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Specific Binding of *Escherichia coli* Ribosomal Protein S1 to *boxA* Transcriptional Antiterminator RNA

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We show that ribosomal protein S1 specifically binds the *boxA* transcriptional antiterminator RNAs of bacteriophage λ and the *Escherichia coli* ribosomal RNA operons. Although S1 competes with the NusB-S10 antitermination complex for binding to *boxA*, it does not affect antitermination by the λ N protein in vitro, and its role, if any, in rRNA synthesis is still unknown.

Translation of mRNA in *Escherichia coli* suppresses the transcriptional termination activity of termination factor Rho because the translating ribosomes prevent Rho from binding the nascent RNA (reviewed in reference 5). Untranslated transcripts, such as those synthesized from the ribosomal RNA (*rrn*) operons, are more readily accessible to Rho. The *rrn* operons are, however, transcribed without premature termination of transcription. The observation that insertion of strong Rho-dependent terminators within *rrnC* caused only a small reduction in the transcription of downstream sequences indicated that a mechanism exists that renders the *rrn* operons resistant to the action of Rho (17). Later experiments suggested that this mechanism is transcriptional antitermination (1).

Transcriptional antitermination has been best characterized in bacteriophage λ (for a review, see reference 8). The λ N protein is able to modify RNA polymerase so that it becomes resistant to both Rho-dependent and Rho-independent terminators (15, 30). A *cis*-acting element called the *nut* site (22) must be transcribed into RNA for N to function (11, 19). The *nut* site consists of two functional elements, *boxA* and *boxB*. *boxB* RNA is able to form a 15-nucleotide stem-loop that binds N (4, 16, 19). *boxA* RNA is a 12-nucleotide sequence 5′ to *boxB* that interacts with host factors (16, 20). The host factors involved in N-mediated antitermination are NusA, NusB, NusG, and ribosomal protein S10 (NusE) (6, 27). It is thought that N, the Nus factors, and the *nut* site form a ribonucleoprotein complex that stays associated with elongating RNA polymerase and directs the enzyme to transcribe through termination signals (19).

Antitermination in the *rrn* operons depends on *boxA* sequences that are closely related to the *boxA* elements of λ *nut* sites (13). Moreover, *rrn boxA* RNA has been shown to bind a heterodimer of NusB and S10 (14, 20). NusB was further implicated in *rrn* antitermination by experiments showing that NusB is important for rRNA synthesis in vivo (23) and that a NusB-depleted extract is unable to support antitermination in vitro (25). However, antitermination in the *rrn* operons is known to differ from N-mediated antitermination in three major ways: first, the bacteriophage λ N protein is not involved in *rrn* antitermination; second, *rrn boxA* is capable of supporting antitermination in the absence of *boxB* or any other RNA sequence (2); third, an unidentified factor(s) is required for antitermination in vitro in the *rrn* system but not in the λ system (25). The present study was initiated to attempt to identify this missing factor(s) and other proteins that interact with *boxA* RNA.

To detect *E. coli* proteins that bind *boxA*, S100 extract (7) was incubated in buffer (40 mM HEPES [pH 7.3], 10 mM ammonium sulfate, 15 mM potassium chloride, 0.5 mg of bovine serum albumin/ml, 50 μg of tRNA/ml) with a 35-nucleotide radiolabeled RNA (AGGGAAAGUUCACUGCUCUUUAACAAUUUAGUCGA) containing the 12-nucleotide *rrn boxA* element (underlined), or *boxA* inserted in the reverse orientation as a control, and was run on a nondenaturing 10% polyacrylamide gel (60:1 acrylamide/bisacrylamide ratio, 2% glycerol, 0.5× Tris-borate-EDTA). The electrophoretic mobility of the RNA probe containing *boxA* was reduced in a concentration-dependent manner when it was incubated with various amounts of extract, whereas the mobility of the control probe containing reversed *boxA* was not (Fig. 1, compare lanes 1 to 4 with lanes 5 to 8). This shifted band was not a consequence of NusB and S10 binding the probe, as this band still formed when antibody against NusB was used to deplete the extract of NusB (data not shown). Furthermore, the band had a mobility different from that of the band that appeared when purified NusB and S10 were incubated with the probe (see Fig. 5).

