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Domain I of 23S rRNA competes with a paused transcription complex for ribosomal protein L4 of *Escherichia coli*

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ABSTRACT

Ribosomal protein L4 of *Escherichia coli* regulates expression of its own eleven gene S10 operon both by inhibiting translation and by stimulating premature termination of transcription. Both regulatory processes presumably involve L4 recognition of the S10 leader RNA. To help define L4's regulatory target, we have investigated the protein's cognate target on 23S rRNA. Binding of L4 to various fragments of the 23S rRNA was monitored by determining their ability to sequester L4 in an *in vitro* transcription system and thereby eliminate the protein's effect on transcription. Using this approach we identified a region of about 110 bases within domain I of 23S rRNA which binds L4. A two base deletion within this region, close to the base to which L4 has been cross-linked in intact 50S subunits, eliminates L4 binding. These results also confirm the prediction of the autogenous control model, that L4 bound to its target on rRNA is not active in regulating transcription of the S10 operon.

INTRODUCTION

Ribosomal protein (r-protein) L4 of *E.coli* functions not only as a component of the 50S subunit, but also as a regulatory protein controlling expression of its own eleven gene S10 operon (1–3). R-protein L4 regulates the S10 operon by two genetically distinct mechanisms: it inhibits transcription by inducing RNA polymerase to prematurely terminate within the S10 leader (4–6) and it inhibits translation by blocking initiation of translation of the most proximal gene of the S10 operon (2,5,7). Both of these regulatory responses are elicited when the synthesis of L4 exceeds the synthesis of its 'normal' target, 23S rRNA.

We have been focusing on L4-mediated transcription control, to learn how a ribosomal protein affects the activity of RNA polymerase. *In vivo* studies showed that transcription inhibition results from termination about 140 bases from the start of transcription (4,6), at an attenuator hairpin that resembles a rho-independent terminator (8). Only the first 150 bases of the S10 leader are required, and translation is not involved (6,9). *In vitro* studies showed that L4-mediated transcription control requires NusA (9,10), a transcription factor needed for efficient N- and Q-dependent antitermination in bacteriophage lambda (11,12). Recently, we reported that NusA is necessary to promote RNA polymerase pausing at the S10 leader termination site: such paused complexes are then stabilized by r-protein L4 (13). This suggests that L4-stabilization of the paused complex is the basis for the r-protein's stimulation of premature transcription termination *in vivo*. *In vivo* measurements of transcription rates indicate that such stable paused complexes are rapidly converted into *bona fide* termination complexes (13,14). However, the *in vitro* system is partially deficient in this step, since efficient release of the nascent transcript is not observed (13).

One of the key questions about L4-mediated autogenous control is what is the regulatory target for the ribosomal protein? Given the known function of the r-protein as a 'primary' 23S rRNA binding protein (15) and the protein's specificity for its own operon, we assume that the L4 target is comprised, at least in part, of S10 leader RNA. Indeed, the generic model for autogenous control by r-proteins assumes that the regulatory r-protein recognizes a target on its own message that is structurally similar to its binding site on rRNA. However, we have failed to detect an interaction between L4 and the S10 leader RNA by standard techniques such as filter binding and gel retardation assays.

One limitation to identifying the L4 target on its own mRNA has been the lack of information about the protein's target on 23S rRNA. In early experiments the L4 binding site was localized to the 5′ 1200 bases of 23S (16). Also, cross-linking studies of intact 50S subunits showed that L4 could be cross-linked to two different sites in 23S, one in domain I (17) and the other in domain II (18). To further define the L4 target, we analyzed the effect of addition of 23S rRNA, or deletion derivatives, on L4-stimulated attenuation in the *in vitro* transcription system. As previously reported (10), RNA containing the proximal 840 bases of 23S rRNA eliminates L4's ability to stabilize the paused transcription complex, presumably because the 23S rRNA fragment binds L4 and thereby sequesters it from the transcription complex. We have now exploited this system to define in more detail the L4 target on 23S rRNA. By testing smaller 23S rRNA fragments in the *in vitro* transcription system, we have narrowed the L4 target to a 110 base region within domain I of 23S. A two base deletion within this region strongly reduces the affinity for L4. In addition, we analyzed the kinetics of the competition for L4 between 23S rRNA and the transcription complex. The results are compatible with the idea that L4 is associated semi-stably with the paused transcription complex.

MATERIALS AND METHODS

Plasmids

Plasmids pT723 and pT714 carrying the 23S and 16S rRNA genes (19), respectively, under control of the T7 RNA polymerase promoter were described previously (10). Plasmid pT725 was derived from pT723 by digestion with *Sst* I and religation. Plasmid pT726 was constructed by digesting pT723 DNA with *Hind* III and *Sna*B I, filling-in the *Hind* III-generated single-stranded ends with Klenow fragment, and religating. Maps of pT723, pT725 and pT726 are shown in Fig. 1.

