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Role of attenuation in growth rate-dependent regulation of the S10 r-protein operon of *E. coli*

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We have investigated the transcription of the 11 gene S10 ribosomal protein operon of *Escherichia coli* under various growth conditions. The differential synthesis rate of structural gene message increases 2- to 2.5-fold immediately after a shift-up from glycerol minimal medium to glucose plus amino acids. After the initial increase, the transcription rate goes through several oscillations before reaching the new steady-state rate. By comparing the rates of transcription of leader and structural genes, we conclude that these oscillations are due predominantly to changes in the level of read-through at the S10 attenuator. This regulation of attenuation can account for most of the variations in protein synthesis from the S10 operon after a shift. We also measured the level of read-through in cells growing exponentially in different growth media. Over a 2.5-fold range in growth rates, the read-through changed $<50\%$. Thus, regulation of attenuation cannot explain the growth-dependent regulation of ribosomal protein synthesis during steady-state growth. Apparently, additional mechanisms are required to control the expression of the S10 operon in exponentially growing cells.

Key words: attenuation/*E. coli*/ribosomes/shift-up/transcription

Introduction

Ribosome synthesis in bacteria is regulated. Physiological experiments, pioneered by Maaloe and his co-workers more than 25 years ago, have shown that the hallmark of this regulation is a correlation between the growth rate and the rate of ribosome synthesis. In exponentially growing cells, the differential rate of ribosome synthesis increases with the growth rate; i.e., fast growing cells invest a larger fraction of their resources in ribosome synthesis than do slow growing cells. Also, after a shift from a relatively poor growth medium to a richer medium (shift-up), the differential rates of rRNA and ribosomal protein (r-protein) synthesis increase almost instantly to the values typical of steady-state growth in the post-shift medium (for reviews, see Gausing, 1980; Kjeldgaard and Gausing, 1974; Maaloe and Kjeldgaard, 1966).

More recently, research on ribosome biosynthesis has focused on the molecular mechanisms underlying its regulation. Experiments in several laboratories have revealed that r-protein synthesis is regulated autogenously. That is, each r-protein operon is repressed by a specific protein encoded by that operon (for a review, see Lindahl and Zengel, 1982). In the 11 gene S10 r-protein operon, autogenous regulation is mediated by L4, the product of the third gene of the operon (Zengel *et al.*, 1980; Yates and Nomura, 1980). We have shown that excessive accumulation of this protein results in premature termination of transcription (attenuation) at a site ~30 bases upstream from the initiation codon of the first gene of the S10 operon (Figure 1; Lindahl *et al.*, 1983). As a result, in the presence of excess L4 the transcription of the 11 r-protein genes in this operon is decreased 5- to 10-fold.

Even in the absence of excessive L4 accumulation, i.e., during exponential growth, some transcripts of the S10 operon are prematurely terminated. In glycerol minimal medium, only one of every two or three transcripts initiated at the S10 promoter continues through the attenuator (Lindahl *et al.*, 1983). Thus, increased read-through at the attenuator could conceivably be responsible for the increased differential rate of r-protein synthesis from the S10 operon in faster growing cells. To test this possibility, we measured the degree of attenuation under various growth conditions. Our results show that the degree of read-through at the S10 attenuator changes dramatically immediately after a shift-up, indicating that attenuation control plays an important role in the growth rate-dependent regulation during the transition period following a change in the growth conditions. However, during steady-state growth the degree of attenuation does not vary enough to account for the growth rate-dependent regulation of r-protein synthesis. Under these conditions, additional mechanisms apparently are required to coordinate the synthesis of r-proteins with the cell growth rate.