Column chromatography was used to purify and identify the protein that was retarding the mobility of bound *boxA* in the mobility shift experiment (Fig. 2). S100 extract was first passed over a DEAE-cellulose column (Whatman DE52), and the column was washed with buffer (10 mM Tris-acetate [pH 7.8], 14 mM magnesium acetate, 1 mM dithiothreitol) containing 0.14 M KCl. The protein with *boxA*-binding activity was then eluted with buffer containing 0.25 M KCl. Fractions containing the protein were pooled (Fig. 2, lane 3) and loaded onto a phenyl-Sepharose column. The protein was eluted with buffer containing 0.05 M KCl, and fractions containing the protein were again pooled (Fig. 2, lane 4) and loaded onto a poly(U)-agarose column (Pharmacia Biotech). The protein remained bound when this column was washed with 1 M KCl and was eluted with 6 M urea. After these purification steps, only one major polypeptide with an apparent molecular mass of 70 kDa and a minor, 60-kDa polypeptide were evident on a sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue (Fig. 2, lane 5). Gel purification and renaturation (10) of

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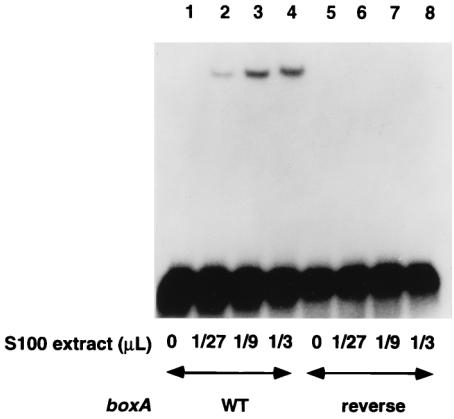

FIG. 1. A factor in a crude *E. coli* extract binds *rrn boxA* RNA. The indicated amount of S100 extract was incubated with radiolabeled *boxA* RNA (lanes 1 to 4) or reverse *boxA* RNA (lanes 5 to 8) and electrophoresed on a nondenaturing polyacrylamide gel. WT, wild type.

the 70-kDa protein confirmed that it was responsible for the *boxA*-binding activity (data not shown).

To identify the 70-kDa protein, its N-terminal sequence was determined for 19 amino acids, providing the identity of the amino acids at all positions except 14 and 16. This sequence was identical to that predicted for ribosomal protein S1 (Fig. 3). S1 is a component of the 30S subunit of the ribosome, where it is thought to interact nonspecifically with the nascent mRNA (26). S1 contains six copies of an approximately 70-amino-acid motif that has been implicated in binding RNA (9). S1 motifs have been found in a variety of proteins, including NusA (3). Considering that NusA has also been implicated in binding *boxA* RNA (16), it is possible that this interaction is mediated through the S1 domain of NusA. Since the S1 gene

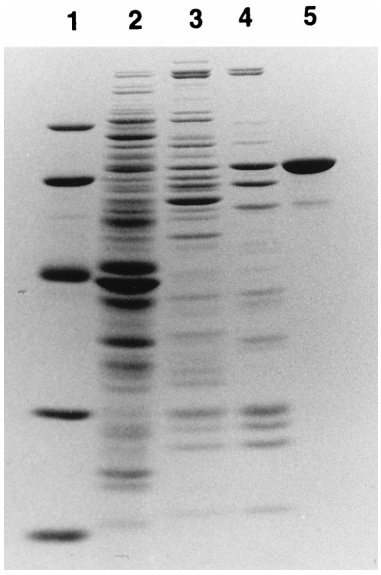

FIG. 2. Purification of a 70-kDa protein that binds *boxA* RNA. Fractions containing *boxA*-binding activity were pooled after DE52 (lane 3), phenyl-Sepharose (lane 4), and poly(U)-agarose (lane 5) column chromatography and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane 1 contains protein molecular mass standards (97.4, 66.2, 45.0, 31.0, and 21.5 kDa from top to bottom), and lane 2 contains *E. coli* S100 extract.

