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Feedback Regulation of the *spc* Operon in *Escherichia coli*: Translational Coupling and mRNA Processing

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The *spc* operon of *Escherichia coli* encodes 10 ribosomal proteins in the order L14, L24, L5, S14, S8, L6, L18, S5, L30, and L15. This operon is feedback regulated by S8, which binds near the translation start site of L5 and inhibits translation of L5 directly and that of the distal genes indirectly. We constructed plasmids carrying a major portion of the *spc* operon genes under *lac* transcriptional control. The plasmids carried a point mutation in the S8 target site which abolished regulation and resulted in overproduction of plasmid-encoded ribosomal proteins upon induction. We showed that alteration of the AUG start codon of L5 to UAG decreased the synthesis rates of plasmid-encoded distal proteins, as well as L5, by approximately 20-fold, with a much smaller (if any) effect on mRNA synthesis rates, indicating coupling of the distal cistrons' translation with the translation of L5. This conclusion was also supported by experiments in which S8 was overproduced in *trans*. In this case, there was a threefold reduction in the synthesis rates of chromosome-encoded L5 and the distal *spc* operon proteins, but no decrease in the mRNA synthesis rate. These observations also suggest that transcription from ribosomal protein promoters may be special, perhaps able to overcome transcription termination signals. We also analyzed the state of ribosomal protein mRNA after overproduction of S8 in these experiments and found that repression of ribosomal protein synthesis was accompanied by stimulation of processing (and degradation) of *spc* operon mRNA. The possible role of mRNA degradation in tightening the regulation is discussed.

It is known that the synthesis of many of the ribosomal proteins (r-proteins) in *Escherichia coli* is coordinately and stoichiometrically balanced with the assembly of mature ribosomes and that a posttranscriptional feedback mechanism is largely responsible for this regulation (for reviews, see references 17, 21, and 28). Key r-proteins have been identified which, when synthesized in excess of the need for ribosome assembly, act as translational feedback inhibitors on their polycistronic mRNA to control the synthesis of all r-proteins within their regulatory unit.

How does a single repressor r-protein inhibit the synthesis of more than one r-protein? This question was first studied with the L11 operon, which encodes the genes for L11 and L1 and is regulated by L1. It was demonstrated that L1 interacts with mRNA at a single target site near the translation start site of L11 and inhibits translation of both L11 and L1 (2, 43). The inhibition of L11 translation by L1 takes place directly and that of L1 translation takes place only indirectly as a result of inhibition of L11 synthesis. It was shown that the synthesis of L1 does not take place unless the preceding L11 cistron is translated, that is, L1 synthesis is translationally coupled to L11 synthesis, and this is the basis of coregulation of the two genes by the single translational repressor L1 (2, 35, 43). Recent experiments have also demonstrated the presence of translational coupling as a basis of coregulation of r-protein genes in the α r-protein operon by the translational repressor S4 (37).

The *spc* r-protein operon encodes 10 r-proteins and is regulated by a translational repressor, S8, encoded by the operon (8, 41) (Fig. 1). We have identified the target site for S8 near the translation initiation site of the third cistron, L5, and shown that interaction of S8 with this site on the polycistronic *spc* mRNA leads to the inhibition of synthesis of all r-proteins distal to L5 (L. C. Mattheakis, Ph.D. thesis, University of Wisconsin-Madison, 1988; D. P. Cerretti, L. C. Mattheakis, K. R. Kearney, L. Vu, and M. Nomura, J. Mol. Biol., in press). In addition, the same interaction also achieves regulation of the two proximal genes, L14 and L24, by a novel mechanism ("retroregulation"; Mattheakis, Ph.D. thesis; L. C. Mattheakis and M. Nomura, manuscript in preparation). From analogy with the regulation of the L11 and α operons, the coregulation of synthesis of distal r-proteins can be explained by the presence of translational coupling between all distal r-protein cistrons. However, a priori, translational coupling is not the only possible mechanism to achieve coregulation of distal cistrons in regulatory units. One possible mechanism is that inhibition of translation of the first cistron in a regulatory unit by the repressor r-protein causes termination of transcription, thus preventing the expression of all distal cistrons in the operon. The presence of such a mechanism, a transcriptional polar effect, has been well established for several non-r-protein operons (1). Therefore, possible involvement of this mechanism should be examined especially for r-protein operons such as the *spc* operon, in which the distance between the target site and the last known regulated cistron (L15) is very long (about 2,500 nucleotides). Related to this mechanism is the attenuation mechanism proposed for the S10 r-protein operon (20). Although the presence of translational regulation by L4 encoded by this operon was originally demonstrated in vitro (42), in vivo analysis of the effects of overproduced L4 led to the conclusion that L4 regulates the expression of distal genes by causing transcription termination at a leader region in vivo (20). (More recent mutational analysis of the target site for L4 suggested that L4-mediated attenuation and translational repression are independent processes and may work in parallel in vivo [11]. In addition, it was shown that