Results

Measurements of read-through at the S10 attenuator

The level of read-through at the S10 attenuator is calculated from the relative rates of transcription of the leader and of the proximal structural genes. These rates can be measured by hybridizing [^{3}H]uridine pulse-labeled RNA to single-stranded DNA from M13 phages carrying a fragment from the S10 leader or from the proximal (S10′-L3′) structural genes (Figure 1; Lindahl *et al.*, 1983). For some of the experiments reported here we used a strain containing the plasmid pLL36 carrying the promoter and the proximal one and a half genes of the S10 operon (Figure 1). Cells with plasmid pLL36 overproduce by ~5-fold transcripts from the proximal part of the S10 operon. This increased rate of transcription allows us to use short pulse times (30 s) and still get enough radioactivity incorporated into S10 message to yield reproducible data with short hybridization probes. The degree of attenuation is not affected by the presence of this plasmid (Lindahl *et al.*, 1983; see also below), which lacks the gene for the regulator (L4) of attenuation. It should also be noted that, since the read-through is determined from synthesis rates of transcripts from the same template (the plasmid-borne part of the S10 operon), the read-through measurements (but not the differential synthesis rate measurements) are independent of variations in plasmid copy number.

Shift-up

We first analyzed attenuation in pLL36-containing cells

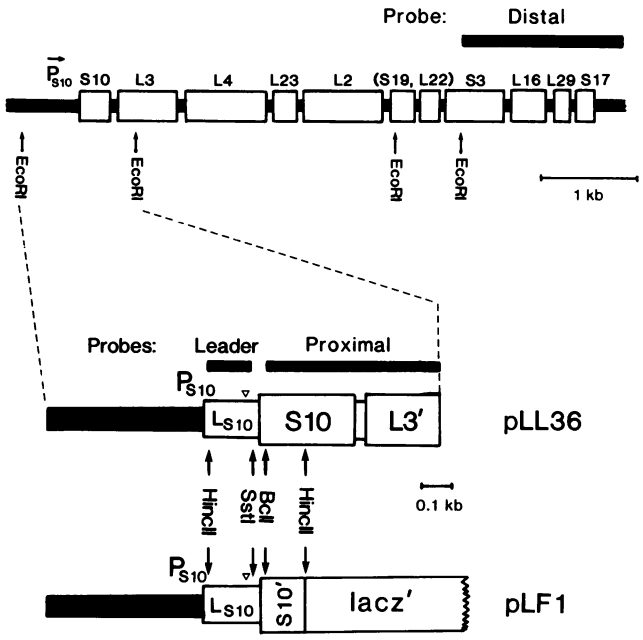

Fig. 1. Map of the S10 operon. The top line shows the entire S10 operon (Zengel *et al.*, 1980). The 1.2-kb *Eco*RI fragment from the proximal end of the operon was cloned into pSC101 to construct pLL36 (Zengel *et al.*, 1980). An expanded map of this fragment is shown below. The fragment contains the promoter (P_{S10}), the leader (L_{S10}), the intact S10 gene (S10) and a portion of the L3 gene (L3'). The approximate position of the attenuator is shown with an open arrow head. Pertinent restriction enzyme sites are indicated with closed arrows. The two bars above the map of the 1.2-kb fragment show the DNA fragments carried by M13 hybridization probes used for measuring leader and proximal structural gene (S10'-L3') transcripts. The bar above the map of the entire S10 operon shows the DNA fragment carried by the M13 hybridization probe used to measure transcripts from the distal structural genes (S3'-L16-L29-S17). The plasmid pLF1 (bottom line) is a derivative of pLL36 which carries the S10 promoter and leader sequences followed by an S10'/*lacz'* gene fusion.