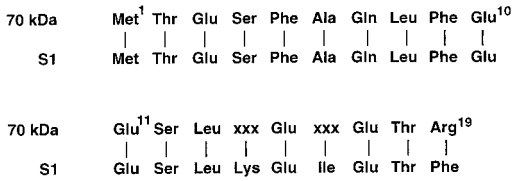

FIG. 3. Comparison of the N-terminal sequence of the 70-kDa protein with the known sequence of ribosomal protein S1. xxx, unknown amino acid.

is essential for the growth of *E. coli* (12), presumably because of the role of S1 in translation, participation by S1 in other processes might easily have escaped attention. S1 is an essential subunit of the replicase of bacteriophage Qβ (29). Recently, S1 has also been shown to be complexed with NusA and recombination protein β of phage λ, although the significance of this complex is unclear (28). Nevertheless, we have found that NusA alone does not bind *rrn boxA* or *nut* site RNA in a gel mobility shift assay (16, 20) and does not affect the binding of S1 to *boxA* RNA (data not shown).

In order to characterize further the specificity of the interaction between S1 and *rrn boxA*, gel mobility shift experiments were performed with mutant *boxA* probes (Fig. 4 and Table 1). Certain point mutations in *boxA* at positions 1, 5, and 7 did not affect the amount of S1 required to shift the mutant probe (Table 1; compare, e.g., Fig. 4A lanes 1 to 4 with lanes 5 to 8). A second class of mutations at positions 2, 4, 6, and 11 substantially affected the interaction between S1 and *boxA* so that approximately nine times more S1 was required to shift the mutant probe (Table 1; compare, e.g., Fig. 4A lanes 1 to 4 with lanes 9 to 12). A third class of mutations at positions 3, 8 plus 10, 9, and 12 affected the interaction between S1 and *boxA* so that at least 30 times as much S1 was required to shift the mutant probe (Table 1; compare, e.g., Fig. 4A lanes 1 to 4 with lanes 13 to 16). An almost undetectable amount of probe containing reverse *boxA* was shifted even at the highest concentration of S1 used (Fig. 4D, lane 16). Approximately equal amounts of S1 were needed to shift probes containing the λ *nutR boxA* sequence and wild-type *rrn boxA* (Fig. 4E, compare lanes 1 to 4 with lanes 5 to 8). Thus, the interaction of S1 with

TABLE 1. Binding of ribosomal protein S1 to mutant *boxA* RNAs

Probe	*boxA* sequence[a]	Binding to S1[b]	Binding to NusB-S10[c]
rrn boxA	UGCUCUUUAACA	+++	+
*boxA*U1G	**G**GCUCUUUAACA	+++	+/−
*boxA*G2U	U**U**CUCUUUAACA	++	−
*boxA*C3G	UG**G**UCUUUAACA	+	−
*boxA*U4G	UGC**G**CUUUAACA	++	−
*boxA*C5G	UGCU**G**UUUAACA	+++	−
*boxA*U6G	UGCUC**G**UUAACA	++	−
*boxA*U7A	UGCUCU**A**UAACA	+++	+/−
*boxA*U8GA10C	UGCUCUU**G**A**C**CA	+	−
*boxA*A9C	UGCUCUUU**C**ACA	+	−
*boxA*C11A	UGCUCUUUAA**A**A	++	+
*boxA*A12G	UGCUCUUUAAC**G**	+	+/−
λ *boxA*	**C**GCUCUU**AC**ACA	+++	−
boxA (reversed)	ACAAUUUCUCGU	+/−	−

[a] Boldface indicates substitutions.

[b] +++, wild-type ability to bind S1; ++, apparent ninefold decrease in affinity; +, apparent 30-fold decrease in affinity; +/−, almost undetectable level of binding.

