JOURNAL OF BACTERIOLOGY, Mar. 1996, p. 1607–1613 Vol. 178, No. 6
0021-9193/96/$04.00+0

Posttranscriptional Osmotic Regulation of the σ^S Subunit of RNA Polymerase in *Escherichia coli*

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Received 21 September 1995/Accepted 15 January 1996

The σ^S subunit of RNA polymerase (encoded by the *rpoS* gene) is a master regulator in a complex regulatory network that governs the expression of many stationary-phase-induced and osmotically regulated genes in *Escherichia coli*. *rpoS* expression is itself osmotically regulated by a mechanism that operates at the posttranscriptional level. Cells growing at high osmolarity already exhibit increased levels of σ^S during the exponential phase of growth. Osmotic induction of *rpoS* can be triggered by addition of NaCl or sucrose and is alleviated by glycine betaine. Stimulation of *rpoS* translation and a change in the half-life of σ^S from 3 to 50 min both contribute to osmotic induction. Experiments with *lacZ* fusions inserted at different positions within the *rpoS* gene indicate that an element required for σ^S degradation is encoded between nucleotides 379 and 742 of the *rpoS* coding sequence.

Like most single-cell organisms, bacteria must be able to cope with extreme fluctuations in the composition and physical parameters of their environments. One of these parameters is the osmolarity of the surrounding medium. When *Escherichia coli* cells experience a shift to high osmolarity, influx of potassium ions and synthesis of glutamate are strongly stimulated. This rapid response is followed by uptake from the medium and/or synthesis of compatible solutes and osmoprotectants like glycine betaine, proline, or trehalose (for a review, see reference 5). In parallel, the induction of numerous proteins can be observed by two-dimensional O'Farrell gel electrophoresis (4, 14). Several corresponding genes have been identified, for instance, by isolating hyperosmotically inducible *lacZ* or *phoA* gene fusions (1, 3, 6, 8–10, 35).

With respect to the regulatory mechanisms involved, two systems have been studied in detail. One is the *proU* operon, which encodes a glycine betaine uptake system (for a recent review, see reference 24), whereas the other is the *ompF*/*ompC* porin system, which is controlled by a typical two-component regulatory system consisting of the membrane-bound sensory histidine kinase EnvZ and the response regulatory OmpR (15, 17, 26, 27). However, the regulatory mechanisms involved do not seem to play a general role in the osmotic regulation of many genes, and efforts to identify a global osmotic regulator have failed.

By contrast, several other hyperosmotically induced genes (*otsBA*, *treA*, *osmB*, *osmY*, and *bolA*) are under the control of σ^S, a sigma subunit of RNA polymerase in *E. coli* that is encoded by the *rpoS* gene (13, 14, 19, 34). This seems to implicate σ^S as a global regulator in the osmotic control of gene expression. In fact, this would be a second global regulatory role for σ^S, which is usually regarded as a stationary-phase-specific sigma factor since the genes mentioned above, as well as many other σ^S-dependent genes, are induced during entry into stationary phase (for recent reviews, see references 11, 12, and 23). Besides being growth phase regulated at the levels of transcription, translation, and σ^S protein stability, *rpoS* has also been shown to be hyperosmotically induced by an unknown mechanism that, in contrast to the osmoregulatory systems mentioned above, operates at the posttranscriptional level (21).

In the present report, we present an analysis of the osmotic regulation of σ^S. By using a combination of gene fusion analysis and immunoblot and pulse-chase experiments, we came to the conclusion that osmotic induction of σ^S is based on stimulation of *rpoS* translation, as well as on inhibition of σ^S turnover, and that a sequence element encoded in the second third of *rpoS* is involved in σ^S degradation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are derivatives of MC4100 [F$^-$ Δ(*arg-lac*)*U169 araD139 rpsL10 ptsF25 flbB5301 rbsR deoC relA1*) (32). Strains RO35, RO90, and RO91 are single-copy lysogens of λRZ5 (29) carrying the translational fusions *rpoS70*::*lacZ*, *rpoS379*::*lacZ*, and *rpoS742*::*lacZ*, respectively. Strain RO200 carries a transcriptional version of *rpoS742*::*lacZ*. The numbers in the fusion designations indicate the nucleotides within the *rpoS* coding region after which the fusions are inserted. The isolation of these fusions has been described before (21).

Cells were grown aerobically in minimal medium M9 (25) with glycerol (0.4%) as the carbon source. For osmotic shift experiments, the cultures were grown for three or more generations before they were divided into two aliquots to one of which 0.3 M NaCl or 0.464 M sucrose was added.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Preparation of samples, SDS-PAGE, and immunoblot analysis were performed as previously described (21). For immunoblot analysis, the antiserum against σ^S described by Lange and Hengge-Aronis (21) was used. This antibody was obtained against σ^S purified in our own laboratory (21) and is not identical to an antiserum described earlier (14) that had been isolated by using a purified protein not prepared in our laboratory. Although originally described as a σ^S antibody, the latter antibody was later found to recognize not σ^S but the product of gene *nlpD*, which is located immediately upstream of *rpoS*, has a molecular weight similar to that of σ^S, and is also not expressed in the Δ*rpoS360* mutant (22).

