

In Vitro Properties of RpoS (σ^S) Mutants of *Escherichia coli* with Postulated N-Terminal Subregion 1.1 or C-Terminal Region 4 Deleted

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Derivatives of the stationary-phase sigma factor σ^S of *Escherichia coli* lacking either of two conserved domains, the postulated N-terminal subregion 1.1 or the C-terminal region 4, were shown to be competent in vitro for transcription initiation from several σ^S -dependent promoters on supercoiled DNA templates. Unlike wild-type σ^S , however, the deletion derivatives were inactive on relaxed templates. The anomalous slow electrophoretic mobility of σ^S on denaturing gels was corrected by deletion of subregion 1.1, suggesting that this domain in σ^S may be structurally and functionally analogous to subregion 1.1 of σ^{70} ; substitutions in which have previously been shown to rectify the anomalous electrophoretic migration of σ^{70} (V. Gopal and D. Chatterji, Eur. J. Biochem. 244:614–618, 1997).

Enterobacterial strains, including *Escherichia coli*, possess two primary and several alternative σ factors that are mutually exclusive in their association with RNA polymerase core enzyme (E) to constitute different holoenzyme species, each with a different promoter specificity in the initiation of transcription (reviewed in references 15, 18, and 27). The two primary σ factors in *E. coli* are RpoD, or σ^{70} , with 613 amino acid residues, which is the housekeeping or indispensable σ factor (6, 27), and RpoS, or σ^S , with 330 amino acid residues, which is important for gene expression under particular conditions of growth, such as the stationary phase, high osmolarity, or low temperature (17, 18, 42).

Based on sequence alignment of a large number of σ factors from both gram-positive and gram-negative bacteria, four conserved regions (numbered 1 to 4, beginning from the N-terminal end) have been delineated in σ^{70} , and each is further divided into subregions (27). Regions 2 and 4 are the most highly conserved among the various σ factors and bind, respectively, to the -10 and -35 promoter sequence motifs (8, 30). σ^{70} mutants from which region 4 has been deleted are still proficient for transcription in vitro at promoters that are not dependent for their activity on a good fit to consensus at the -35 region (22, 23).

Region 1 in σ^{70} has further been divided into two subregions, 1.1 and 1.2, of which subregion 1.1 (more N-terminal) is highly acidic and appears to be conserved only in the housekeeping σ factors of different bacteria and to a lesser extent

perhaps in σ^S (27, 34). Subregion 1.1 in *E. coli* σ^{70} (comprising the segment from approximately residues 30 through 100) has been ascribed various functions and activities, including those of masking the DNA-binding domain(s) in free σ (10, 11) and mediating the binding of σ with core enzyme (16, 32), thereby influencing promoter binding and the efficiency of transcription initiation by holoenzyme (4, 5, 13, 31, 46, 47). Deletion of subregion 1.1 of σ^{70} is associated with a dominant lethal phenotype in *E. coli* (10, 38). Interestingly, the σ^{70} polypeptide exhibits on denaturing polyacrylamide gels an anomalous slow electrophoretic mobility whose basis is not completely understood (6), but this anomaly was corrected in two different mutants with single amino acid substitutions in subregion 1.1 (13).

The second primary σ factor in *E. coli* σ^S also has the conserved regions 1 through 4 (Fig. 1). The N-terminal acidic stretch of 60 amino acids has been postulated to constitute its subregion 1.1 (27, 34), but Wilson and Dombroski (47) undertook sequence alignment of the housekeeping σ factors from nine bacteria to identify invariant and conserved residues in subregion 1.1, less than half of which are present in σ^S (34); furthermore, unlike the case of σ^{70} (10, 11, 47), σ^S from which this N-terminal region has been deleted appears to retain substantial functional activity in vivo (34). It has also been speculated that region 4 in σ^S may be dispensable for promoter recognition (19), since the recognition specificity is apparently dictated mainly by the -10 hexamer and the $-13/-14$ sequences at the promoter (2, 21, 41).