Plasmids pT727 and pT728 were constructed by digesting pT723 DNA with *Bss*H II, treating the linearized DNA with mung bean nuclease (20), and religating. The ligated DNA was again treated with *Bss*H II (to select against plasmids which had not been digested with the mung bean nuclease), and used for transformation. The extent of mung bean nuclease-generated deletion at the *Bss*H II site was determined by sequencing of double-stranded DNA. Plasmid pT727 had the expected four base deletion; plasmid pT728 was apparently incompletely digested with the nuclease and had only a two base deletion.

Plasmid pLL226 carrying the S10 promoter and S10 leader upstream of the *rrnC* terminator (10) was used as template for *E.coli* RNA polymerase transcription reactions.

In vitro transcription reactions with T7 RNA polymerase

Reactions were performed using standard conditions (10). To quantitate the transcription products, 1 μCi [5,6-^{3}H]UTP was included in the reaction. The extent of incorporation was then calculated as the ratio of ice cold TCA-precipitable radioactivity divided by total input radioactivity. RNA products were processed as described (10).

T7 RNA polymerase templates were also synthesized by the PCR technique (20). The 5′ oligonucleotide was a 43-mer containing 23 bases from the T7 promoter (TAATACGACTC-ACTATAGGGAGA) proximal to a 20 base sequence corresponding to bases 50–69 (oligo 'O23') or bases 265–284 (oligo 'O24') of the mature 23S rRNA sequence. The 3′ oligonucleotide (oligo 'O21') corresponded to bases 366–386 of the 23S sequence.

In vitro transcription reactions with *E.coli* RNA polymerase

Conditions for transcription reactions have been described (13). The standard 40 μl reaction contained 20 nM supercoiled pLL226 plasmid DNA, 20 nM RNA polymerase, and 40 nM NusA. Where indicated, r-protein L4 or S7 was added to 120 nM. As described previously (13), these reaction components were mixed together with 500 μM each GTP and CTP, and incubated for 10 min at 37° to allow formation of an initiation complex and incorporation of the first three nucleotides (pppGGC). A single round of transcription elongation was then started by addition of ATP (to 500 μM), UTP (to 100 μM), and 5–10 μCi [α-^{32}P]UTP. In early experiments rifampicin (to 10 μg/ml) was added with the UTP and CTP to prevent additional initiation events. However, in practice, the addition of rifampicin was unnecessary, probably because under our reaction conditions essentially all polymerases that can initiate will have initiated during the pre-incubation, and those polymerases are not efficiently recycled during the course of the reaction. Competitor rRNA was added at the indicated times to a final concentration of 60–200 nM.

The RNA products were processed and analyzed as described previously (10,13).

RESULTS

Experimental strategy

E.coli RNA polymerase transcribing the S10 operon leader *in vitro* pauses briefly at the S10 attenuator (9,10,13). This pausing requires the addition of transcription factor NusA, and is markedly prolonged by the addition of purified r-protein L4 (13). Our assay for L4 binding to a fragment of 23S rRNA involves adding the fragment to the *in vitro* transcription reaction and monitoring the effect on L4-stimulated pausing of *E.coli* RNA polymerase at the attenuator. Sequestering of L4 by binding to the RNA fragment abrogates the L4-mediated stabilization of the paused transcription complex (10).

RNA molecules containing various portions of 23S rRNA were synthesized *in vitro* using T7 RNA polymerase and T7 promoter-containing templates in which the 23S gene was truncated at various restriction sites. The starting plasmid was pT723 [Fig. 1A; (10)], a derivative of plasmid pT7-2 (US Biochemicals) carrying the entire 23S and 5S rRNA genes, as well as the 3′ half of the spacer between 16S and 23S. RNA molecules extending for various distances into the 23S gene were then synthesized from this template after linearization with the indicated restriction enzymes (Figs. 1A and 2). Since we could not directly analyze RNAs from *Sst* I-linearized DNA because of the problem of templates with protruding 3′ termini (21), we deleted the *Sst* I fragment from pT723 to create pT725, which was then linearized with *Eco*RI (Figs. 1A and 2). Note that transcripts synthesized from pT723 and pT725 contain at their 5′ ends about 260 bases of the 16S-23S spacer sequence. To synthesize 23S rRNA lacking domain I, we constructed pT726, a deletion derivative of pT723 (Fig. 1A). This plasmid directs the synthesis of RNA starting at the *Sna*B I site in 23S (Fig. 2). The positions of the various restriction enzyme sites relative to the secondary structure of the 23S rRNA are shown in Figs. 3 and 4. As a negative control, we also synthesized RNA from plasmid pT714 (10), carrying the 16S gene under control of the T7 promoter (Fig. 2).