Pulse-labeling of cells and immunoprecipitation. The procedure used for pulse-labeling of cells with L-[^{35}S]methionine and immunoprecipitation of σ^S was previously described (21). For labeling, the optical density at 578 nm (OD_{578}) of the samples was adjusted by using supernatant freshly prepared from the same cultures. Pulse-labeling was performed, and the samples were further processed as previously reported (21). Polyclonal antisera against σ^S (see above) and β-galactosidase were used for immunoprecipitation. As a σ^S-deficient control, strain RH90 was used (labeled in exponential-phase samples harvested at an OD_{578} of 0.5). Immunoprecipitated proteins were quantified directly from the dried gels by using a PhosphorImager (Molecular Dynamics) or by densitometric quantification of bands on autoradiographs (which yielded similar results).

β-Galactosidase assay. β-Galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galactopyranoside as a substrate and is reported as micromoles of *o*-nitrophenol per minute per milligram of cellular protein (25).

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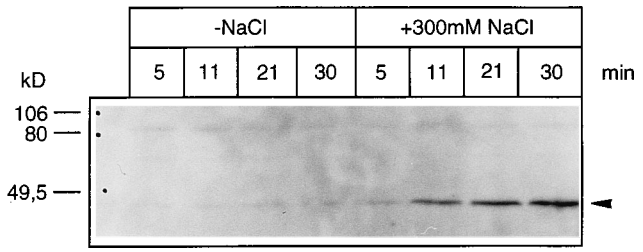

FIG. 1. Cellular levels of σ^s protein after osmotic upshift. Strain MC4100 was grown in M9 medium with 0.4% glycerol. At an OD_{578} of 0.3, the culture was divided into two aliquots. NaCl (0.3 M) was added to one aliquot (lanes in the right half of the gel), whereas the control aliquot did not receive any supplement (lanes in the left half of the gel). Samples were withdrawn at 5, 11, 21, and 30 min after addition of NaCl and subjected to immunoblot analysis with an antiserum against σ^s as described in Materials and Methods. The σ^s band is marked by the arrowhead. Size standards are indicated to the left. kD, kilodaltons.

RESULTS

Characterization of the osmotic regulation of *rpoS*. The expression of many σ^s-controlled genes is stimulated in response to an osmotic upshift in the growth medium (14). Figure 1 shows that this is accompanied by a substantial increase in the expression of σ^s itself. With Fig. 1, we also correct our previous view that the level of σ^s protein was not increased in response to osmotic upshift (14) (see Materials and Methods for technical differences from our previous experiments). The finding of strongly increased cellular σ^s content under conditions of osmotic upshift is consistent with the previously reported osmotic regulation of the translational *rpoS742*::*lacZ* fusion (encoding a hybrid protein containing the amino-terminal 247 amino acids of σ^s). By contrast, osmoinduction was not found for a transcriptional fusion with the same fusion joint (21). These results indicate that osmotic regulation of *rpoS* operates at the posttranscriptional level.

rpoS expression is not only stimulated in response to osmotic upshift but constantly elevated under conditions of continuous incubation at increased osmolarity. Approximately fourfold higher levels of σ^s were found by immunoblot analysis of samples obtained from cultures continuously growing in the presence of 0.3 M NaCl (data not shown). Similar results were obtained with *rpoS*::*lacZ* fusions under the same conditions (Fig. 2). Again, higher expression in the presence of NaCl was observed only for the translational *rpoS742*::*lacZ* fusion (Fig. 2A and B) and not for the corresponding transcriptional fusion (Fig. 2C and D). The expression of the transcriptional fusion was even somewhat lower in the presence of 0.3 M NaCl. Like

