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Identification of Transcriptional Start Sites and the Role of ppGpp in the Expression of *rpoS*, the Structural Gene for the σ^S Subunit of RNA Polymerase in *Escherichia coli*

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***rpoS* is the structural gene for the σ^S subunit of RNA polymerase which controls the expression of a large number of genes in *Escherichia coli* that are induced during entry into stationary phase or in response to increased medium osmolarity. Using a combination of primer extension experiments and a 5′ deletion analysis of the region upstream of *rpoS*, we show that *rpoS* transcription is mainly driven by a single promoter (*rpoSp*$_1$) located within the *nlpD* gene upstream of *rpoS* (the two relatively weak *nlpD* promoters contribute to the low level of *rpoS* expression during early exponential phase). In addition, we demonstrate that the expression of both transcriptional and translational *rpoS*::*lacZ* fusions as well as the level of *rpoS* mRNA originating at *rpoSp*$_1$ is strongly reduced in ppGpp-deficient *relA spoT* mutants. However, experiments with the 5′ deletion constructs indicate that a lack of ppGpp does affect transcriptional elongation rather than initiation.**

The σ^S subunit of RNA polymerase controls the expression of more than 30 genes or operons that are involved in starvation survival, multiple stress resistance during stationary phase, and coping with a high osmolarity environment. σ^S is coded for by the *rpoS* gene (formerly also designated *katF* or *appR*; for recent reviews, see references 4, 5, and 10). Expression of *rpoS* is induced during entry into stationary phase (7, 8, 11, 12, 15, 19) and in response to an increase in medium osmolarity (8, 14). Whereas starvation stimulates *rpoS* expression at both the transcriptional and posttranscriptional levels (8, 11, 12), high osmolarity influences only the posttranscriptional control of *rpoS* (8).

In the present study, we investigated transcription of the *rpoS* gene. *nlpD*, the structural gene for a lipoprotein, is located upstream of *rpoS* and is transcribed in the same direction (counterclockwise on the *Escherichia coli* chromosome) (6, 9). While *nlpD* is not stationary phase induced, its two closely spaced promoters contribute to the basal level of expression of *rpoS* in exponentially growing cells (9). In a recent study, the subcloning of small fragments of the coding region of *nlpD* into a promoter probe vector was reported, and the authors suggested that at least four promoters for the expression of *rpoS* were present within the *nlpD* gene (22). In contrast, our primer extension experiments for the determination of transcriptional start sites, as well as a deletion analysis presented here, lead to the conclusion that there is only one major *rpoS* promoter within *nlpD*.

The intracellular signals and the signal transduction pathways involved in the control of *rpoS* expression have remained largely unknown. It has been shown that *relA spoT* double mutants exhibit a pleiotropic phenotype very similar to that observed for *rpoS* mutants and contain strongly reduced levels of σ^S protein. Therefore, guanosine-3′,5′-bispyrophosphate (ppGpp) has been implicated in the control of *rpoS* expression as a positively acting signal molecule, although its mechanism of action has not been studied in detail (3). In the present study, we therefore investigated whether ppGpp plays a role in the transcription of *rpoS*.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The strains were constructed by P1 transduction as described previously (13). Strains carrying *relA* and *spoT* mutant alleles were freshly constructed before the experiments were performed and were repeatedly tested for nongrowth on minimal plates (which is due to the multiple-amino-acid auxotrophy of *relA spoT* double mutants [24]) in order to avoid the appearance of second-site suppressor mutations. *relA*$^+$ derivatives of MC4100 (which is *relA1*) were obtained by P1 transduction of the *relA*$^+$ allele linked to *fuc-3072*::Tn*10* (21) from an MG1655 derivative into which *fuc-3072*::Tn*10* had been first introduced by P1 transduction. Phenotypic testing of the *relA* allele was done by the serine hydroxamate sensitivity assay (17). Plasmid pRL1-25, which carries the translational *rpoS70*::*lacZ* fusion, has been described previously (8). Plasmid pRH320 is a pBR322 derivative carrying *rpoS* on a 4.3-kb *Cla*I insert (7).