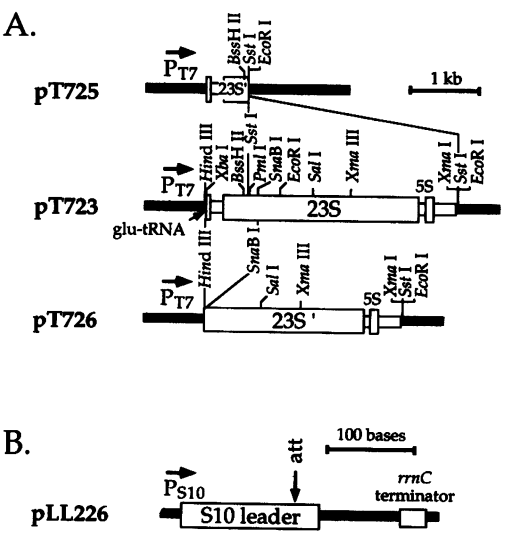

Figure 1. Plasmid maps. (A) Maps of plasmids used for T7 RNA polymerase-directed synthesis of 23S fragments. Plasmid pT723 has been described (10). The construction of deletion derivatives pT725 and pT726 is described in Materials and Methods. Pertinent restriction endonuclease sites are indicated. (B) Map of plasmid pLL226 (10,13), the template for *E.coli* RNA polymerase transcription.

The template for analysis of L4-mediated attenuation control was pLL226 (Fig. 1B). This plasmid carries the S10 operon promoter and first 165 bases of the leader, followed by the efficient *rrnC* terminator.

The proximal 360 bases of 23S rRNA contain the L4-binding site

The various fragments of 23S rRNA and the 16S rRNA control were tested for their effect on L4-mediated attenuation. Typical gel electrophoresis patterns and their quantitation are shown in Fig. 5A and B. The results from these and other experiments are summarized in Fig. 2. The 23S derivatives containing the proximal 360 bases of mature 23S (see the pT725 *Eco*R I lane in Fig. 5B) bind L4, as evidenced by their ability to eliminate an L4 effect on transcription pausing at the attenuator. However, RNA molecules only 45 bases shorter (pT723 *Bss*H II, Fig. 5A and B) did not relieve the L4 effect, nor did an even shorter 23S fragment (pT723 *Ava* I, Fig. 5B) or RNA from the 16S gene (10 and data not shown). These results indicate that domain I contains a target for L4 binding, and, more specifically, that the region at or near the L4 cross-linking site in domain I (17, see Fig. 3) is required.

Because L4 has also been cross-linked to domain II (18, Fig. 3), we tested whether a second independent binding site was present in this region of 23S rRNA, using plasmid pT726 in which domain I was deleted. None of the tested RNAs from this

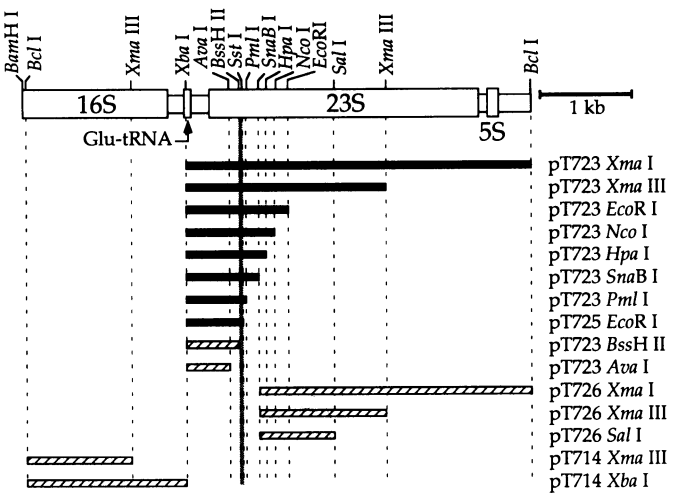

Figure 2. Map of *in vitro* synthesized RNAs used for competition experiments. The map of the *rrnB* transcription unit with relevant restriction endonuclease sites is shown at the top. Transcription is from left to right. Below are the various transcripts synthesized from a T7 RNA polymerase promoter using the linearized templates indicated to the right. Solid bars represent RNA molecules which bind L4 as defined by the *in vitro* transcription assay. Hatched bars represent RNAs which have no effect on L4-stimulated pausing. The vertical gray bar indicates the boundary between L4-binding and non-binding molecules.

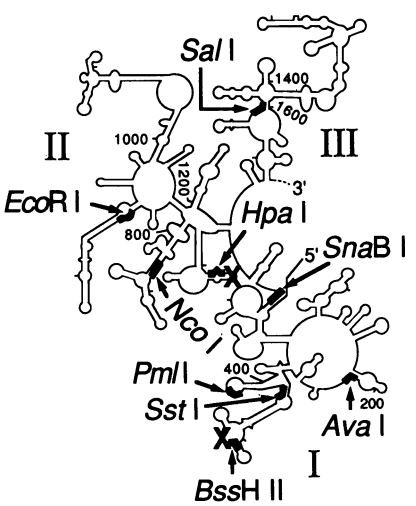

Figure 3. Secondary structure of the three promoter proximal domains of *E. coli* 23S rRNA. The structure shown is from Egebjerg et al. (30), and is based on the structure of Noller et al. (31). Relevant restriction enzyme sites are indicated by black boxes. The sites of L4 cross-linking [near the *Bss*H II site in domain I (17) and near the *Hpa* I site in domain II (18)] in intact 50S ribosomes are indicated by X's.

