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Sequences in the −35 Region of *Escherichia coli rpoS*-Dependent Genes Promote Transcription by $E\sigma^S$

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σ^S is an alternate sigma factor which functions with RNA polymerase to activate transcription of genes that are involved in a number of stress responses, including stationary-phase survival and osmoprotection. The similarity of the σ^S protein to σ^D (*Escherichia coli*'s major sigma factor) in the regions thought to recognize and bind promoter sequences suggests that σ^S- and σ^D-associated RNA polymerases recognize promoter DNA in a similar manner. However, no promoter recognition sequence for σ^S holoenzyme ($E\sigma^S$) has been identified. An apparent conservation of cytosine nucleotides was noted in the −35 region of several σ^S-dependent promoters. Site-directed mutagenesis and reporter gene fusions were used to investigate the importance of the −35 cytosine nucleotides for σ^S-dependent transcription. Substitution of cytosine nucleotides for thymidine at the −35 site of the σ^D-dependent *proU* promoter effectively abolished transcription by $E\sigma^D$ but allowed $E\sigma^S$ to direct transcription from the mutant promoter. Inclusion of the σ^D consensus −10 hexamer strengthened transcription by $E\sigma^S$, demonstrating that both $E\sigma^D$ and $E\sigma^S$ can recognize the same −10 sequences. Conversely, replacement of −35 site cytosine nucleotides with thymidine in the σ^S-dependent *osmY* promoter reduced transcription by $E\sigma^S$ and increased transcription by $E\sigma^D$. Our data suggest that DNA sequences in the −35 region function as part of a discriminator mechanism to shift transcription between $E\sigma^D$ and $E\sigma^S$.

Bacterial cells must be able to efficiently induce transcription of a number of genes whose products are required to survive environmental stresses. *Escherichia coli* can survive environments characterized by nutrient scarcity, limited oxygen availability, toxic chemicals, and high osmolarity. Several of the genes induced by environmental stress rely on the *rpoS* gene for induction (2, 9, 22–25, 30, 36, 61, 64).

rpoS encodes an alternate sigma factor (47) which acts as a principal regulator of *E. coli*'s stress response by modifying the promoter recognition capacity of core RNA polymerase. Stationary-phase induction is characteristic of *rpoS*-dependent genes (9, 22–24, 30, 34–36, 45, 58, 60, 61, 64). Several *rpoS*-dependent genes (*bolA*, *otsBA*, *treA*, *osmB*, *osmY*, and *pexB*) are also induced by hyperosmotic stress during exponential growth in a defined minimal medium (24, 25, 30, 34, 35, 38, 64). The cellular content of the σ^S protein correlates with σ^S-dependent gene induction, increasing during stationary phase and following hyperosmotic shock (37). Thus far, no DNA-binding protein that acts specifically as an activator of σ^S-dependent genes has been identified. However, a mutation in *hns*, which encodes a nonspecific DNA-associated protein, leads to accumulation of the σ^S protein during exponential growth (4, 63). An increase in the level of the alarmone ppGpp correlates with increased amounts of σ^S protein (16). UDP-glucose and homoserine lactone have also been proposed as signal molecules that influence production of σ^S (5, 27).

σ^S, like most known sigma factors, has notable homology to *E. coli*'s primary sigma factor, σ^D (σ^{70}) (39, 47). σ^D, which is encoded by the *rpoD* gene, is the chief determinant of promoter recognition by RNA polymerase during exponential growth. A large body of statistical and genetic evidence has identified the σ^D consensus promoter as two hexamers centered at about −10 and −35 nucleotides upstream relative to the start site of transcription (19, 20, 66). The ability of certain *rpoD* mutants to specifically suppress mutations at the −10 and −35 sites of the *E. coli lac* and P22 *ant* promoters strongly suggests that promoter recognition is determined by direct interactions between certain amino acids of regions 2.4 and 4.2 of the σ^D protein and nucleotides in the conserved −10 and −35 promoter hexamers (15, 52, 59).

Studies of promoters used by secondary sigmas in a variety of bacteria, including *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, have also identified two regions of conserved sequence believed to function as promoter recognition elements (10, 21, 67). The homology between σ^S and σ^D in the regions thought to determine promoter specificity is sufficient to predict that σ^S holoenzyme will interact with promoter DNA in a manner resembling that of σ^D holoenzyme. Specifically, one would suppose that σ^S promoters would consist of two conserved sequence elements.

