

Transcription of the S10 Ribosomal Protein Operon Is Regulated by an Attenuator in the Leader

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Summary

Previous studies have shown that ribosomal protein L4 specifically inhibits the expression of its own operon, the 11-gene S10 operon. To elucidate the mechanism for this regulation, we have examined the effect of protein L4 on transcription of the S10 operon. Hybridization and gel electrophoresis studies indicate that in the presence of excess L4 only RNA molecules about 140 bases long are transcribed from the S10 operon. These short RNA molecules contain the leader, but not structural gene, sequences. Our results suggest that protein L4 stimulates premature termination (attenuation) of transcription about 30 bases upstream from the start of the first structural gene of the S10 operon. The attenuation appears to be independent of the regulation of translation of the operon. We suggest that attenuation of transcription plays a primary role in the autogenous regulation of the S10 operon.

Introduction

The synthesis of ribosomes in *Escherichia coli* is regulated as a function of growth rate and of aminoacyl tRNA availability. Many questions about the molecular mechanisms underlying the regulation of ribosome synthesis remain obscure. However, a model accounting for one aspect of this regulation, the coordinate expression of the 16 or more ribosomal protein (r-protein) operons, has evolved from work in a number of laboratories. The principle of this model is that a specific protein in each operon autogenously regulates its own operon. That is, in addition to being a structural component of the ribosome, the protein also inhibits the expression of its own operon when it accumulates in excess of other r-proteins. Since all r-proteins (with the exception of L7/L12) are incorporated into ribosomes in equimolar amounts, such an autogenous regulation could effectively coordinate the expression of the various r-protein operons (for a review, see Lindahl and Zengel, 1982).

The autogenous regulation of the S10 r-protein operon is mediated by protein L4, a component of the 50S ribosomal subunit (Zengel et al., 1980; Yates and Nomura, 1980). We have previously reported that the excessive accumulation of r-protein L4 inhibits transcription of the entire S10 operon, thereby leading to a reduced synthesis of all 11 r-proteins encoded by the operon (Zengel et al., 1980; Lindahl et al., 1982). Other studies have shown that L4 also acts at the level of translation (Yates and Nomura, 1980).

We have investigated the mechanism by which protein L4 inhibits the transcription of the S10 operon. The results suggest that there is a transcription terminator in the leader region of the S10 mRNA. When protein L4 is oversynthesized, S10 mRNA transcription terminates at a site upstream from the ribosome-binding site for the first gene of the operon. This attenuation of the transcription is apparently independent of the translation of the S10 operon.

Results

Protein L4 could regulate the transcription of the S10 operon by two obvious mechanisms: modulation of the frequency of transcription initiation or modulation of premature termination of mRNA elongation (attenuation). These two models generate different predictions for the effect of L4 on the transcription of the leader of the S10 operon. If protein L4 inhibits mRNA synthesis at the level of initiation, transcription of the leader and of the structural genes should be inhibited to the same extent. Alternatively, if the L4-mediated regulation is caused by premature termination upstream from the structural genes, transcription of the structural gene message should be inhibited, whereas the synthesis of the leader transcript should be relatively unaffected.

To determine which of these two models is correct, we examined the transcription of the S10 operon in cells in which we could induce an increased L4 accumulation. These cells carried a plasmid (pLL133) containing an L4 gene which had been removed from its natural promoter and fused to the *lac* operator/promoter. Addition of isopropylthiogalactoside (IPTG) to these cells increases the rate of L4 synthesis approximately 4-fold (Zengel et al., 1980). The transcription rates of the S10 leader and structural genes were measured by hybridizing RNA from cells which had been pulse labeled with ³H-uridine for 0.1, 0.3, or 1.0 min to DNA probes specific for these regions of the S10 operon. These hybridization probes were single-stranded DNA from M13 phages into which we had inserted a 144-base pair (bp) Hinc II-Sst I fragment containing most of the leader sequence or a Bcl I-Eco RI fragment of approximately 550 bp containing parts of the structural genes for S10 and L3 (Figure 1). (Note that there is no overlap of sequences on the *lac*-L4 fusion plasmid pLL133 and the two hybridization probes.) Since we expected only a small fraction of the radioactive RNA to hybridize to such short probes, in most experiments we used a strain which in addition to pLL133 contains a plasmid, pLL36, carrying the promoter, leader, and first one and one-half structural genes of the S10 operon (Figure 1). The pLL36 containing cells synthesize about 5-fold more mRNA from the proximal part of the S10 operon (i.e. the region of the message complementary to the hybridization probes) than do haploid cells (Lindahl et al., 1982). Using these cells, we could obtain reliable hybridization data with ³H-RNA from cells which had been labeled for as short as 0.1 min.