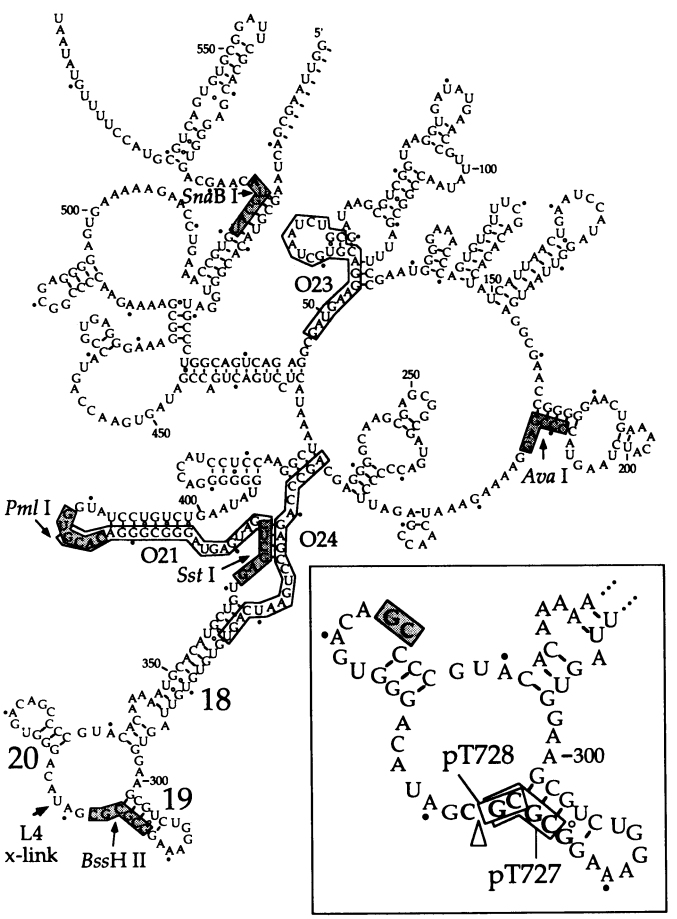

Figure 4. Detailed structure of domain I of 23S rRNA. Restriction enzyme sites are indicated by shaded boxes. The site of L4 cross-linking (17) and the sequences used to design oligonucleotides O21, O23, and O24 (used for synthesis of T7 polymerase templates by PCR) are also indicated. The helices in the region of bases 280–360 ('18', '19' and '20') are numbered according to Egebjerg et al. (30). The inset shows the region containing bases 292 to 348, including the hexanucleotide (bases 320–325) containing the base to which L4 has been cross-linked. The open arrowhead shows the site of *Bss*H II cleavage of the corresponding DNA. The two and four bases deleted in plasmids pT728 and pT727, respectively, after *Bss*H II digestion of pT723 are indicated by open boxes. Phylogenetic studies (23) suggest that bases 333–334 (shaded box) pair with bases 317–318 to form a pseudoknot.

