides without any apparent sequence specificity to facilitate proper folding (Zeilstra-Ryalls et al., 1991), provide a useful precedent for rationalizing our observations that a single RNA molecule interacts with at least four different proteins.

Our studies to date have not provided evidence of a functional role for 10Sa RNA. The observations indicating that activities of all the DNA-binding proteins we tested, though small in number, are modulated by 10Sa RNA suggests that 10Sa RNA may serve as a global regulator. Variations in the levels of 10Sa RNA could regulate expression of genes either by altering the effective levels of a number of regulator proteins or by controlling action of a single protein that, in turn, regulates expression of a number of genes. Levels of 10Sa RNA could be changed by regulating *ssrA* transcription, altering the RNA structure, or degrading the RNA.

Although results from our studies do not lead to a definitive model of 10Sa RNA action, the following is one of the more attractive models we have entertained. According to this model, 10Sa RNA acts competitively to sequester proteins from specific binding sites. The need to consider nonspecific binding in formulating a quantitative description of repressor binding was recognized by Kao-Huang et al. (1977). The fact that 10Sa RNA outcompetes a 10-fold excess of E. coli DNA for λ cI (data not shown) is consistent with a model in which 10Sa RNA serves as a source of nonoperator binding sites. Two observations argue against this idea. First, the amount of 10Sa RNA in the bacterium, ~1000 copies of a 362 nt RNA per haploid genome, is obviously significantly less than that of total cellular DNA. Second, von Hippel et al. (1974) determined that 7% or less of LacI in the cell is not associated with the nucleoid, suggesting that those repressor tetramers not bound specifically are bound to nonoperator sites on the DNA. The assay employed in the latter study, however, may not have

scored LacI bound to 10Sa RNA as nonnucleoid. If there were a limited number of proteins affected by 10Sa RNA, this model would be more attractive, since it is difficult to see how 1000 RNA molecules could sequester a large number of different DNA-binding proteins. At this time we have no idea of the number of DNA-binding proteins whose actions in vivo are influenced by 10Sa RNA. However, studies to date limit the proteins able to bind to 10Sa RNA to proteins with the helix-turn-helix motif (J. Withey and D. I. F., unpublished data). We also do not know if or how 10Sa RNA levels may vary. It is possible that under some yet to be determined conditions there are significantly more (or less) than 1000 copies of 10Sa RNA per haploid genome. Alternatively, 10Sa RNA might alter the binding equilibrium by selectively facilitating release of proteins bound to specific sites. If nonspecific DNA serves as a sink for unbound repressor, the relative increase in binding at the nonspecific sites would decrease the availability of proteins for binding at specific sites.

Computer analysis indicates that 10Sa RNA has the potential to form complex secondary structures (B. Shapiro, personal communication). This secondary structure could provide a double-stranded nucleic acid, with associated loops, as a target for the DNA-binding proteins. Our studies suggest that both the preprocessed and mature forms of 10Sa RNA may be functional. Komine et al. (1994) have shown that the 5′ end of E. coli 10Sa RNA is processed by RNase P and that the fully processed form can potentially form an acceptor stem resembling that of alanine tRNA. Further, these workers showed that this RNA could be charged in vitro with alanine. Examination of the sequence of the 10Sa RNA homologs identified in other species show that those RNAs may also form similar tRNA-like structures (Ushida et al., 1994). Whether 10Sa RNAs are charged in vivo is unknown, but it is possible that either the association of 10Sa RNA with the alanyl–tRNA synthetase or the charging of 10Sa RNA could influence its structure and binding potential. In fact, a Neurospora mitochondrial tyrosyl–tRNA synthetase bound to group I introns promotes splicing (Mohr et al., 1994; Guo and Lambowitz, 1992; Lambowitz and Perlman, 1990). An enhancement of 10Sa RNA activity following association with alanyl–tRNA synthetase, or charging with alanine, might explain the apparently paradoxical findings with the *sipB391* mutant. This mutant, which exhibits a less stringent phenotype than the *ssrA::cat* mutant, expresses a 10Sa RNA that is approximately the same size and is present in relatively the same abundance as that found in the otherwise isogenic bacterium wild-type at the *ssrA* locus (Retallack et al., 1994). The *sipB391* 10Sa RNA has a different 3′ end (Retallack et al., 1994) and may not be chargeable. Thus, uncharged 10Sa RNA may be only partially active in facilitating derepression. Alternatively, the difference in the 3′ end of the preprocessed form of the *sipB391* 10Sa RNA might influence the ultimate folding of the processed form, leading to a structural isomer with functional properties different from the wild-type processed form.