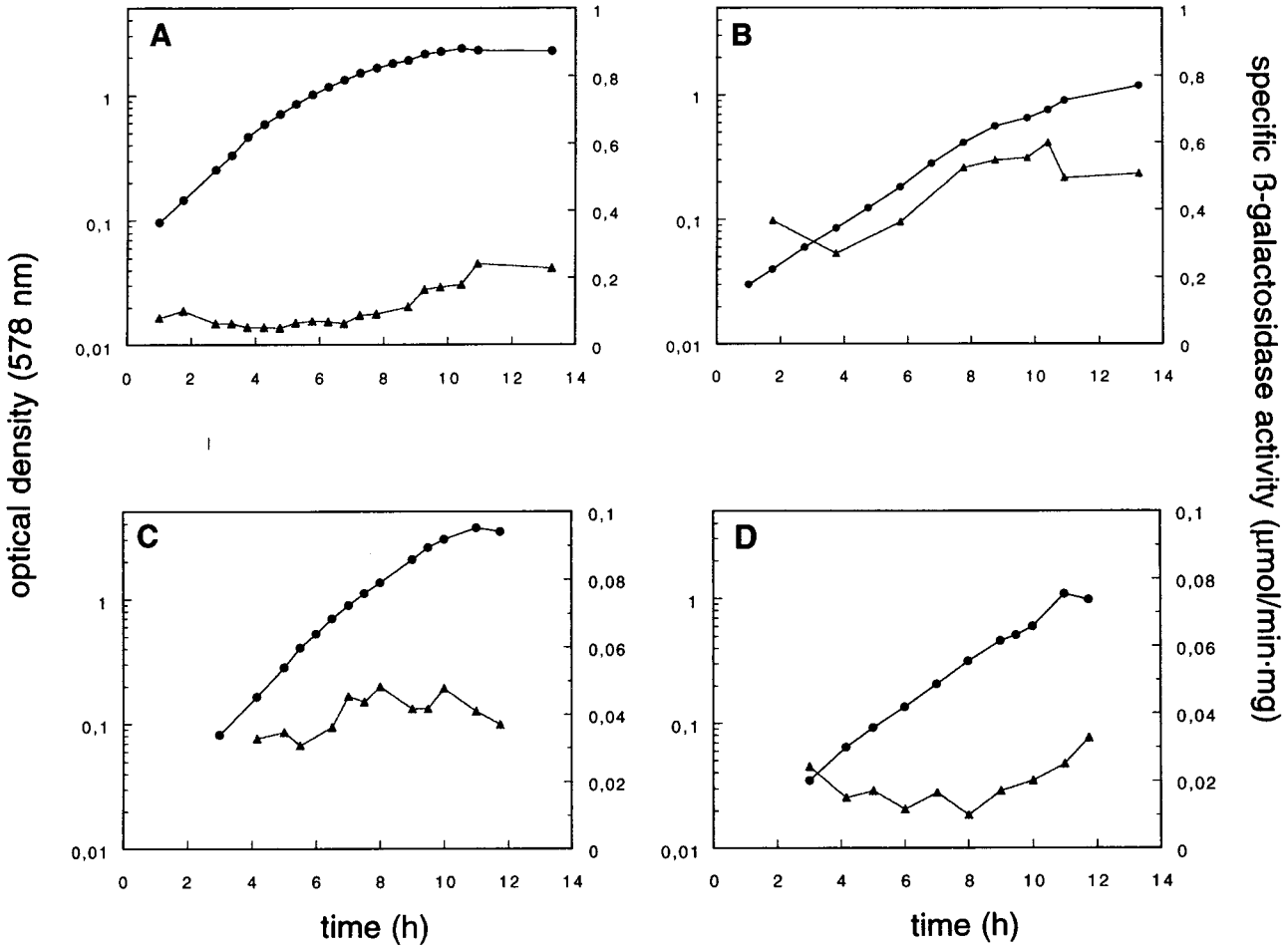

FIG. 2. Expression of translational and transcriptional *rpoS*::*lacZ* fusions during continuous growth in the absence or presence of 0.3 M NaCl. Strains RO91 (A and B) and RO200 (C and D), carrying the translational and transcriptional *rpoS742*::*lacZ* fusions, respectively, were grown in M9 with 0.4% glycerol (A and C) or M9 with 0.4% glycerol and 0.3 M NaCl (B and D) (also, the cultures used for inoculation had been grown in the presence or absence of NaCl). Optical densities (●) and β-galactosidase specific activities (▲) were determined.