In the present study, we prepared His-tagged derivatives of full-length and truncated σ^S polypeptides and tested their properties in vitro. Our studies provide direct evidence that holoenzyme bearing σ^S lacking either postulated subregion 1.1 or region 4 retains at least partial activity at σ^S -dependent promoters on supercoiled DNA templates. The deletion mutants were inactive on relaxed templates, whereas full-length σ^S under the same conditions was equally active on both supercoiled and relaxed templates. Furthermore, our data dem-

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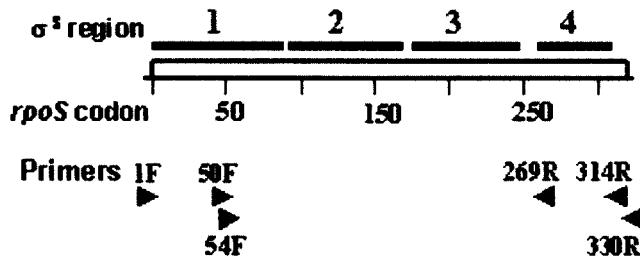


FIG. 1. Conserved regions in σ^S and construction of N- and C-terminally truncated σ^S mutants. The *rpoS* coding region is shown by the open bar, and the lines above demarcate the segments encoding the conserved regions 1 through 4 in the σ^S polypeptide. Aligned below are arrowheads depicting the positions for annealing to the *rpoS* locus of forward and reverse PCR primers (suffixed F and R, respectively) employed in construction of the various His-tagged σ^S derivatives: σ^S NH, 1F and 330R; σ^S NH Δ 2-50, 50F and 330R; σ^S NH Δ 2-54, 54F and 330R; σ^S NH Δ 315-330, 1F and 314R; and σ^S NH Δ 270-330, 1F and 269R. The sequences of the primers (5' to 3'; mismatches to *rpoS* sequence are shown in italics) were as follows: 1F, GGAGGCCACCATA TGAGTCAGAATAC; 50F, GTTATCGCAG *CATATGACACAGC* GTGTG; 54F, GAGCCACACAGC *ATATGTTGGACGC*; 269R, CG TGCCAGC *GCGGCCGCCTATTG*GG; 314R, CCCCTGCGTTGCG *GCGCTTAGGCAA*; and 330R, CTGACAGGCGGCCGCTTACT CGCGG.

onstrate that the anomalously slow electrophoretic mobility of σ^S on denaturing polyacrylamide gels (25, 42) is a property that is conferred by the N-terminal region of the polypeptide, providing additional support to the hypothesis that this region is structurally and functionally analogous to subregion 1.1 in σ^70 .

Evidence for expression in vivo of a partially functional σ^S Δ 2-54 polypeptide from an *rpoS* allele with amber mutation in codon 33. The ancestral *E. coli* K-12 strain and several of its derivatives have been shown to bear an amber mutation in codon 33 of the *rpoS* gene (1, 45). In the course of studies on PCR amplification and sequencing of the *rpoS* gene from different strains, we noted that strain W3350 and several stocks of strain W3110 (19) also carried this mutation.

The *rpoS* (Am) allele from W3350 was cloned downstream of the *lac* promoter on a pBR322-based plasmid vector, and the resulting plasmid (designated p25*rpoS*) was introduced into the suppressor-free null *rpoS*:Kan strain ZK1000 (obtained from R. Kolter). The following lines of evidence indicated that the mutant *rpoS* gene on the plasmid was able to direct the synthesis of a σ^S variant polypeptide that lacks the major part of the postulated subregion 1.1 but that still retains at least partial σ^S activity in vivo. (i) In Western blot experiments with anti- σ^S antibody, cell extracts of ZK1000/p25*rpoS* exhibited a cross-reacting band of apparent M_r of 34,000 (that is, smaller than the native σ^S band of apparent M_r of 43,000) whose intensity was approximately 20 to 30% of that for native σ^S from a haploid *rpoS*⁺ strain; as expected, no immunoreactive band was detected in cell extracts of either ZK1000 or W3350 (data not shown). (ii) The ZK1000/p25*rpoS* cell extract also possessed a moderate level of catalase HPII activity (encoded by the σ^S -dependent *katE* gene), as assayed by the method of Visick and Clarke (45); the activity was approximately 60% of that in the haploid *rpoS*⁺ strain (data not shown). (iii) Finally, N-terminal sequence analysis indicated that the protein immunoprecipitated by anti- σ^S antibody from the cell extract has an

N-terminal Met residue followed by a sequence which is identical to that downstream of residue 54 in native σ^S . We refer to this protein as σ^S Δ 2-54 and infer that it has been synthesized from the mutant gene by reinitiation of translation from codon 54 (GUG) in *rpoS* mRNA (eleven nucleotides upstream of which also is the sequence AGGGAG that represents a ribosome-binding-site motif).