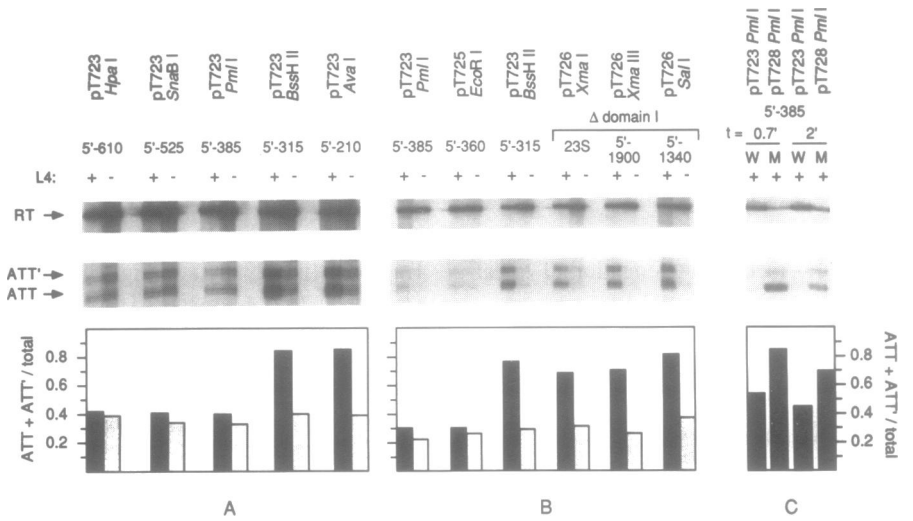

Figure 5. Effect of competitor 23S RNA on L4-mediated attenuation. **(A)** and **(B)** Single-round transcription reactions were performed as described in Materials and Methods. Each reaction contained either r-protein L4 (+) or, as a control, r-protein S7 (−). RNA from the indicated templates (60 nM final concentration) was added at 1 min before the addition of ATP, UTP and rifampicin. Reactions were terminated after 5 min and the products were analyzed by gel electrophoresis (13). Autoradiograms of the gels are shown. Bands corresponding to read-through (RT) and attenuated (ATT and ATT′) molecules are indicated. [Two attenuated RNA bands are observed in the *in vitro* system; the ATT molecules are the same size as the *in vivo* RNAs, the ATT′ are several bases longer; see (9,13)]. These bands were excised and the radioactivity in each was determined in a scintillation counter. The fraction of RNA polymerases at the attenuator region ('ATT + ATT′/total') was then calculated as the ratio of radioactivity in the ATT and ATT′ bands divided by the radioactivity in ATT, ATT′ and RT bands . The region of 23S rRNA contained in the various competitor RNAs is indicated above the autoradiogram. **(C)** The transcription reactions were performed and analyzed as for (A) and (B) except that all reactions received L4, and RNA was added to 100 nM. Reactions were terminated at the indicated times after the start of elongation. W: wild-type RNA transcribed from pT723; M: mutant RNA transcribed from pT728 containing a two base deletion at the *Bss*H II site (see Fig. 4).

plasmid could block the L4 effect on transcription (Figs. 2 and 5B), indicating that only domain I bound L4.

Kinetic analysis of the effect of competitor RNA on L4-mediated attenuation

Before continuing with the strategy of using the transcription reaction to indirectly monitor 23S RNA-L4 binding, we wanted to confirm that the competition was indeed reflecting L4 sequestering, and not, for example, the sequestering of NusA, the other component necessary for a stable pausing by RNA polymerase. Previously published experiments showed that, in the presence of NusA, whether or not L4 was added, essentially all RNA polymerases pause briefly at the attenuator site (13). The paused transcription complex is further stabilized by L4, whether the r-protein is present from the beginning of the reaction or is added after RNA polymerase has already reached the pause site. On the other hand, in the absence of NusA, RNA polymerase exhibits little or no pausing at the attenuator site, and L4 has no effect (13). We reasoned that, if the added RNA were interacting only with L4, then the early kinetics of the reaction should be identical to the kinetics of a reaction never receiving L4. If the competitor RNA were interacting with NusA or some other (unknown) component that affected the efficiency of RNA polymerase pausing, then the kinetics should resemble the kinetics of a reaction never receiving NusA.

The results of the kinetic analysis are consistent with a specific effect of competitor RNA on L4 availability. The addition of competitor RNA from plasmid templates pT723 *Sna*B I or pT725 *Eco*R I, both of which reduced the effect of L4 in earlier experiments (Fig. 5A and B), essentially eliminated the effect of L4 addition, without affecting the NusA-dependent pause itself [compare curves in panels (A) and (B) of Fig. 6 with the -L4 curve in panel (D)]. The kinetics of transcription after addition of RNA from the domain I deletion template pT726 *Sal* I, which did not appear to sequester L4 in earlier experiments (Fig. 5B) were the same as those from the −RNA control reactions [Fig. 6, panels (C) and (D)].

RNAs synthesized from PCR fragments also contain the L4 binding site

To further define the L4 binding domain in 23S rRNA, we constructed templates for T7 RNA polymerase-directed transcription using the PCR technique (20). By incorporating a T7 RNA polymerase promoter sequence in the 'upstream' primer, we could transcribe RNA directly from the PCR DNA products without first cloning into a promoter vehicle. Two different T7 promoter-containing oligonucleotides, O23 and O24 (Fig. 4), were used to define the promoter proximal end of the 23S sequence (see Materials and Methods). Oligonucleotide O21 (Fig. 4) was used to delineate the distal end. The resulting PCR products were used in the T7 RNA polymerase transcription assays. The O23−O21 template generated an approx. 340 base long RNA corresponding to bases 48 to ~386 of 23S. The O24−O21 template generated an approx. 120 base long RNA corresponding to bases 265 to ~386 of 23S.

The effects of addition of these short RNAs to the *in vitro* transcription reaction are shown in Fig. 7. The 340 base long RNA from O23−O21 was essentially as effective as the control pT723 *Sna*B I RNA (containing the 5′ 525 bases) in sequestering L4. Although the shorter RNA, corresponding to bases 265−386, was less effective, it still showed significant L4-binding activity when compared to the reaction without any RNA (Fig. 7). These results, together with the results summarized in Fig. 5, indicate that the L4 binding site in 23S rRNA is contained within the structure defined by oligo O24 on the 5′ end and the *Sst* I site on the 3′ end (Fig. 4).