It has been demonstrated through in vitro assays that transcription by σ^S holoenzyme is determined by recognition of nucleotides downstream from position −17 of *rpoS*-dependent promoters (56). We have investigated the in vivo requirements for transcription by $E\sigma^S$. We show here that $E\sigma^S$ can use the same −10 hexamer preferred by $E\sigma^D$. Furthermore, nucleotides in the −35 region of *rpoS*-dependent promoters play a prominent role in σ^S-dependent transcription.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study are listed in Table 1. All strains containing promoter-*lacZ* fusions are derivatives of MC4100. TE2680 was used to transfer promoter-*lacZ* fusions to the *E. coli* chromosome (14). DH5α (New England Biolabs) was host for all plasmid constructions.

Recombinant DNA techniques. Plasmid DNA was isolated either by alkaline lysis (3) or by the boiling method (26). Standard methods were used for restriction digests, gel electrophoresis, ligations, and transformation of *E. coli* DH5α (3). PCR was done as described by Innes et al. (28). The primers were either obtained from Operon Technologies or synthesized on a Millipore DNA synthesizer. DNA sequencing was done by the dideoxynucleotide chain termination method using the Sequenase enzyme and protocols from U.S. Biochemical Corp. P1*vir* transductions were done according to protocols described by Silhavy et al. (53).

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TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Genotype or description	Reference or source
Strains		
MC4100	F⁻ D(*argF-lac*)*U169 thiA*	7
RH90	MC4100 *rpoS359*::Tn*10*	36
TE2680	F⁻ λ⁻ IN(*rrnD-rrnE*)*1* D(*lac*)*X74 rpsL galK2 recD*::Tn*10d* Tet^r *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lacZ*]	14
JM409	MC4100 *trpDC700*::*proU-lacZ*	44
RB409	RH90 *trpDC700*::*proU-lacZ*	This study
RB110	MC4100 *trpDC700*::$proU_{CC}$-*lacZ*	This study
RB120	RH90 *trpDC700*::$proU_{CC}$-*lacZ*	This study
VR110	MC4100 *trpDC700*::$proU_{CCTATAAT}$-*lacZ*	This study
VR120	RH90 *trpDC700*::$proU_{CCTATAAT}$-*lacZ*	This study
AW446	MC4100 *trpDC700*::$proU_{446}$-*lacZ*	This study
AW447	RH90 *trpDC700*::$proU_{446}$-*lacZ*	This study
AW448	MC4100 *trpDC700*::$proU_{CC446}$-*lacZ*	This study
AW449	RH90 *trpDC700*::$proU_{CC446}$-*lacZ*	This study
RB10	MC4100 *trpDC700*::*osmY-lacZ*	This study
RB11	RH90 *trpDC*::*700*::*osmY-lacZ*	This study
RB12	MC4100 *trpDC700*::$osmY_{TT}$-*lacZ*	This study
RB13	RH90 *trpDC700*::$osmY_{TT}$-*lacZ*	This study
MG12	MC4100 *trpDC700*::$osmY_{AA}$-*lacZ*	This study
MG13	RH90 *trpDC700*::$osmY_{AA}$-*lacZ*	This study
Plasmids		
pTZ18U	pUC18 derivative	U.S. Biochemical Corp.
pRS551	pBR322 derivative with promoterless *lacZ* gene	54
pKK232-8	pBR322 derivative with promoterless *cat* gene	Pharmacia Biotech
pBP33U	pTZ18U containing the *proU* promoter region positions (377 to 786)	49
pJM409	pRS551 containing the *proU* promoter region	44
pAW409	pRS551 containing the $proU_{CCTATAAT}$ promoter	This study
pAW411	pK232-8 containing the 0.4-kb $proU_{CCTATAAT}$ promoter	This study
pAW412	pK232-8 containing the 0.4-kb $proU_{TATAAT}$ promoter	This study
pAN446	pRS551 containing the 0.4-kb $proU_{446}$ promoter	This study
pDY1.4	pTZ18U containing the *osmY* promoter on a 1.4-kb fragment	64
pRB10	pRS551 containing the *osmY* promoter on a 0.9-kb fragment	This study
pRB11	pRS551 containing the $osmY_{TT}$ promoter	This study