Based on studies on an *IS10* insertion mutation in *rpoS*, a recent report had also similarly concluded that a variant of σ^S whose N-terminal 50 amino acids were expected to have been substituted by six amino acids encoded by the *IS10* end is partially active in vivo (34). Accordingly, in the experiments described below, we tested two N-terminally truncated σ^S polypeptides (Δ 2-50 and Δ 2-54) for their properties in vitro. We also tested two C-terminal deletions of σ^S (Δ 315-330, with removal of residues downstream of region 4 in the segment that has been termed the C-terminal extension [33], and Δ 270-330, with removal of all of region 4).

Expression and purification of His-tagged full-length or truncated σ^S derivatives. Procedures for PCR and molecular cloning were as described previously (37). Appropriate forward and reverse oligonucleotide primer pairs were used along with a proofreading-proficient thermostable DNA polymerase to PCR amplify, from a chromosomal DNA preparation of wild-type *E. coli*, *rpoS* sequences that represented full-length σ^S or σ^S derivatives with N- or C-terminal truncations (Fig. 1). Each amplified *rpoS* sequence was flanked at its upstream and downstream ends by *Nde*I and *Not*I sites, respectively, and the products were then cloned into the *Nde*I-*Not*I sites of plasmid vector pET28a (Novagen, Madison, Wis.) so that the vector-encoded sequence provided an N-terminal fusion of 20 amino acid residues (including a six-residue His tag) to each of the full-length or truncated σ^S derivatives. The authenticity of each plasmid construct was confirmed by DNA sequencing (data not shown). Our decision to place the His tag at the N-terminal ends of the polypeptides was based on an earlier report that N-terminally His-tagged σ^S is fully functional in vitro (46).

The plasmids so constructed were introduced into one of two overexpression host strains, BL21-SI (12) or BL21(DE3) (40), and overproduction of the σ^S derivatives was achieved in 250-ml cultures by induction, respectively, with both isopropyl- β -D-thiogalactopyranoside and NaCl (3, 12) or with isopropyl- β -D-thiogalactopyranoside alone (40). Cell lysates were prepared after suspension in lysis buffer (50 mM Tris-Cl [pH 8], 1 mM EDTA, 100 mM NaCl) as previously described (25, 26), and the overproduced protein (present predominantly in the insoluble fraction) was purified from each of them in a single step by column chromatography through a nickel-nitrilotriacetic acid resin column (Qiagen, Chatsworth, Calif.) in the presence of 6 M guanidine hydrochloride (following the manufacturer's instructions). In each case, the His-tagged protein was eluted in a 2-ml volume with 100 mM imidazole, and the chaotrope was then removed by dialysis at 4°C against two changes of one liter each of storage buffer (10 mM Tris-Cl [pH 7.6], 200 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol). The dialysates were centrifuged at 12,000 \times g for 10 min at 4°C, and the supernatants, which were judged to be substantially pure for the full-length and truncated σ^S polypeptides (see Fig. 2), were stored at -30°C. The His-tagged polypeptides so prepared included those of full-

