The cellular concentration of the σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability

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The second vegetative sigma factor σ^S (encoded by the *rpoS* gene) is the master regulator in a complex regulatory network that governs the expression of many stationary phase-induced and osmotically regulated genes in *Escherichia coli*. Using a combination of gene-fusion technology and quantitative immunoblot, pulse-labeling, and immunoprecipitation analyses, we demonstrate here that *rpoS*/σ^S expression is not only transcriptionally controlled, but is also extensively regulated at the levels of translation and protein stability. *rpoS* transcription is inversely correlated with growth rate and and is negatively controlled by cAMP–CRP. In complex medium *rpoS* transcription is stimulated during entry into stationary phase, whereas in minimal media, it is not significantly induced. *rpoS* translation is stimulated during transition into stationary phase as well as by an increase in medium osmolarity. A model involving mRNA secondary structure is suggested for this novel type of post-transcriptional growth phase-dependent and osmotic regulation. Furthermore, σ^S is a highly unstable protein in exponentially growing cells (with a half-life of 1.4 min), that is stabilized at the onset of starvation. When cells are grown in minimal glucose medium, translational induction and σ^S stabilization occur in a temporal order with the former being stimulated already in late exponential phase and the latter taking place at the onset of starvation. Although σ^S does not control its own transcription, it is apparently indirectly involved in a negative feedback control that operates on the post-transcriptional level. Our analysis also indicates that at least five different signals [cAMP, a growth rate-related signal (ppGpp?), a cell density signal, an osmotic signal, and a starvation signal] are involved in the control of all these processes that regulate *rpoS*/σ^S expression.

[*Key Words*: σ-factor; *rpoS*; stationary phase; starvation; osmotic regulation; post-transcriptional regulation; *rpoH*]

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In many bacterial species starvation for an essential nutrient triggers the onset of complex developmental programs resulting in the formation of highly stress-resistant spores that can survive long periods of starvation. Although *Escherichia coli* is usually classified among nondifferentiating bacteria, clear structural and physiological alterations can be observed during entry into stationary phase, which ultimately produce smaller and almost spherical cells exhibiting multiple stress resistance (for review, see Kolter et al. 1993). At least several dozen proteins are induced with distinct kinetics during the transition from the decelerating growth phase to late stationary phase, indicating a complex sequence of regulatory events (Groat et al. 1986; Almirón et al. 1992; Weichart et al. 1993).

A key regulatory gene for these processes is *rpoS*, which encodes a second vegetative σ-subunit of RNA polymerase (σ^S, Mulvey and Loewen 1989; Lange and Hengge-Aronis 1991; Tanaka et al. 1993). As a component of RNA polymerase, σ^S recognizes a set of promoters that are poorly recognized in vivo by polymerase holoenzyme containing the "housekeeping" σ-factor σ^{70} (Hengge-Aronis 1993b). Although growing cells contain a certain low amount of σ^S, σ^S is of primordial importance during stationary phase. *rpoS* mutants die off rapidly under starvation conditions and do not develop the multiple stress resistance so characteristic for stationary phase (Lange and Hengge-Aronis 1991; McCann et al. 1991). To date >20 genes or operons have been shown to

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be under the direct or indirect control of σ^S. The respective gene products have a wide variety of functions and can be found in all cellular compartments (for review, see Hengge-Aronis 1993a, 1993b).

A subset of σ^S-controlled genes also exhibits hyperosmotic induction during exponential growth. Although transcriptional induction of some of these genes can be as high as 50-fold, *rpoS* transcription is not significantly stimulated by increased medium osmolarity (Hengge-Aronis et al. 1993). This indicated that some post-transcriptional mechanism of σ^S expression or the activity of σ^S are influenced by changes in osmolarity or that other regulatory factors other than σ^S participate in osmotic regulation of some σ^S-dependent genes. Additional global regulatory proteins besides σ^S are also involved in the fine modulation of expression of many stationary phase-induced genes. Among these modulating factors are the 3′, -5′-cyclic AMP–cAMP receptor complex (cAMP–CRP) cAMP–CRP complex, Lrp, and integration host factor (Lange et al. 1993; Weichart et al. 1993). All of these regulatory factors together constitute a complex regulatory network.

It is still unknown what the external and internal stationary phase or starvation signals are, and how this information is transmitted into the control of expression or of activity of the various regulatory components involved in the stationary-phase response. As a first step in solving this problem we analyzed the regulation of *rpoS* expression. Earlier studies based on the analysis of gene fusions have shown that in general, *rpoS* expression increases during transition into stationary phase (Mulvey et al. 1990; Lange and Hengge-Aronis 1991; Schellhorn and Stones 1992). Also, some evidence for a post-transcriptional regulation was presented (Loewen et al. 1993; McCann et al. 1993). Sometimes contrasting results were reported, for example, with regard to a negative or a positive role of cAMP–CRP (Lange and Hengge-Aronis 1991; McCann et al. 1993) or to autoregulation or *rpoS* (Schellhorn and Stones 1992; McCann et al. 1993). In most cases, however, these studies were not comparable to each other because of the use of different strain backgrounds, different kinds of gene fusions in single or multicopy constructs, and varying growth and starvation conditions. Therefore, a clear picture of the apparently complex regulation of *rpoS* has not yet emerged.

In the present study we systematically investigated the regulation of *rpoS*/σ^S expression on the levels of transcription, translation, and protein stability by using a combination of gene fusion technology and quantitative immunoblot and pulse-chase immunoprecipitation analysis. Our results demonstrate that *rpoS* expression is regulated both on the transcriptional and post-transcriptional levels. Transition into stationary phase as well as an increase in medium osmolarity in growing cells strongly stimulate *rpoS* translation. In addition, σ^S was found to be a very unstable protein in exponentially growing cells. At the onset of starvation, σ^S half-life increases more than sevenfold. Protein stabilization is thus a major factor contributing to the strong increase of σ^S content in stationary-phase cells.

Results

Transcriptional and post-transcriptional control of rpoS *in relation to growth phase as determined by gene fusion and immunoblot analyses*

To experimentally separate transcription and translation of *rpoS*, a series of transcriptional and translational *lacZ* gene fusions was constructed. As a first step, Tn*lacZ* (Manoil 1990) insertions in *rpoS* were isolated on a plasmid (pRH320; Lange and Hengge-Aronis 1991) that carries >1.25 kb upstream of *rpoS*. This upstream region contains the entire adjacent gene (*nlpD*) as well as all sequence determinants involved in transcriptional control of *rpoS* (Lange and Hengge-Aronis 1994). For this study two of these translational *lacZ* fusions, an "early" and a "late" fusion (encoding hybrid proteins carrying the amino-terminal 23 and 247 amino acids, respectively, of a total of 329 amino acids of σ^S) were chosen. Transcriptional fusion derivatives with identical fusion joints and upstream sequences were constructed from the translational fusions. All fusions were crossed onto λRZ5 (Ostrow et al. 1986) and integrated at the *att* site in the chromosome (see Material and methods for details).

The expression of these four chromosomal *rpoS*::*lacZ* fusions was tested along the growth curve in cultures grown in minimal medium with a limiting concentration of glucose (Fig. 1). Both transcriptional fusions exhibited very little, if any, induction during entry into stationary phase (Fig. 1A,B). This corresponds to our results reported previously, obtained with a transcriptional fusion in the chromosomal copy of *rpoS*, which is not significantly induced in cells grown in minimal medium and starved for carbon (Lange and Hengge-Aronis 1991), nitrogen, or phosphate (Weichart et al. 1993). In contrast, the early and late translational fusions were induced two- and fivefold, respectively, during transition into stationary phase. The specific activity of β-galactosidase synthesized from these two fusions continued to increase for at least 3 hr after the onset of glucose starvation (Fig. 1C,D).

When the cells were grown in complex Luria–Bertani (LB) medium, stronger induction of all four *rpoS*::*lacZ* fusions was observed. Whereas the transcriptional fusions were induced fivefold, specific β-galactosidase activity of the early and late translational fusions increased by factors of 10 and 30, respectively (Fig. 2).

Corresponding increases in cellular σ^S content were found in immunoblot experiments (Fig. 3). Densitometric quantitation of the immunoblot data shown in Figure 3 yielded at least a 4.5- and a 6.5-fold increase in σ^S content in cells grown in minimal medium and in LB, respectively (lanes 1 and 7 were compared in Fig. 3A,B). When the cells were grown in minimal glucose medium, σ^S content continued to increase for at least 2 hr in stationary phase (Fig. 3A). In LB medium, however, σ^S reached its maximum cellular concentration at the beginning of the stationary phase (Fig. 3B).