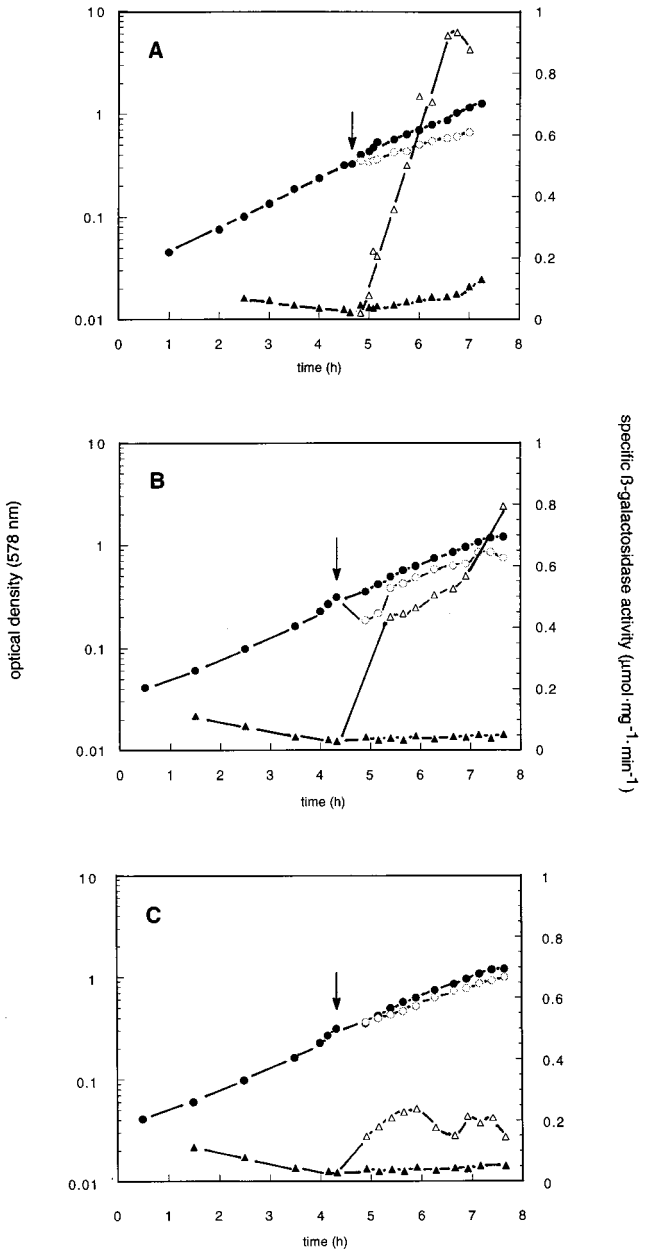

FIG. 3. Osmotic induction of *rpoS* by NaCl and sucrose and effect of glycine betaine. Strain RO91 (carrying the late translational *rpoS*::*lacZ* fusion) was grown in M9 medium containing 0.4% glycerol. At optical densities of approximately 0.3, the cultures were each divided into two aliquots, one of which received either 0.3 M NaCl (A), 0.464 M sucrose (B), or 0.3 M NaCl together with 2 mM glycine betaine (C) (arrows). Optical densities (● and ○) and β-galactosidase specific activities (▲ and △) were determined in the absence (closed symbols) or presence (open symbols) of the above-mentioned supplements.

the osmotic shift experiments reported earlier (21), these findings indicate that osmotic induction of *rpoS* occurs at the posttranscriptional level.

rpoS expression was also stimulated in response to the addition of a nonionic, osmotically active substance. As demonstrated in Fig. 3A and B, *rpoS* expression was stimulated when the osmolarity was increased by adding either NaCl (0.3 M) or sucrose (0.464 M), indicating true osmotic regulation. This is further substantiated by the finding that glycine betaine, when added simultaneously with NaCl, interfered with osmotic induction of *rpoS* (Fig. 3C). Glycine betaine is a potent osmoprotectant that is known to reduce the osmotic induction of, e.g., the *proU* operon (18, 24).

The rate of σ^s expression and the half-life of σ^s are changed in response to osmotic upshift. We have previously shown that two distinct mechanisms contribute to the growth phase-dependent posttranscriptional regulation of σ^s. Already during the late exponential growth phase, *rpoS* translation is stimulated. This increase in translation can be observed only in cultures that have reached a certain density (above an OD_{578} of 0.5 or 0.6) but are not yet starved and therefore appears to be a cell density-dependent process. In addition, σ^s turnover is inhibited after the onset of starvation (21).

In pulse-chase experiments, changes in *rpoS* expression and in σ^s turnover in response to osmotic upshift were detected (Fig. 4). Only 8 min after addition of 0.3 M NaCl to an exponentially growing culture of strain MC4100, the rate of σ^s expression was increased by a factor of 3.5 (compare the intensities of the σ^s bands in the first lanes of Fig. 4A and B). At 45 min after osmotic upshift, the rate of σ^s expression was twofold higher than before NaCl treatment (Fig. 4C). Since our previously published experiments (21) with transcriptional and translational *rpoS*::*lacZ* fusions indicate that *rpoS* mRNA levels are not affected by osmotic upshift, the increased σ^s expression shown here reflects an increased rate of *rpoS* translation.

Figure 4 also shows changes in σ^s stability that occur as a consequence of osmotic upshift. Densitometric quantification of the bands obtained after various chase times yielded σ^s half-lives of 3 min before osmotic upshift, more than 50 min directly after osmotic upshift, and 16 min after prolonged incubation with 0.3 M NaCl (Fig. 5D). Taken together, these data demonstrate that an increase in medium osmolarity affects both the rate of *rpoS* translation and the rate of σ^s turnover. Moreover, these effects are somewhat alleviated during prolonged incubation in the presence of NaCl, indicating a negative feedback or adaptation mechanism.