Regardless of the molecular mechanism of 10Sa RNA action, we suspect that further studies will identify other DNA-binding proteins whose affinity for their target sites are influenced by 10Sa RNA or other RNAs with similar function. Indeed, recent studies from S. Gottesman's laboratory have identified another small RNA in E. coli that appears to modulate silencing of the *rcsA* promoter by the H-NS DNA-binding protein (Sledjeski and Gottesman, 1995).

Experimental Procedures

Bacteria, Plasmids, and Phage

Bacteria used in this study and their relevant genotypes are as follows: K5316, containing a λ*cI857–P$_R$–boxAcon–nutR–gal* fusion (derived from OR1150; Reyes et al., 1979; Dambly-Chaudiere et al., 1983); K7823, an *ssrA::cat* (Kirby et al., 1994) derivative of K5316; K37, *ssrA*$^+$ (Friedman et al., 1973); K7649, *sipB391* (derived from K37) (Retallack et al., 1994); K6678, *ssrA::cat* (derived from K37) (Retallack et al., 1994); JL2497, (N99) *Δlac F′lacZ::Tn9* (Lin and Little, 1988); K8216, JL2497 cured of F′; K8220 (derived from K8216), *ssrA*$^+$ λ*sulA::lacZ*; K8221 (derived from K8220), *ssrA::cat,* λ*sulA::lacZ*. The pSP100 plasmid is a derivative of pBR325 that contains the wild-type E. coli *ssrA* gene (Retallack et al., 1994). The phage λ*sulA::lacZ* (Lin and Little, 1988) was used to construct K8220.

Media

TB and LB agars and broths were prepared as previously described (Friedman et al., 1990).

Galactokinase Assay

Overnight cultures of each bacterial strain tested were grown in LB broth, one each at 32°C and 42°C. These cultures were diluted 40-fold in LB broth and again grown at either 32°C or 42°C for 2.5 hr. A 5 ml aliquot from each culture was used to measure galactokinase levels essentially as described by Adhya and Miller (1979). Results are presented as a percentage of the galactokinase level determined for the congenic *ssrA*$^+$ strain.

β-Galactosidase Assay

Overnight cultures of bacterial strains grown in LB broth were diluted 30-fold and divided into aliquots. Aliquots were grown to log phase at 37°C in the presence or absence of IPTG as indicated, and β-galactosidase activity was measured according to Miller (1992).

Mitomycin C Treatment

Overnight cultures of bacteria containing the λ*sulA::lacZ* fusion were diluted 30-fold, divided into four aliquots, and grown for a total of 2 hr at 37°C. Mitomycin C was added to aliquots, yielding a final concentration of 5 μg/ml at the staggered times indicated in Figure 6. β-Galactosidase activity was measured according to Miller (1992).

Construction of Vectors with the *ssrA* Gene for In Vitro Transcription

Polymerase chain reaction (PCR) was used to generate double-stranded DNA fragments containing the coding region for both the mature 10Sa and precursor 10Sa forms of 10Sa RNA. The plasmid pSP100, which contains the intact wild-type *ssrA* gene, was used as template with the primers described in Figure 3. Primers were synthesized by the University of Michigan Core Facilities. A reaction buffer of 50 mM KCl, 10 mM Tris–HCl (pH 7), 2.5 mM $MgCl_2$, 0.01% gelatin, and 0.2 mM dNTPs was used for each PCR reaction, along with 50 pmol of each of the appropriate primers and 0.1 μg of template DNA. PCR was performed in a COY thermocycler for 20 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min. The products of these reactions were gel purified, digested with HindIII and BamHI, and ligated to pGEM-3Z (Promega) linearized with HindIII and BamHI. The resulting clones contain mature 10Sa and preprocessed 10Sa under control of the SP6 promoter for in vitro transcription.