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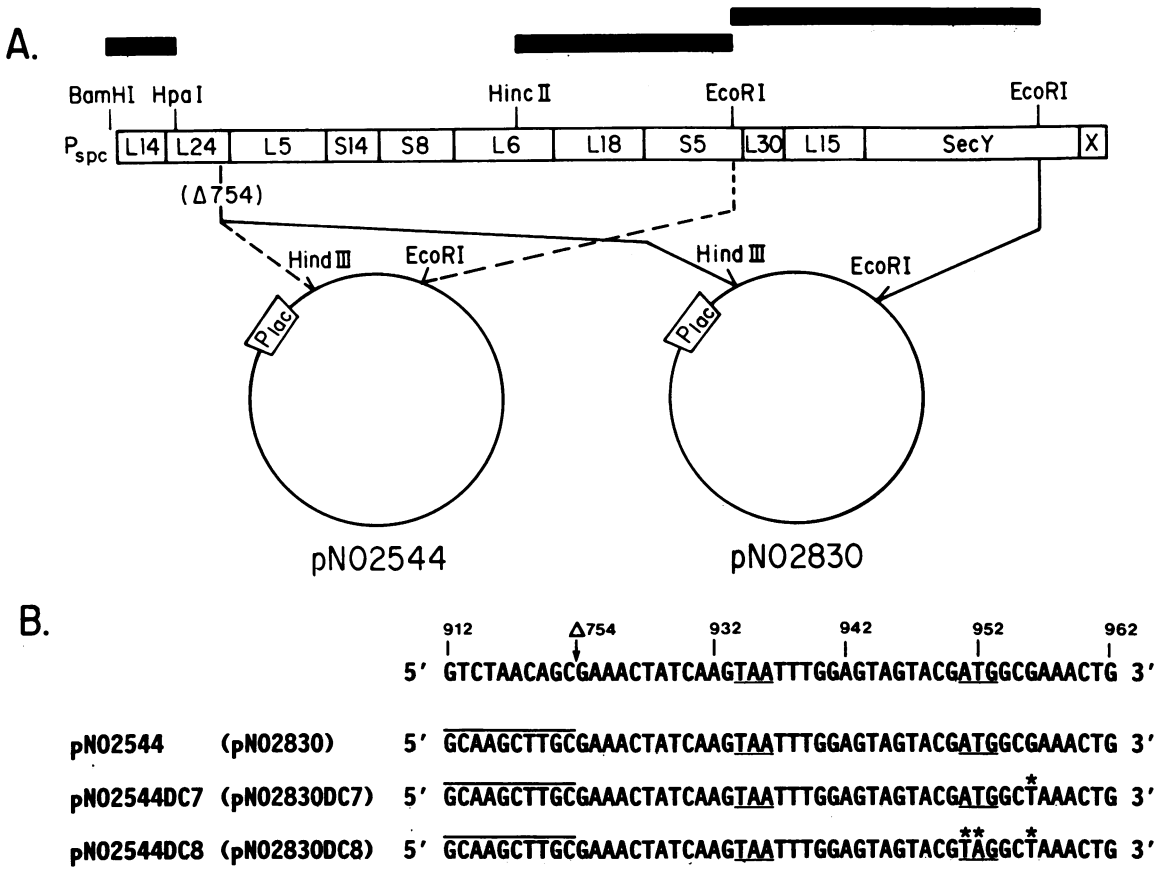

FIG. 1. (A) Physical map of the *spc* operon in *E. coli* and construction of expression plasmids for analyzing effects of site-directed mutations on regulation. P_{spc} and P_{lac} refer to the *spc* and *lac* promoters, respectively. Some pertinent restriction enzyme sites used in the construction of the expression plasmids as well as the hybridization probes are shown. *secY* is a gene involved in protein secretion (16, 32). X is the gene for a protein associated with 50S ribosomal subunits (protein B [38, 39]). Solid bars and restriction enzyme sites below indicate regions of the *spc* operon used as probes for hybridization experiments. Δ754 indicates the deletion endpoint in L24 ligated to the expression vector by using *Hin*dIII linkers. (B) Nucleotide sequence of the L24/L5 intercistronic region and nucleotide sequences of the same region from the various expression plasmids. Numbering of nucleotides is according to reference 4. Deletion endpoint Δ754 was constructed by *Bal*31 exonuclease digestion (Cerretti et al., in press). The termination site for L24 (TAA) and start site for L5 (AUG) are underlined. Overlined are nucleotide sequences derived from the *Hin*dIII linker used for plasmid construction. The nucleotides altered by the DC7 and DC8 mutations are indicated by asterisks.

the effects of L4 on transcription observed in vivo were not a consequence of the absence of translation in this case [20].)

Another possible mechanism to regulate the expression of distal cistrons is that inhibition of translation of the first cistron by repressor might expose naked mRNA to nuclease attack, leading to the degradation of distal mRNA and hence inhibition of translation. In the original gene dosage effect experiments with strains diploid for the *spc* and α operons, it was found that the rate of transcription measured with a probe covering both operons increases about twofold, but the excess r-protein mRNA synthesized was preferentially degraded (9, 29). Although specific inhibition of the synthesis of at least some distal r-proteins was demonstrated in the absence of mRNA degradation in vitro, for example, for the L11 operon (2), mRNA degradation may play a role to prevent escape of distal cistrons from translational coupling, as was suggested previously (6).

In this paper, we describe the results of experiments designed to study the mechanism of regulation of distal cistrons in the *spc* operon by S8. Specifically, we have examined the effects of S8 overproduction in *trans* on the transcription of distal genes in the *spc* operon and confirmed the conclusion that repression of translation of distal cistrons by S8 is not due to the inhibition of transcription. We have also shown that translation of distal cistrons on the polycistronic *spc* mRNA depends on the translation of the proximal cistron, that is, distal cistrons are translationally coupled to proximal cistrons. In addition, we have observed that processing (and degradation) of *spc* mRNA is stimulated by the overproduction of S8 repressor and discuss its significance in tightening the regulation.

MATERIALS AND METHODS

Strains and media. Strain GM1 (Table 1) and its derivatives carrying various plasmids were used. Supplemented AB-glucose medium was AB minimal medium (5) containing 0.4% glucose, thiamine (1 μg/ml), all amino acids except lysine (40 μg/ml each), and ampicillin (50 μg/ml). Supplemented MOPS medium was minimal MOPS medium (27) containing 0.4% glycerol, all amino acids except lysine and cysteine (50 μg/ml each), thiamine (1 μg/ml), and ampicillin (50 μg/ml).