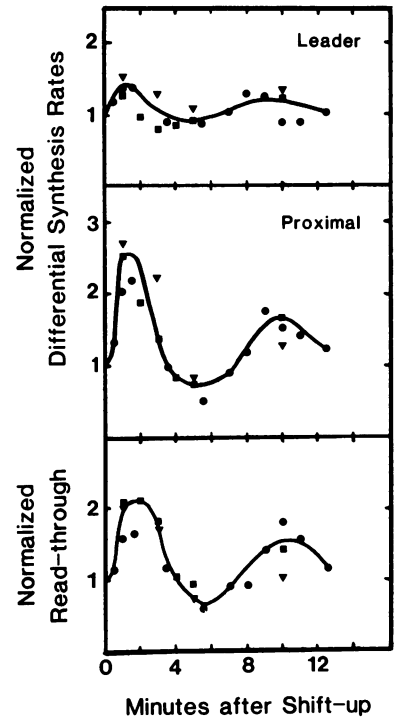

Fig. 2. Transcription of the S10 operon after a nutritional shift-up in a strain with a multicopy plasmid carrying the proximal part of the operon. The shift-up was induced by adding glucose to 0.2% and 19 amino acids (minus methionine) to 20 μg/ml each to a culture of strain LL308/pLL36 growing exponentially in glycerol minimal medium at 37°C. At different times after the shift, samples of the culture were labeled with [^{3}H]uridine for 30 s and the amounts of radioactivity incorporated into transcripts from the leader and from the S10 and L3 genes were determined by hybridization. The differential rates of transcription of the leader and of the proximal structural genes were calculated by dividing the radioactivity hybridizing to a specific probe by the total amount of acid-precipitable radioactivity added to the hybridization assay. These values were then normalized to the respective synthesis rates measured for the pre-shift glycerol cells. The top panel shows the results using the leader probe. The middle panel shows the results using the probe for the proximal (S10'-L3') structural genes. The read-through at the S10 attenuator after the shift-up was calculated by dividing the radioactivity in proximal structural gene message by the radioactivity in leader transcript. The resulting quotients were then normalized to the pre-shift value. The results are shown in the bottom panel. The different symbols indicate results obtained in three independent experiments.

responding to a nutritional shift-up. The shift was induced by adding glucose and either 19 amino acids (minus methionine) or casamino acids to a glycerol minimal culture. The rates of transcription of leader and structural genes were measured at different times after the shift and normalized to the pre-shift rates. Since the culture samples were collected within 10 min of the shift, we did not expect any significant change in the plasmid copy number and the relative differential rates of synthesis of individual transcripts should therefore be directly comparable.

The differential synthesis rate of structural gene message showed an immediate and dramatic response to the shift, going through two strong oscillations within the first 15 min (Figure 2, middle panel). The first peak showed a 2.5-fold increase in the structural gene mRNA synthesis within 1 – 1.5 min of the shift. Since the differential rate of leader transcription showed much less change (Figure 2, top panel), the variations in the structural gene transcription are predominantly due to regulation of the read-through at the attenuator (Figure 2, bottom panel). Given that only one of every two or three transcripts continues through the S10 attenuator in the pre-shift glycerol culture (Lindahl *et al.*, 1983), the 2-fold increase in the read-through measured at the peaks of the structural gene mRNA synthesis after the shift-up implies that essentially all transcripts are elongated past the attenuator at these times.

To ensure that the effect on read-through observed with the partial S10 operon carried on plasmid pLL36 is also characteristic of the intact chromosomal S10 operon, we repeated the shift-up experiment using a haploid strain. In this case, the cells were labeled with [^{3}H]uridine for 45 s. Again, we found that the cells responded to the shift-up with an immediate adjustment in the level of attenuation (Figure 3). Furthermore, by using a probe specific to the distal structural genes (Figure 1), we could show that the oscillation in the transcription rate of the proximal genes is propagated down the entire S10 operon (Figure 3).

The post-shift variations in structural gene mRNA synthesis are strikingly similar to the oscillations previously reported for the differential rate of total r-protein synthesis after a shift-up (Gausing, 1980). This correlation suggests that the regulation of attenuation is important for the control of r-protein synthesis from the S10 operon during the transition phase after the shift. To confirm this point we measured the rate of synthesis of both mRNA and S10 protein in the same