Cells were grown aerobically in Luria-Bertani (LB) medium or in M9 minimal medium (13). For the minimal medium, glucose (0.2%) was used as a carbon source. For plasmid-containing strains, the medium was supplemented with 50 μg of ampicillin per ml. In experiments that involved *relA spoT* mutants, the cultures were supplemented with 0.1% Casamino Acids. The growth of the bacterial cultures was monitored by measuring the optical density at 578 nm.

DNA manipulations. For DNA manipulations, such as those involving restriction digests, ligation, transformation, and agarose gel electrophoresis, standard procedures were followed (18, 20). Plasmid preparation and the preparation of DNA fragments from agarose gels were performed with the Jetstar and Jetsorb kits, respectively (Genomed). DNA was sequenced with the Sequenase kit (U.S. Biochemical) and α-^{35}S-thio-dATP (>1,000 Ci/mmol; Amersham). Nonradioactive sequencing was performed with the ΔTaq cycle sequencing kit (U.S. Biochemical). Oligonucleotides were purchased from MWG-Biotech.

Construction of 5′ deletions upstream of *rpoS*. Plasmid pRL1-25 contains a 1,470-kb fragment of chromosomal DNA extending from the *Cla*I restriction site located shortly upstream of the 3′ end of the *pcm* gene to nucleotide 70 in the *rpoS* structural gene (8). Plasmids carrying deletions between *Cla*I and various positions farther downstream (pRL49, -47, and -29) were constructed by subcloning fragments extending from various restriction sites in this region to restriction sites within *lacZ* into pJL28, a pBR322-based *lacZ* fusion vector with a multiple cloning site upstream of the eighth codon of *lacZ* (kindly provided by J. Lucht and E. Bremer). pRL30 and pRL31 were constructed previously by subcloning fragments obtained from pRL1-25 by *Cla*I digestion followed by exonuclease III treatment into pJL28 (9). The control plasmid pRL48, which contains the entire upstream region as it is present in pRL1-25, was constructed by subcloning an *Eco*RI-*Sac*I fragment from pRL1-25 into pJL28 (the *Eco*RI site is located in the vector immediately upstream of *Cla*I, and the *Sac*I site is located in *lacZ*).

RNA preparation and primer extension. RNA was prepared by hot phenol extraction of cells growing in LB medium supplemented with 50 μg of ampicillin per ml. Samples were taken during the transition into stationary phase at optical densities of between 2.5 and 4. For the primer extension experiments, the fol-

TABLE 1. Bacterial strains

Strain	Relevant genotype	Reference
MC4100	F$^-$ Δ(*arg-lac*)*U169 araD139 rpsL150 ptsF25 flbB5301 rpsR deoC relA1*	20
RO91	MC4100 [λRZ5: *rpoS742*::*lacZ*(Hyb)][a]	8
RO200	MC4100 (λRZ5: *rpoS742*::*lacZ*)[a]	8
CF1652	MG1655 Δ*relA251*::*kan*	24
CF1693	MG1655 Δ*relA251*::*kan* Δ*spoT207*::*cat*	24
RO206	MC4100 *relA*$^+$ *fuc-3072*::Tn*10*	This study
RO98	MC4100 Δ*relA251*::*kan* Δ*spoT207*::*cat*	This study
RH137	RO91 *relA*$^+$ *fuc-3072*::Tn*10*	This study
RH139	RO91 Δ*relA251*::*kan* Δ*spoT207*::*cat* *fuc-3072*::Tn*10*	This study
RH140	RO200 *relA*$^+$ *fuc-3072*::Tn*10*	This study
RH142	RO200 Δ*relA251*::*kan* Δ*spoT207*::*cat* *fuc-3072*::Tn*10*	This study

[a] The fusion constructs present in RO91 and RO200 are located in single copies at the *att*(λ) site in the chromosome, and both contain the entire region upstream of *rpoS* (starting with the *Cla*I restriction site) that is involved in the transcriptional control of *rpoS*.