Plasmid construction and site-directed mutagenesis. Plasmids used in this study are described in Table 1 and Fig. 1. Construction of plasmids pNO2544 and pNO2544DC7 will

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Description	Reference
E. coli GM1	Δ*lac-pro thi*/F′ *lacI*^{q} *lacp*L8 *pro*$^+$	7
Plasmids		
pBR322		3
pBGP120	Cloning vector carrying *lac* promoter	30
pNO1018	Derivative of pBGP120 carrying S8 gene under transcriptional control of *lac* promoter-operator	8
pJJS300	Cloning vector carrying *lac* promoter	34
pNO1567	Derivative of pJJS300	37
pNO2544	Carries *spc* operon DNA from Δ754 deletion in L24 to *Eco*RI site in S5 ligated to *Hin*dIII-*Eco*RI sites of pNO1567; see Fig. 1	
pNO2544DC7 (=pNO2610)	Derivative of pNO2544 with DC7 single mutation (G-956 to T); see Fig. 1	
pNO2544DC8 (=pNO2682)	Derivative of pNO2544 with DC8 triple mutation (G-956 to T, T-952 to A, and A-951 to T); see Fig. 1	
pNO2830	Same as pNO2544 but carrying in addition 1.5-kb *Eco*RI fragment containing genes for S5, L30, L15, and part of *secY* inserted into *Eco*RI site; see Fig. 1	
pNO2830DC7 (=pNO2831)	Derivative of pNO2830 carrying DC7 mutation; see pNO2544DC7	
pNO2830DC8 (=pNO2832)	Derivative of pNO2830 carrying DC8 mutation; see pNO2544DC8	
M13 phages		
M13mp7		23
M13mp8		24
M13NO8066	L11-L12 probe; M13mp8 derivative containing a 2.1-kb *Eco*RI fragment from λ*rif*^{d}18. The fragment contains the genes for L1 and L10 and part of the L11 and the L7/L12 genes in the *rif* region	36
M13NO7047	L6-S5 probe; M13mp7 derivative containing a 1.0-kb *Hin*cII-*Eco*RI fragment from L6 to S5 inserted into *Hin*cII site by forced blunt-end ligation; see Fig. 1	
M13NO7007	S5-L15 probe; M13mp7 derivative containing a 1.5-kb *Eco*RI fragment containing the genes for L30, L15, and part of S5 and *secY*; see Fig. 1	

be described elsewhere (Cerretti et al., in press). Plasmid pNO2544DC8 is a derivative of pNO2544 constructed by the same oligonucleotide-directed mutagenesis procedures to be described elsewhere (Cerretti et al., in press). Plasmids pNO2830, pNO2830DC7, and pNO2830DC8 are derivatives of pNO2544, pNO2544DC7, and pNO2544DC8, respectively. Each of these derivatives was constructed by cutting pNO2544 (and mutant derivatives DC7 and DC8) with *Eco*RI and ligating a 1.5-kilobase (kb) *Eco*RI fragment containing the structural genes for L30, L15, and part of S5 and *secY* (Fig. 1) in the correct orientation.

Protein synthesis rate measurements. To determine individual r-protein synthesis rates under specified experimental conditions, cells (about 2×10^8/ml) were pulse-labeled with [^{3}H]lysine (40 μCi/ml; 87 Ci/mmol) for 1 min, followed by a 1-min chase with excess nonradioactive lysine (500 μg/ml, final concentration). Cells were rapidly chilled and mixed with an equal volume of [^{14}C]lysine-labeled carrier cells. These carrier cells were prepared by growing cells for several generations in the presence of [^{14}C]lysine (3 μCi/ml; 0.36 Ci/mmol). Cells were lysed by heating in sodium dodecyl sulfate (SDS) buffer, and selected r-proteins were immunoprecipitated as described, followed by SDS-polyacrylamide gel electrophoresis (41). Locations of labeled r-proteins were determined by autoradiography of dried gels. The desired protein bands were then cut from the gels and oxidized in a Packard sample oxidizer, and ^{14}C and ^{3}H were determined separately. The ^{3}H/^{14}C ratio for each r-protein was divided by the ^{3}H/^{14}C ratio for total protein in the sample. This value represents the differential synthesis rate (synthesis rate of a protein/total protein synthesis rate) of the pertinent protein normalized to that of the same protein in ^{14}C-labeled reference cells. The value obtained in this way under one condition normalized to that under another condition is equal to the ratio of differential synthesis rates under these two conditions and is called the relative differential synthesis rate. The rationale for the use of ^{14}C-labeled reference cells to eliminate various errors and the accuracy of the method generally attained were described in detail previously (37).

Measurements of mRNA synthesis by RNA-DNA hybridization. Cells (about 2×10^8/ml) were pulse-labeled with [^{3}H]uridine (16 μCi/ml; 39.5 Ci/mmol) for the time periods indicated in the table footnotes. The samples were immediately added to boiling SDS lysis buffer and heated for 2 min, and the RNA was isolated by phenol extraction followed by ethanol precipitation, as described previously (18). RNA-DNA hybridization was carried out with DNA probes (described in the tables) immobilized on Millipore nitrocellulose filters as described before (18). Background counts (usually less than 10%) observed with control filters carrying M13mp8 DNA were subtracted from experimental counts. Several different volumes of RNA samples were analyzed for each sample to confirm that DNA was in excess. The data were first expressed as the fraction of total radioactive RNA that was hybridized to the specific probes and then normalized in appropriate ways.

Northern (RNA) blot hybridization. Total RNA was isolated from exponentially growing cells by the method described in Miller (25). RNA was denatured in the presence of formaldehyde and formamide at 55°C for 20 min as described in Maniatis et al. (22), and duplicate samples were electrophoresed through a formaldehyde–0.9% agarose gel for 15 h at 40 V as described before (22). Afterwards, the gel containing RNA was directly blotted to a nitrocellulose membrane filter overnight in the presence of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described (40). For hybridization, the nitrocellulose was cut into strips (each strip containing one set of duplicate RNA samples that were electrophoresed) and hybridized with radioactive probes as described before (40). The probes were gel-purified restriction fragments labeled by the random primer method de-

scribed by Feinberg and Vogelstein (10). Autoradiography was used to visualize RNA bands which hybridized to radioactive DNA probes.

RESULTS

Translational coupling of L5 and downstream S14, S8, L6, and L18 cistrons in the *spc* operon. As described in the Introduction, S8 inhibits the translation of L5 directly and the synthesis of r-proteins (S14, S8, L6, L18, S5, L30, and L15) from genes ("distal cistrons") downstream from the L5 gene indirectly. In order to examine whether distal cistrons are translationally coupled with L5, we designed experimental systems in which conditional expression of L5 and the distal cistrons can be achieved from a plasmid operon and the effects of inhibition of L5 translation on distal cistron expression can be easily studied.