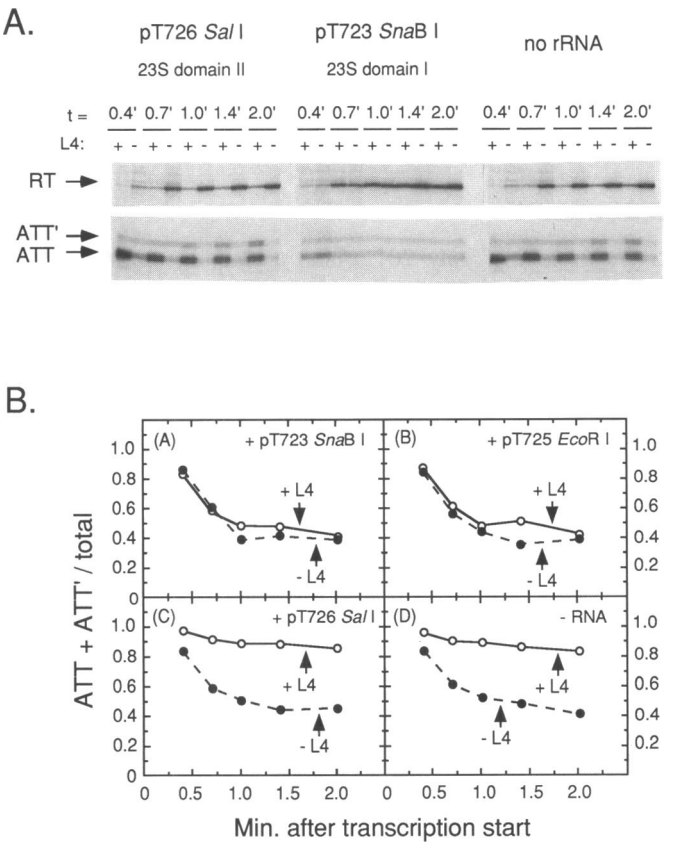

Figure 6. Early kinetics of transcription elongation after addition of competitor 23S RNA. The transcription reactions were as described in the legend to Fig. 5, except that competitor RNA from the indicated plasmid templates was added (to 100 nM) 0.6 min before the addition of ATP and UTP (no rifampicin). Aliquots of each transcription reaction were removed and processed at the indicated times after the start of elongation. (A) shows an autoradiogram of the gel. (B) shows the quantitation of the fraction of RNA polymerases at the attenuator region (see legend to Fig. 5).

A two base deletion near the L4 cross-linking site abolishes L4 binding

The L4 binding target defined by the results reported above contains the hexanucleotide (bases 320–325) containing the base to which L4 has been cross-linked in intact 50S ribosomal subunits (17). To analyze the importance of this region we exploited the presence of a unique *Bss*HII site to introduce small deletions. Two plasmids were generated, pT727 containing a four base deletion and pT728 containing a two base deletion at the *Bss*H II site (see inset in Fig. 4). Analysis of L4 binding to the RNA containing the smaller deletion, shown in Fig. 5C, indicates that this region is indeed critical for L4 binding: the ability of the mutant RNA to block the L4 effect on transcription is significantly reduced compared to the wild-type RNA. Similar results were obtained with the four base deletion (data not shown).

Addition of 23S RNA containing the L4 binding domain to paused transcription complexes destabilizes the complex

Our previous experiments showed that r-protein L4 can stabilize a NusA-dependent paused transcription complex even if the protein is not added to the reaction until RNA polymerase has already reached the pause site (13). These results suggest that L4 might mediate its effect by binding to the paused transcription

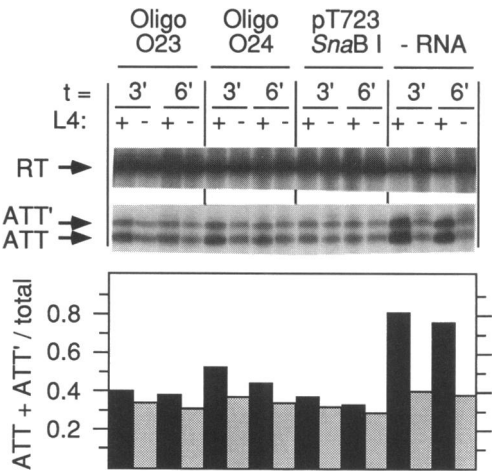

Figure 7. Effect of RNA synthesized from PCR products on L4-mediated attenuation. The transcription reactions were as described in the legend to Fig. 5, except the competitor RNA was added to 200 nM, and no rifampicin was added with the ATP and UTP. Aliquots of each reaction were removed and processed after 3 and 6 min of transcription elongation. The PCR-generated templates for competitor RNA synthesis were constructed using the indicated oligonucleotides (see Materials and Methods and Fig. 3).