lowing oligonucleotides were used as primers: 5′-TTATAGACGATGCGTC C-3′, 5′-ATGTACTGCTGACAGTC-3′, 5′-CTTGTTGTTCCCGGACC-3′, and 5′-CTCCTACCCGTGATCCC-3′ (these oligonucleotides are complementary to various regions within *nlpD* and the intergenic region between *nlpD* and *rpoS*). The reactions were performed with 50 μg of total RNA at 42°C for 30 min with 12.5 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and α-^{35}S-thio-dATP (>1,000 Ci/mmol; Amersham), and this was followed by a 30-min chase with all four nucleotides (at 1 mM each). Alternatively, for the nonradioactive primer extension experiment shown in Fig. 5, a digoxigenin-labeled primer (with a sequence corresponding to that of the second oligonucleotide mentioned above) was used. As a reference, double-strand sequencing reactions were performed with the same primers as those used for the primer extension experiments.

β-Galactosidase assay. β-Galactosidase activity was assayed with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) being used as a substrate and is reported as micromoles per minute per milligram of cellular protein (13).

RESULTS AND DISCUSSION

Identification of transcriptional start sites upstream of *rpoS*. Total RNA was prepared from strain MC4100 containing pRL1-25 (a pBR322 derivative carrying *rpoS70*::*lacZ* [8]) and was subjected to primer extension analysis. For the primer extension reaction, several primers complementary to various regions within *nlpD* were used, so putative start sites at any position within the *nlpD* gene should have been detected. Only two reverse transcripts of *rpoS* mRNA could be found (Fig. 1). One indicates the presence of a promoter that overlaps with a *Sna*BI restriction site and that was also identified and referred to as P2 by Takayanagi et al. (22). The second putative start site is located in a region farther downstream (near an *Nru*I site) that, according to the same authors, conferred weak promoter activity when it was cloned into a promoter probe vector. However, no reverse transcript that corresponded to the most upstream P1, as it was localized by these authors, could be found. The fact that the rather large fragment (>500 bp) that seemed to carry P1 also contains the *nlpD* promoters farther upstream (9) probably accounts for its activity in the promoter probe vector (22). Our experiments shown here thus provide evidence for at most two putative promoters within the *nlpD* gene. Whereas a typical, probably σ^{70}-dependent promoter sequence (*rpoSp*$_1$) is readily apparent for the first one (Fig. 1A), the second putative start site is not preceded by a promoterlike sequence (Fig. 1B).

5′ deletion analysis of the upstream regulatory region of *rpoS*. In order to clarify the relative contributions of these two putative promoters to *rpoS* expression, we introduced 5′ deletions (starting from the *Cla*I restriction site upstream of the *nlpD* promoters) that consecutively eliminate the potential promoters. The restriction sites used for the construction, the positions of the putative promoters, and the regions upstream of *rpoS* present in the deletion plasmids are shown in Fig. 2. The plasmid from which the deletion constructs were derived was a pBR322 derivative carrying a *lacZ* fusion after nucleotide 70 within the *rpoS* gene as a reporter gene (8). We have previously shown that the deletions present in pRL30 and pRL31 that eliminate the *nlpD* promoters (either present on plasmids or in single copies in the chromosome) result in a 40% reduction of expression of this *rpoS*::*lacZ* fusion during steady-state exponential growth (determined after prolonged growth) (9). This indicates that a substantial fraction of the low level of σ^s in growing cells is synthesized from a polycistronic mRNA that also comprises the *nlpD* message. However, stationary-phase induction of *rpoS* is not affected by the deletion of the *nlpD* promoters (compare pRL48 and pRL49 in Fig. 3A and B, respectively), which is consistent with *nlpD* not being a stationary-phase-inducible gene (9). The finding that the stationary-phase induction of *rpoS*::*lacZ* present on pRL48 and pRL49 is very similar (Fig. 3A and B) again provides evidence against the reportedly stationary-phase-inducible P1 promoter