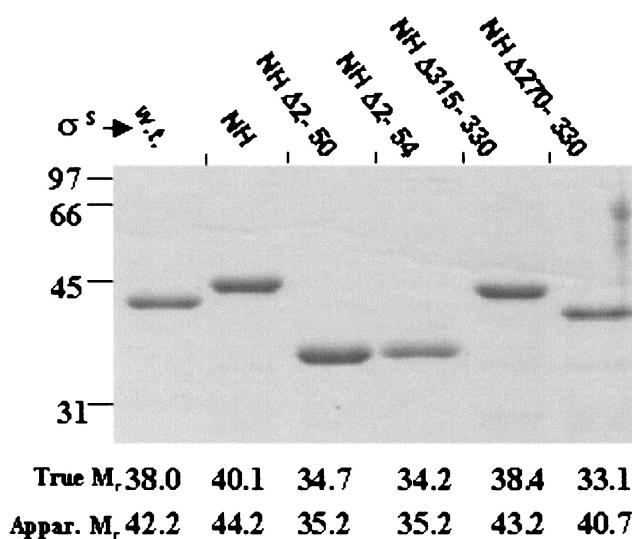


FIG. 2. Electrophoretic migration of σ^S and its derivatives. The indicated σ^S derivatives were electrophoresed (37) on a 10% polyacrylamide gel with sodium dodecyl sulfate and visualized after staining with Coomassie blue. At left are shown the positions of migration of marker proteins of indicated M_r . For each σ^S derivative, the true and apparent (Appar.) M_r values, the latter as calculated from the migration rate on the gel, are given below the corresponding lane. w.t., wild-type (native) σ^S .

length σ^S (σ^S NH), N-terminal truncations of amino acid residues from 2 to 50 (σ^S NHΔ2-50) or from 2 to 54 (σ^S NHΔ2-54), and C-terminal truncations of amino acid residues from 270 to 330 (σ^S NHΔ270-330) or from 315 to 330 (σ^S NHΔ315-330).

Electrophoretic mobility of σ^S derivatives on denaturing polyacrylamide gels. It is known that σ^S , like several other σ factors (25), exhibits anomalously slow migration properties during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the electrophoretic mobility of the various purified His-tagged σ^S preparations were compared by standard procedures (37) with that of native σ^S , it was observed that σ^S NH, as well as each of its C-terminal truncated derivatives σ^S NHΔ270-330 and σ^S NHΔ315-330, exhibited an anomalous mobility similar to that of native σ^S , whereas in the N-terminally truncated derivatives σ^S NHΔ2-50 and σ^S NHΔ2-54, the anomaly had largely been corrected (Fig. 2). The difference is particularly striking, for example, between σ^S NHΔ270-330 and either of the N-terminally truncated derivatives, since the former is smaller and yet migrates less rapidly than the latter. The results therefore indicate that deletion of the postulated N-terminal subregion 1.1, but not of C-terminal region 4, is associated with correction of the anomalous electrophoretic mobility of σ^S .

In vitro transcription with σ^S derivatives on supercoiled templates. Each of the σ^S derivatives was reconstituted as previously described (26) with highly purified core RNA polymerase at a ratio of 4:1 or higher, and the holoenzyme preparations were used in in vitro transcription experiments. The DNA templates in the reactions were plasmids pHYD351 (36) or pHYD406, supercoiled preparations of which were obtained with the aid of a commercial plasmid preparation kit (Qiagen)

from transformants of the strain DH5 (37). The two plasmids are derivatives of plasmid vector pCU22 (43) and carry the -60 to +116 and the -121 to +70 regions of the σ^S -dependent *proU* P1 (35) and *glgS* P2 (2) promoters, respectively, from *E. coli*, cloned about 50 bp upstream of the first of a tandem pair of Rho-independent transcription terminators present in the vector (that are themselves 40 bp apart).

Single-round in vitro transcription reactions with [α -³²P] UTP were performed, and the products were analyzed by autoradiography following electrophoresis on urea-polyacrylamide gels, as earlier described (36); the results are shown in Fig. 3. $\text{E}\sigma^S$ NH, carrying the N-terminal His-tagged full-length σ^S derivative, was indistinguishable from $\text{E}\sigma^S$ (Fig. 3A, compare lanes 1 and 2), while the negative control reaction with core enzyme E alone yielded no transcripts under these conditions for both pHYD406 (Fig. 3B, lane 6) and pHYD351 (data not shown). The two N-terminally truncated σ^S derivatives and the two C-terminally truncated derivatives exhibited substantial transcriptional activity at both the *proU* P1 and *glgS* P2 promoters (Fig. 3A and B, respectively). All the deletion derivatives were active also for transcription of RNA-I (whose promoter, in the pCU22 vector-derived sequence common to both plasmids, is known to be recognized by both $\text{E}\sigma^{70}$ and $\text{E}\sigma^S$ [26, 35, 36]), although there was some variation in its relative band intensity between different experiments, even for the same σ^S mutant preparation (compare, for example, lanes 3 and 2 in panels A and B, respectively, of Fig. 3 for σ^S NHΔ2-50).