We measured the transcription rates for the S10 operon before and 10 min after induction of L4 oversynthesis. The

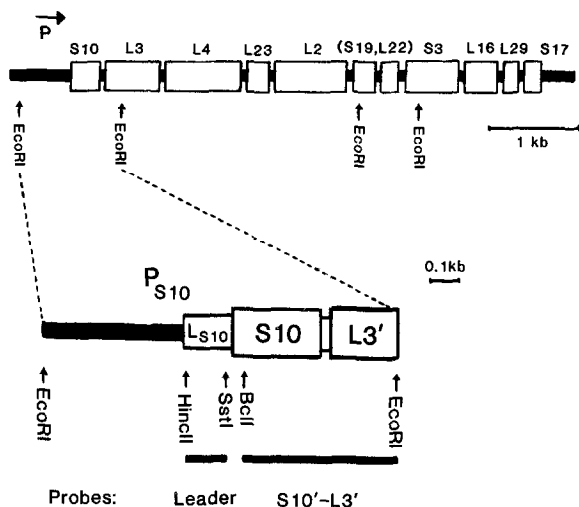


Figure 1. Map of the S10 Operon

The top line shows the entire operon with genes drawn approximately to scale (Lindahl et al., 1977; Zengel et al., 1980). An expanded map of the 1.2-kb EcoRI fragment based on the DNA sequence of Olin and Nomura (1981) has been drawn below. This 1.2-kb EcoRI fragment is carried by pLL36 (see text). The fragment contains the promoter (P_{S10}), the leader (L_{S10}), the intact S10 gene (S10), and a portion of the L3 gene (L3'). Only pertinent restriction enzyme sites are indicated. The two bars below the map of the 1.2-kb fragment show the DNA fragments carried by the M13 hybridization probes used for measuring leader and structural messenger (S10'-L3') transcripts.

results (Figure 2) show that excess protein L4 caused a 5- to 10-fold reduction in mRNA synthesis from the structural genes for S10 and L3. In contrast, little effect on the leader synthesis was observed, particularly for the 0.1- and 0.3-min pulses. As a control, we also measured the synthesis of mRNA from other r-protein operons (the *spc*, α , and L11 operons) by hybridizing the ³H-RNA to single-stranded M13 DNA carrying inserts from these operons. The transcription from the other r-protein operons was not affected by L4. Thus, the effect of L4 on transcription is specific for the structural genes of the S10 operon. We repeated this detailed kinetic analysis of ³H-uridine incorporation into α mRNA and S10 leader and structural gene message (data not shown). We also did four independent analyses of RNA from cells labeled for 0.5 min (the results from one such experiment are shown in Table 1). Although the hybridization efficiencies for these various experiments differed as much as 2-fold, the conclusion from all these experiments was the same: L4 strongly inhibits transcription of the structural genes, but not the leader, of the S10 operon.

It is important to note that the differential effect of L4 on incorporation of ³H-uridine into leader transcript and structural mRNA was observed even when the cells were labeled for only 0.1 min. Since it is unlikely that substantial turnover of radioactive transcripts takes place during such a short pulse, the incorporation data are probably reasonable estimates of synthesis rates. These results therefore indicate that L4 inhibits synthesis of the structural message in the S10 operon by causing premature termination of

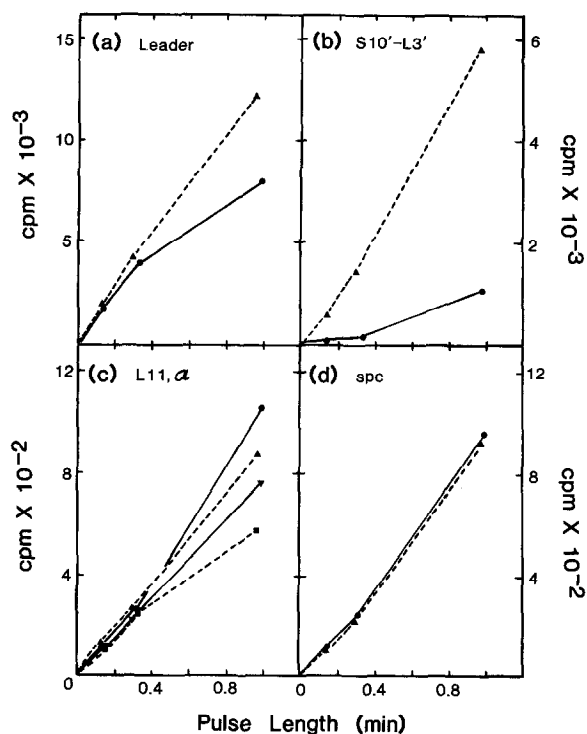


Figure 2. Effect of L4 Overproduction on the Synthesis of Specific r-Protein Messengers

Cells containing pLL36 and pLL133 were grown exponentially in glycerol minimal medium. At a density of about 10^6 cells/ml, a portion of the culture was mixed with ³H-uridine ($t = 0$). At the indicated times after mixing with radioactivity, samples were withdrawn and immediately lysed. Overproduction of L4 was then induced by adding IPTG to the remainder of the (nonradioactive) main culture. Ten minutes later, the labeling protocol was repeated with a sample of the induced cells. RNA was purified from the radioactively labeled samples and aliquots were hybridized to filters containing a large excess of the indicated DNA probes. The amount of radioactive RNA hybridizing to each probe was normalized by dividing by the length (in kilobases) of each probe. The normalized results are shown as a function of the time elapsed between addition of ³H-uridine and cell lysis. The amount of RNA used for each hybridization represents the extract of 0.5 ml culture. The hybridization results have been corrected for variations in recovery of total trichloroacetic acid-precipitable radioactivity during purification. Broken lines connect data for uninduced cells. Solid lines connect data for cells induced to oversynthesize L4. (a) Normalized radioactivity hybridizing to leader probe; (b) normalized radioactivity hybridizing to S10'-L3' structural genes probe; (c) normalized radioactivity hybridizing to α operon probe (●, ▲) and L11 operon probe (▼, ■); (d) normalized radioactivity hybridizing to *spc* operon probe.

transcription. Interestingly, we also observed that, even in the absence of excessive L4 accumulation, the leader sequence was transcribed at a rate two to three times the rate of structural mRNA synthesis (Figure 2). This suggests that some attenuation occurs even in exponentially growing cells.