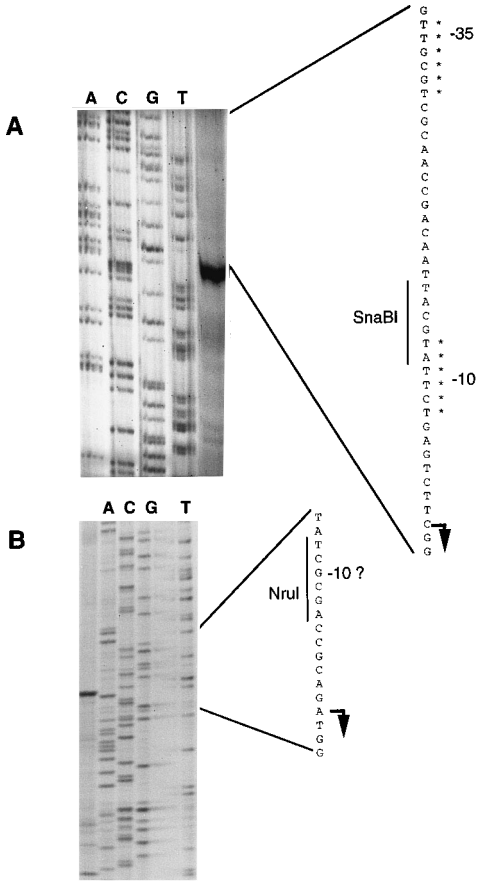

FIG. 1. Primer extension analyses for the determination of *rpoS* transcriptional start sites (A and B). RNA was prepared from strain MC4100 carrying pRL1-25 and was used for primer extension reactions as described in Materials and Methods. Sequencing reactions performed with pRL1-25 and the same primers are shown as a reference (lanes A, C, G, and T). Sequences corresponding to the coding strand are given for the relevant regions, and the putative promoter sequence is indicated by asterisks (A). For the locations of these sequences within *nlpD* and in relation to the *rpoS* structural gene, see Fig. 2.

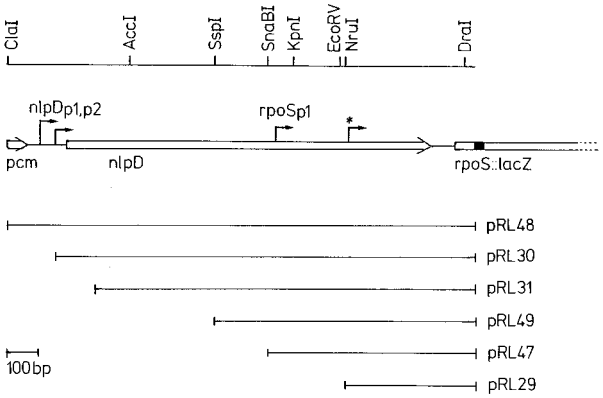

FIG. 2. Physical map and locations of deletions in the region upstream of *rpoS*. Plasmids carrying 5′ deletions in the region upstream of *rpoS* were constructed as described in Materials and Methods. Chromosomal DNA present in the deletion constructs is indicated as closed lines. The 5′ ends of chromosomal DNA in pRL49, pRL47, and pRL29 correspond to the *Ssp*I, *Sna*BI, and *Nru*I restriction sites, respectively. An asterisk indicates the second putative transcriptional start site for *rpoS* within *nlpD* that, however, is not preceded by an apparent promoter sequence.

that was proposed to be located between the *Acc*I and *Ssp*I restriction sites (Fig. 2) (22).