Taken together, these results indicate that *rpoS* expression is not only controlled on the level of transcription but also on a post-transcriptional level. This is par-

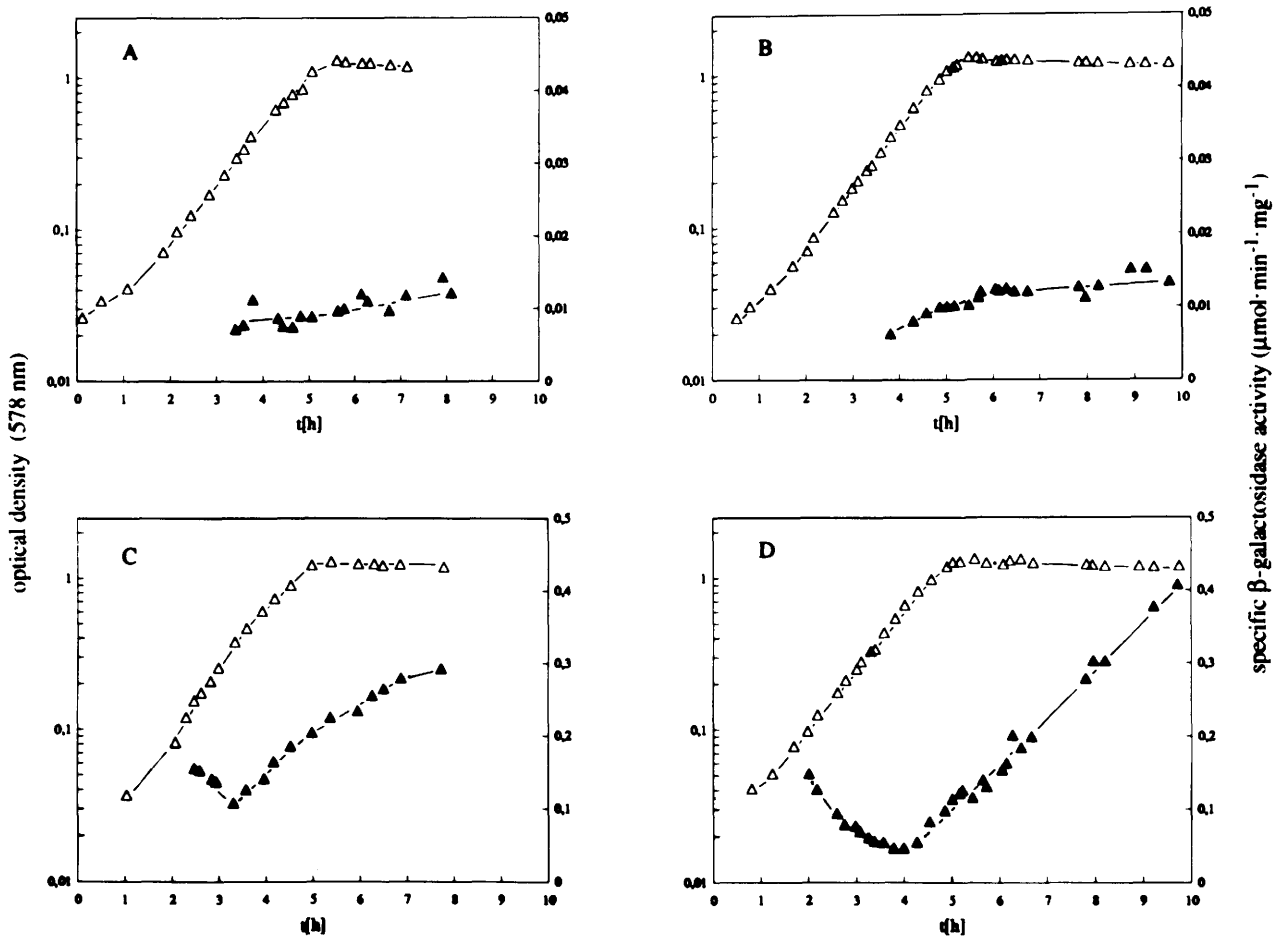

Figure 1. Expression of *rpoS::lacZ* fusions in cells growing in minimal glucose medium. MC4100 derivatives carrying single-copy early (*A*) and late (*B*) transcriptional *rpoS::lacZ* fusions, as well as early (*C*) or late (*D*) translational *rpoS::lacZ* fusions (strains RO77, RO200, RO35 and RO91, respectively) were grown in M9 medium with 0.1% glucose as a carbon source. OD_{578} was followed (△) and β-galactosidase activities were determined along the growth curve (▲).

ticularly evident when the nearly constant expression of the transcriptional *rpoS::lacZ* fusions during entry into stationary phase in minimal medium (Fig. 1A,B) is compared with the strong increase of σ^S protein under the same conditions (Fig. 3A).

Post-transcriptional osmotic induction of rpoS *expression*

Whereas several σ^S-controlled genes exhibit strong and rapid transcriptional induction in response to increased medium osmolarity, an early translational *rpoS::lacZ* fusion was not osmotically induced in parallel, indicating that *rpoS* transcription is not under osmotic control (Hengge-Aronis et al. 1993). This result is confirmed here with the two transcriptional and the early translational *rpoS::lacZ* fusions (Fig. 4A–C). However, the late translational *rpoS::lacZ* fusion was clearly osmotically induced (Fig. 4D). This induction was ~14-fold and occurred rapidly with kinetics very similar to those found for osmotic induction of σ^S-dependent genes such as *osmY* (*csi-5*; Hengge-Aronis et al. 1993). In immunoblot experiments, a similar increase of cellular σ^S content in response to increased osmolarity was observed (data not shown).

These results indicate that *rpoS* expression is subject to a novel mechanism of osmotic regulation that operates at a post-transcriptional level and involves sequences in the *rpoS* mRNA downstream of the fusion joint of the early *rpoS::lacZ* fusion.

Rate of σ^S synthesis and changes in σ^S stability in relation to growth phase

The rate of σ^S synthesis and the rate of total protein synthesis were determined for cells grown in glucose-limited minimal medium. The cells were pulse-labeled with [^{35}S]methionine, and extracts were immunoprecipitated with a polyclonal serum against σ^S followed by SDS–gel electrophoresis and autoradiography (Fig. 5A). For quantitation (Fig. 5B), radioactivity was directly counted from the gel in the σ^S bands and, as an internal standard, in the bands representing the late *rpoS::lacZ*-encoded hybrid protein (a standard aliquot of a cell ex-