The N- and C-terminally truncated σ^S derivatives were also active in vitro on the σ^S -dependent *proU* P1 promoter of *Salmonella enterica* serovar Typhimurium carried on a supercoiled pCU22 derivative plasmid (35; data not shown). As expected, none of the σ^S derivatives was active on the exclusively σ^{70} -dependent *proU* P2 promoter (36; data not shown).

Ohnuma et al. (33) have previously demonstrated that holoenzyme carrying a mutant σ^S with deletion of the C-terminal residues from 315 to 330 is rendered salt sensitive in that it is transcriptionally inactive in the presence of 0.2 M potassium glutamate, whereas the activity of $\text{E}\sigma^S$ itself is known to be actually stimulated under these conditions (26, 36). Using the various holoenzyme preparations of the His-tagged full-length and N- or C-terminally truncated σ^S derivatives on supercoiled plasmid pHYD351 (with *E. coli* *proU* P1) as template, we were able to confirm that it is only the holoenzyme reconstituted with either of the C-terminally truncated mutants (σ^S NHΔ315-330 or σ^S NHΔ270-330) whose activity is sensitive to a high potassium glutamate concentration in vitro (data not shown). Similarly, only the two C-terminal truncation mutants were consistently associated with a reduction in the background of high-molecular-weight RNA species that is a common feature of in vitro transcription with supercoiled plasmid DNA templates (35, 36, 43) (see Fig. 3A and B); a plausible explanation for this observation is given in the following section.

In vitro transcription with σ^S derivatives on linear templates. Previous studies have shown both that the superhelical density of DNA in *E. coli* cells is less in the stationary phase than it is in the exponential phase and that σ^S -directed transcription in vitro from a σ^S -dependent promoter *osmY* is indeed maximal from a relaxed DNA template (26). We examined the ability of the His-tagged σ^S full-length and truncated derivatives to support transcription from linear DNA tem-