In contrast, the deletion upstream of the *Sna*BI restriction site that eliminates $rpoSp_1$ (pRL47) reduced the expression of the *rpoS*::*lacZ* reporter fusion approximately 20-fold, both during exponential phase and during entry into stationary phase (Fig. 3C; note the differences in scale). This demonstrates that $rpoSp_1$ is the major promoter involved in the control of *rpoS* expression. Further deleting to the *Nru*I restriction site, and thereby eliminating also the putative second promoter within the *nlpD* coding region (pRL29), does not significantly affect the residual expression that can be observed in the absence of $rpoSp_1$ (compare Fig. 3C and D). This indicates that the putative second transcriptional start site identified by primer extension does not play a significant role in the expression of *rpoS*. Also, since no sequence with at least some similarity to the −10 consensus for recognition by σ^{70} is apparent in this region (Fig. 1B), it seems more likely that the mRNA giving rise to the reverse transcript observed in Fig. 1B is a stable degradation product of the primary mRNA originating at $rpoSp_1$. Both the primary *rpoS* transcript and the putative degradation product have the potential to form extended stem-loop secondary structures close to their 5′ ends that may play a role in the stabilization of these transcripts (data not shown).

We also tried to transfer the fusion constructs carrying the various deletions into the chromosome in order to obtain single-copy constructs (by recombination onto λRZ5 [16], as previously described [8]). Whereas similar results were obtained with single-copy fusion derivatives of pRL48 and pRL49, no blue plaques, i.e., λRZ5 derivatives carrying the respective fusions, could be obtained with the constructs that are devoid of $rpoSp_1$ (pRL47 and pRL29), which also indicates that a putative second promoter around the *Nru*I site or any other region present farther downstream does not activate expression in a single-copy situation (data not shown). The low-level expression observed in the multicopy constructs could be due to some sequence downstream from the *Nru*I site that artificially might act as a weak promoter or due to some weak transcriptional readthrough from the vector into the insert in pRL47 and pRL29. The residual stationary-phase induction of these constructs may be caused by a posttranscriptional mech-