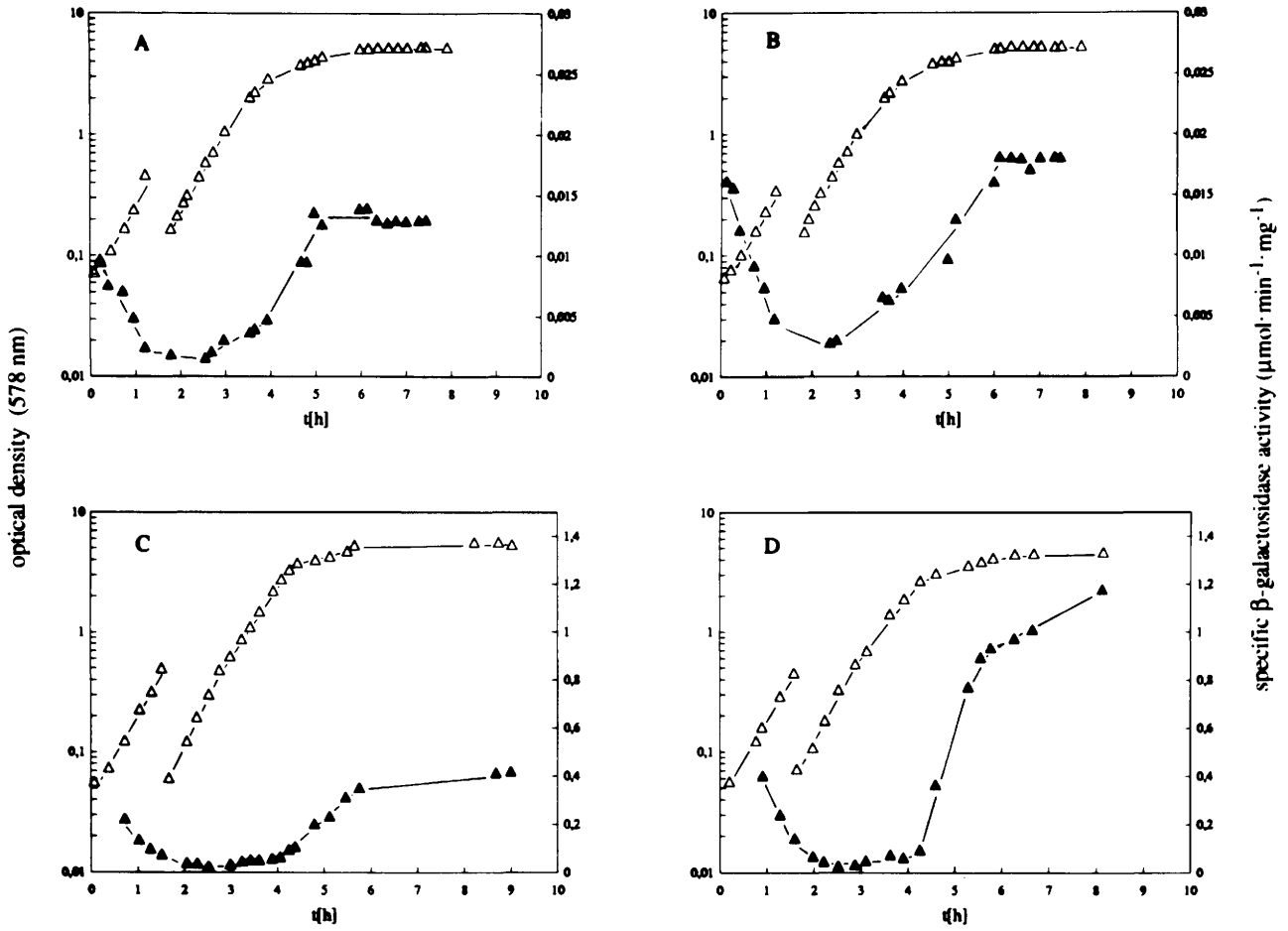

Figure 2. Expression of *rpoS*::*lacZ* fusions in cells growing in LB medium. The strains used and their order of appearance are the same as in Fig. 1. At an OD_{578} of ~0.5, the cultures were diluted fivefold into fresh medium to prolong the exponential phase of growth. OD_{578} and β-galactosidase activities are shown with the same symbols as in Fig. 1.

tract prepared from the *rpoS* deletion strain RH100 carrying the plasmid with the late translational *rpoS*::*lacZ* fusion was added to each sample). Figure 5 shows a certain low rate of σ^S synthesis also during steady-state exponential growth. During late exponential phase σ^S synthesis started to increase and reached a maximum (fivefold higher than the basal level in growing cells) at the onset of starvation. After entry into stationary phase, the rate of σ^S synthesis rapidly decreased again (Fig. 5), although the cellular concentration of σ^S continued to increase for at least 2 hr after the onset of starvation under these conditions (Fig. 3A).

An explanation for this apparent discrepancy was found when we analyzed σ^S stability in relation to growth phase. Cells were grown in minimal glucose medium and pulse-labeled with [^{35}S]methionine either during late exponential phase or shortly after the onset of starvation. Chase times with nonradioactive methionine varied between 0.25 and 10.25 min. Autoradiographs obtained after immunoprecipitation and SDS-gel electrophoresis are shown in Figure 6, A and B. Whereas exponential-phase cells had a high turnover of σ^S protein, σ^S was much more stable in stationary phase. Half-life of σ^S was determined as 1.4 min in exponentially growing cells and ~10.5 min in stationary phase (Fig. 6C). This stabilization of σ^S accounts for the continously increasing cellular content of σ^S in glucose-starved nongrowing cells despite the reduced rate of synthesis and is thus a major factor in the control of the cellular σ^S level.

The role of cAMP–CRP in the control of rpoS *expression*

We reported previously that exponential-phase expression of a transcriptional *rpoS*::*lacZ* fusion was strongly increased in *cya* or *crp* strains and could be decreased by the external addition of cAMP (Lange and Hengge-Aronis 1991). This suggested that cAMP–CRP was a direct or indirect negative regulator for *rpoS* transcription. Yet, it was also reported that expression of another transcriptional *rpoS*::*lacZ* fusion was reduced in a *cya* mutant background and was no longer induced during entry into stationary phase (McCann et al. 1993).

Here, we determined the cellular σ^S contents in *cya*$^+$ and Δ*cya* strains. Densitometric quantitation of the data shown in Figure 7 indicated that σ^S levels in the *cya*$^+$

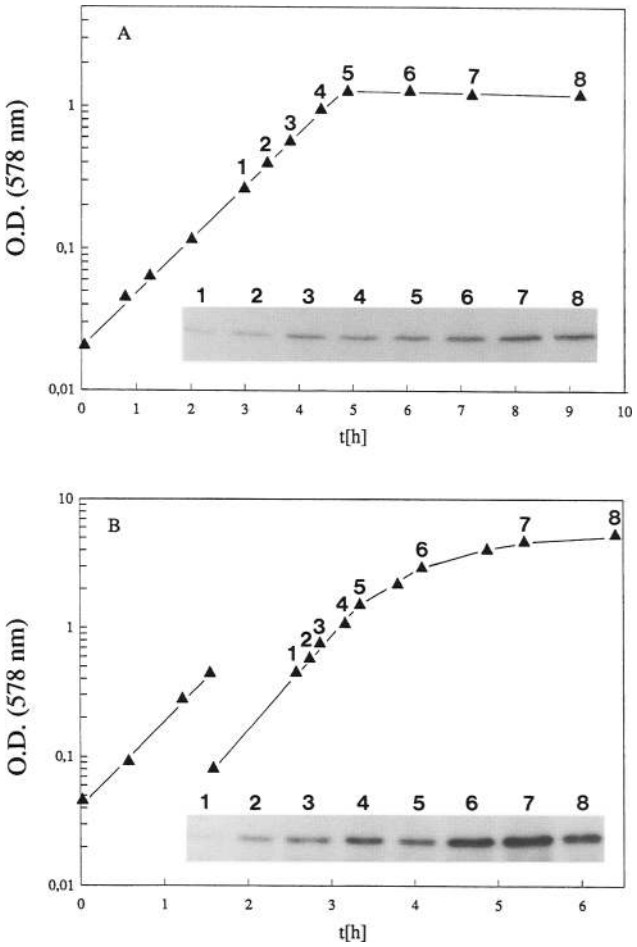

Figure 3. Growth phase-dependent increase in cellular σ^S content. Strain MC4100 was grown in M9 medium with 0.1% glucose (*A*) and in LB medium (*B*). Samples were taken along the growth curve as indicated by the numbers, normalized for equal total protein concentration, and subjected to immunoblot analysis with a polyclonal serum against σ^S (shown in the autoradiographs).

strain increased approximately fourfold during transition into stationary phase in glucose-limited minimal medium, whereas the Δ*cya* mutant had a high σ^S content even in exponential phase which remained unchanged after the onset of starvation. This corresponds to our observations with a transcriptional *rpoS*::*lacZ* fusion (Lange and Hengge-Aronis 1991).

A sequence upstream of *rpoS* exhibits some similarity to the σ^F consensus (Mulvey and Loewen 1989). σ^F is an intermediate regulator in the regulatory cascade that governs the expression of flagellar and motility genes and is itself under the control of a regulator encoded by *flbB* (Helmann 1991). This cascade is also positively controlled by cAMP–CRP. Because the parental strain used in our studies (MC4100) carries the *flbB5301* mutant allele, we might have missed a potential positive effect of cAMP–CRP on *rpoS* expression. Therefore, a *flbB*$^+$ derivative of MC4100 was used for the experiment shown in Figure 7. However, the results were similar to those obtained with MC4100 and its isogenic Δ*cya* derivative (data not shown). In addition, we did not find a significant difference in the expression of *rpoS*::*lacZ* fusions in *flbB*$^+$ and *flbB5301* strains (data not shown). These results demonstrate that cAMP–CRP negatively affects the expression of σ^S and also suggest that, at least under the conditions tested, σ^F is not involved in the regulation of *rpoS*.

Figure 8 demonstrates that the total cellular content of σ^S decreased significantly within a few minutes after the addition of cAMP to the Δ*cya* strain. This degradation reflects the instability of σ^S protein because the short half-life in exponential phase of σ^S is identical in *cya*$^+$ and Δ*cya* strains (data not shown) and confirms that the addition of cAMP to the Δ*cya* mutant strongly inhibits synthesis of σ^S. The slow growth of the Δ*cya* mutant accelerated after the addition of cAMP only after the cellular σ^S content was strongly decreased and then corresponded to the σ^S content of *cya*$^+$ cells (Fig. 8B).

Discussion

In the present paper we demonstrate that the expression of *rpoS*/σ^S during entry into stationary phase is not only controlled by transcriptional regulation but also by several distinct post-transcriptional processes. Based on the analysis of *rpoS*::*lacZ* fusions, it was suggested previously that *rpoS* is subject to translational control (Loewen et al. 1993; McCann et al. 1993). Our results demonstrate that we have to distinguish translational control as well as changes in σ^S stability.