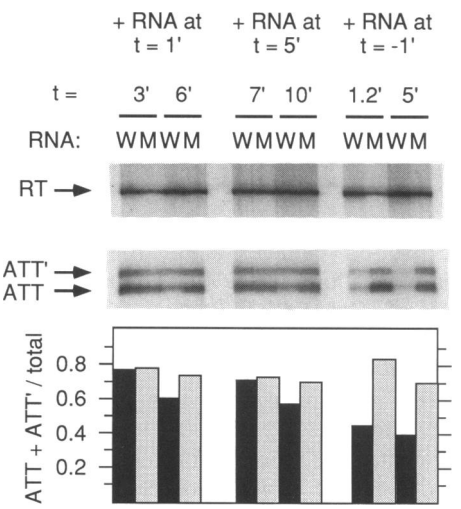

Figure 8. Effect of late 23S addition on the L4-mediated transcription pausing. All reactions contained r-protein L4. Competitor RNA from plasmid pT723 *Pml* I ('W') and plasmid pT728 *Pml* I ('M') was added 1 min before or 1 or 5 min after the addition of ATP and UTP to start transcription elongation. The RNA concentration was 100 nM. The reactions were terminated at the indicated times after elongation started, and analyzed as described above.

complex, to become an integral part of the paused structure. An alternative possibility is that L4 plays a transient role, modulating a component of the transcription complex, such as RNA polymerase, and then becoming dispensable. To learn more about the role of L4 in stabilizing the paused complex, we asked what effect competitor 23S rRNA has when added to transcription complexes already paused in the presence of r-protein L4. If L4 has a transient role, late addition of the competitor RNA should have no effect on the stability of the paused complex. On the other hand, if L4 is part of the paused complex and if its removal destabilizes the complex, then late addition of 23S rRNA might

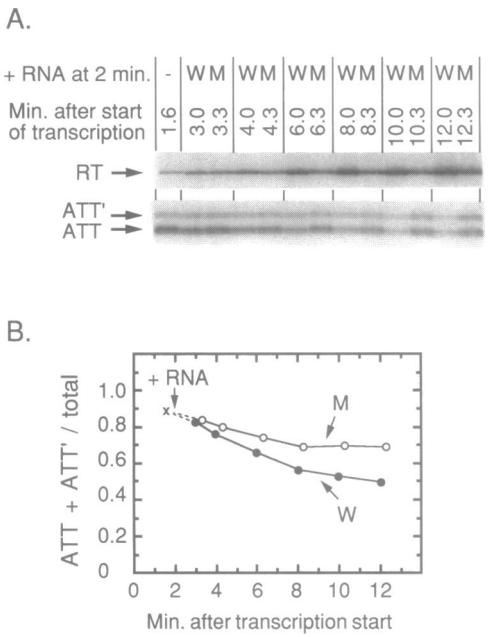

Figure 9. Kinetic analysis of the effect of late 23S addition on the L4-mediated transcription pausing. A single-round transcription reaction containing r-protein L4 was started at t = 0 min. One aliquot was removed and processed at 1.6 min. The remainder of the reaction was divided into two parts. One part was added at t = 2 min to a tube containing RNA from plasmid pT723 *Pml* I ('W'; final concentration 100 nM); the other was added at t = 2.3 min to a tube containing competitor RNA from pT728 *Pml* I ('M'; final concentration 100 nM). At the indicated times aliquots were removed, processed and analyzed by gel electrophoresis. (A) shows an autoradiogram of the RNA products; (B) shows the quantitation.

disrupt the paused complex and promote resumption of transcription elongation.

The results of a late 23S addition experiment are shown in Fig. 8. Two different RNAs were used: the wild-type RNA ('W') from pT723 *Pml* I and a control RNA ('M') from pT728 *Pml* I which contains the two-base deletion at the *Bss*H II site that reduces L4 binding (see Fig. 5C). The competitor RNAs were added to L4-containing transcription reactions either 1 min or 5 min after the start of transcription elongation. Although the effects are very subtle, the wild-type RNA caused a reproducible decrease in the stability of the paused complex. The effect was not apparent within 2 min of the addition of RNA (see the 3 min and 7 min time points in Fig. 8), but could be seen at the later time point (the 6 min and 10 min lanes in Fig. 8). The late addition of competitor RNA was clearly less effective than its addition 1 min prior to the start of elongation (Fig. 8).

Further evidence that the destabilizing effect of late addition of competitor RNA is real was provided by a second experiment in which we followed the kinetics of transcription in more detail after late addition of 23S rRNA. In this experiment, competitor RNA was added to the L4-containing reaction 2 min after the start of elongation, and aliquots were removed for analysis at the indicated times. Again, we observed a relative decrease in the stability of the paused complex with wild-type (W) RNA compared to mutant (M) RNA (Fig. 9), even though the effect was still relatively small compared to the effect of RNA added before the start of elongation. Furthermore, the effect was not immediate: a clear difference between the mutant and wild-type RNA was not observed until at least 2 min after addition. In contrast, RNA added as little as 0.6 min before transcription start was fully effective in eliminating the L4-stimulated pause (see, e.g., Fig. 6). These results are consistent with a model in which L4-mediated stabilization of the paused transcription complex depends on a 'permanent' association of L4.

DISCUSSION

L4, a protein of only 201 amino acids, functions both as a component of the ribosome and as a regulator of both transcription and translation of its own operon (1–3). Since L4 is a known rRNA-binding protein (15,16), its regulatory target is probably also RNA. Our genetic studies have implicated the region of the S10 operon leader containing two critical hairpin structures as potential L4 binding sites (5 and unpublished results). However, we have no direct evidence for L4's interaction with its own mRNA, nor do we know if the protein recognizes similar binding sites on the two target RNAs. Indeed, we can only surmise that L4's mRNA target for transcription control is the same as its mRNA target for translation control.