In the first experimental system, we used a plasmid, pNO2544, which carries a portion of the *spc* operon under control of the *lac* promoter-operator (Fig. 1), and its two mutant derivatives, pNO2544DC7 and pNO2544DC8. Plasmid pNO2544 carries the region of the *spc* operon from deletion endpoint Δ754 to the *Eco*RI site in S5 (Fig. 1) and has the target site for S8 repressor still kept intact (Cerretti et al., in press) (Table 1). The second plasmid, pNO 2544DC7, carries a single point mutation changing the G residue at position 956 to T (Fig. 1B), which disrupts translational feedback regulation by S8. The third plasmid, pNO2544DC8, contains the same point mutation as pNO 2544DC7 but in addition carries another mutation which changes the AUG start codon of L5 to the termination codon UAG (Fig. 1B). Thus, not only is the hybrid operon on this plasmid not regulated by S8 as pNO2544DC7, the gene for r-protein L5 in the plasmid cannot be translated. Previous experiments had shown that because of the DC7 mutation, r-proteins encoded by pNO2544DC7 are overproduced after induction of the operon with a *lac* operon inducer, isopropylthiogalactopyranoside (IPTG) (Cerretti et al., in press). We wished to see the effects of the second mutation (AUG to UAG) in pNO2544DC8 on the expression of distal genes.

Strains carrying these plasmids were grown in a synthetic medium, the hybrid operons carried by plasmids were induced by IPTG, and synthesis rates of selected r-proteins were determined before and 10 min after induction. Differential synthesis rates of r-proteins after induction normalized to those before induction are shown in Table 2. It can be seen first that in the strain carrying pNO2544, the synthesis rates of L5 through L18 increased only slightly, on the average 33%, even though the rate of transcription of this region increased close to 10-fold (see below). On the other hand, the synthesis rates of S5 and L30 decreased by 50%, reflecting the fact that these two proteins are encoded only by the chromosome, whereas the other proximal proteins are encoded by both the chromosome and the plasmid. Calculation shows that the proximal proteins (L5 through L18) were synthesized mainly (about 63%; [1.33 − 0.49]/1.33 = 0.63) from the plasmid mRNA and partly (about 37%; 0.49/1.33 = 0.37) from mRNA derived from the chromosome. (These values indicate that protein synthesis from the chromosome-derived mRNA is more efficient than that from the plasmid-derived mRNA. The plasmid-derived mRNA is an artificial mRNA lacking both 5′ and 3′ parts of the intact, chromosome-derived mRNA, and this may be responsible for the observed apparent differences in translational efficiency between the two mRNAs, e.g., because of differences in mRNA half-life under these conditions.)

TABLE 2. Relative differential synthesis rates of *spc* operon proteins after induction of hybrid operons present on pNO2544 and its mutant derivatives[a]

Group	r-Protein	Relative differential synthesis		
		pNO2544	pNO2544DC7	pNO2544DC8
I	L3	1.06	1.15	1.05
	L22	1.13	1.37	1.07
II	L5	1.25	5.8	0.78
	S14	1.36	5.4	1.12
	S8	1.31	4.3	1.08
	L6	1.30	5.2	1.12
	L18	1.41	3.9	1.24
	Mean ± SD	1.33 ± 0.06	4.9 ± 0.79	—[b]
III	S5	0.39	0.17	0.91
	L30	0.58	0.36	0.90
	Mean ± SD	0.49	0.27	—

[a] Derivatives of strain GM1 carrying the indicated plasmids were grown and protein synthesis rates were measured as described in Materials and Methods. Differential synthesis rates (see text) of selected r-proteins relative to that by noninduced cells are shown. Group I proteins are not encoded by the *spc* operon; group II proteins are *spc* operon proteins encoded by the plasmid as well as the chromosome; and group III proteins are *spc* operon proteins encoded only by the chromosome.

[b] The average of the values for S14, S8, L6, and L18 is 1.14. This value was used to estimate the average synthesis rate of these four r-proteins from plasmid-encoded mRNA (see text).

For strains carrying the mutant plasmid pNO2544DC7, there was an overproduction of the proximal proteins encoded by the plasmid, indicating the failure of the regulation of synthesis of these proteins by S8. On the other hand, stronger repression of the synthesis of genes S5 and L30 was observed, indicating that mRNA derived from the chromosome is sensitive to S8 and the degree of repression increased by the increased production of S8 repressor. Calculation shows that the synthesis rates of r-proteins L5 through L18 from the plasmid-encoded mRNA were about 4.6-fold (4.9 − 0.27 = 4.6) higher than the synthesis rate of these r-proteins from the chromosome-encoded mRNA before induction.

In the strain carrying pNO2544DC8, the synthesis of L5 was entirely from the chromosome-encoded mRNA, and the effects of the absence of L5 translation on the synthesis of L5-distal proteins (S14, S8, L6, and L18) from plasmid-encoded mRNA can be calculated. The average relative differential synthesis rates of these proteins (S14, S8, L6, and L18) was 1.14 (Table 2), while the average value for the proteins (S5, L30, and L5) synthesized from the chromosome-encoded mRNA was 0.86. The difference between these two values (1.14 − 0.86 = 0.28) represents the average synthesis rate of proteins S14, S8, L6, and L18 from plasmid-encoded mRNA. Thus, although some translation of L5-distal proteins appears to have still taken place from the plasmid-encoded mRNA, the level of this translation was very small compared with the synthesis rate from mRNA derived from pNO2544DC7, that is, only about 6% (0.28/4.6 = 0.061). It is clear that the absence of translation of L5 led to a very strong reduction (94%) in the synthesis of S14, S8, L6, and L18. The question was then whether this reduction was due to transcriptional polarity caused by the absence of L5 translation. We tested this possibility by measuring mRNA synthesis rates with a probe covering the distal portion of the mRNA extending from the *Hin*cII site of L6 to the *Eco*RI site of S5 (M13NO7047; Table 1 and Fig. 1). As a

TABLE 3. Relative amounts of pulse-labeled L6-S5 *spc* mRNA after induction of plasmid-encoded hybrid *spc* operon[a]