Translational control of rpoS *expression*

Our conclusion that *rpoS* expression is post-transcriptionally regulated is based on the analysis of various *rpoS*::*lacZ* gene fusions and visualization of wild-type σ^S protein in immunoblot and pulse-labeling experiments. Post-transcriptional control is most readily apparent in minimal medium, where expression of the transcriptional fusions remained nearly constant during transition into stationary phase, whereas the expression of the corresponding translational fusions (Fig. 1), the rate of σ^S synthesis (Fig. 5), and the total cellular level of σ^S protein (Fig. 3A) were clearly increased. In addition, the factor of induction was always higher for the late translational fusion (encoding a hybrid protein with 247 amino acids of σ^S) than for the early translational fusion (encoding a hybrid protein with 23 amino acids of σ^S). This indicated that sequences in *rpoS* downstream of the fusion joint of the early fusion are involved in this post-transcriptional control of *rpoS* expression. This conclusion is considerably strenghtened by our finding that among our four fusions, only the late translational *rpoS*::*lacZ* fusion exhibited osmotic induction (Fig. 4). To our knowledge, this osmotic regulation of *rpoS* expression is the first example of a post-transcriptional osmoregulation in *E. coli*. The expression of another osmotically regulated

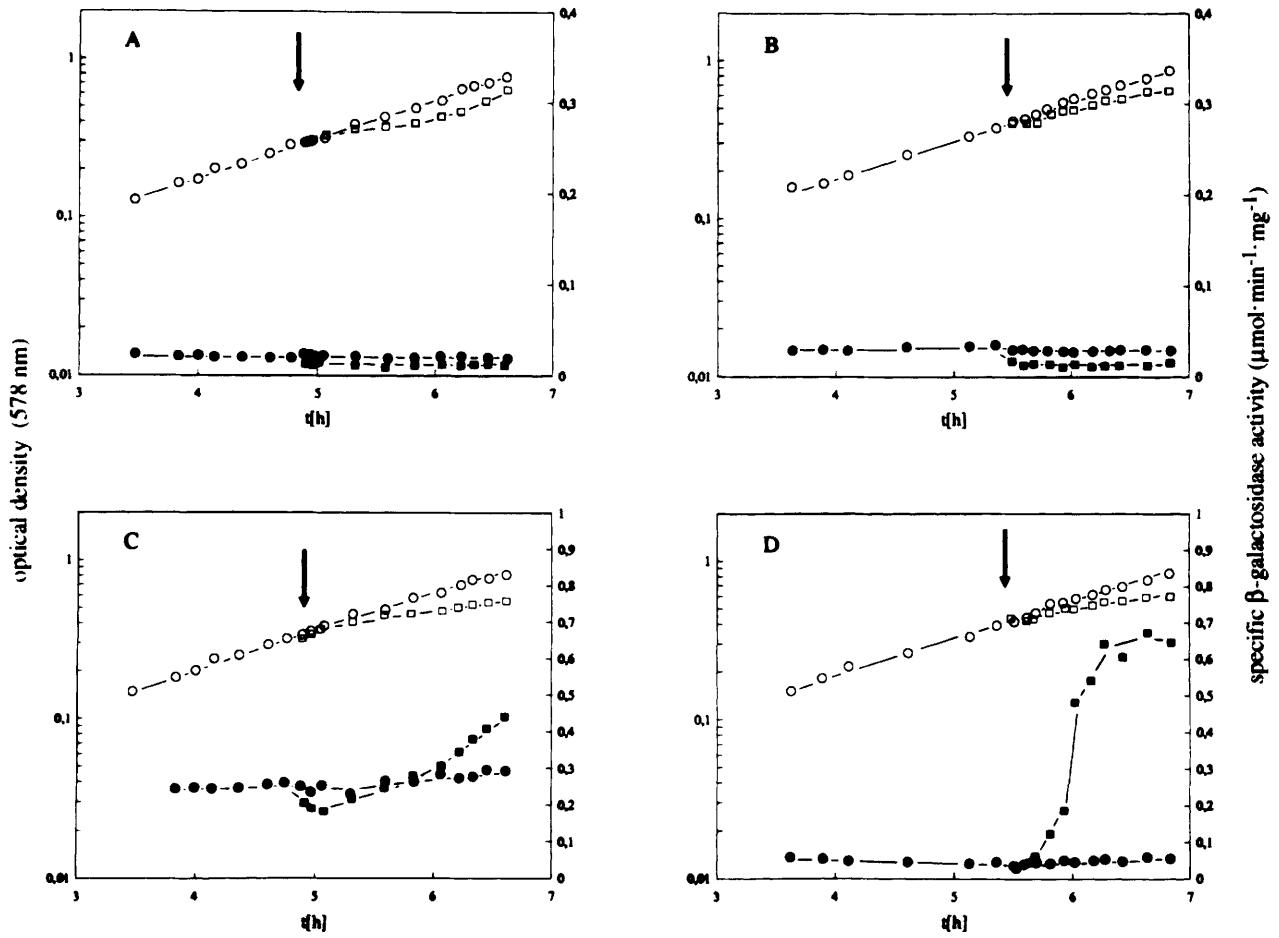

Figure 4. Expression of *rpoS::lacZ* fusions in response to increased medium osmolarity. The strains used and their order of appearance are the same as in Fig. 1. The cells were grown in M9 medium with 0.4% glycerol as a carbon source. At an OD_{578} of ~0.3, the cultures were divided and 0.2 M NaCl was added to one of the two aliquots in each case. OD_{578} (open symbols) and β-galactosidase activities (solid symbols) are shown in the absence (circles) and in the presence (squares) of NaCl.

gene, *ompF*, is controlled by several factors, one being an antisense RNA (*micF*, Mizuno et al. 1984), but *micF* does not appear to be specifically involved in osmotic regulation of *ompF* (Csonka 1989).

In some cases, quantitative differences in the expression of gene fusions might also be explained by differences in mRNA stability. However, our corresponding transcriptional and translational fusions differ only by a 90-bp insertion upstream of the eighth codon of *lacZ* (this insertion provides stop codons in all three reading frames, a Shine and Dalgarno sequence, and an initiation codon followed by six additional codons). Even if this short insertion were to influence mRNA stability, this could not explain the clearly differential osmotic regulation of the two late fusions, because neither of the two early fusions (that differ by the same insertion) is osmotically regulated. We therefore conclude that our results strongly argue for a translational control mechanism in the regulation of σ^S expression.

A similarly complex translational control was described for σ^{32}, the *rpoH*-encoded alternative σ-factor involved in the heat shock response in *E. coli* (Kamath-Loeb and Gross 1991; Nagai et al. 1991). A model based on interaction of two regions within the *rpoH* mRNA in secondary structure formation has been proposed. Region A comprises the initiation codon and the downstream box [a sequence with complementarity to nucleotides 1469–1483 of 16S rRNA (Sprengart et al. 1990)], whereas region B consists of nucleotides 110–210 in *rpoH* and functions as a *cis*-acting antisense RNA that is complementary to region A (Yuzawa et al. 1993). Figure 9 shows the predicted secondary structure around the 5′ regions of *rpoS* and *rpoH*. The energetically most stable secondary structure of *rpoS* mRNA is characterized by long and branched stem and loop structures and base-pairing between a downstream sequence and the translational initiation region of the mRNA. Figure 9 demonstrates that this structure is strikingly similar to that obtained for *rpoH*. We have made similar mRNA structure predictions for 5′ regions of similar lengths of other genes, but we have not found a comparable structure for any of these. Among the genes tested were other osmotically and/or growth phase-regulated genes (*proU*, *osmY*, *otsBA*, *osmB*, *hns*), another *E. coli* σ-factor gene (*rpoD*)

and several genes containing downstream boxes (*lacZ*, *lysU*, *glnS*, *tufA*).