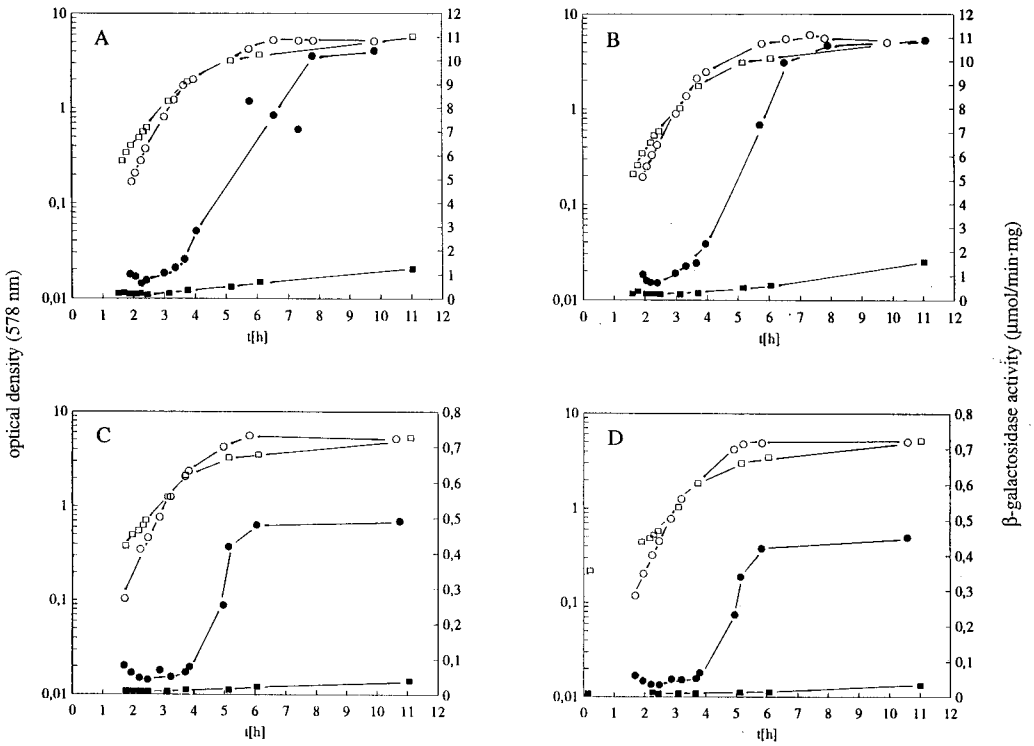

FIG. 3. Expression of *rpoS*::*lacZ* present on plasmids carrying various deletions in the region upstream of *rpoS*. Strains RO206 (*relA*$^+$ *spoT*$^+$; circles) and RO98 (Δ*relA* Δ*spoT*; squares) carrying pRL48 (A), pRL49 (B), pRL47 (C), and pRL29 (D) (see Fig. 2 for plasmid details) were grown in LB medium containing ampicillin (50 μg/ml). Optical densities (open symbols) and specific β-galactosidase activities (solid symbols) were determined along the growth curve.

anism. The *rpoS*::*lacZ* fusion used in these constructs is an early translational fusion that, in contrast to that for a transcriptional fusion with the same point of insertion in *rpoS*, exhibits weak stationary-phase induction (whereas strong stationary-phase induction as well as osmotic induction can be observed for a late translational fusion only) (8).

Taken together, our results indicate that apart from the two relatively weak and non-growth-phase-regulated *nlpD* promoters, there is just one promoter ($rpoSp_1$) within the *nlpD* structural gene and that this promoter is responsible for the increased transcription of *rpoS* during the entry into stationary phase in cells grown in rich LB medium. $rpoSp_1$ corresponds to P2 as identified by Takayanagi et al. (22). The sequence of this promoter conforms relatively well to the σ^{70} consensus sequence (−35, TTGCGT; −10, TATTCT, with a spacing of 17 bp). Consistent with transcription at $rpoSp_1$ being initiated by σ^{70}, transcriptional autoregulation of *rpoS* has been excluded previously (8, 19).

How could Takayanagi et al. (22) come to the conclusion that *rpoS* was preceded by four stationary-phase-inducible promoters? The first and second fragments derived from the *nlpD* region that conferred activity in their promoter probe vector carry the *nlpD* promoters (beside the region that contains the purported P1) and $rpoSp_1$, respectively. A third fragment that contained the putative promoters P3 and P4 conferred very low activity, which might be due to a cloning artifact. Unfortunately, no attempt to assay the promoter activity of these segments in a single-copy situation was made, and it seems likely that the apparent threefold stationary-phase induction observed with the first and third fragments was due to a growth-phase-dependent copy number increase of the plasmids carrying these constructs.

The role of ppGpp in the expression of *rpoS*. ppGpp-free *relA spoT* mutants exhibit reduced σ^s levels and a pleiotropic phenotype reminiscent of that of *rpoS* mutants. In addition, artificial overproduction of ppGpp was found to result in an increased σ^s level (3). ppGpp thus appears to be a positively acting signal molecule in the control of *rpoS* expression. ppGpp interferes with the transcription of many stringently controlled genes, and the cellular level of ppGpp increases in response to starvation for amino acids or sources of carbon, phosphorus, or nitrogen (1, 3). It thus seemed possible that transcription of *rpoS*, which is also an inversely growth-rate-controlled gene (7), is under positive stringent control. We therefore tested the expression of single-copy transcriptional and translational *lacZ* fusions inserted at the same position within *rpoS* (8) in genetic backgrounds that were either wild type with respect to ppGpp synthesis ($relA^+$ $spoT^+$) or deficient for ppGpp (Δ*relA* Δ*spoT*). Fig. 4 shows that the expression of both fusions was strongly reduced in the ppGpp-deficient strains.