To facilitate our analysis of the L4 binding site on the S10 leader RNA, we have investigated the protein's binding site on 23S. In earlier experiments with intact 50S ribosomes, Brimacombe and coworkers (17,18) showed that L4 could be cross-linked to both domain I (sequence region 320–325) and domain II (sequence region 613–617) of 23S rRNA. These results indicate that, in the three-dimensional structure of the ribosome, L4 is in close contact with two regions of 23S rRNA which are widely separated in the RNA secondary structure. However, cross-linking sites are not necessarily synonymous with binding targets. After trying unsuccessfully to detect an interaction between L4 and 23S rRNA by standard procedures (e.g., gel retardation and filter binding studies), we have used an *in vitro* transcription system as a functional assay for L4-23S rRNA binding. Using this procedure, we have localized the L4 target to the same region as the domain I cross-link detected by Brimacombe and coworkers (17). Although our assay system may not be sensitive enough to detect weak interactions with domain II, we conclude that this other cross-link site is probably not the primary binding site for L4. Thus, the primary sequence similarities between domain II and the S10 leader (22) are probably fortuitous, a conclusion consistent with genetic studies showing that this region of the S10 leader could be mutated with no effect on L4 regulation (5,6).

Our competition results indicate that a fragment of 23S domain I containing helices 18, 19 and 20 (the O24–O21 product; see Fig. 4) is sufficient for L4 recognition (although our assay is not sufficiently quantitative to determine if this fragment binds L4 as tightly as do the larger RNA molecules). Furthermore, mutant RNA containing a deletion of bases 316 and 317 has a significantly reduced affinity for L4. Together with the cross-linking data (17), these results indicate that the region around helices 19 and 20 is critical for L4 recognition of 23S RNA. Phylogenetic studies suggest that this region assumes a complex tertiary structure that includes a pseudoknot structure formed by base-pairing between bases 317–318 and 333–334 (23, see inset in Fig. 4) as well as pairing of bases 319 and 323 (24,25). Since this complex structure would be disrupted by the two base deletion that reduces L4 binding, these tertiary interactions may be necessary for L4 binding.

The simplest model for autogenous regulation by a r-protein assumes that the regulatory protein recognizes a target on its own

messenger RNA that resembles its target on rRNA. Now that we have identified an L4 binding site in domain I of 23S rRNA (i.e., the region around helices 18, 19 and 20), we have looked for primary or secondary structure features shared by this region and the S10 leader. No similarity is obvious, but until we understand more about the tertiary structures of both RNA targets, such similarities may not be apparent. Further definition of the leader region required for L4 regulation of transcription and translation may facilitate our identification of such similarities.

One obvious approach to finding the putative L4 binding site on its mRNA would be to use leader RNA instead of 23S RNA as competitor in the *in vitro* transcription reaction. Unfortunately, addition of leader RNA did not affect the L4-stimulated attenuation, even at a concentration that was 20-fold higher (1.2 μM) than the effective concentration of 23S fragment (data not shown). A possible explanation is that the input S10 leader RNA (which was synthesized using T7 RNA polymerase and contains all of the S10 leader including the sequence downstream of the L4-stimulated termination site) does not have the appropriate conformation for L4 binding. For example, the nascent RNA might form a transient structure with a higher affinity for L4 than the complete 'mature' leader structure. Also, protein components of the transcription complex (e.g., NusA or RNA polymerase) might contribute to the L4 target, either directly by protein-protein interactions or indirectly by affecting the leader structure.

During the *in vitro* assembly of 50S subunits, r-protein L4 is one of 5 'early assembly proteins' which are essential for formation of the obligatory early intermediate 'RI_{50}*' particle (26,27). Interestingly, all five proteins (L4, L13, L20, L22 and L24) interact with the 5′ portion of 23S rRNA (27). The key protein in this assembly step is L24, one of two 'assembly initiator proteins' (the other is L3) which are believed to initiate formation of critical assembly domains (28). Protein L24 has been cross-linked (in intact 50S) to domain I of 23S rRNA, at bases 99–107 (17). Later foot-printing studies concluded that the L24 interacts with two other sites in domain I (29). One site, around the unpaired A residues in helix 18 (see Fig. 4), is thought to be the primary L24 attachment site; the other site is within two A-rich interhelical regions near bases 450- 500 (Fig. 4) and may be involved in L24's critical role in initiating assembly (29). The role of L4 in the early assembly steps leading to RI_{50}* synthesis is still poorly defined, although it is known to stimulate the binding of L22 as well as other proteins added later (27). Our observation that L4 binds to the same region of domain I as L24 raises the possibility that the two proteins interact during assembly, although no such interaction is evident from the current 50S assembly map (27). In any case, our mapping studies refute the presumption that L24 is the only primary binding protein that associates with domain I (29).

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