A region between nucleotides 379 and 742 within *rpoS* encodes an element required for σ^s turnover. The absolute levels of expression and the osmotic regulation of translational *lacZ* fusions inserted at different positions in the *rpoS* gene are markedly different. The *rpoS742*::*lacZ* fusion exhibited relatively rapid and more than 10-fold osmotic induction (Fig. 3A and 5B). By contrast, a fusion with a shorter *rpoS* part, *rpoS379*::*lacZ*, showed a basal level of expression in a steadily growing culture that was nearly as high as the osmotically induced level observed for *rpoS742*::*lacZ* (Fig. 5A). In addition to this high basal level of expression, *rpoS379*::*lacZ* was induced approximately twofold in response to NaCl addition. These data indicate that a region located between nucleotides 379 and 742 of the *rpoS* structural gene may encode an element that downregulates σ^s expression and that the mechanism that makes use of this element is involved in the osmotic induction of σ^s.

To clarify the role of this region in the regulation of σ^s, we compared the rates of synthesis and turnover of RpoS::LacZ hybrid proteins that either contain or do not contain this region. The relative rates of synthesis before and shortly after osmotic upshift were determined for the hybrid proteins encoded by *rpoS70*::*lacZ*, *rpoS379*::*lacZ*, and *rpoS742*::*lacZ*. Labeling and chase times were short (60 and 30 s, respectively), such that potential differences in hybrid protein stability should not play a role. Figure 6 demonstrates that the osmotic induction of expression and, therefore, translational regulation is very similar for the hybrid proteins encoded by *rpoS379*::*lacZ* and *rpoS742*::*lacZ*, whereas the small hybrid protein encoded

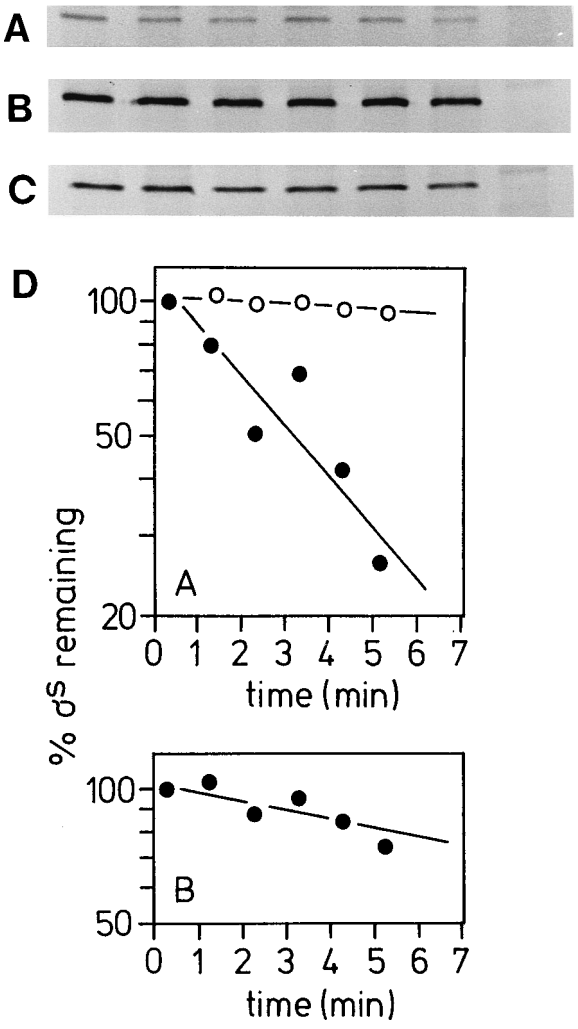

FIG. 4. σ^s synthesis and turnover before and after osmotic upshift. Strain MC4100 was grown in M9 plus 0.4% glycerol to an OD_{578} of 0.3. Aliquots were pulse-labeled for 1 min with [^{35}S]methionine immediately before (A) and 8 (B) and 45 (C) min after addition of 0.3 M NaCl. The chase times with nonradioactive methionine were 0.25, 1.25, 2.25, 3.25, 4.25, and 5.25 min. Samples were further processed and subjected to immunoprecipitation and SDS-PAGE as described in Materials and Methods. As a control, *rpoS* mutant strain RH90 was used (last lanes in A, B, and C). Autoradiographs are shown in A, B, and C, and densitometric quantification of the autoradiographs is shown in D. (D) Graph A, data from panels A (solid symbols) and B (open symbols); graph B, data from panel C.

by *rpoS70*::*lacZ* exhibited weaker osmotic induction. These data indicate that the region encoded between nucleotides 379 and 742 in *rpoS* is not involved in the control of σ^s synthesis.