Pulse label (min)	Relative *spc* mRNA synthesis								Ratio of plasmid-derived mRNA, DC8/DC7 (i)
	pNO2544DC7				pNO2544DC8				
	IPTG			Plasmid derived (d)	IPTG			Plasmid derived (h)	
	−(a)	+(b)	+/−(c)		−(e)	+(f)	+/−(g)		
0.5	0.50	4.2	8.4	7.4	0.41	1.78	4.3	3.3	0.45
2.0	0.47	4.9	10.4	≥9.4*	0.32	0.95	3.0	2.0	≤0.21*
6.0	0.46	2.1	4.6	≥3.6*	0.40	0.65	1.62	0.62	≤0.17*

[a] Derivatives of strain GM1 carrying either pNO2544DC7 or pNO2544DC8 were grown, pulse-labeled with [^{3}H]uridine, and hybridized as described in the text. Cells were labeled before (−) and 10 min after (+) addition of IPTG, and the ratio of these values is shown (+/−). To correct for variations in the efficiency of hybridization from sample to sample, samples were incubated simultaneously with two kinds of DNA filters, one for L6-S5 and the other for L11-L7/L12 mRNA, and the amounts of [^{3}H]RNA hybridized to the former DNA were normalized to those hybridized to the latter DNA and are shown in the table. (An example of such hybridization data and normalization can be seen in Table 6.) The amounts of L6-S5 *spc* mRNA derived from the plasmid operons were calculated as follows. For example, with the strain containing pNO2544DC7, after a 0.5-min pulse, the total amounts of radioactive L6-S5 *spc* mRNA that hybridized to the probe increased 8.4-fold (column c). Assuming that the rate of transcription of the chromosomal *spc* operon does not change after induction (though there may be a slight stimulation; see the text), the amount of radioactive L6-S5 *spc* mRNA derived from the plasmid is 7.4 (column d) relative to the amount before induction (which is 1.0). However, with plasmid pNO2544DC7, repressor S8 is overproduced and the degree of repression of (chromosomal) *spc* mRNA should increase, leading presumably to faster decay of *spc* mRNA derived from the chromosome. Therefore, the values calculated for 2 and 6 min (indicated with an asterisk in column d) are lower estimates compared with the 0.5-min value. (However, the error caused by this is small. For example, the value 9.4 shown for the 2-min pulse may be a lower estimate, but should not exceed 10.4.) This complication does not exist with pNO2544DC8, which does not stimulate S8 synthesis because of the AUG to UAG mutation and hence does not inhibit translation of chromosomal mRNA. The values given in column i are obtained by dividing the values in column h by the corresponding values in column d, and hence, the values for 2 and 6 min (indicated with asterisks) are subject to the small errors mentioned.

control, an L11-L12 probe specific for L11, L1, L10, and L7/L12 mRNA was used (M13NO8066; Table 1).

Strains carrying pNO2544DC7 or pNO2544DC8 were grown under the same conditions used in the experiments described above. Cells were labeled with [^{3}H]uridine for 0.5, 2, or 6 min both before and 10 min after addition of IPTG, radioactive RNA was isolated, and the *spc* mRNA synthesized was quantified by hybridization to the probes mentioned above. The lengths of pulse labeling were varied from 0.5 to 2 and 6 min to assess the relative contribution of mRNA stability to the measurements of mRNA synthesis rates. The results are shown in Table 3.

It can be seen that the *spc* mRNA synthesis rate in the strain carrying pNO2544DC8 measured with a 30-s pulse increased 4.3-fold after induction even though the increase in the synthesis rates of the pertinent *spc* r-proteins (S14, S8, L6, and L18) was very small, as mentioned above. On the other hand, the increase in the strain carrying pNO2544DC7 was 8.4-fold, which can be compared with the 4.3-fold increase in the synthesis rates of the *spc* r-proteins. The calculations shown in Table 3 indicate that the rate of transcription of the plasmid-encoded *spc* operon from pNO2544DC8 was 45% of that from pNO2544DC7. In addition, similar calculations made for the values obtained after 2-min and 6-min pulses showed that mRNA synthesized from pNO2544DC8 was more unstable than mRNA synthesized from pNO2544DC7. Although we do not have enough data to calculate the real transcription rates, it is clear that the difference in transcription rates between pNO2544DC7 and pNO2544DC8 must be significantly less than the 55% calculated from the 0.5-min pulse values. Thus, the almost 20-fold decrease in the synthesis rates of the r-proteins (S14, S8, L6, and L18) from pNO2544DC8 relative to pNO 2544DC7 cannot be explained by decreased transcription of these genes. We conclude that translation of these r-proteins in the *spc* operon does not take place independently but is coupled to the translation of L5.

Translational coupling of L5, S5, L30, and L15 cistrons. The above experiments were carried out with a set of plasmids lacking the distal cistrons S5, L30, and L15. We examined translational coupling of these three distal cistrons with L5 by using three plasmids, pNO2830, pNO2830DC7, and pNO2830DC8. These plasmids carry genes for S5, L30, and L15 and were constructed from pNO2544, pNO 2544DC7, and pNO2544DC8, respectively (Fig. 1).

Relative differential synthesis rates of r-proteins were measured after induction of the plasmid-encoded hybrid operon as was done in the experiments shown in Table 2. It can be seen from the results given in Table 4 that relative differential synthesis rates of S5, L30, and L15 were affected by the DC7 and DC8 mutations in the same way as those of the other r-proteins (S14 through L18), and the results were similar to those shown in Table 2. The DC7 target site mutation caused a large derepression of the hybrid operon and led to a three- to fivefold stimulation of S5, L30, and L15 synthesis, and the additional mutational alteration of L5 initiation codon (AUG to UAG in the DC8) abolished this stimulation nearly completely. Although we did not measure transcription rates for the mRNA segment covering S5 through L15, we can assume that the transcription rates measured with the L6-S5 probe (Table 3) may also represent

TABLE 4. Relative differential synthesis rates of *spc* operon proteins after induction of hybrid operons present on pNO2830 and its mutant derivatives[a]

Group	r-Protein	Relative differential synthesis		
		pNO2830	pNO2830DC7	pNO2830DC8
I	L3	0.96	1.13	1.07
II	L5	1.12	5.1	0.77
	S14	1.22	5.1	1.14
	S8	1.09	4.8	1.05
	L6	1.17	5.0	1.08
	S5	1.09	3.2	1.02
	L30	1.31	4.1	1.10
	L15	1.29	5.0	1.09
Mean ± SD	S14–L15	1.20 ± 0.10	4.5 ± 0.7	1.08 ± 0.04

[a] Derivatives of strain GM1 carrying the indicated plasmids were grown and pulse-labeled as described in the text. The group I protein L3 is a control not encoded by the *spc* operon. Group II proteins are encoded by the r-protein genes carried by the plasmids. L18 is also encoded by the plasmids, but its synthesis rates were not measured.