The S10 messenger measured in the experiments described above was derived predominantly from the partial S10 operon carried on the multicopy plasmid. To confirm that the transcription of the (intact) chromosomal operon is regulated in the same way, we measured the effect of L4 on the synthesis of leader transcript and structural mRNA in a strain carrying only the chromosomal S10

Table 1. Effect of L4 Overproduction on the Transcription of the S10 Operon in Haploid and Polyploid Strains^a

Plasmids	IPTG	Input RNA (cpm × 10 ⁻⁵)	RNA Hybridized (cpm)			Specific Hybridization (cpm)	
			Leader	S10'-L3'	Control	Leader	S10'-L3'
pLL127	-	3.6	232	239	79	153	160
	+	3.8	276	119	69	207	50
pLL36 and	-	3.3	649	893	67	582	826
pLL133	+	3.0	719	144	70	649	74

^a Cells containing pLL127 were grown in glucose minimal medium supplemented with casamino acids; cells containing pLL36 and pLL133 were grown in glycerol minimal medium. Both pLL127 and pLL133 carry an L4 gene fused to the lactose promoter (see Experimental Procedures for details). Samples of the cultures were labeled for 0.5 min with ³H-uridine before and 10 min after induction of L4 oversynthesis. RNA was extracted and hybridized to the indicated DNA probes as described in the legend to Figure 2. The control hybridization probe was single-stranded DNA from an M13 clone carrying the S10'-L3' genes (Bcl I-Eco RI fragment; see Figure 1) inserted in the nonhybridizing orientation (see Experimental Procedures). "Specific hybridization" was calculated by subtracting the amount of radioactivity bound to the control filter. Note that the data in this table have not been normalized to DNA probe length. The relative counts per minute for leader and structural genes therefore differ from the values in Figure 2 in which the data were normalized (see legend to Figure 2).

operon. Cells containing the *lac*-L4 plasmid (but not pLL36) were pulse labeled for 0.5 min before and 10 min after induction of L4 oversynthesis, and the RNA was hybridized to the S10 leader and the S10'-L3' structural gene probes. The results (Table 1) showed that the haploid strain responds to excess L4 in the same way as the pLL36-containing strain. The synthesis of structural message was inhibited strongly by L4, whereas little effect on the synthesis of leader transcript was observed. Thus, the L4-induced attenuation of transcription from the S10 operon is a normal phenomenon.

The hybridization data suggested that cells with an excess of protein L4 terminate transcription of the S10 operon somewhere near the beginning of the first structural gene of the operon. To more precisely map the site of premature termination, we wanted to size the transcripts from the S10 operon by electrophoresis on a denaturing urea-polyacrylamide gel. Since mRNA is rapidly degraded in procaryotes, the standard blotting technique of bulk RNA ("Northern analysis") would not permit us to distinguish between primary transcripts, processed transcripts, and degradation intermediates. Instead, we analyzed RNA which had been pulse labeled with ³H-uridine for 0.5 min and therefore probably represents primary transcripts. To identify transcripts from the S10 operon, the gel under each slot was cut into slices, and RNA from each slice was eluted and hybridized to filters carrying either the leader probe or the S10'-L3' structural gene probe. By including molecular weight markers on the gel, we could estimate the molecular weights of the various RNA species containing leader and/or structural gene sequences.

The gel analysis of mRNA revealed that there is indeed an attenuated transcript. The results obtained with the strain containing pLL36 (Figure 3, a and b) and the haploid strain (Figure 3, c and d) again were very similar, except for the expected differences in the amounts of mRNA. In the RNA from cells oversynthesizing L4, almost all the leader transcripts were found in a single class of molecules with an apparent length of 140 to 155 bases (Figure 3, b and d). (The heterogeneous tail of transcripts smaller than the attenuated leader transcript probably represents chains which had not reached full size at the time the cells were

harvested.) As expected, little structural gene mRNA was found. This indicates that in the presence of excess L4 only an attenuated transcript is formed.

Since the hybridization analysis of RNA from gel samples is time-consuming and laborious, we routinely cut only 20 to 25 fractions/lane. Only one or two fractions contained the attenuated transcript, allowing only a rough approximation of the molecular weight. To increase the accuracy of the molecular weight determination of the attenuated transcript, we repeated the gel electrophoresis of RNA from induced cells and cut out smaller fractions from the region of the gel containing this transcript. The results (see insert in Figure 3b) showed that the peak fraction falls in the range of apparent molecular weight between 140 and 145 bases.