The overall similarity of the most likely secondary structures of the *rpoS* and *rpoH* mRNAs suggests immediately that the mechanisms for translational control are similar for the two genes. In both cases, the translational initiation region would be sequestered by base-pairing with an "antisense-element" located farther downstream in the mRNA. This model makes precise predictions about the location of the downstream antisense-element in *rpoS* (between nucleotides 160 and 210 in

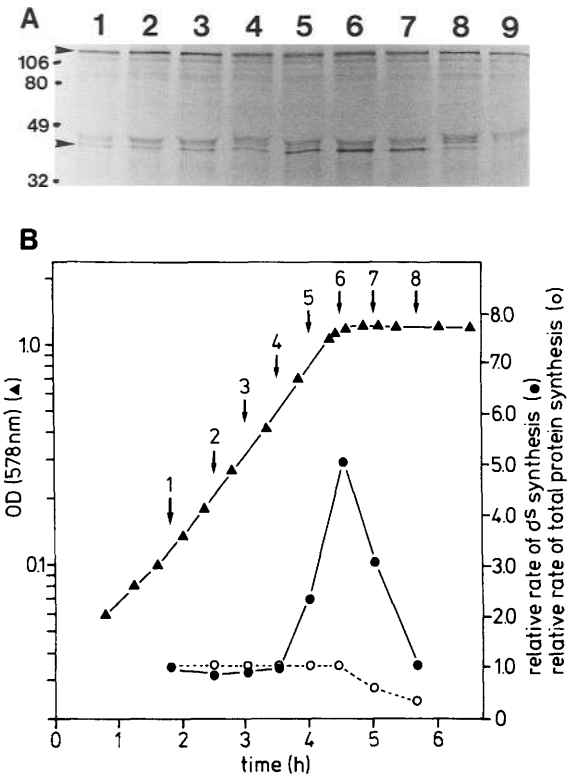

Figure 5. Rate of σ^S synthesis along the growth curve. Strain MC4100 was grown in M9 medium with 0.1% glucose as a carbon source. Samples were taken along the growth curve, adjusted to equal optical densities, and pulse-labeled with [^{35}S]methionine. Extracts were prepared and, after the addition of an internal standard, were immunoprecipitated with a polyclonal serum against σ^S (see Materials and methods for details). Immunoprecipitate samples were analyzed by SDS-PAGE. An autoradiograph of the dried gel is shown in *A*. The lane numbers correspond to the numbers in *B* and indicate the sampling times. Lane *9* shows the internal standard alone and serves as a control for the identification of the σ^S band. Arrowheads indicate the positions of σ^S and the hybrid protein encoded by the late translational *rpoS*::*lacZ* fusion. Numbers at *left* indicate the molecular mass (in kD) of size standard proteins. The rate of σ^S synthesis was determined by directly counting radioactivity in the σ^S and the fusion protein bands on the dried gel (the latter was used as an internal standard for the correction of the former) and is shown in *B* relative to the initial rate of σ^S synthesis in exponential phase (●). In addition, the rate of [^{35}S]methionine incorporation into total cellular protein is shown (normalized for the initial rate in exponential phase; ○ in *B*).

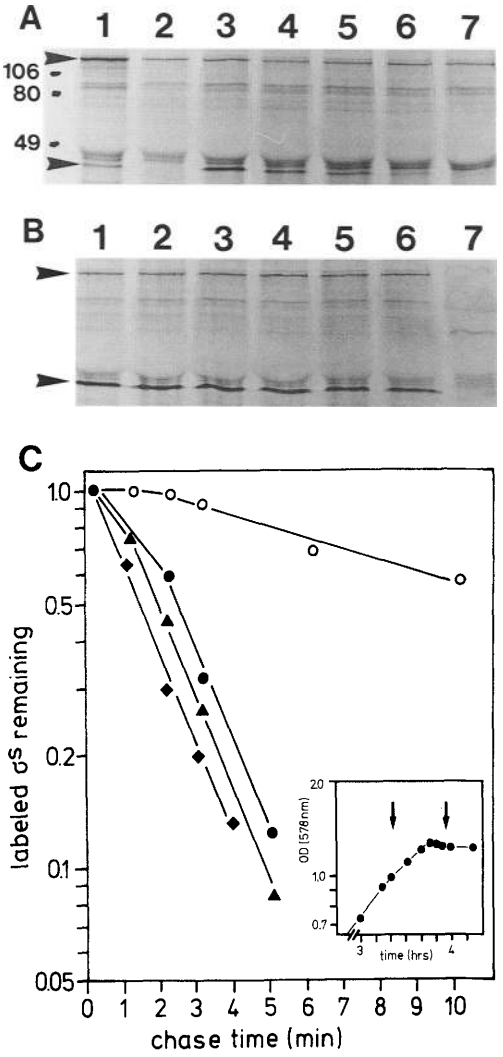

Figure 6. Determination of the half-life of σ^S. Strain MC4100 was grown in M9 medium with 0.1% glucose as a carbon source. Samples were taken in late exponential phase (at an OD_{578} of 1.0) and 15 min after the onset of starvation. The inset in *C* shows the relevant section of the growth curve with the exact sampling times (arrows). Samples were pulse-labeled for 1 and 3 min, respectively. Chase times varied between 1 and 10.25 min. Immunoprecipitation, SDS-PAGE, and quantification of the radioactivity in the σ^S bands were as described in Fig. 5. Autoradiographs obtained with exponential-phase cells (*A*) and stationary-phase cells (*B*) are shown. In *A* the chase times were 0.25 min (lane *3*), 2.25 min (lane *4*), 3.25 min (lane *5*), 5.25 min (lane *6*), and 7.25 min (lane *7*). As controls, MC4100(pRL44) (lane *1*) and the *rpoS* deletion mutant RH100(pRL44) (lane *2*) were used (for details, see Materials and methods). In *B* the chase times were 0.25 min (lane *1*), 1.25 min (lane *2*), 2.25 min (lane *3*), 3.25 min (lane *4*), 5.25 min (lane *5*), and 10.25 min (lane *6*). As a control the *rpoS* mutant RH90 was used (lane *7*). Arrowheads indicate the positions of σ^S and the hybrid protein encoded by the late translational *rpoS*::*lacZ* fusion. Small numbers indicate the molecular mass (in kD) of size standard proteins. In *C* the fraction of labeled σ^S remaining (corrected with the internal standard and expressed relative to the initial value) is shown as a function of the chase time. Results are given for the experiments shown as autoradiographs in *A* and *B*, (● and ○, respectively) as well as for two additional experiments made with exponentially growing cells also harvested at an OD_{578} of 1.0 (▲ and ■).

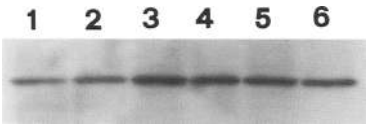

Figure 7. σ^S content of a Δ*cya* mutant. Strains RO79 (*cya*$^+$) (lane *1*–*3*) and RO78 (Δ*cya*) (lane *4*–*6*) were grown in M9 medium with 0.1% glucose as a carbon source. Samples were taken at ODs of 0.335 (lane *1*), 1.05 (lane *2*) and 1.1 (lane *3*) for RO79, and 0.26 (lane *4*), 0.51 (lane *5*), and 0.96 (lane *6*) for RO78. Lanes *1* and *4* correspond to early exponential phase; lanes *2* and *5* to late exponential phase; lanes *3* and *6* to stationary phase. Immunoblot analysis was as described for Fig. 3.

Figure 9), that can now be tested experimentally. In our early translational fusion this element is not present (Tn*lacZ* is fused to *rpoS* after the G at position 96 in Fig. 9). Therefore, a higher level of expression might be expected for this early fusion, which is indeed observed under certain conditions (cf. Fig. 4C and D). However, the predicted mRNA secondary structure for the early fusion construct suggests the formation of a local stem and loop structure (between nucleotide 20 and 48 in Fig. 9; data not shown) that involves the initiation codon and the downstream box, and this structure probably reduces translational efficiency.

Because the environmental signals that stimulate translation of *rpoS* and *rpoH* are different, we speculate that specific proteins may be involved in translational induction of *rpoS* and *rpoH*, whose activity is dependent on changes in temperature and on growth phase and/or osmolarity. These proteins could either stabilize the mRNA secondary structure under noninducing conditions or they might actively resolve it under inducing conditions.

In the case of *rpoS*, it is also possible that yet another mechanism is involved in translation regulation. Also the early translational fusion, which does not contain the sequences downstream of codon 23 in *rpoS* that are involved in translational control, exhibits weak stationary phase induction not found for the transcriptional fusions. The nature of this potential second translation control mechanism is unknown.

In addition, there is evidence for a negative feedback control exerted by σ^S on a post-transcriptional level of its own expression. In contrast to transcriptional and early translational *rpoS*::*lacZ* fusions, late translational fusions show a higher level of expression in a *rpoS* mutant than in the wild-type background (Schellhorn and Stones 1992; McCann et al. 1993; R. Lange and R. Hengge-Aronis, unpubl.). Because σ^S itself is a transcription factor, it seems likely that an unidentified σ^S-dependent protein is involved in this control.

Growth phase-dependent control of σ^S stability

We have also shown here that in exponentially growing cells σ^S is an unusually unstable protein that is degraded with a half-life of only 1.4 min. At the onset of glucose starvation σ^S half-life increases more than sevenfold (Fig. 6). This is in pronounced contrast to a stimulation of general protein turnover during entry into stationary phase (Kolter et al. 1993). Again, this stabilization of σ^S is reminiscent of a similar phenomenon observed with σ^{32}, which also has a short half life at low temperature but is stabilized in response to heat shock (Straus et al. 1987). The molecular mechanisms of rapid degradation as well as starvation-induced or heat-induced stabilization are presently unknown in both cases.