[c] +, wild-type ability to bind NusB-S10; +/−, apparent ninefold decrease in affinity; −, no detectable binding (20).

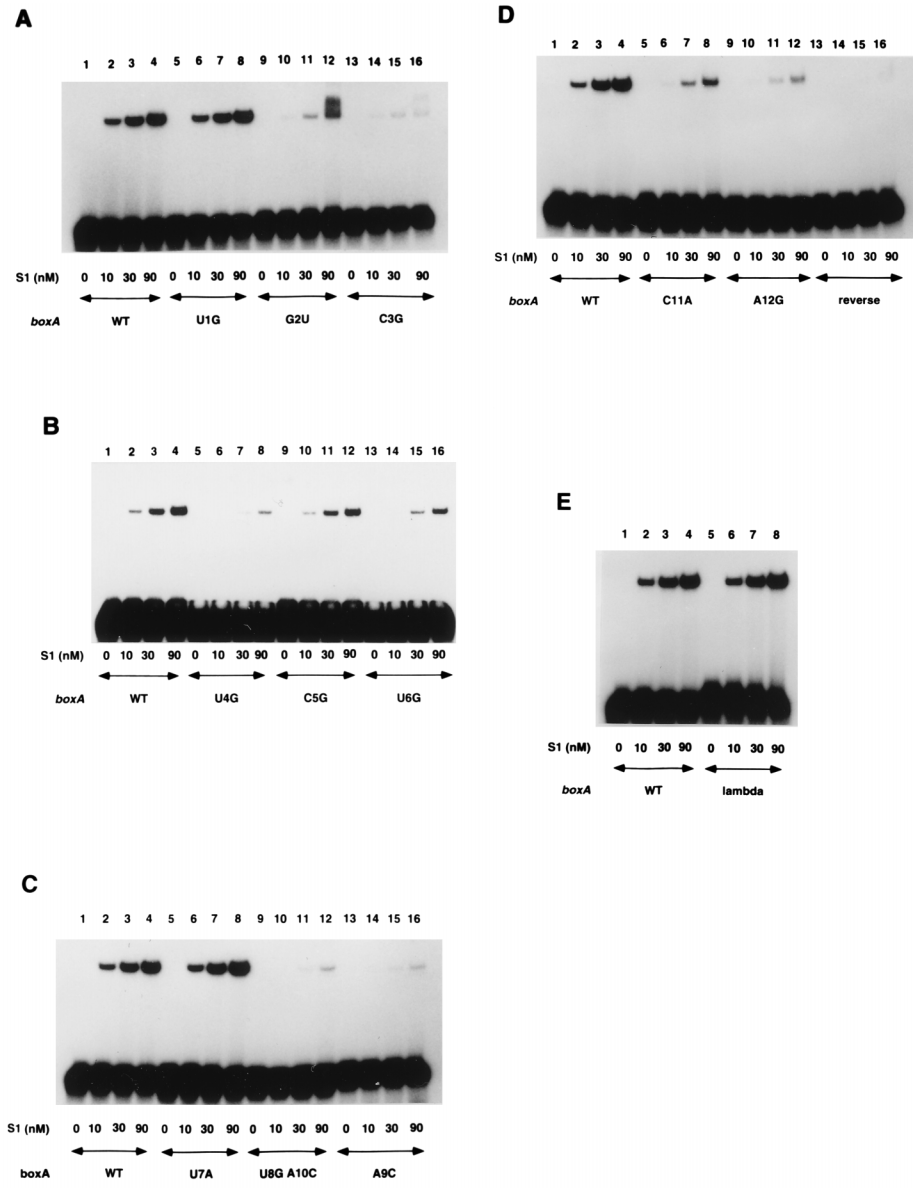

FIG. 4. Mutations in *boxA* affect its ability to bind ribosomal protein S1. Various concentrations of S1 were incubated with radiolabeled RNAs containing wild-type (WT) or mutant *boxA* as indicated, and the reaction mixtures were electrophoresed on nondenaturing polyacrylamide gels.