The RNA from the uninduced sample also contained the attenuated leader transcript, but in a lower amount than that found in the induced cells (Figure 3, a and c). In addition, the RNA from the uninduced cells contained longer molecules carrying both leader and structural mRNA sequences. This indicates that the exponentially growing cells synthesize both attenuated and nonattenuated transcripts, in agreement with our conclusion from the kinetic labeling experiment (Figure 2). The nonattenuated transcripts in the uninduced samples (Figure 3, a and c) form a heterogeneous distribution of molecules larger than the attenuated transcript. This heterogeneous distribution probably represents growing chains, since the labeling time (0.5 min) is relatively short compared to the synthesis time of a full length transcript.

Since the presumed Shine-Dalgarno sequence for the S10 gene is 159 to 162 bases from the start of the transcript, the length of the attenuated transcript suggests that the termination site is upstream from the ribosome-binding site for the S10 gene (Figure 4). Alternatively, transcription could terminate downstream from the ribosome binding site if, in spite of the short labeling time, the original 5' end had been removed by processing. We could rule out the possibility that the attenuated transcript contains the intact ribosome-binding site by analyzing the mRNA synthesized from a derivative of pLL36 carrying a 15-bp deletion which includes the Shine-Dalgarno se-

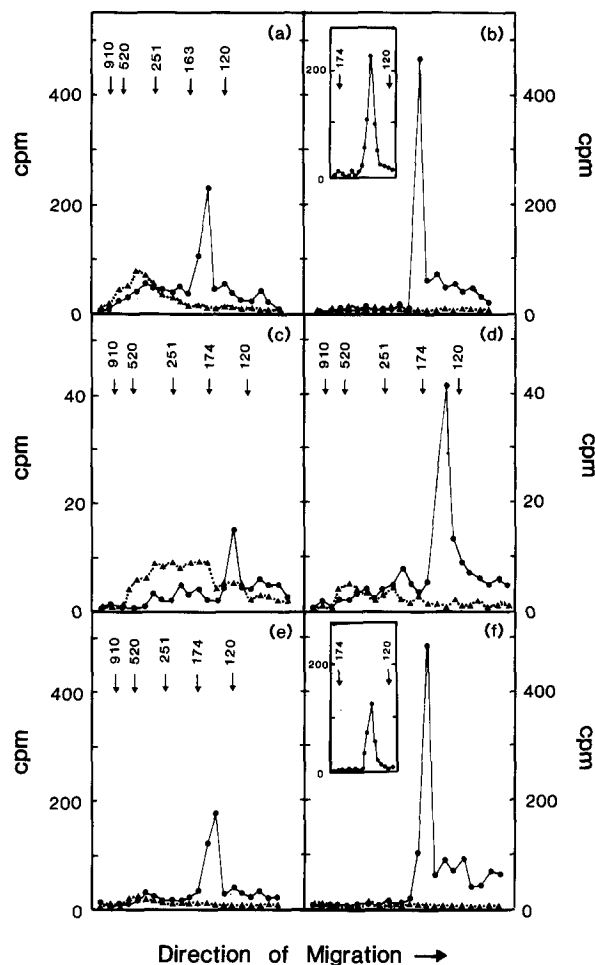


Figure 3. Gel Electrophoresis of Transcripts from the S10 Operon

Cells containing pLL36 and pLL133 (a and b) or pLL36 Δ Sac2 and pLL133 (e and f) were grown exponentially in glycerol minimal medium. Cells containing pLL127 (c and d) were grown in glucose minimal medium with casamino acids. Samples of the cultures were labeled with 3 H-uridine for 0.5 min before (a, c, and e) and 10 min after (b, d, and f) induction of L4 oversynthesis. RNA was extracted from the labeled cells, denatured by heating in formamide and urea, and run on a urea-acrylamide gel. After the electrophoresis, segments from each lane were placed in formamide hybridization buffer together with filters carrying the indicated DNA probes. The graphs show the amount of radioactivity from each gel segment which hybridized to the leader (●) or the S10'-L3' structural gene (▲) probe. All slots were loaded with 5×10^5 cpm of TCA-precipitable material. The inserts in panels (b) and (f) show hybridizable radioactivity from another gel in which the gel in the region containing the attenuated transcript was cut into slices one-fourth the size of the slices from the main gel. The arrows indicate the position of denatured size markers run in parallel tracks. The following markers were used: Alu I-digested pBR322 DNA (only 910, 520, and 251 base fragments are indicated), a 174-base Hind III-Eco RI DNA fragment from the M13 bacteriophage carrying the S10 leader fragment (see Experimental Procedures) or a 163-base Bam HI-Hind III DNA fragment from the same phage, and 5S rRNA of *E. coli* (120 bases).

quence for the S10 gene (Lindahl et al., 1982; Figure 4). If the attenuator were downstream from the ribosome-binding site of the S10 gene, we would expect the attenuated transcript from the deletion plasmid (pLL36 Δ Sac2) to be shorter than the attenuated transcript from the wild-type plasmid. However, when we electrophoresed RNA from

the pLL36 Δ Sac2 and from the wild-type plasmid in adjacent lanes in a gel, we found that the attenuated transcripts from the two plasmids have the same length (Figure 3, a, b, e, and f). If the transcript from the deletion plasmid had been 15 bases shorter, the position of the peaks shown in the inserts of Figure 3, b and f, should have been separated by about four fractions. These results suggest that the site of attenuation is upstream from the deletion, i.e. upstream from the ribosome-binding site for the S10 gene.