These results seemed to indicate that ppGpp positively affects the transcription of *rpoS*. Since this effect was considerable, we speculated that the major promoter $rpoSp_1$ might require ppGpp for full activity. However, when the various promoter deletion constructs described above were examined in the *relA spoT* double mutant background, a more than tenfold reduction was found with all deletion plasmids, i.e., also with those that did not carry $rpoSp_1$ (squares in Fig. 3). These results indicate that the *relA spoT* double mutation does not specifically influence transcription initiation at any of the promoters upstream of *rpoS* but rather affects elongation or transcript stability. With *lacZ* being used as a model system, it was shown that in response to amino acid starvation, transcription and translation become uncoupled in a ppGpp-deficient mutant, which then results in premature transcriptional termination and therefore reduced levels of *lacZ* mRNA (2, 23). This

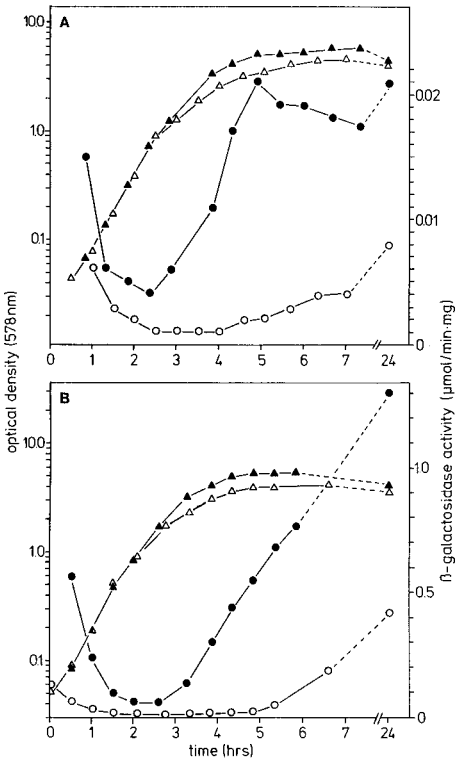

FIG. 4. The expression of transcriptional and translational *rpoS*::*lacZ* fusions is reduced in ppGpp-free strains. Strains RH140 (carrying a transcriptional *rpoS*::*lacZ* fusion in a $relA^+$ $spoT^+$ background; solid symbols in panel A) and RH137 (carrying a translational *rpoS*::*lacZ* fusion in a $relA^+$ $spoT^+$ background; solid symbols in panel B) as well as the corresponding Δ*relA* Δ*spoT* derivatives RH142 and RH139 (open symbols in panels A and B, respectively) were grown in LB medium. Optical densities (triangles) and specific β-galactosidase activities (circles) were determined along the growth curve.

effect may account for the lower expression of our various *rpoS*::*lacZ* fusions, especially during entry into a starvation situation. While this ppGpp effect has been described for *lacZ* (2, 23), it is probably of a more general nature. Figure 5 demonstrates that the level of *rpoS* wild-type mRNA was also significantly reduced in the *relA spoT* mutant (to an extent that was similar to that observed for *rpoS*::*lacZ* mRNA) (data not shown). Taken together, these data indicate that ppGpp plays a role in transcriptional elongation of *rpoS* (and probably other genes as well) similar to that observed for *lacZ*.

In addition, we have evidence that ppGpp also acts as a positive signal in the signal transduction pathway that controls osmotic induction of *rpoS* (14). Since osmotic control of *rpoS* operates at the posttranscriptional level (8), this indicates a

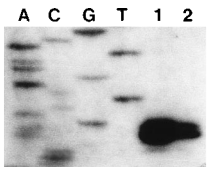

FIG. 5. The amount of transcript originating at $rpoSp_1$ is reduced in a Δ*relA* Δ*spoT* strain. RNA was prepared from strains MC4100 (lane 1) and RO98 (Δ*relA* Δ*spoT*; lane 2) carrying pRH320 grown in LB medium containing ampicillin (50 μg/ml) and was subjected to nonradioactive primer extension analysis as described in Materials and Methods. As a reference, sequencing reactions performed with the same primer and pRH320 are shown (lanes A, C, G, and T).

second, not-yet-defined role for ppGpp in the regulation of *rpoS*. Further work to elucidate the various functions of ppGpp in the control of σ^s expression is in progress.

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