λ or *rrn boxA* RNA is highly specific and could potentially play a role in λ and/or *rrn* antitermination.

To determine if S1 could enter a ribonucleoprotein complex with NusB, S10, and *rrn boxA* RNA, a gel mobility shift experiment was performed in which purified NusB, S10, and S1 were incubated at various concentrations with RNA containing *boxA* (Fig. 5). Probe shifted by S1 had a lower electrophoretic mobility than probe shifted by the NusB-S10 complex (Fig. 5A, compare lanes 2 and 5). As S1 was added in increasing amounts to reaction mixtures containing NusB and S10, the NusB-S10-RNA complex disappeared and no supershifted band was observed (Fig. 5A, lanes 5 to 8). Thus, S1 apparently competes with NusB and S10 for binding to *boxA* RNA. This result is consistent with our observations that the nucleotides most important for binding S1 (Table 1) are also important for binding NusB and S10 (20) (Table 1). In a similar experiment, addition of increasing amounts of NusB-S10 to reaction mixtures containing S1 decreased the amount of S1-RNA complex without producing a supershifted complex (Fig. 5B, lanes 2 to 6), again demonstrating that NusB-S10 and S1 cannot simultaneously bind *boxA* RNA.

Our binding data (Fig. 5) indicated that the affinity of *rrn boxA* RNA for S1 is at least 200 times greater than its affinity for the NusB-S10 complex. This raised the possibility that S1 might be an inhibitor of antitermination. Nevertheless, adding purified S1 did not inhibit *rrn boxA*-mediated antitermination in reactions containing crude *E. coli* extract (25), nor did it make possible *rrn* antitermination in vitro when it was added to reactions containing purified Nus factors (24). Therefore, the significance of the specific interaction we have described between *rrn boxA* and ribosomal protein S1 is still unclear.

The existence of an *E. coli* inhibitor of N-mediated antiter-

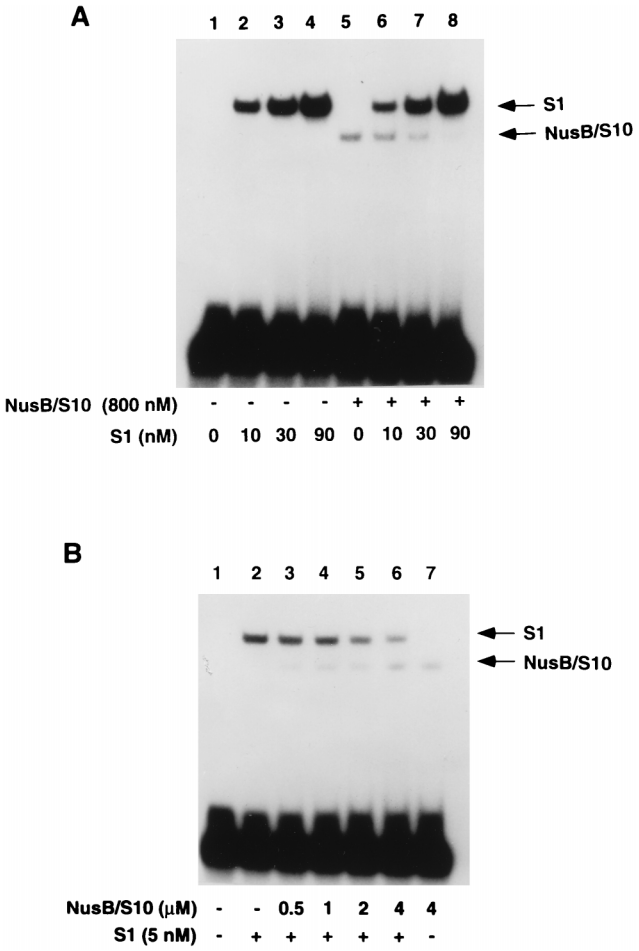

FIG. 5. S1 competes with NusB-S10 for binding of *boxA*. S1, NusB, and S10 (as indicated) were incubated with radiolabeled *boxA* RNA. The reaction mixtures were electrophoresed on a nondenaturing polyacrylamide gel. +, present; −, absent.