There is, however, a pronounced difference in stability between the hybrid proteins encoded by *rpoS379*::*lacZ* and *rpoS742*::*lacZ*. Whereas the former was stable during a 10-min chase period, that latter was nearly completely degraded during this time (Fig. 7A). The half-life of the unstable fusion protein was estimated to be in the same range as that of σ^s itself. After osmotic upshift, both hybrid proteins were stable (Fig. 7B). In addition, we found that the *rpoS742*::*lacZ*-encoded hybrid protein was also stable in the absence of NaCl when expressed in mutants that were deficient in either RssB or ClpP (data not shown), i.e., components that are known to be required for rapid turnover of σ^s in nonstressed cells (28, 31). The turnover of the hybrid protein encoded by *rpoS742*::*lacZ* is thus mediated by the same mechanism that is responsible for the degradation of σ^s itself. Since the shorter hybrid protein encoded by *rpoS379*::*lacZ* is not subject to this turnover, we conclude that a putative "turnover element" encoded between nucleotides 379 and 742 in the *rpoS* structural gene is essential for σ^s turnover and may function as a recognition site for a component of the degradative pathway.

DISCUSSION

The σ^s subunit of RNA polymerase has received a great deal of attention because of its central role in the control of expression of stationary-phase-induced genes (11, 12, 23). More recently, however, it has been shown that σ^s is also required for hyperosmotic induction of various genes in exponentially growing cells (13, 14, 34). Here we demonstrate that σ^s itself is strongly induced upon osmotic upshift (Fig. 1) and is expressed at elevated levels in cells growing continuously at increased osmolarity (Fig. 2). Induction of σ^s can be triggered by addition of NaCl, as well as of sucrose, indicating true osmotic regulation rather than a salt effect (Fig. 3). We conclude that besides being a master regulator for stationary-phase-induced genes, σ^s is also a global regulator in the osmotic control of gene expression in *E. coli.*

In contrast to that of nearly all other osmotically regulated systems in *E. coli*, the osmotic control of σ^s occurs at the posttranscriptional level (to our knowledge, the only other posttranscriptional osmoregulation is that of *ompF* by the *micF* antisense RNA during a shift from low to intermediate osmolarity [30]). In the case of σ^s, increased translation and inhibition of σ^s turnover contribute to osmotic induction (Fig. 4). These two processes occur simultaneously. This is unlike the situation during the transition into stationary phase, in which translation is already stimulated during the late exponential phase when the culture reaches a certain cell density but σ^s turnover is inhibited only at the onset of starvation (21). Figure 8 summarizes the different levels of σ^s regulation, as well as the conditions affecting these levels of control.

It seems reasonable to assume that the translational control of *rpoS* involves mRNA secondary structures that may prevent binding of the ribosome to the translational initiation region (TIR) of the *rpoS* mRNA. Under inducing conditions, the mRNA secondary structures might be dissolved or changed in such a way that translational initiation can take place. We have previously suggested that the *rpoS* TIR may base pair to a sequence located between nucleotides 154 and 185 in the *rpoS* coding region (21). This would be consistent with the reduced osmotic induction of *rpoS70*::*lacZ* which does not contain the proposed downstream antisense element (21 and Fig. 6). However, it seems that osmotic regulation of *rpoS70*::*lacZ* is not totally abolished (Fig. 6), indicating that sequences upstream of nucleotide 70 in *rpoS* may also be involved in translational control. We have recently found that alternative secondary structures, for instance, between the TIR and a region located directly upstream of the TIR, are also possible. Site-directed mutagenesis of the complementary elements that may base pair with the *rpoS* TIR are required to unequivocally identify the sequence elements that are crucial for the translational control of *rpoS.*

A second sequence element involved in the osmotic regulation of *rpoS* was shown to be located between nucleotides 397 and 742 in the *rpoS* coding sequence. This element down-regulates σ^s, and part of the osmotic induction appears to be a relief of this down-regulation. While we have shown that translational control of *rpoS* is not dependent on the presence of this element (Fig. 6), several lines of evidence indicate that the