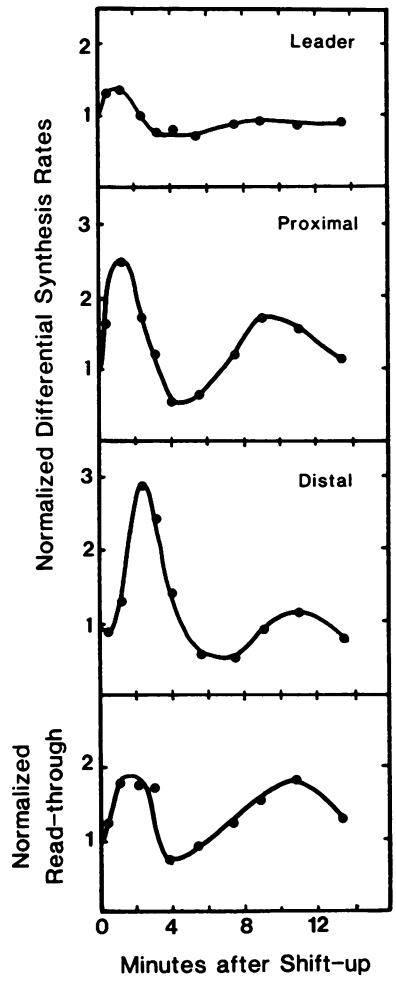

Fig. 3. Transcription of the S10 operon after a nutritional shift-up of a haploid strain. The experiment was performed as described in the legend to Figure 2, except that a haploid strain (LL308) was used, and the [^{3}H]-uridine labeling was extended to 45 s. Incorporation of radioactivity into transcripts from the distal genes of the operon was followed in addition to transcripts from the leader and proximal genes. The read-through was calculated as described in the legend to Figure 2.

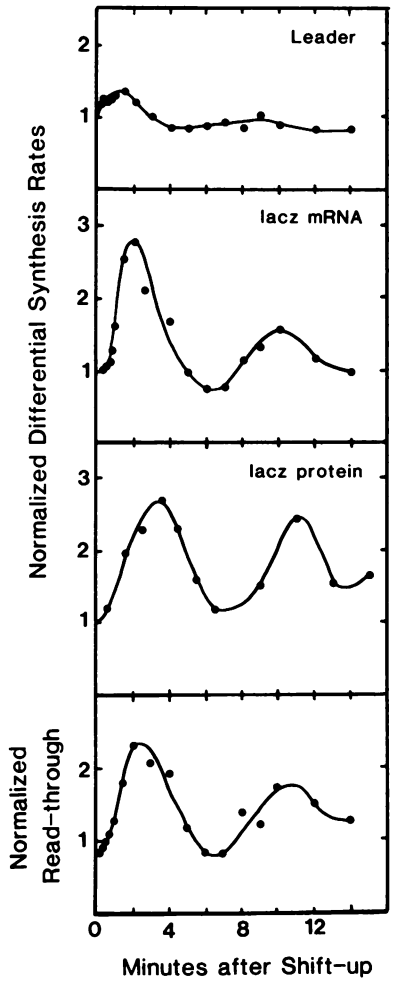

Fig. 4. Messenger and protein synthesis from the S10 operon after a nutritional shift-up. The strain used for these experiments was LL308/pLF1 (Figure 1). The normalized differential synthesis rates of leader and structural gene RNA were determined as described in the legend to Figure 2, except that λp*lac* (Ippen *et al.*, 1971) was used as a probe for the structural gene message. The read-through was calculated by dividing the radioactivity in *lac* messenger by the radioactivity in leader transcript, and then normalizing to the pre-shift value. The differential rate of synthesis of the S10′/*lacz*′ fusion protein was measured by labeling with [^{35}S]methionine for 30 s followed by a 6 min chase with non-radioactive methionine. The total extract from the labeled cells was then analyzed by gel electrophoresis. The radioactivity in the fusion protein band in each lane was determined (Lindahl and Zengel, 1979) and normalized to the total amount of acid-precipitable radioactivity loaded into the respective well.

experiment. To bypass the need for two-dimensional gel electrophoresis, we used a plasmid (pLF1) containing the S10 promoter and leader followed by a fusion of the S10 and *lacz* genes (Figure 1). Because the fusion protein synthesized from this plasmid is very large (~110 000 daltons) we could determine the differential synthesis rate of this protein by one-dimensional gel electrophoresis of a total extract of cells pulse-labeled with [^{35}S]methionine. It should be pointed out that the expression of the S10′/*lacz*′ fusion gene is regulated in response to excessive L4 accumulation in the same way as the wild-type S10 protein (L.P. Freedman, J.M. Zengel and L. Lindahl, unpublished data).