TABLE 5. Effect of S8 overproduction in *trans* on the synthesis rates of *spc* operon proteins[a]

Group	Protein	Synthesis rate after IPTG addition	
		7 min	25 min
—	S8	2.72	2.51
I	L1	1.21	1.48
	L3	1.10	1.48
II	L14	0.68	1.20
	L24	0.69	1.16
	Mean ± SD	0.69	1.18
III	L5	0.31	0.37
	L6	0.26	0.31
	S5	0.35	0.36
	L15	0.32	0.33
	Mean ± SD	0.31 ± 0.04	0.34 ± 0.03

[a] Derivatives of strain GM1 carrying pBGP120 or pNO1018 were grown, induced with IPTG, and pulse-labeled with [^{3}H]lysine or [^{3}H]uridine to measure mRNA synthesis rates. In addition, the strain carrying pNO1018 was pulse-labeled with [^{3}H]lysine again at 25 min. The relative differential synthesis rate values shown are the ratios of the differential synthesis rates of r-proteins in the strain carrying pNO1018 relative to those of the same proteins in the strain carrying control plasmid pBGP120. Group I proteins are control proteins not encoded by the *spc* operon, group II are proteins encoded by cistrons which are upstream from the S8 target site, and group III are proteins encoded by cistrons distal to the S8 target site. The results of mRNA synthesis rate measurements are given in Table 6.

the rates for S5 through L15. Therefore, we conclude that these three distal cistrons are also translationally coupled to L5.

Effects of overproduction of S8 in *trans* on the synthesis of *spc* r-proteins and mRNA from the chromosomal *spc* operon. In the early studies which identified S8 as a translational feedback repressor for the *spc* operon, it was shown that overproduction of S8 in vivo from a plasmid caused specific repression of the synthesis of *spc* operon proteins L5 through L15 (8). In these experiments, the effects of S8 overproduction on mRNA synthesis were not studied. The experiments described in the previous sections demonstrated the presence of translational coupling as the basis of the coregulation of *spc* operon r-protein gene expression (L5 through L15) by S8. Nevertheless, we wished to confirm this conclusion by measuring the mRNA synthesis rate under conditions in which synthesis of *spc* r-proteins from the chromosomal *spc* operon is inhibited by S8 overproduced from a plasmid. In addition, we wished to study the mRNA degradation process which is presumably stimulated under these conditions, as suggested by earlier studies (9; see also reference 6 for the L11 operon and reference 33 for the α operon). Thus, we repeated the earlier experiments and simultaneously measured the effects of S8 overproduction on the rates of both *spc* operon protein synthesis and distal *spc* mRNA synthesis. For these studies, we used the same plasmid used in the original in vivo experiments. This plasmid, pNO1018, carries the S8 gene under transcriptional control of the *lac* promoter-operator.

Derivatives of strain GM1 carrying this plasmid or the control plasmid pBGP120 were grown to mid-log phase and the *lac* operon inducer IPTG was added to the cultures to induce synthesis of S8 from the plasmid. At 7 min after IPTG addition, the cultures were divided and the synthesis rates of *spc* r-proteins were measured in one culture and those of distal *spc* mRNA were measured in the other culture. The protein synthesis rates were also measured at 25 min after IPTG addition. The results for protein synthesis rates are shown in Table 5 and those for mRNA synthesis rates are given in Table 6.

It can be seen from Table 5 that there was a 2.7-fold and 2.5-fold increase in the synthesis rate of S8 at 7 and 25 min after IPTG addition, respectively. This resulted in an approximately threefold decrease in the synthesis rates of proteins L5, L6, S5, and L15 shown in group III. There was also a weak decrease (about 30%) in the synthesis rate of L14 and L24 at 7 min which was not observed at 25 min after IPTG addition. The inhibition of L14 and L24 by S8 is due to a retroregulation mechanism, which will be reported in a separate communication (Mattheakis and Nomura, manuscript in preparation). It should also be noted that the synthesis of control proteins L1 and L3 was not inhibited but in fact gradually stimulated after induction of S8 overproduction. A similar general stimulation in r-protein synthesis rates was observed previously after induction of S4, which is the repressor for r-proteins in the α operon (36). This phenomenon was interpreted to be due, presumably, to a general derepression of r-protein mRNA synthesis which resulted from a defect in ribosome assembly caused by S4 overproduction. We note that the assembly of ribosomes was in fact very severely inhibited under the present experimental conditions, that is, after overproduction of S8 in *trans* (A. Miura and M. Nomura, unpublished experiments).

Measurements of *spc* mRNA synthesis rates were carried out by analyzing radioactive RNA labeled during a 0.5-min pulse with an M13 DNA hybridization probe (M13NO7007) carrying the 1.5-kb *Eco*RI fragment containing the distal L30 and L15 genes (Table 1 and Fig. 1). As a control, we used the L11-L12 probe (M13NO8066). The results (Table 6) showed that there was no specific inhibition of *spc* mRNA synthesis at 7 min after S8 overproduction. Thus, the threefold decrease in the synthesis rates of L15 and the other *spc* operon r-proteins distal to L5 cannot be due to any transcriptional effects, confirming our expectation mentioned above.