In vitro studies have shown that L4 also regulates the translation of the S10 operon (Yates and Nomura, 1980). Since reduced translation in some cases leads to premature termination of transcription (Rosenberg and Court, 1979), it was possible that the L4-mediated attenuation could be a secondary result of the inhibition of translation. However, our mapping of the site of transcription termination suggests that the attenuated transcript does not have the S10 gene ribosome-binding site. This implies that the regulation of attenuation is independent of the regulation of the translation of the S10 gene. We tested this idea directly by analyzing how L4 oversynthesis affects the synthesis of leader and structural mRNA from the deletion plasmid pLL36 Δ Sac2. The expression of the S10 gene is inhibited 10–20-fold by the deletion on pLL36 Δ Sac2 (L. Freedman and L. Lindahl, unpublished experiments). If attenuation were a result of inhibition of translation of the S10 gene, we would expect that mRNA synthesized from pLL36 Δ Sac2 should always be attenuated, irrespective of the rate of L4 accumulation. However, the data presented in Figure 5 show that this prediction is not borne out: transcription from pLL36 Δ Sac2 is regulated by L4 in the same way that transcription from the wild-type S10 operon is regulated. That is, the synthesis of structural mRNA is inhibited by L4, but the synthesis of leader sequences is essentially unaffected. It appears then that attenuation is not affected by decreased translation of the S10 gene. This conclusion is also supported by the gel electrophoresis experiments described above (Figure 3, e and f).

Discussion

The S10 operon codes for 11 r-proteins. The expression of all 11 genes is repressed in vivo by the r-protein L4, the product of the third gene of the S10 operon (Zengel et al., 1980). Our studies have shown that synthesis of mRNA from the S10 operon is inhibited in response to excessive L4 accumulation (Zengel et al., 1980; Lindahl et al., 1982). We have now analyzed the mechanism of the L4-mediated regulation of transcription. Our data suggest that L4 inhibits synthesis of mRNA from the S10 operon by stimulating termination of transcription at a site about 30 bases upstream from the initiation codon of the first gene in the operon. This conclusion is based on two types of evidence. First, specific measurements of the rates of mRNA synthesis showed that L4 (directly or indirectly) inhibits transcription of the structural genes but not of the leader. Second, gel analysis of pulse-labeled RNA showed that the synthe-

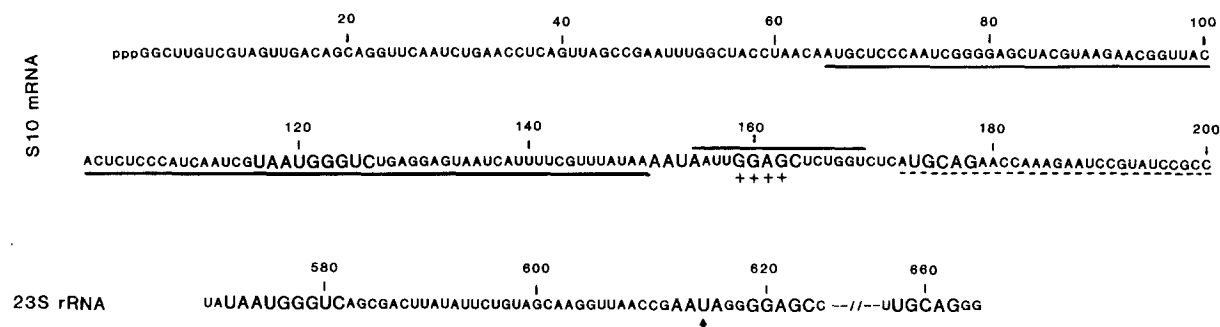


Figure 4. Nucleotide Sequence of the 5' End of the Transcript of the S10 Operon

The two top lines show the nucleotide sequence of the first 200 bases of the transcript of the S10 operon. The start site and sequence of the transcript were determined by Olins and Nomura (1981). Our unpublished experiments with S10'-lacZ' fusions (L. Freedman and L. Lindahl) have shown that there are no promoters for the S10 gene other than the promoter identified by Olins and Nomura (1981). A 32-codon open reading frame in the leader is indicated with solid underlining. The beginning of the S10 gene is shown by dashed underlining. The presumed Shine-Dalgarno sequence preceding the S10 gene is identified by + + + +. The Δ Sac2 deletion is indicated by solid overlining. The bottom line shows a portion of the sequence of 23S rRNA which includes the L4-binding site. The arrow indicates the U residue to which L4 has been cross-linked by irradiation with ultraviolet light (Maly et al., 1980). Large size bases indicate identical sequence segments on the S10 mRNA and 23S rRNA (Olins and Nomura, 1981).