The low level of σ^S in exponential phase cells is thus the result of a dynamic equilibrium between a certain low rate of synthesis determined by transcriptional and translational activities and rapid degradation of σ^S. To increase the cellular content of σ^S during transition into stationary phase, the cells make use of alterations both in the rate of synthesis and the rate of degradation. Interestingly, there is a sequential order of the occurrence of these processes along the growth curve when the cells are grown in glucose-limited minimal medium. During late exponential phase, translation is stimulated but the half-life of σ^S remains unchanged, whereas at the onset of glucose starvation, the rate of translation is reduced again but σ^S turnover is now inhibited. The result is a continuous increase in σ^S concentration from late exponential phase until at least 2 hr after the onset of starvation. In LB medium transcriptional and translational

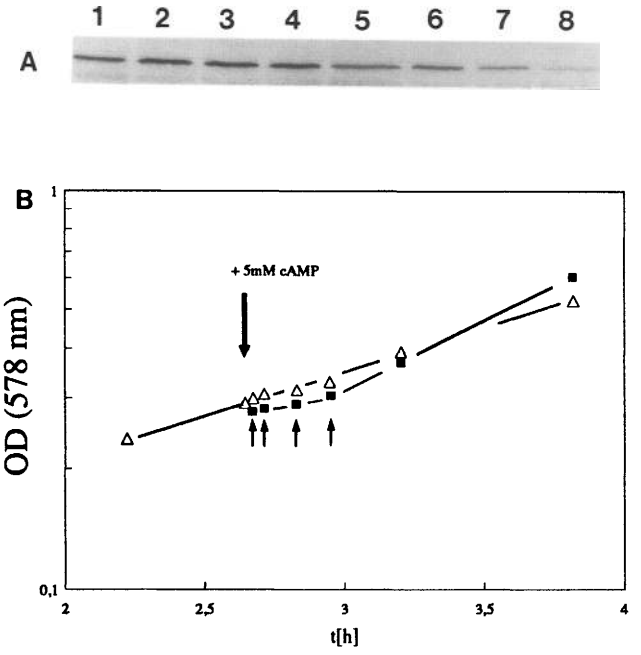

Figure 8. σ^S content of a Δ*cya* mutant after the addition of cAMP. The Δ*cya* strain RO78 was grown in M9 medium with 0.1% glucose as a carbon source. At an O_{578} of ~0.3 the culture was divided into two aliquots, one of which was supplemented with 5 mM cAMP. Immunoblot analysis with the σ^S antiserum (*A*) was performed with samples taken at the time points indicated by the small arrows in *B* from the cAMP-free culture (lanes *1*–*4* in *A*, △ in *B*) and from the cAMP-containing culture (lanes *5*–*8* in *A*, ■ in *B*).

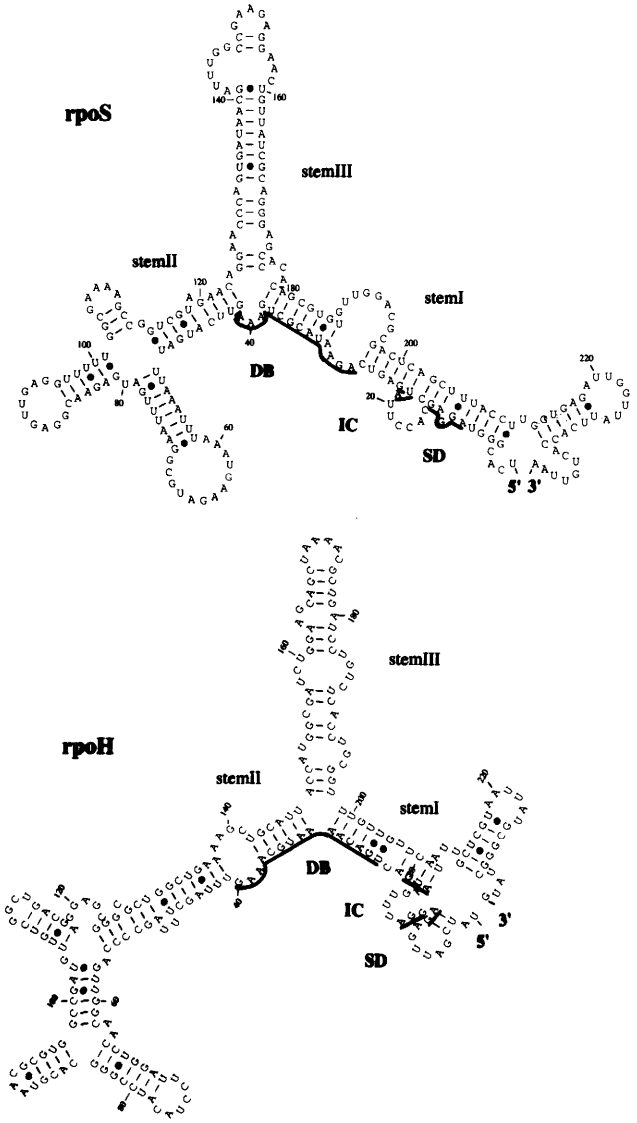

Figure 9. Predicted secondary structure of the *rpoS* mRNA in the translational initiation region. The 5′ region of *rpoS* mRNA (nucleotides −20 to 220), and for comparison, the 5′ region of *rpoH* mRNA (nucleotides −20 to 214) were analyzed. The structure for *rpoH* corresponds to that reported previously (Nagai et al. 1991; Yuzawa et al. 1993). The Shine and Dalgarno sequences (SD), the initiation codons (IC), and the downstream boxes (DB) are indicated. Black dots indicate G-U pairs.

induction and maybe also stabilization of σ^S seem to occur in parallel during entry into stationary phase.

Signals involved in the control of σ^S expression

Our results indicate that *rpoS*/σ^S expression is controlled by several different signals (summarized in Fig. 10). First, a growth rate-related signal controls *rpoS* transcription. It was shown that the expression of transcriptional *rpoS*::*lacZ* fusions is only stimulated when the growth rate decreases continuously as, for instance, during entry into stationary phase in LB medium (Fig. 2; Mulvey et al. 1990; Lange and Hengge-Aronis 1991). In addition, the expression of transcriptional and early translational fusions correlates in a linear way with the doubling time when the growth rate of the cultures is experimentally manipulated, for instance, by adding α-methylglucoside to cultures growing on glucose or by adding various low concentrations of cAMP to cultures of a Δ*cya* strain (Lange and Hengge-Aronis 1991; R. Lange and R. Hengge-Aronis, unpubl.). The signal involved could be guanosine-5′-diphosphate -3′-diphosphate (ppGpp), because an increase in ppGpp concentration has been found in slowly growing nutrient-limited cultures (Chesbro 1988). Also, a *relA spoT* double mutant that does not synthesize ppGpp has a strongly decreased cellular σ^S content (Gentry et al. 1993).

The second signal occurs during late exponential phase and influences *rpoS* translation. This signal cannot be growth rate related because its effect, that is, the induction of translational *rpoS*::*lacZ* fusions, can be observed at a time when the growth rate of the culture is still unchanged (Fig. 1). It may be a cell density-related signal, for example, a certain extracellular concentration of a substance released by the cells during growth. This is suggested by our observation that the translational fusions were not induced in late exponential phase when the cells were grown in minimal medium containing only 0.05% glucose where they entered stationary phase at a lower cell density (R. Lange and R. Hengge-Aronis, unpubl.). A third unidentified signal reflects changes in medium osmolarity and also affects *rpoS* translation. The fourth signal plays a role at the actual onset of glucose starvation characterized by an abrupt stop in cellular mass increase. This signal triggers the process that results in σ^S stabilization. The fact that these signals control processes in the regulation of *rpoS*/σ^S, which now can be experimentally distinguished, will hopefully allow their identification in the future.