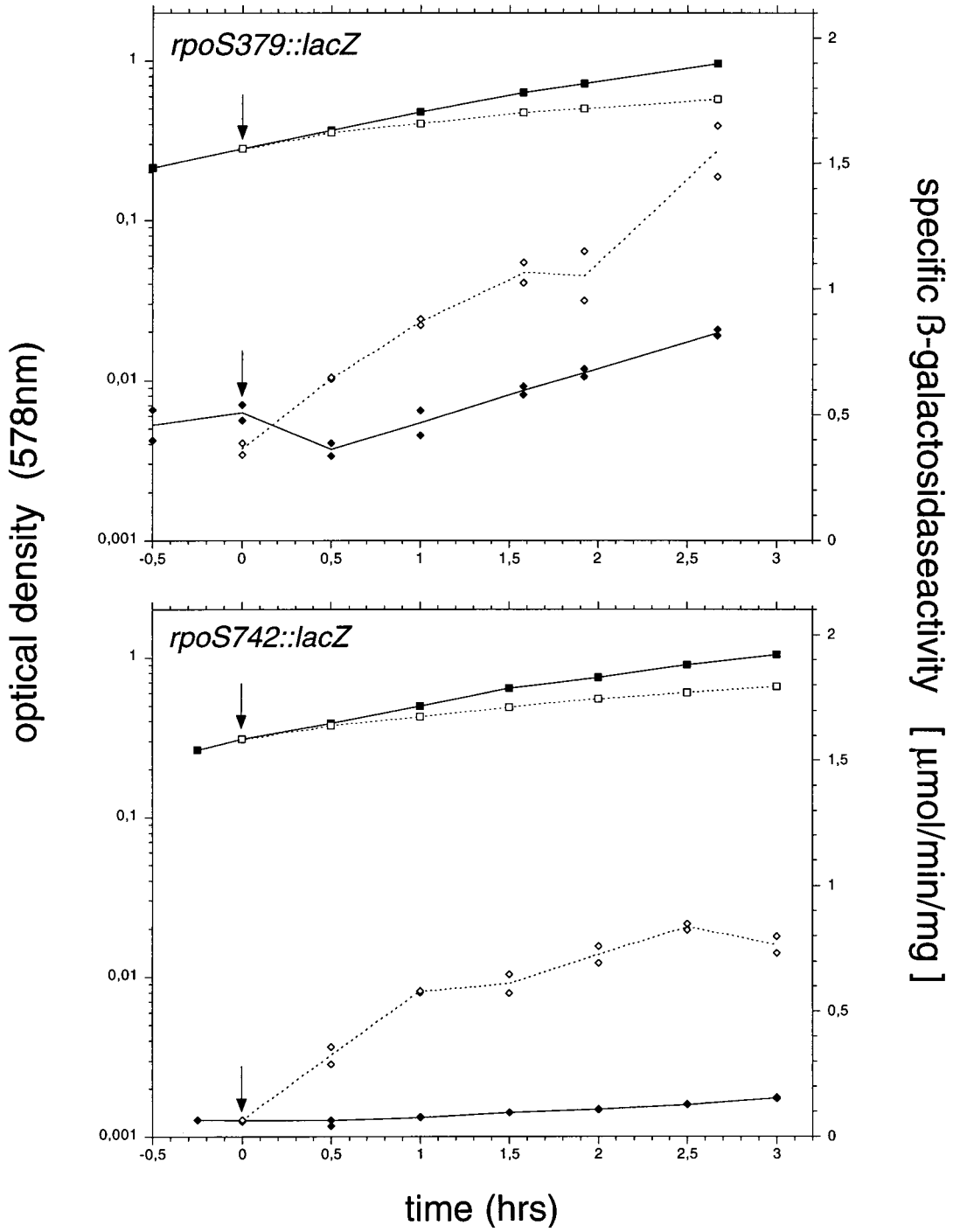

FIG. 5. Osmotic induction of different translational *rpoS*::*lacZ* fusions. Strains RO90 and RO91 carrying *lacZ* fusions inserted after nucleotides 379 and 742, respectively, of the *rpoS* coding region were grown and osmotically induced with 0.3 M NaCl as described in the legend to Fig. 3. Optical densities (squares) and β-galactosidase specific activities (diamonds) were determined in the NaCl-free (closed symbols) and NaCl-treated cultures (open symbols).

region encoded between nucleotides 379 and 742 in *rpoS* is crucial for σ^s turnover. First, a hybrid protein containing this region is subject to turnover, whereas a shorter hybrid protein not containing the turnover element is stable (Fig. 7A). Remarkably, the RpoS742::LacZ hybrid protein is totally degraded, whereas in general, only the N-terminal extension is cleaved off in vivo from LacZ hybrid proteins and the resulting β-galactosidase is usually stable. It seems that the presence of the turnover element results in recognition by a protease machinery that fully degrades a protein regardless of its biochemical properties. Second, in response to osmotic upshift and just like σ^s itself, the RpoS742::LacZ hybrid protein becomes stable (Fig. 7B). Third, we have recently identified a novel response regulator, RssB, that is required for σ^s degradation and thus may be part of the protease machinery. Whereas *rssB* mutations result in a strong increase of expression of

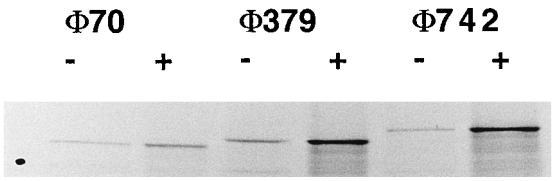

FIG. 6. Synthesis of β-galactosidase hybrid proteins containing σ^s parts of various lengths before and after osmotic upshift. Strains RO35, RO90, and RO91 carrying translational fusions *rpoS70*::*lacZ*, *rpoS379*::*lacZ*, and *rpoS742*::*lacZ*, respectively, were grown in M9 with 0.4% glycerol to an OD_{578} of 0.3. Aliquots were pulse-labeled for 1 min with [^{35}S]methionine immediately before (−) and 12 min after (+) the addition of 0.3 M NaCl, followed by a 30-s chase with nonradioactive methionine. Samples were further processed and subjected to immunoprecipitation with a polyclonal antibody against β-galactosidase and SDS-PAGE as described in Materials and Methods.