TABLE 6. Effects of S8 overproduction in *trans* on the synthesis rates of *spc* operon mRNA[a]

Probe	GM1(pBGP120)			GM1(pNO1018)		
	cpm hybridized	Fraction of input	Ratio, *spc*/L11-L12	cpm hybridized	Fraction of input	Ratio, *spc*/L11-L12
L11-L12	1,176	3.9×10^{-4}	0.69	1,406	3.2×10^{-4}	0.67
Distal *spc*	818	2.7×10^{-4}		946	2.2×10^{-4}	

[a] Radioactive RNA synthesized during the 0.5-min pulse was hybridized to a DNA probe specific for distal *spc* genes S5, L30, and L15 (M13NO7007) or to the L11-L12 probe (M13NO8066). The amounts of input [^{3}H]RNA were 3.00×10^6 cpm for GM1(pBGP120) and 4.36×10^6 cpm for GM1(pNO1018). The background ^{3}H cpm bound to M13mp8 filters (about 40 cpm) were subtracted. The values representing the fractions of total radioactive RNA that were specifically hybridized to the DNA probes are shown as fraction of input. In addition, the ratios of these values obtained from the distal *spc* probe relative to the L11-L12 probe are also shown (ratio, *spc*/L11-L12).

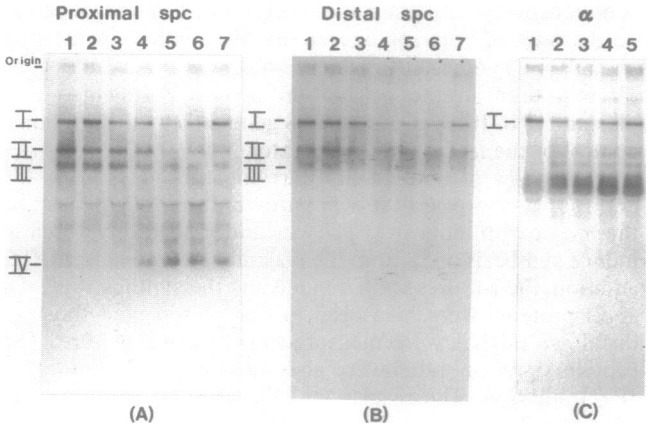

FIG. 2. Northern blot analysis of r-protein mRNA after S8 overproduction. Strain GM1 and its derivatives carrying pBGP120 or pNO1018 were grown as described in the text, and IPTG was added to a final concentration of 1 mM. Cells were harvested either before or at various times after IPTG addition, and growth was stopped by the addition of sodium azide to a final concentration of 20 mM and cooling on ice. Total RNA was isolated, and duplicate samples containing 10 μg of RNA were run on a denaturing formaldehyde-agarose gel. After blotting onto nitrocellulose membrane, the membrane was cut into strips and hybridized with different radioactive probes. The proximal *spc* (L14) and distal *spc* (S5-*secY*) probes are the *Bam*HI-*Hpa*I and *Eco*RI-*Eco*RI restriction fragments, respectively (Fig. 1). The α probe is a 1.8-kb *Sph*I restriction fragment from the beginning of S13 through the S11 and S4 genes and to the middle of the gene encoding the α subunit of RNA polymerase. (A) Proximal *spc* probe hybridized to RNA isolated from GM1 (lane 1), GM1 carrying pBGP120 (lane 2), GM1 carrying pNO1018 before IPTG addition (lane 3), and GM1 carrying pNO1018 at 5, 10, 15, and 30 min after IPTG addition (lanes 4, 5, 6, and 7, respectively). (B) Distal *spc* probe hybridized to the same RNAs as in panel A. (C) Hybridization to the α probe. Lanes 1 to 5 correspond to the same RNA samples shown in lanes 1, 4, 5, 6, and 7, respectively, in panels A and B.

Northern blot analysis of r-protein mRNA after S8 overproduction. We used Northern blot analysis to examine changes in the state of *spc* mRNA after S8 overproduction in *trans*. The experimental conditions were the same as described in the previous section. Total RNA was isolated from derivatives of strain GM1 carrying pBGP120 or pNO1018 at various times after IPTG addition. Equal amounts of RNA were subjected to electrophoresis on a denaturing formaldehyde-agarose gel. The gel was then blotted onto a nitrocellulose membrane, and RNA containing *spc* operon mRNA was detected by hybridization with radioactive DNA probes. Three different probes were used. The proximal *spc* probe was a 481-nucleotide *Bam*HI-*Hpa*I fragment (Fig. 1) covering the region from the *spc* mRNA leader sequence to the beginning of L24 (Fig. 2A). The distal *spc* probe used was a 1.5-kb *Eco*RI fragment covering the region from the end of S5 to *secY* (Fig. 2B). Finally, we used a probe that covered a region of the adjacent α operon (Fig. 2C), since previous results had shown that the *spc* and α operons were cotranscribed (4) and we wished to see the state of contiguous *spc*/α transcripts. This probe, a 1.8-kb *Sph*I fragment, covered a region of the α operon from the beginning of the S13 gene to the middle of the gene encoding the α subunit of RNA to polymerase.

As shown in Fig. 2, these probes hybridized to several bands. In the absence of S8 overproduction (lanes 1, 2, and 3, Fig. 2A and B; lane 1, Fig. 2C), three major RNA bands (I, II, and III) were detected by both the proximal and the distal *spc* probes. Band I was also seen with the α probe, but bands II and III were not. The size of the band I RNA was estimated to be about 8.4 kb from size markers run in parallel. This size is in close agreement with the predicted size (8.6 kb) of the contiguous *spc*/α transcript based on DNA sequence data. The size estimates for band II and III RNAs were about 5.5 and 4.4 kb, respectively. These bands may represent processed products of the full-length transcript, although we cannot exclude the possibility that they represent primary transcripts produced by transcription termination events. Assuming that both of these RNAs carry the same 5′ end as the full-length *spc*/α transcript, the 3′ end of band III RNA is estimated to be near the beginning of *secY* and that of band II RNA is estimated to be near the end of *secY*.