The role of cAMP–CRP in the regulation of rpoS

We have shown previously that Δ*cya* and Δ*crp* mutants exhibit higher expression in exponential phase of a transcriptional *rpoS*::*lacZ* fusion than the isogenic wild-type

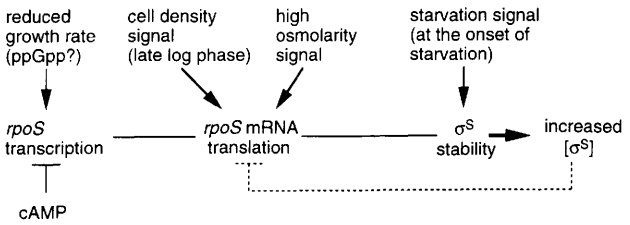

Figure 10. Model for the regulation of expression of *rpoS*/σ^S. The model summarizes the results of the present study with respect to the control of transcription and translation of *rpoS* and of σ^S stability in relation to growth phase. In addition, putative signals that influence these processes are indicated (see text for discussion).

strain, and no increase in β-galactosidase activity can be observed during entry into stationary phase (Lange and Hengge-Aronis 1991). Here, we demonstrate by immunoblot analysis that the cellular content of σ^S follows this pattern of expression (Fig. 7). This indicates that cAMP–CRP negatively affects the transcription of *rpoS*. The addition of cAMP to the Δ*cya* mutant seems to inhibit de novo synthesis of σ^S completely, because rapid degradation of preexisting σ^S within a few minutes can be observed (Fig. 8). This degradation is attributable to a similar short half-life of σ^S in the Δ*cya* mutant as in an isogenic *cya*$^+$ strain (data not shown). Because the inhibition of *rpoS* transcription after the addition of cAMP to the Δ*cya* mutant occurs immediately and before any change in growth rate can be observed (Fig. 8), it seems likely that cAMP–CRP directly inhibits *rpoS* transcription and does not only act indirectly by influencing the growth rate. Inhibition by cAMP–CRP may also explain the lack of transcriptional induction of *rpoS* at the onset of glucose starvation, where a burst of cAMP production occurs (Buettner et al. 1973). Taken together, cAMP is a signal involved in the control of *rpoS* transcription.

Conclusion and perspectives

In this paper we describe the complex regulation of *rpoS*/σ^S expression on the levels of transcription, translation, and protein stability. We were able to specify the relative contributions of the various mechanisms to increased expression in relation to growth phase, medium composition, and osmolarity. Figure 10 integrates these data in a schematic model that also defines the questions about *rpoS*/σ^S regulation for future work. What are the molecular mechanisms of translational control, negative feedback control of translation, and control of σ^S stability? What is the nature of the unknown signals that reflect cell density, medium osmolarity, and starvation? How are these signals recognized and transduced? Finding the answers to these questions will be an exciting challenge for the future.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The construction of plasmids and gene fusions is described in detail below. Cultures were grown at 37°C under aeration either in LB medium or in minimal medium M9 (Miller 1972). M9 was supplemented with glucose or glycerol as a carbon source (in concentrations as indicated in the figure legends) and with vitamin B_1 (1 μg/ml). Growth was monitored by measuring the optical density at 578 nm (OD_{578}).

DNA manipulations

For DNA manipulations such as plasmid DNA preparations, restrictions digests, ligation, transformation, and agarose gel electrophoresis, standard procedures were followed (Silhavy et al. 1984; Sambrook et al. 1989). DNA was sequenced with the Sequenase Kit (U.S. Biochemical) and [α-^{35}S]thio-dATP (>1000 Ci/mmole; Amersham).

Isolation of rpoS::lacZ *gene fusions*

Translational *lacZ* fusions were isolated by random insertion mutagenesis of pRH320 with the Tn*5* derivative Tn*lacZ* (Manoil 1990). Plasmid pRH320 is a pBR322 derivative and contains a 4.35 kb *Cla*I fragment of chromosomal DNA that, in addition to the whole *rpoS* gene, carries ~1.25 kb upstream of *rpoS* as well as nearly 2 kb of downstream sequences (Lange and Hengge-Aronis 1991). pRH320 derivatives carrying Tn*lacZ* insertions were isolated in strain CC311 as described by Manoil (1990). Plasmids that no longer complemented a chromosomal *rpoS* mutation were analyzed further with respect to the location of the fusion joint on pRH320. Two plasmids with Tn*lacZ* insertions that created active in-frame fusions either early or late in *rpoS* were chosen for the present study. To remove the region encoding the transposase and the kanamycin resistance of Tn*lacZ*, a *Hin*dIII blip-out was done that eliminates the mate-

Table 1. *Bacterial strains*

Strain	Relevant genotype	Reference
MC4100	F$^-$ Δ(*arg–lac*)*U169 araD139 rpsL 150 ptsF25 flbB5301 rpsR deoC relA1*	Silhavy et al. (1984)
CC311	Δ(*ara–leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsL recA1* pOxgen::Tn*lacZ*	Manoil (1990)
RO77	MC4100 (λRZ5: *rpoS70::lacZ*)	this study
RO35	MC4100 [λRZ5: *rpoS70::lacZ* (hybr)]	Hengge-Aronis et al. (1993)
RO200	MC4100 (λRZ5: *rpoS742::lacZ*)	this study
RO91	MC4100 [λRZ5: *rpoS742::lacZ* (hybr)]	this study
RO74	MC4100 (λp1048: Φ*tyr′::lacY*)[a]	this study
RH76	MC4100 Δ*cya851*	Lange and Hengge-Aronis (1991)
RO40	RH76 [λRZ5: *rpoS70::lacZ* (hybr)]	this study
MM335	F$^-$ Δ(*arg–lac*)*U169 araD139 rpsL 150 ptsF25 rpsR deoC relA1 malT*^{c}	M. Manson (Texas A & M University, College Station)
RO79	MM335 *malT*$^+$	this study
RO78	RO79 Δ*cya851*	this study
RH90	MC4100 *rpoS359::*Tn*10*	Lange and Hengge-Aronis (1991)
RH100	MC4100 Δ(*nlpD–rpoS*)*360 zfi-3251::*Tn*10*	Hengge-Aronis et al. (1993)
JU6	MC4100 Δ(*nlpD–rpoS*)*360* Φ(*csi-5::lacZ*) (λp*lac*Mu55)	Lange et al. (1993)

[a]λp1048: Φ*tyr′::lacY* is described in Berman and Jackson (1984).

rial of Tn*lacZ* downstream of the first *Hin*dIII site in IS50$_L$ to the *Hin*dIII site in the vector part of pRH320. The *rpoS*::*lacZ* fusion joints, as well as the *rpoS* regions upstream of the fusion joints, were verified by sequencing. The fusion joints of the two *rpoS*::*lacZ* fusions are after the nucleotides at positions 70 and 742 within the *rpoS* open reading frame, that is, the amino-terminal 23 and 247 codons, respectively, in the fusions are the same as in wild-type *rpoS*. To these *rpoS* segments, 58 nucleotides from the left end of Tn*lacZ* , followed by a *Bam*HI site, a C, and the eighth codon of *lacZ*, are fused. The plasmids carrying these early and late fusions were termed pRL1-25 and pRL1-43, respectively.

Preliminary β-galactosidase assays demonstrated that both *lacZ* fusions were stationary phase induced, but the late fusion generated only low activity. However, when transformed into a *supE* strain, both fusions yielded similar high activities, indicating the presence of an amber mutation in *rpoS* on pRH320 between the positions specified by the two fusion joints. Many *E. coli* strains carry amber mutations in *rpoS* with MC4100 being one of the rather rare exceptions (Strøm and Kaasen 1993). The *rpoS* allele on pRH320 is derived from a cosmid gene bank made on strain BHB2600 (Birkenbihl and Vielmetter 1989), and sequencing demonstrated that pRL1-43 carries a TAG stop codon instead of the CAG codon for Gln-33 in wild-type *rpoS*. The corresponding *rpoS* wild-type region was recombined onto pRL1-43 by transformation into RO74 and screening for colonies able to metabolize lactose efficiently when grown on BTB lactose plates (M9 medium supplemented with 0.5% lactose, 0.075% yeast extract, 0.1% vitamin B_1, and 0.02% bromothymol blue). One of the resulting plasmids was termed pRL-44, and its wild-type *rpoS* sequence upstream of the fusion joint was confirmed by sequencing.

The size of the hybrid proteins was verified by immunoblotting and pulse-labeling experiments, followed by immunoprecipitation. As with most β-galactosidase hybrid proteins, the amino-terminal part of the hybrid protein encoded by the late *rpoS*::*lacZ* fusion is partially cleaved off in vivo, whereas the remaining β-galactosidase moiety is stable. Usually, specific β-galactosidase activity is not influenced by the presence of amino-terminal extensions (an exception are hybrid proteins of exported proteins that are jammed in the membrane). Therefore, it can be expected that β-galactosidase activity of these *rpoS*::*lacZ* fusion correctly reflects *rpoS* expression.