The synthesis of *lacz* mRNA in the pLF1-containing cells folowed the same kinetics after the shift-up as did the synthesis of proximal and distal structural gene mRNA in the previous experiments. That is, the synthesis of *lacz* message went through two oscillations within the first 15 min after the shift-up. Furthermore, the peaks in the rate of *lac* mRNA synthesis were followed within ~1 min by similar peaks in the rate of *lac* fusion protein synthesis (Figure 4). It is important to note that the kinetics of the fusion protein synthesis immediately after the shift are virtually identical to the kinetics of the synthesis of genuine S10 reported by Dennis (1974a) after a similar shift-up. The close correlation between mRNA synthesis and protein synthesis strongly suggests that the modulation of read-through at the attenuator plays a crucial role in regulating the synthesis of r-proteins from the S10 operon after a shift-up.

Steady-state growth

We also analyzed cells growing exponentially at various rates. Experiments by Dennis (1974b) have shown that the differential rate of protein S10 synthesis increases ~2-fold when the steady-state growth rate increases from 0.65 to 1.9 doublings/h. If modulation of attenuation were responsible for this effect we would expect to find a similar increase in the read-through over this range of growth rates. However, as shown in Figure 5, we found only a slight change in the attenuation. Even at high growth rates (1.5–1.8 doublings/h)

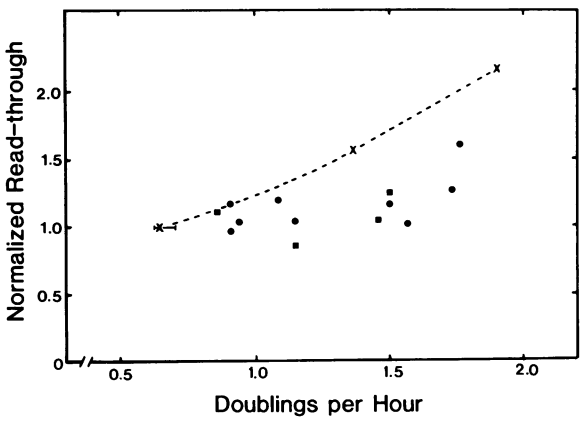

Fig. 5. Read-through at the S10 attenuator during steady state growth in different media. Strain LL308 containing plasmid pLL36 (circles) or pLF1 (triangles) was grown exponentially at 37°C in minimal medium supplemented with glycerol, glycerol and 19 amino acids (minus methionine), glucose, glucose and 19 amino acids, or glucose and casamino acids (0.2% or 1%). The read-through at the S10 attenuator was calculated from the relative transcription rates of the leader and of the structural genes (S10 and L3 in the case of pLL36; *lacz'* in the case of pLF1; Figure 1). To obtain these rates we labeled the cultures for 30 s with [^{3}H]uridine and determined the amount of radioactivity incorporated into the pertinent transcripts by hybridization. Each experiment involved a glycerol minimal culture and one or more cultures growing in other media. The read-through values determined for each culture were normalized to the value obtained for the glycerol culture in the same experiment. Thus the relative read-through in glycerol minimal medium is defined as 1. Most of the points represent the average of two or three labelings of a given culture. The dashed line illustrates the differential rate of r-protein synthesis (α_r) at different growth rates; the three Xs on the dashed line represent actual measurements of the differential rate of synthesis of protein S10 at the indicated growth rates (Dennis, 1974b).

we observed at most a 40–50% increase in the read-through relative to the more slowly growing cells. Again, we found similar results using both haploid cells (data not shown) and cells carrying pLL36 or pLF1. The discrepancy between the growth rate dependence of attenuation and of protein S10 synthesis suggests, therefore, that regulation at the S10 attenuator is not sufficient to account for all of the growth rate-dependent regulation of the S10 operon in exponentially growing cells.

Discussion

We have examined the contribution of the regulation of attenuation of transcription in the S10 operon to the overall growth rate-dependent regulation of protein synthesis from this operon. To approach this problem, we have determined how the read-through at the S10 attenuator varies with the growth conditions.

Shift-up

During a nutritional shift-up we found that attenuation, and consequently the synthesis of mRNA from the r-protein structural genes, changes in a manner which can explain most of the variation in the r-protein synthesis from the S10 operon. Regulation of the read-through at the S10 attenuator thus appears to make a very significant contribution to the control of protein synthesis from this operon immediately after a nutritional shift-up.