Cloning

Standard cloning procedures were employed as detailed in Sambrook et al. (1989). Restriction enzymes and buffers were obtained from New England Biolabs, Bethesda Research Laboratories, or Promega and used according to the instructions of the manufacturers.

DNA Sequencing

Mature and preprocessed 10Sa clones were sequenced to ensure that no mutations were introduced by the PCR reactions. The same primers used for the PCR reaction were used for sequencing using the *fmol* sequencing system (Promega). Primers were end labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (New England Biolabs). Dideoxy sequencing reactions were carried out according to the instructions of the manufacturer and run on a 6% polyacrylamide gel for varying lengths of time. Both strands of DNA were sequenced.

In Vitro Transcription

Derivatives of pGEM-3Z containing the coding sequences for mature 10Sa and preprocessed 10Sa, as well as a derivative with a HindIII–BamHI fragment containing the 5′ 360 bp of the *tetR* gene from pBR322, were linearized with AvaI. Linearization resulted in the addition of six nucleotides, CCUAGG, to the 3′ end of each RNA. The linearized DNAs were gel purified and used as templates for in vitro transcription. The Riboprobe in vitro transcription kit (Promega) employed in the transcription reactions was used with SP6 polymerase and [α-^{32}P]CTP according to the instructions of the manufacturer. Unincorporated nucleotides were removed using a Sephadex-G50 Quick Spin Column (Boehringer Mannheim Biochemicals).

RNA Mobility Shift Assays

Binding reactions contained 10 mM Tris base (pH 7), 50 mM KCl, 1 mM MgCl, 0.1 mM NaEDTA, 100 μg/ml BSA, and 400 pmol of E. coli tRNA. The appropriate amounts of purified protein and 10Sa RNA (labeled or unlabeled) were mixed and allowed to react for 15 min at 4°C. Bound and unbound RNAs were separated on a 5% native polyacrylamide gel. The gel was dried and exposed to film.

Competition for λ cI Binding between Preprocessed 10Sa RNA and the λ O_R2 Site

Oligonucleotides corresponding to both strands of the wild-type or mutant λ O_R2 sequence were purchased from the University of Michigan Core Facilities and hybridized to produce 25 bp double-stranded fragments containing the O_R2 sites. Any single-stranded DNA present after hybridization was removed by digesting with S1 nuclease (Boehringer Mannheim Biochemicals) for 45 min. The resultant double-stranded DNA was phenol-extracted, ethanol-precipitated, and end labeled using [γ-^{32}P]ATP (Amersham) and T4 kinase (New England Biolabs). Preprocessed 10Sa RNA was produced in vitro essentially as described above, but in the absence of [α-^{32}P]CTP. Binding reactions were prepared essentially as described for RNA mobility shift analysis, but tRNA was added to the reactions only when indicated above the figure. DNA and RNA were added together in reaction mixtures containing both molecules. The amount of the fragment containing O_R2 bound to protein was determined from data obtained by scanning the gel using the Ambis 4000 Radioanalytic Imaging System.

Competition for P22 C1 Binding between Preprocessed 10Sa and P22 P_{RE}

A 400 bp EcoRV fragment containing the P22 P_{RE} site isolated from a pKO1 derivative with a cloned fragment containing the wild-type P22 P_{RE} site (Retallack et al., 1993) was used as a probe. This fragment was dephosphorylated using calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals) and end labeled using [γ-^{32}P] ATP and T4 kinase (New England Biolabs). Unlabeled preprocessed 10Sa RNA was prepared and binding reactions were performed as described in the previous section. The amount of the fragment containing P_{RE} bound to protein was determined from data obtained by scanning the gel using the Ambis 4000 Radioanalytic Imaging System.

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