For assaying transcriptional activity alone, the two translational *rpoS*::*lacZ* fusions were converted into transcriptional fusions. In the case of the early translational fusion, the *Pst*I restriction site in the *bla* gene and the *Bam*HI site directly upstream of *lacZ* were used. A 760-bp *Pst*I–*Bam*HI fragment of the promoter probe vector pMLB1010, which is similar to pMLB1034 (Silhavy et al. 1984) except for the insertion of ~0.7 kb of *trpA'* between the *Bam*HI site and *lacZ*, was replaced by the 2.25-kb *Pst*I–*Bam*HI fragment (carrying the 5' part of *rpoS* and the fusion joint) from pRL1-25. This construction results in a transcriptional fusion (on the plasmid termed pRL36). To remove the *trpA'* material, which is a frequent source of exceedingly high basal levels of expression and even of pseudopromoters arising by spontanous mutation, a 2.75-kb *Bam*HI–*Sac*I fragment of pRL36 carrying the *trpA'* material and part of the *lacZ* gene was replaced by a corresponding *Bam*HI–*Sac*I fragment of the promoter probe vector pTAC3575 (Atlung et al. 1991). This fragment provides the ribosomal binding site of *lacZ*, does not contain any *trp* sequences, and restores the *lacZ* gene. The resulting plasmid was termed pRL37. The late transcriptional fusion was constructed by replacing the 2.070-kb *Sca*I–*Bam*HI fragment in the early transcriptional fusion plasmid pRL37 by the 2.741-kb *Sca*I–*Bam*HI fragment of pRL1-44 (these *Sca*I–*Bam*HI fragments carry the upstream regions and the 5' parts of *rpoS* as well as the fusion joints of the early and late fusions, respectively). The resulting plasmid was termed pRL45. The sequences around and upstream of the fusion joints of pRL37 and pRL45 were verified by sequencing. In summary, the transcriptional fusions differ from their translational counterparts by the presence of a 90-bp insertion upstream of the eighth codon of *lacZ*, which contains stop codons in all three reading frames, a ribosomal binding site, and an initiation codon followed by six additional codons.

Cloning of rpoS *under* tac *promoter control*

A 1207-bp fragment carrying *rpoS* was isolated from chromosomal DNA of strain MC4100 by the polymerase chain reaction (PCR) using the oligonucleotides 5'-CCGCAGCGAgAAtTCGGCGGAA-3' and 5'-TTTACCGGAagCTTAATTACCTGTGTG-3'. The resulting fragment carries the ribosomal binding site of the first of two potential start codons, the *rpoS* structural gene and a potential *rho*-independent terminator at the end of *rpoS*. For the amplification of DNA fragments by PCR, *Vent* polymerase (New England Biolabs) was used and protocols given in Innis et al. (1990) were followed. After digestion with *Eco*RI and *Hin*dIII, this fragment was ligated into pRH800 treated with the same restriction enzymes. pRH800 carries *lacI*^{q}, the *tac* promoter, and a multiple cloning site, followed by *rrnB* and its two terminators, and was obtained (R. Hengge-Aronis and D. Fischer, unpubl.) by replacing a *Eco*RI–*Hin*dIII fragment carrying *dnaA* in pLSK5 (Schauzu et al. 1987) by the multiple cloning site from pSPTBM20 (Boehringer Mannheim). The resulting plasmid (pRL40.1) carries *rpoS* downstream of the *tac* promoter.

Overproduction and purification of σ^S *protein and preparation of a polyclonal antiserum*

For the purification of σ^S protein, strain JU6 carrying pRL40.1 was used. Cells growing in LB medium with ampicillin (50 μg/ml) were induced for the synthesis of σ^S by adding IPTG (1 mM) at an OD_{578} of 0.8. After an additional growth period of 2.5 hr the cells were harvested at an OD_{578} of 2.3. The cells were washed twice in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, and 20 μg/ml of PMSF and resuspended in buffer A (50 mM Tris-HCl (pH 8.2), 100 mM NaCl, 2 mM EDTA, 20 μg/ml of PMSF, 1 μg/ml of pepstatin). The cells were disrupted by two passages through a French press cell at 103,500 kiloPascal (kPa). The cell lysate was centrifuged at 17,000*g* for 30 min, and DNA was precipitated from the supernatant with streptomycinsulfate (1.5%) for 30 min on ice. Centrifugation was repeated and the clear supernatant was precipitated with ammonium sulfate (20%). The pellet of the precipitate was resuspended in buffer A and applied to a desalting column (Bio-Rad Europac 10DG) equilibrated previously with buffer A. The protein-containing fractions were pooled, and ~4.7 mg of protein was loaded onto a MonoQ HR5/5 column (Pharmacia) and eluted with a linear gradient of 100 mM to 500 mM NaCl in buffer A; σ^S eluted at 300 mM NaCl. Approximately 450 μg of protein obtained from the fractions most enriched for σ^S (>80% of this protein was σ^S) were run on preparative SDS gels. The gels were incubated in 1 M sterile KCl (Bergmann and Jörnvall 1987) to visualize the σ^S band. This band was excised from the gels, prepared for immunization as described (Harlow and Lane 1988), and used to produce a polyclonal antiserum in a rabbit. The antiserum was tested by immunoblot analysis of whole cell extracts of strains

carrying a deletion, a Tn*10* insertion, and translational *lacZ* fusions in *rpoS* as controls.

SDS-PAGE and immunoblot analysis

For immunoblot analysis samples from different growth stages were taken from cultures growing in LB or minimal glucose medium. Samples corresponding to 40 μg of total cellular protein were precipitated with 10% trichloroacetic acid (TCA). The protein pellets were resuspended in 40 μl of SDS-PAGE sample buffer and boiled. Samples (15 μl) were separated on 12% or 10% SDS–polyacrylamide gels and directly electroblotted onto polyvinylidene difluoride membranes (Immobilon, Millipore). This amount of total cellular protein was found to be optimal for producing σ^S bands from cultures grown under the conditions used here, which were in the linear range for densitometric quantitation. SDS-PAGE and electroblotting were performed using the respective modules of the Bio-Rad Mini-PROTEAN II system. Blots were blocked overnight in TBSTM [20 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.05% Tween (Fluka), 5% skimmed milk powder], probed with the polyclonal antiserum against σ^S, washed with TBST, and incubated with goat anti-rabbit IgG alkaline-phosphatase conjugate (Sigma). The blots were developed either with Lumi-Phos 530 (Boehringer Mannheim) as a chemoluminescent substrate or with a chromogenic substrate (BCIP/NBT; Boehringer Mannheim).

Pulse labeling of cells and immunoprecipitation

For pulse-labeling experiments, cultures were grown in M9 containing 0.1% glucose and 0.01% vitamin B_1. For the determination of synthesis rates of σ^S in relation to total protein synthesis, 1-ml samples were adjusted to an OD_{578} of 0.3 with supernatant from their own culture obtained by filter sterilization immediately before taking the samples. The samples were labeled with 10 μCi of L-[^{35}S]methionine for 1 min followed by a chase with 0.2 mM methionine for 15 sec (all steps done at 37°C) and TCA (10%) precipitation. For the determination of the half-life of σ^S, 3-ml samples adjusted to an optical density of 0.6, as described above, were labeled with 60 μCi of L-[^{35}S]methionine followed by the addition of nonradioactive methionine (2 mM) after 1 min (exponential cells) and 3 min (stationary-phase cell). Aliquots (0.5 ml) were removed at the times indicated in the figure legends and precipitated with TCA (10%).

In addition, MC4100(pRL44) and the *rpoS* deletion mutant RH100(pRL44) were pulse-labeled to be used as controls, and in the case of the latter, also an an internal standard during immunoprecipitation. These strains were grown in M9 medium containing 0.2% glucose and 50 μg/ml of ampicillin. At an OD_{578} of ~1.0, 3-ml samples concentrated to an OD_{578} of 1.5 were labeled with 150 μCi of L-[^{35}S]methionine for 1.5 min followed by a 1-min chase with 2 mM methionine and TCA precipitation as described above.

After TCA precipitation, total cell extracts were prepared (1 ml for the σ^S synthesis and half-life samples; 3 ml for the control samples), and total cellular incorporation of [^{35}S]methionine was determined. Aliquots (0.1 ml) of the RH100(pRL44) extract were added as an internal standard to 0.5-ml samples of the other extracts. σ^S and the *rpoS*::*lacZ*-encoded hybrid protein were isolated by immunoprecipitation as described (Hengge-Aronis and Boos 1986), using the polyclonal serum against σ^S, and analyzed by SDS-PAGE. Gels were dried and subjected to autoradiography. For quantitative analysis the radioactivity in the σ^S bands and the hybrid protein bands was determined directly from the gels using a PhosphorImager (Molecular Dynamics) (Johnston et al. 1990).

β-Galactosidase assay

β-Galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as micromoles of *o*-nitrophenol per minute per milligram of cellular protein (Miller 1972).

mRNA secondary structure prediction

mRNA secondary structure predictions were done according to published methods (Jaeger et al. 1989; Zuker 1989) with the MULFOLD and LOOPVIEWER programs developed by Don Gilbert, Indiana University, Bloomington (published on Internet) on a Macintosh Centris 650.

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