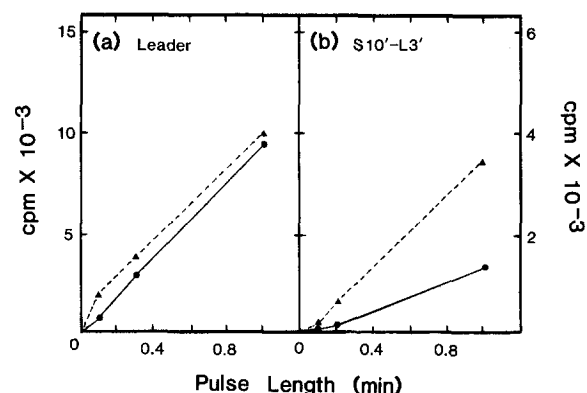


Figure 5. Effect of L4 Overproduction on the Transcription of the S10 Operon in the Absence of Translation

Cells containing the plasmids pLL36 Δ Sac2 and pLL133 were grown in glycerol minimal medium. Samples of the culture were labeled with 3 H-uridine before (broken lines and \blacktriangle) and 10 min after (solid lines and \bullet) induction of L4 overproduction. Radioactive RNA was hybridized to leader (a) or structural gene (b) probes. For details, see the legend of Figure 2.

sis of long transcripts is inhibited in the presence of excess L4. Only an attenuated transcript about 140 bases long and containing just leader sequences is synthesized.

An alternative interpretation of our data is that L4 accelerates the degradation of structural mRNA but not of leader transcript. This could happen by various combinations of endo- and exonucleolytic attacks. We cannot exclude such models but we find them very unlikely because the differential effect of L4 on incorporation of 3 H-uridine into leader and structural gene transcripts is observed even when the labeling period is as short as 0.1 min. Substantial turnover of labeled RNA in such a short labeling period would require that any hybridizable sequence of bases transcribed from the structural genes be degraded within a few seconds of its synthesis. Accelerated degradation of mRNA in response to increased synthesis of regulatory r-proteins has previously been observed (Fallon et al., 1979a, 1979b), but in these cases, the degradation rate was only slightly increased, and synthesis and degradation

were easily measured. We therefore think that ultrarapid degradation of mRNA is an unlikely interpretation of our results.

We found a significant degree of attenuation even before L4 oversynthesis was induced. This may be due to a low concentration of free L4 (i.e. not bound to ribosomal particles) in exponentially growing cells. If so, the attenuation is sensitive to very low concentrations of L4. Consistent with this hypothesis, Delcuve and Dennis (1981) observed an increase in transcription of the S10 operon in a strain synthesizing less L4 because of a nonsense mutation with a polar effect on L4 translation. It will be interesting to measure the attenuation rate in such a mutant to see if there is in fact decreased attenuation in response to a decreased level of L4.

In addition to the *in vivo* transcriptional control of the S10 operon described in this report, L4 has also been shown to inhibit the *in vitro* translation of the proximal four genes (Yates and Nomura, 1980). The L4-mediated regulation of the S10 operon therefore apparently involves modulation of both transcription and translation. We considered the possibility that the reduced transcription is a secondary effect of inhibition of translation (Nomura et al., 1982). However, two pieces of evidence indicate that the regulation of transcription of the S10 operon is independent of the L4-mediated inhibition of translation. First, in a mutant lacking the Shine-Dalgarno sequence in front of the S10 gene, translation of the gene is strongly reduced but transcription continues beyond the attenuator. In this mutant, L4 still stimulates attenuation in the mutated S10 operon. Second, since the site of termination of transcription is upstream from the beginning of the S10 gene, the transcript contains no structural genes and therefore is not translatable. It follows from the latter point that the inhibition of translation operates only on messenger molecules already present at the time of increased L4 concentration. Once these pre-existing messengers have decayed, the attenuation of transcription would pre-empt any further translational regulation.

It is interesting to speculate about why both transcription and translation of the S10 operon are regulated. Inhibition of translation has the advantage of yielding an immediate response, while transcriptional inhibition allows several minutes of residual protein synthesis from pre-existing mRNA. However, transcriptional inhibition may be necessary for regulating long multicistronic operons. It has been proposed that translation of a downstream cistron in an r-protein operon is coupled to the translation of upstream genes; direct inhibition of translation of the proximal gene by binding of the regulatory r-protein to the mRNA would thereby result in indirect inhibition of translation of downstream cistrons (Yates and Nomura, 1981; Yates et al., 1981). One potential difficulty with this model is that, if the proposed "coupling" of the translation of a downstream cistron to translation of the upstream sequence is not absolute, then the amount of breakthrough translation would increase progressively through an operon. In a long operon, such as the 11-gene S10 operon, this could result in inadequate regulation of distal genes. In fact, both *in vitro* and *in vivo* experiments are consistent with this possibility. First, in a cell-free system, L4 inhibits the translation of only the proximal four or five genes (Yates and Nomura, 1980). The distal genes are not regulated *in vitro*. Although the lack of regulation of downstream cistrons could be due to other causes (such as artificial fragmentation of mRNA *in vitro*; Nomura et al., 1982), this observation may reflect that translation of a downstream gene is not absolutely dependent on the translation of upstream genes. [It should be mentioned that in at least one other respect the *in vitro* system does not accurately reflect the *in vivo* situation: for unknown reasons L4 does not inhibit the transcription of the S10 operon (Yates and Nomura, 1980).] Second, Cabezon et al. (1980) showed that *in vivo* a nonsense mutation in the L3 gene affects the translation of the succeeding three genes, but not of the six most distal genes. Thus, the inhibition of translation caused by the nonsense mutation in the L3 gene is not propagated to the end of the operon. If translational regulation is not effective in tightly regulating a long operon, then transcriptional control is essential to ensure that the entire operon is regulated.