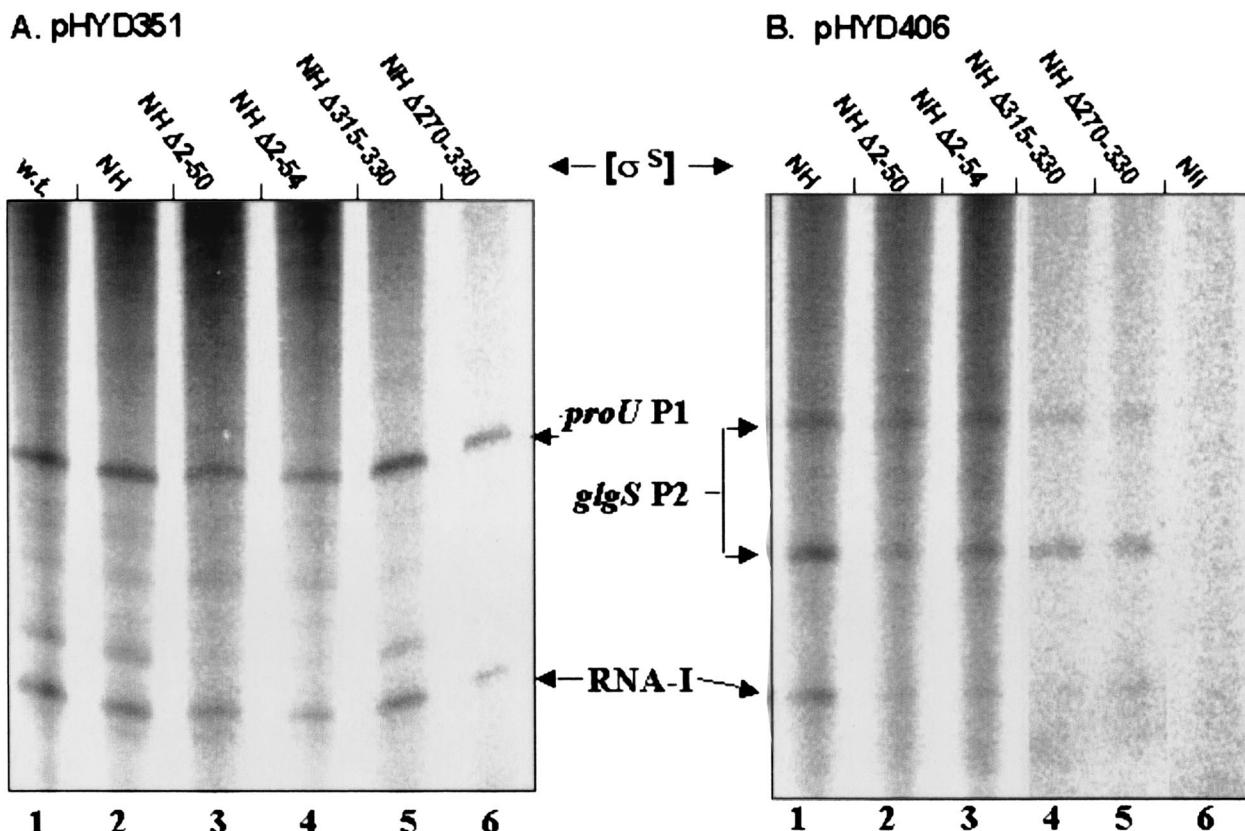


FIG. 3. Single-round in vitro transcription with indicated $E\sigma^S$ derivatives on supercoiled pHYD351 (A) and pHYD406 (B) templates, carrying the *proU* P1 and *glgS* P2 promoters, respectively. Bands corresponding to RNA-I and to the transcripts initiated from *proU* P1 and *glgS* P2 are marked. The reaction represented on lane 6 (labeled Nil) in panel B was done with core enzyme alone (without any σ^S).

plates carrying the *E. coli* *proU* P1 and *glgS* P2 promoters. For this purpose, the template plasmids pHYD351 and pHYD406 were digested with *Pst*I, which cuts each plasmid at a single site immediately upstream of the promoter-bearing DNA fragment cloned into the pCU22 vector.

The results of these experiments are shown in Fig. 4. For either promoter tested, the full-length σ^S derivative σ^S NH was as effective for transcription from a linear template as it was from a supercoiled template (Fig. 4, compare lane 1 with lane 2 in each of the two panels). On the other hand, the N- and C-terminally truncated σ^S derivatives were all inactive for transcription from both *proU* P1 and *glgS* P2, as well as from the RNA-I promoter, carried on the linear templates. These data indicate that the truncations have compromised the property of supercoiling insensitivity of promoter-specific transcription that is associated with full-length σ^S .

We also observed an interesting difference in behavior between the σ^S derivatives with N-terminal truncations, on the one hand, and those with C-terminal truncations, on the other. The former behaved like σ^S NH on either DNA template in yielding a pair of bands (designated by the term "end loading" in Fig. 4) whose sizes (approximately 230 and 270 bases) are consistent with transcription initiated from the upstream *Pst*I-cut end (29) and proceeding through the cloned promoter fragment to the tandem terminators situated downstream. On the other hand, the C-terminal deletion derivatives did not

yield such products (Fig. 4, compare lanes 1 to 3 with lanes 4 and 5 in each of the panels A and B). Therefore, the loss of postulated subregion 1.1 in σ^S does not apparently affect the ability of holoenzyme to load nonspecifically at a DNA nick or end and initiate transcription (29); the inability of the C-terminal deletion mutants to do so may perhaps explain the reduced background of high-molecular-weight RNA species in the cognate transcription reactions with supercoiled template preparations (Fig. 3), which are expected also to have a proportion of nicked and linear DNA molecules.

Discussion. In this study, we have investigated the in vitro properties of mutant σ^S derivatives with N-terminal deletions of the postulated subregion 1.1 and with two different C-terminal deletions. In a previous study (33), the stretch of 16 amino acid residues at the extreme C terminus of σ^S was taken to be separate from, and downstream of, its subregion 4.2, as defined by Lonet et al. (27). We found in this study, however, that in a variety of assays, the transcriptional activity of a mutant σ^S lacking its C-terminal 16 amino acid residues is indistinguishable from that with a larger deletion of all of region 4, suggesting that the extreme C terminus is also a part of region 4 in the polypeptide.