The level of read-through at the S10 attenuator increases 2-fold immediately after a shift-up (bottom panels of Figures 2, 3 and 4). We have previously shown that the level of read-through is decreased 5- to 10-fold in response to excessive accumulation of the regulatory r-protein 14 (Lindahl *et al.*, 1983). Taken together, these results indicate that the bacterial cell can achieve a 10- to 20-fold variation in the rate of transcription of the S10 operon, simply by modulating the level of read-through at the attenuator. Therefore, attenuation affords the cell a powerful mechanism for regulating the synthesis of r-proteins from the S10 operon. In this connection, we should note that preliminary experiments indicate that transcription from other r-protein operons is not affected as dramatically after a nutritional shift-up (J.M. Zengel and L. Lindahl, unpublished data). Thus, attenuation control is clearly not the only mechanism involved in growth rate-dependent control of r-protein synthesis (see below for further discussion).

In agreement with Gausing's (1980) protein synthesis measurements, we found that attenuation and r-protein synthesis oscillate for some time after a shift-up before the new steady-state level characteristic of the post-shift medium is achieved. This observation raises two questions: (i) how is the change in growth medium converted into a signal for decreased attenuation (and therefore increased r-protein synthesis) from the S10 operon? (ii) why does the r-protein synthesis go through oscillations instead of simply changing immediately to the rate dictated by the new medium?

Since the concentration of 'free' L4, that is, L4 not incorporated into ribosomal particles, determines the level of read-through at the S10 attenuator (Lindahl *et al.*, 1983), the variations in attenuation in response to the growth medium shift probably reflect changes in the concentration of free L4. Because L4 is a rRNA binding protein, the pool size of free L4 may depend in turn on the amount of rRNA 'targets' available for binding of L4. If so, then changes in the rate of rRNA synthesis after a shift-up would elicit changes in the rate with which newly synthesized L4 is 'consumed' from the cytoplasm, thereby affecting the level of L4 mediated attenuation. What determines the rate of rRNA synthesis immediately after a change in the growth medium is not clear. The increase in rRNA synthesis induced by the shift might be related to the finding of Friesen *et al.* (1975) that the concentration of ppGpp decreases below the level of detection immediately after a shift-up of either *relA*$^+$ or *relA*$^-$ strains. Ribosomal RNA synthesis may also be affected by the concentration of ribosomes not bound to mRNA. Jinks-Robertson *et al.* (1983) have presented evidence suggesting that free ribosomes may inhibit the synthesis of rRNA. Thus, if the shift results in an increase in the rate of total mRNA synthesis, more ribosomes may get engaged in protein synthesis, thereby stimulating rRNA synthesis.

The reason for the oscillations observed after a shift-up is also not clear. As pointed out previously by Lindahl and Zengel (1982) and by Maaloe (see Ingraham *et al.*, 1983), they may be related to the fact that it takes several minutes to assemble a ribosome (Lindahl, 1975). As discussed above, the first result of the shift-up is a rapid increase in the synthesis of ribosomal components. However, since it takes 2–3 min before the increased rate of synthesis of ribosomal components results in an increased rate of accummulation of functional ribosomes, the cells may 'over-shoot' the synthesis of ribosomal components after the shift. The result would be a surplus of ribosomes relative to the rate of synthesis of resources for protein synthesis (amino acids, triphosphates, etc.). Thus the cells may momentarily find themselves in a

down-shift situation, resulting in a decrease in the rate of synthesis of ribosomal components. Several corrections in the rate of synthesis of ribosomal components may be necessary to achieve the new rate characteristic of the post-shift growth medium.