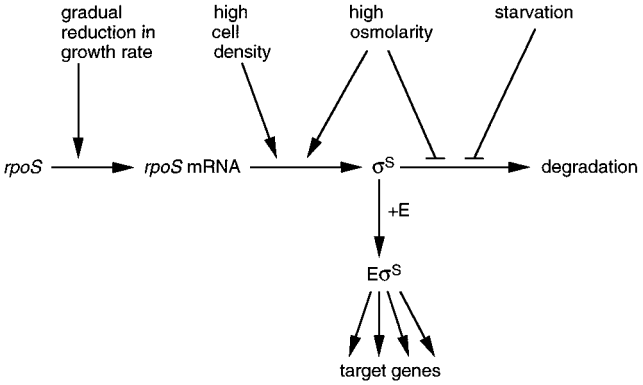

FIG. 8. Levels of regulation and environmental conditions affecting cellular σ^s content E, RNA polymerase core enzyme.

rpoS742::*lacZ*, they do not affect *rpoS379*::*lacZ*, indicating again that a putative target site for RssB-mediated σ^s degradation is encoded between nucleotides 379 and 742 (28). Fourth, we have observed that degradation of RpoS742::LacZ does not take place in a *clpP* mutant background. Recently, Schweder et al. have demonstrated that the ClpXP protease is involved in σ^s turnover, and by using a similar hybrid protein approach, they have also come to the conclusion that a region encoded approximately in the middle of *rpoS* plays a role in σ^s degradation (31). Finally, an element with a similar function (region C [36]) has also been located within the *rpoH* gene (encoding heat shock sigma factor σ^{32}, which also exhibits regulated turnover [33]), and comparable *rpoH*::*lacZ* fusions show regulatory behavior similar to that of our *rpoS*::*lacZ* fusions (36).

We have also observed that the initial stimulation of translation and the strong inhibition of σ^s turnover triggered by osmotic upshift are somewhat alleviated during prolonged incubation under conditions of high osmolarity. This indicates an adaptive mechanism activated with some delay after the initial osmotic upshift. Consistent with this, we observed increased steady-state levels of σ^s, as well as increased expression of the *rpoS*::*lacZ* fusion under conditions of continuous growth in high-osmolarity medium, but the absolute levels of expression were not as high as during the 2-h period directly after osmotic upshift (compare, e.g., Fig. 2B and 3A). The mechanism of this apparent adaptive regulation is unknown.

The multilayered control of σ^s must involve multiple unknown regulatory factors and, probably, complex signal transduction pathways. What are the intracellular signals for all of the environmental conditions (Fig. 8) that result in an increase in the cellular σ^s level? Evidence has been presented that ppGpp (7, 20), a homoserine lactone (16), and UDP-glucose (2) affect σ^s levels. The challenge for future work is to find the links between various changes in the environment, fluctuations in the cellular levels of these and perhaps additional putative signal molecules, and the multiple mechanisms of σ^s control.

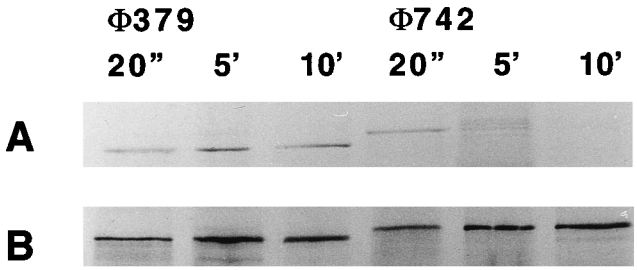

FIG. 7. Turnover of β-galactosidase hybrid proteins containing σ^s parts of various lengths before and after osmotic upshift. Strains RO90 and RO91 were grown and pulse-labeled (A) and then treated with NaCl and again pulse-labeled (B) as described in the legend to Fig. 6. The chase times were 20 s, 5 min, and 10 min. The samples were further processed as described in the legend to Fig. 6.

ACKNOWLEDGMENTS

We thank Winfried Boos (in whose laboratories this work was carried out) for continuous interest in the project and Daniela Fischer for excellent technical assistance. We thank Abdul Matin for communicating results prior to publication.

Financial support was provided by the Deutsche Forschungsgemeinschft (Schwerpunkt-Programm "Regulatory Networks in Bacteria," He-1556-5-1) and by the Fonds der Chemischen Industrie. Andrea Muffler is the recipient of a fellowship from the Graduiertenförderung des Landes Baden-Württemberg.

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