The present data provide direct and conclusive evidence that mutant σ^S polypeptides from which postulated subregion 1.1 or region 4 is deleted, and whose putative occurrence in some naturally occurring *E. coli* strains is not unlikely (1, 19, 34, 45),

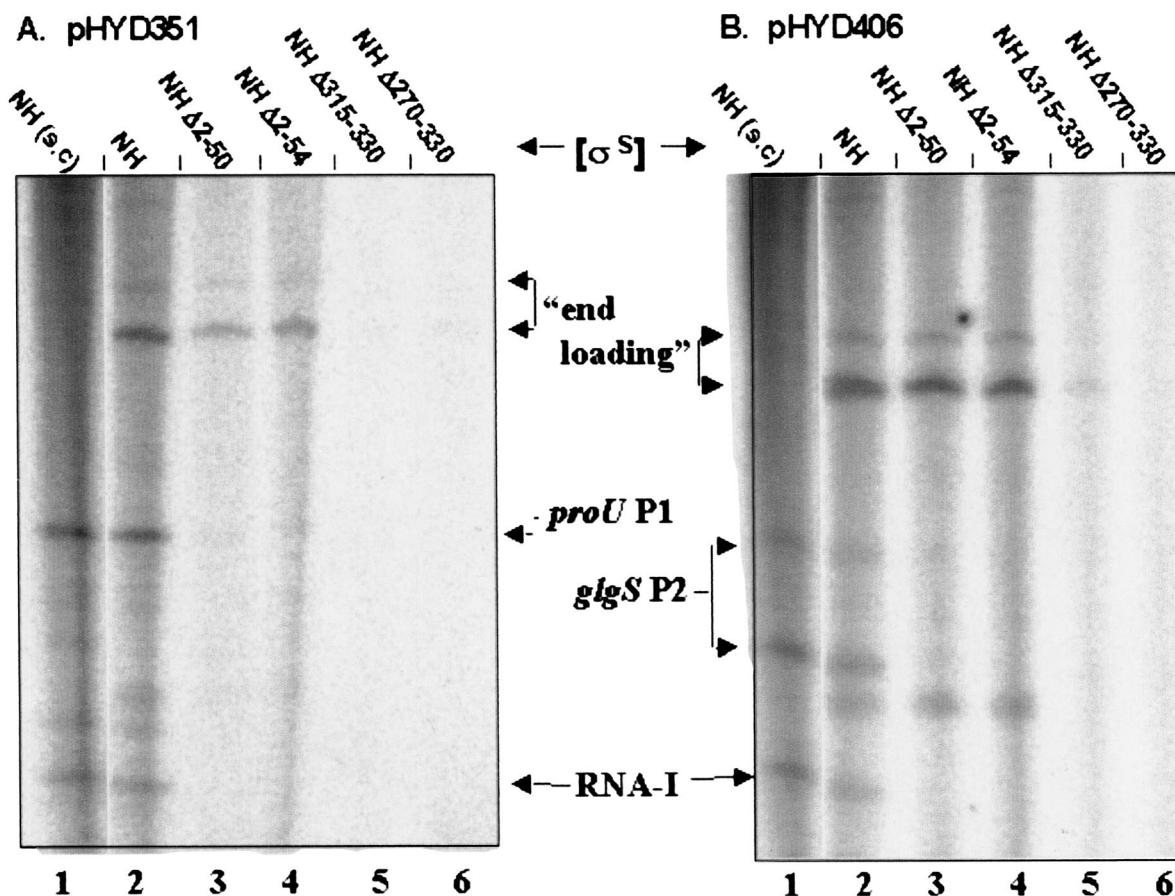


FIG. 4. Single-round in vitro transcription with indicated σ^S derivatives on linear pHYD351 (A) and pHYD406 (B) templates, carrying the *proU* P1 and *glgS* P2 promoters, respectively. Lane 1 in each panel represents a control reaction in which the corresponding supercoiled (s.c.) template was employed. Bands corresponding to RNA-I and to the transcripts initiated from *proU* P1 and *glgS* P2 are marked. Bands labeled "end loading" are discussed in the text.

retain substantial promoter-specific transcription-initiation activity on supercoiled templates. In particular, the behavior of the σ^S mutant lacking region 4 is markedly different from that of the equivalent σ^{70} mutant, which is active in vitro only on the subset of promoters that possess an extended -10 recognition motif (23).