Steady-state

The difference in the level of read-through between cells in balanced growth at rates ranging from 0.67 to 1.75 doublings per hour was only 40–50% (Figure 5). Since Dennis (1974b) has shown previously that the differential rate of S10 synthesis increases ~2-fold over this range of growth rates, we conclude that regulation of attenuation cannot account for the steady-state growth rate-dependent increase in r-protein synthesis from the S10 operon. This conclusion implies that other regulatory processes must assume a role in the regulation of this operon during balanced growth. One process that could contribute to the steady-state control of the S10 operon is the regulation of transcription initiation at the S10 promoter. Regulation of the initiation frequency might be accomplished by a passive control mechanism like the one proposed by Maaloe (see Ingraham *et al.*, 1983). According to this model, the regulation of r-protein synthesis can be explained as a competition for transcriptional activity between promoters for r-proteins and for non-ribosomal proteins. The principle of this model is that the relatively strong r-protein promoters can take better advantage of the increasing transcription activity at higher growth rates than can the relatively weaker (on an average) non-ribosomal promoters.

Other reports suggest that post-transcriptional regulation may also play a role in the growth rate regulation of r-protein synthesis. Experiments by Gausing (1977) and Miura *et al.* (1981) indicate that the rate of r-protein synthesis at different growth rates is not strictly proportional to the rate of r-protein mRNA synthesis, i.e., the number of protein molecules synthesized per messenger molecule apparently varies with the growth rate. Nomura and his co-workers have suggested that this post-transcriptional regulation is accomplished by the autogenous control mediated by operon-specific r-proteins (Miura *et al.*, 1981; Nomura *et al.*, 1982). In fact, in addition to being a regulator of attenuation of the transcription of the S10 operon, L4 also inhibits the translation of at least the proximal four genes of the operon (Yates and Nomura, 1980). Our experiments do not directly test whether L4-mediated translation control contributes to the steady-state growth rate control of the S10 operon.

Growth rate regulation depends on an interaction between several mechanisms

Our data have revealed an interesting difference between the steady-state growth where attenuation control apparently makes a relatively small contribution to the growth rate regulation of the S10 operon (except perhaps at the highest growth rates tested), and the shift-up where changes in attenuation can account for most of the regulation of the expression of the operon. This complex regulatory pattern may indicate that overall growth rate-dependent regulation results from an interaction between several different molecular mechanisms, including r-protein-mediated autogenous control. We believe that the relative contribution of each mechanism varies with the specific growth conditions. Hopefully, future experiments will clarify the contributions made by each mechanism under various physiological conditions.

Materials and methods

Strains, plasmids and media

All experiments were performed with *E. coli* K12 strain LL308 (Zengel *et al.*, 1980) or the same strain carrying a multicopy plasmid. We used two plasmids derived from pSC101: pLL36 (Zengel *et al.*, 1980) and pLF1 (see Figure 1). Cells were grown at 37°C in the AB minimal medium of Clark and Maaloe (1967) supplemented as indicated in the figure legends.

RNA labeling and hybridization

Cells were labeled with 75–150 μCi/ml of [^{3}H]uridine (40–50 Ci/mmol; New England Nuclear or ICN) for 30 s (strains carrying pLL36 or pLF1), or for 45 s (strains which are haploid for the S10 operon). Extraction of RNA from the labeled cells and hybridization to immobilized DNA on nitrocellulose filters were performed as previously described (Zengel *et al.*, 1980; Lindahl *et al.*, 1983). The hybridization probes were denatured λ*plac* (Ippen *et al.*, 1971) or single-stranded DNA from M13 phages carrying inserts from the S10 leader, the S10 and L3 structural genes, or the S3, L16, L29 and S17 genes (Figure 1). Construction of the first two M13 recombinant molecules has been described prevously (Lindahl *et al.*, 1983). The latter hybrid molecule was obtained by transferring an *Eco*RI-*Hin*cII fragment from pNO2003 (Post *et al.*, 1978) to M13 mp9.

Labeling of protein and gel electrophoresis

Cells were labeled with 25 μCi/ml [^{35}S]methionine (700–1000 Ci/mmol; Amersham) for 0.5 min and chased with nonradioactive methionine for 6 min. The labeled cells were lysed in SDS sample buffer (Laemmli, 1970) at 95°C. The total extract was then fractionated by electrophoresis through a 7.5% polyacrylamide-SDS slab gel (Laemmli, 1970). Radioactivity was extracted from pertinent bands and measured as described earlier (Lindahl and Zengel, 1979).

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