Attenuation of transcription in other operons depends on the rapid translation of a leader peptide (e.g. see Yanofsky, 1981) or on the modulation of the activity of transcription termination factors (Lau et al., 1982). The molecular mechanism of attenuation in the S10 operon is still obscure. There is an open reading frame in the S10 leader with capacity to code for a 32-amino acid peptide (Figure 4). However, there is no obvious "Shine-Dalgarno" sequence preceding this open reading frame, and therefore it may not be translated. It is perhaps more interesting that the sequence between 95 and 142 bases from the start of the transcript can be written as a stem-loop structure followed by four U-residues (Figure 6). Similar structures are believed to function as terminators for transcription (e.g. see Platt, 1981). Termination at the UUUU

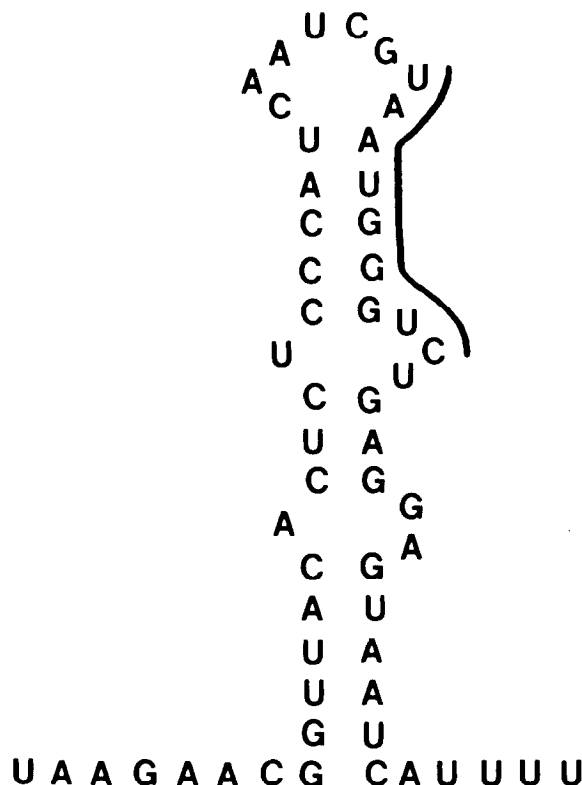


Figure 6. Possible Terminator in the S10 Leader

The figure illustrates a possible secondary structure of the S10 leader between 95 and 142 bases from the start of the transcript. The line indicates a nine-base sequence which is also found in the 23S rRNA close to the L4-binding site (see Figure 4).

sequence would be consistent with the length of the attenuated transcript which we have found. Perhaps the stem-loop structure shown in Figure 6 is intrinsically unstable but is stabilized by binding of L4, thereby enhancing RNA chain termination. In this regard, it is interesting to note that the stem-loop structure contains a nine-base sequence which is identical to a sequence found in the 23S rRNA only 33 bases from a uridine residue to which L4 can be cross-linked (Maly et al., 1980). If the nine-base sequence constitutes part of the binding site for L4 on the 23S rRNA, then it is conceivable that L4 also binds to the same sequence in the S10 leader (Olins and Nomura, 1981).

The L4-binding model assumes that L4 plays a direct role in regulating transcription of the S10 operon. A direct interaction between regulatory r-proteins and their respective messengers has also been implied by models explaining the translational regulation of r-protein operons, based on the similarities between critical portions of r-protein messengers and rRNA (Nomura et al., 1980; Olins and Nomura, 1981). However, only in the case of the L10 protein and β operon mRNA has such an interaction been established experimentally (Johnsen et al., 1982). It remains to be seen whether one can demonstrate that L4 binds to a specific site(s) on the S10 operon mRNA (or

DNA). It would also be interesting to know if proteins other than L4 are involved in the attenuation process. Obvious candidates include other r-proteins, the transcription factors Rho and NusA, and even RNaseIII, since the structure shown in Figure 6 also is reminiscent of some substrate sites for this enzyme (e.g. see Gegenheimer and Apirion, 1981). Finally, it remains to be established whether the attenuation described here for the S10 operon is a general model for all r-protein operons.

Experimental Procedures

Bacterial Strain

All experiments were performed with *E. coli* K12 strain LL308 (Lindahl and Zengel, 1979) carrying the indicated plasmids. Cultures were grown at 37°C in AB minimal medium (Clark and Maaløe, 1967) supplemented with 2.5 µg thiamine/ml, and either 0.4% glycerol or 0.2% glucose and 0.1% casamino acids.

Plasmids

Plasmids pLL36 (Zengel et al., 1980) and pLL36ΔSac2 (Lindahl et al., 1982) are derivatives of pSC101. Plasmids pLL133 (Zengel et al., 1980) and pLL127 (Lindahl and Zengel, 1979) are ColE1 derivatives.