The amount of band I RNA, the *spc*/α transcript, decreased after S8 overproduction. This can be clearly seen with all three probes by comparing the RNA sample at 10 min after IPTG addition (lane 5 in Fig. 2A and B, and lane 3 in Fig. 2C) with the RNA sample before IPTG addition (lane 3 in Fig. 2A and B, and lane 1 in Fig. 2C; see the legend to Fig. 2). The decrease had probably already taken place at 5 min after induction; it was clearly seen with the distal *spc* probe (lane 4 in Fig. 2B), and some reduction can be recognized with the other two probes (lane 4 in Fig. 2A, and lane 2 in Fig. 2C). The reason that a greater reduction was seen only with the former probe in this case is not known. After the initial decrease, however, the band I RNA began to increase between 10 and 15 min after IPTG addition, and at 30 min after induction the amount became nearly the same as before induction, as can be seen with all three probes. We have not studied this recovery phenomenon, but it might be related to the general derepression of transcription of r-protein genes under conditions inhibiting ribosome assembly, as discussed above.

The amounts of band II and III RNAs also appeared to decrease after S8 overproduction, at least transiently (see also below). However, the patterns of RNA bands at later times after induction became complex, suggesting that some new RNA species accumulated as a result of degradation of the primary transcript(s), presumably band I RNA. No further analysis was carried out on these RNAs.

The most striking change seen after induction of S8 overproduction in *trans* was the appearance of a new RNA species (band IV; lanes 4 to 7 in Fig. 2A), which could be detected only by the proximal *spc* probe. The size of this RNA was estimated from the size markers to be approximately 900 nucleotides. The RNA began to appear at the earliest time analyzed, i.e., at 5 min after overproduction of S8. The probable 3′ as well as 5′ ends of this band IV RNA were examined by the S1 mapping method. It was found that this RNA had a 3′-end near (and distal to) the target site where S8 binds. In addition, the amount of this RNA was found to be greatly reduced in strains deficient in RNase III, indicating that the processing event responsible for the formation of the *spc* mRNA fragment requires RNase III (L. C. Mattheakis, F. Sor, and M. Nomura, unpublished experiments). These results as well as their significance will be reported in a separate communication.

It should be noted that with RNA samples from growing cells in the absence of S8 overproduction, there was a broad band detected only by the α probe at a position between bands III and IV. This band may represent RNA molecules processed from band I RNA or those initiated from the α promoter (4). The intensity of this band increased with time

after induction of S8 overproduction. In addition, several RNA bands which existed as faint bands before induction at or near bands II and III position became very prominent after induction. Some of these RNA bands may correspond to the RNA species detected with the distal *spc* probe, which appeared to increase after S8 overproduction, as mentioned above. They may represent processing products derived from the primary *spc*/α transcripts. These results show a correlation between the repression of *spc* r-protein synthesis and stimulation of processing of *spc* operon mRNA.

DISCUSSION

The results presented in this paper show that the regulation of *spc* operon protein synthesis distal to the target site for translational repressor S8 is not the result of polar effects on transcription of the distal genes. Rather, it is based on the presence of translational coupling of the cistrons for these proteins with L5 on polycistronic mRNA. Our results also show that, during translational repression, processing and degradation of mRNA are stimulated. However, mRNA degradation caused by the absence of L5 translation cannot be the primary factor responsible for the observed polar effects on translation of distal cistrons. If initiation of translation of each of the distal seven cistrons, S14 through L15, took place independently, one would expect that the mRNAs for these proteins would be protected by translating ribosomes, especially those encoding the most distal proteins such as L15. However, our experiments show that translation of all the distal proteins, including L15, is almost completely blocked when the AUG initiation codon of L5 is altered and that this is not because of the absence of transcription. (Previous studies on the half-life of the L11-L1 mRNA from hybrid plasmid operons analogous to pNO2830DC7 and pNO2830DC8 showed that transcription rates were not significantly different, but half-life values between the two were different by about 10-fold [6]. These results also support the conclusion made for the present system that the observed decrease of about 50% in the synthesis rate measured by 0.5-min pulse-labeling is probably largely due to the decrease in mRNA stability in the absence of translation, as described in the Results section.) We conclude that the inhibition of L5 synthesis leads to inhibition of distal protein synthesis because of translational coupling of the distal proteins with L5 and suggest that stimulation of processing and degradation of mRNA takes place as a consequence of the absence of translation caused by this primary event.

Nevertheless, we think that mRNA processing and degradation are important to make the regulation tighter. One can imagine, for example, that the binding of S8 with mRNA is not strong, and in the absence of mRNA degradation, inhibition of L5 translation is perhaps only partial unless the S8 concentration in the cellular pool is very high. Processing and degradation could initiate during transient inhibition of translation of a mRNA molecule, leading to irreversible inactivation of the mRNA molecule. Processing and degradation of mRNA may be especially important for regulation of distal cistrons if translational coupling is not complete, as suggested previously (6).

The exact mechanism of mRNA processing and degradation is unknown except that RNase III is somehow involved in some step of the pathway(s) (our unpublished experiments mentioned in the Results section). Even in the absence of RNase III activity, we found at least three major RNA species containing all the *spc* r-protein cistrons (L14 through L15 cistrons) intact in exponentially growing cells. We do not know how these three major species differ with respect to translational efficiency. Thus, although we have observed changes in the amounts of these mRNA species as well as changes in the synthesis rates of r-proteins after S8 overproduction in *trans*, precise interpretation of the data is perhaps premature at this stage.

As discussed above, the major conclusion of the present work is the presence of translational coupling between at least seven r-protein cistrons on a polycistronic mRNA as large as 2,500 nucleotides. It is surprising that the absence of translation along such a large distance does not cause transcription termination. As mentioned earlier in this paper, the same situation also exists with the L11 operon (2). Thus, it is possible that transcription from r-protein promoters may be special, perhaps able to overcome transcription termination signals, as seen with transcription from rRNA promoters (13–15, 19, 26) as well as phage lambda promoters such as p_L (see, e.g., reference 12 for a review). In fact, we have evidence that a significant fraction of transcription started in the S10 operon does not stop at the termination site (31) of the S10 operon and continues through the *spc* operon (F. Sor, L. C. Mattheakis, and M. Nomura, unpublished experiments). Thus, the presence of an antitermination mechanism associated with transcription of r-protein genes is a serious possibility and deserves further study.

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