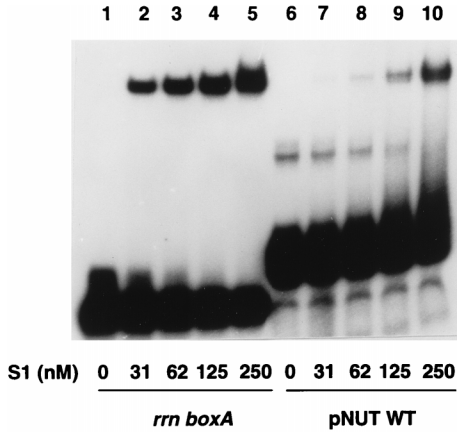

FIG. 6. S1 binds RNA containing a *nut* site. Various amounts of S1 (as indicated) were incubated with probe containing either the *rrn boxA* or λ (pNUT WT) *nut* site. The reaction mixtures were electrophoresed on a nondenaturing polyacrylamide gel.

mination that binds *boxA* has been predicted by Patterson and colleagues on the basis of genetic experiments with deletion and point mutations in *boxA* (21). To determine whether S1 could be this inhibitor, we first compared the strength of binding between S1 and a probe containing *rrn boxA* with that of S1 and a probe containing a λ *nut* site (*boxA* + *boxB*) (Fig. 6). S1 bound the *nut* site probe, although approximately eight times as much S1 was required to shift the *nut* site probe as to shift a similar amount of the *rrn boxA* probe (Fig. 6, compare lanes 2 to 5 with lanes 7 to 10), raising the possibility that *boxB* might partially hinder the S1-*boxA* interaction. Even though S1 could bind *boxA* in the presence of *boxB*, we found that S1 did not inhibit N-mediated nonprocessive antitermination in vitro in reactions in which NusA was the only *E. coli* cofactor (30), nor did it inhibit processive antitermination in reactions containing NusA, NusB, NusG, and S10 (15) (data not shown). The inability of S1 to inhibit antitermination even though it binds *boxA* with an apparently higher affinity than NusB-S10 (which does not bind *nut* site RNA in the absence of N and other factors [16]) suggests that protein-protein interactions within the N-modified transcription complex involving N, RNA polymerase, NusA, and NusG allow NusB-S10 to outcompete S1 for binding *boxA* (8). The ability of S1 to compete with NusB-S10 for binding *boxA* makes it seem unlikely that S1 has a positive role in antitermination. It is conceivable that S1 may be just one component of an antitermination inhibitory complex whose other component(s) remains to be identified.

Alternatively, S1 may be involved in some other kind of *boxA*-mediated process. For example, the processing stalks of the 16S and 23S rRNAs are located near *boxA* sequences in the leader regions of the *rrn* operons and in the spacer regions between the 16S and 23S rRNAs. Morgan (18) has suggested that the processing stalks could be juxtaposed for processing by their adjacent *boxA* sequences. If that is so, one could imagine that *boxA* and S1 might play a role in the processing of rRNA.

Another possibility is provided by the recent observation that the λ N protein can repress the translation of its own mRNA in a process that requires the *nut* site but appears to be independent of NusA, NusB, and S10 (31). The *boxA16* mutation, which alters the fifth nucleotide (underlined) of λ *boxA* (CGCUCUUACACA), prevents antitermination of transcription but not repression of translation, whereas the *boxA5* mutation, which alters the second nucleotide of λ boxA (CGCU CUUACACA), prevents both (31). Interestingly, nucleotides 2 and 5 of *boxA* are both important for the binding of NusB and S10 (20) (Table 1), whereas only nucleotide 2 is important for the binding of S1 (Fig. 3 and Table 1). Translational repression may therefore involve a complex containing the ribosome in which the binding of N to *boxB* and the binding of ribosomal protein S1 to *boxA* at the *nutL* site somehow prevent or aid binding of the ribosome to the AUG initiator codon of the *N* gene located not far downstream of *nutL*.

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