Our observations indicate that in comparison with full-length σ^S , both the N-terminal and the C-terminal deletion mutants are severely compromised for activity at promoters on linear or relaxed templates. Although a mechanistic explanation for this phenomenon was not obtained in this study, we suggest that holoenzymes reconstituted with the mutant σ^S polypeptides are unable to achieve efficient isomerization from the closed to the open complex at the various promoters and that they are able to do so only if the energy of DNA supercoiling is available for facilitating strand opening at the promoter -10 region (9). A similar phenotype of supercoiling sensitivity of transcription in vitro has been reported for mutations in subregion 2.3 of σ^A (the *Bacillus subtilis* housekeeping σ factor) and shown to be associated with a defect in melting of the DNA strands at the -10 region of the promoter (20). Since the stationary growth phase is believed to be associated with relaxation of DNA supercoiling (26), our in vitro

data may also explain why it is that *in vivo*, even the shorter C-terminal deletion mutant of σ^S is inactive (33) and the subregion 1.1 deletions are only partially active (34; this study) at σ^S -dependent promoters.

Anomalous electrophoretic mobility is an unusual property of σ^{70} (6) that is also shared by a variety of other σ factors, including σ^S , σ^H , σ^F , and σ^N of *E. coli* (25, 42) and σ^A of *B. subtilis* (39), but whose basis is not completely understood. It had earlier been suggested (6) that an acidic region between amino acid residues 184 and 215 in σ^{70} may be responsible, but a mutant from which this region has been deleted retains this property (24). Gopal and coworkers have shown that substitution mutations in subregion 1.1 (13) or a mutation in subregion 2.3 (14) of σ^{70} abolish its aberrant migration and that the cognate holoenzymes are defective for transcription initiation on linear templates; interestingly, the residue mutated in subregion 2.3 in the latter study was the equivalent of that whose substitution in *B. subtilis* σ^A conferred supercoiling sensitivity (20).

Gopal and coworkers have therefore suggested that binding to DNA of the free σ^{70} subunit is inhibited by hydrophobic interaction of subregion 1.1 with the -10 promoter DNA-binding subregion 2.3 and that this interaction is also respon-

sible for the aberrant electrophoretic mobility of σ^{70} (13, 14). Their suggestions are contrary to the more prevalent view (10, 11) that autoinhibition of σ^{70} binding to DNA involves direct interaction between subregion 1.1 and domain 4.2 that binds the -35 region of the promoter, but they are consistent with three other findings, namely, (i) that subregion 1.1 of σ^{70} also masks the core-binding sites in the vicinity of subregion 2.3 (16), (ii) that the only mutations or deletions in σ that affect the isomerization step (of holoenzyme bound to promoter) from the closed complex to the open complex map either to subregion 1.1 (13, 46, 47) or to subregion 2.3 (9, 14, 20), and (iii) that the property of supercoiling sensitivity in transcription initiation is shared by subregion 1.1 mutations (in σ^S , as shown in this study) and subregion 2.3 mutations (as shown for *B. subtilis* σ^A [20]). Furthermore, a recent study (7) has excluded the possibility of direct interaction between subregions 1.1 and 4.2 of σ^{70} . It is noteworthy that crystals of σ^{70} (either as free subunit or as part of holoenzyme) have been successfully obtained only if the autoinhibitory subregion 1.1 of the polypeptide is omitted (8, 28, 31, 44), although crystals of the holoenzyme-DNA complex can include this domain (30, 31).

In this context, our finding in this study that deletions of the N-terminal region (but not of region 4) in σ^S abolish aberrant mobility is significant in that (i) it strengthens the case for a distinct subregion 1.1 in σ^S equivalent to that in σ^{70} and (ii) it supports an autoinhibitory role for this subregion in free σ^S similar to that for σ^{70} suggested by Gopal and coworkers (13, 14).

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