Hybridization Probes

The hybridization probes were all obtained by cloning DNA fragments into M13 mp8 or mp9 (Messing and Vieira, 1982). Since these two vectors have linkers containing a series of restriction enzyme sites arranged in opposite sequences, we could obtain pairs of clones carrying the same DNA fragment inserted in opposite orientations. Thus, single-stranded DNA from one clone of such a pair will hybridize mRNA, but the other will not. Note that the *lac* sequences on single-stranded DNA from the two M13 vectors do not hybridize *lac* mRNA.

The probe for measuring structural mRNA from the S10 and L3 genes was obtained by inserting a 0.55-kilobase (kb) Bcl I-Eco RI fragment from pLL36 between the Eco RI and Bam HI sites of M13 mp8 (Figure 1). This probe contains the DNA sequence corresponding to positions 212 through approximately 760 of the S10 operon transcript (Figure 4). (The sequences of the ends of the Eco RI fragment in Figure 1 have not been determined; see Olins and Nomura, 1981). A control probe carrying the same fragment but in the opposite (nonhybridizing) orientation was constructed by using M13 mp9.

To obtain a hybridization probe for measuring transcription from the S10 leader, we first digested pLL36 DNA with Sst I (Figure 1) and removed the single-stranded tails with mung bean nuclease. The resulting linear, blunt-ended molecule was then digested with Hinc II and ligated to M13 mp9 which had been opened at the Hinc II site. DNA from 64 recombinant phages was purified and digested with several restriction enzymes to identify a recombinant carrying the Hinc II-Sst I fragment from the S10 leader. However, single-stranded DNA from this clone did not hybridize to mRNA, indicating that the fragment was inserted in the nonhybridizing orientation. To invert the insert, we digested this DNA with Hind III and Eco RI, which cleave in the linker of M13 mp9 on opposite sides of the insert. The resulting Eco RI-Hind III fragment was then inserted into M13 mp8 which had been digested with the same enzymes. Single-stranded DNA from the clone obtained in this way hybridized to mRNA and was used for measurements of leader transcripts. We confirmed the structure of the leader hybridization probe by DNA sequencing. It contains the DNA sequence corresponding to positions 17 through 160 of the transcript of the S10 operon (Figure 4). Apparently, two base pairs at the Hinc II end of the insert were lost during the cloning procedure.

The probe for measurements of messenger from the α operon was constructed by inserting a 1.3-kb Pst I-Hind III fragment carrying part of the S13 gene as well as the intact S11 and S4 genes (Post et al., 1980) into M13 mp9. This fragment was obtained from a pSC101-derived plasmid carrying the proximal part of the α operon (J. M. Zengel, unpublished experiments). The probe for *spc* messenger was constructed by inserting a 2.2-kb Sal I-Eco RI fragment from the transducing phage λ fus3 (Lindahl

et al., 1977) into M13 mp8. The probe for measuring mRNA from the L11 operon was a gift from Dr. S. Pedersen (University of Copenhagen) and carries a 617-bp Eco RI-Bgl II fragment (Post et al., 1979) inserted into M13 mp9.

Hybridization

The hybridization experiments were performed essentially as described earlier (Zengel et al., 1980) except that the filters were loaded with single-stranded DNA from the M13 recombinant genomes instead of denatured double-stranded DNA. The results were corrected for unspecific binding of 3 H-RNA to the filters. In most experiments, these corrections correspond to less than 20% of the hybridization to the S10 leader, S10'-L3', *spc* and α probes, and 30-40% of the hybridization to the L11 probe.

Gel Electrophoresis and Hybridization of RNA Eluted from Gels

RNA was labeled, extracted, and purified according to the protocol used for hybridization experiments (Zengel et al., 1980). The RNA was then dialyzed against Tris-HCl (10 mM, pH 7.4)/EDTA (0.1 mM), precipitated with ethanol, and redissolved in a few microliters of Tris/EDTA. Before loading the gel, the RNA solution was mixed with an equal volume of loading buffer (95% formamide, 10 mM EDTA, pH 7.0, 1 mg/ml bromophenol blue, and 1 mg/ml xylene cyanol), heated to 100°C for 1 min, and cooled in ice. The samples were then immediately loaded on an 8% acrylamide gel (acrylamide/bisacrylamide, 20:1) containing 8 M urea (Maxam and Gilbert, 1980). The gel was 30 cm long and 0.75 mm thick. Nonradioactive molecular weight markers were loaded in parallel tracks. Electrophoresis was carried out at 300-600 V and 60°C until the xylene cyanol had migrated to about 20 cm from the origin. After the run, the tracks with markers were cut off and stained. The tracks with 3 H-RNA were cut into segments 1 cm long (0.25 cm long for the inserts in Figure 3). The gel pieces were added to 0.5 ml hybridization buffer (Zengel et al., 1980) together with filters carrying single-stranded hybridization probes. The mixture was incubated at 41°C for 3 days while shaking at 50 rpm. Since the hybridization buffer contains 50% formamide, both RNA elution and hybridization take place during the incubation (Achord and Kennell, 1974). After the incubation, the filters were treated with RNase and washed according to the standard hybridization protocol.

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