Mutagenesis of the *proU* and *osmY* promoters. The $osmY_{TT}$ and $proU_{CC}$ mutant promoters were synthesized by a modification of the method described by Kunkel (33). Klenow enzyme was substituted for T4 DNA polymerase to circumvent difficulties in polymerization due to DNA secondary structure. The oligonucleotide 5′-GAATCTGAGGCGGCCCCTGATGGC-3′ was used with plasmid pBP33U to synthesize the $proU_{CC}$ promoter. The oligonucleotide 5′-CGCTGATATCTTGAGCGGTTTC was used with plasmid pDY1.4 to create the $osmY_{TT}$ promoter. Underlined positions in primers correspond to nucleotide modifications in mutant promoters.

The sequential PCR method (3) was used to create the $proU_{CCTATAAT}$, $proU_{CC446}$, $proU_{TATAAT}$, and $osmY_{AA}$ promoters. End primers used to amplify mutant promoter fragments either matched sequences upstream from the cloning sites of pTZ18U (M13 Reverse Primer; Stratagene) or were complementary to a region downstream from the cloning sites (U primer; U.S. Biochemical Corp.). Mutagenic primers used to incorporate the consensus −10 sequence in the $proU_{TATAAT}$ and $proU_{CCTATAAT}$ mutant promoters were 5′-CAGTATGT TATAATAGAAAAAAGTG-3′ and its exact complement. Templates used in construction of these variants of the *proU* promoter were pBP33U and a pTZ18U derivative containing the $proU_{TATAAT}$ promoter. pAN446 was used as template with primer 5′-GAATCTGAGGCGGCCCCTGATGGC-3′ and its exact complement to create the $proU_{CC446}$ promoter. The $osmY_{AA}$ promoter was created by using pRB10 as a template with primer 5′-GAAACCGCTCTT GATATCAG-3′ and its exact complement. Mutant promoter fragments were cloned into pTZ18U to allow confirmation of the desired mutation through DNA sequencing.

Strain construction. Mutant *proU* promoter fragments were removed from pTZ18U derivatives with an *Eco*RI-*Bam*HI double digest. pTZ18U derivatives containing the *osmY* promoter and its variants were digested with *Eco*RI and *Bst*EII, resulting in a 0.9-kb promoter fragment. Promoter fragments were cloned with transcription oriented toward the promoterless *lacZ* gene of pRS551. TE2680 was transformed to kanamycin resistance with linearized pRS551 derivatives. Transformants were screened for loss of ampicillin and chloramphenicol resistance to affirm proper integration into the chromosome (14, 54). P1*vir* transduction was used to move promoter-*lacZ* fusions from the chromosome of TE2680 to the chromosomes (*trpDC* locus) of MC4100 and RH90.

To clone the $proU_{TATAAT}$ and $proU_{CCTATAAT}$ promoters into pKK232-8, the *Eco*RI sites of the appropriate pTZ18U derivatives were first replaced with *Bam*HI. The resulting plasmids were digested with *Bam*HI, and promoter fragments were cloned into the *Bam*HI site preceding the promoterless *cat* gene of pK232-8. DH5α was transformed with the resulting plasmids with selection for ampicillin resistance. The transformants were screened for their ability to grow on chloramphenicol. Only plasmids with promoters oriented towards the *cat* gene allowed growth on chloramphenicol. The resulting pKK232-8 derivatives, pAW411 and pAW412, were moved by transformation into MC4100 and RH90 for promoter analysis.

Growth conditions. Growth was monitored with a Klett-Summerson colorimeter. For assays of stationary-phase promoter activity, an overnight culture of cells was diluted 1:1,000 in Luria-Bertani (LB) broth made with 5 g of NaCl per liter (46) and then grown through the 2nd or 3rd h of stationary phase. Osmotic induction of promoter activity was measured after growth in M9 minimal medium (46) supplemented with 0.2% glucose, 0.05% Casamino Acids, and 1 μg of thiamine and 40 μg of tryptophan per ml. When the cells reached exponential phase (about 40 Klett units), the cultures were divided. One half of the divided culture was subjected to hyperosmotic shock by the addition of NaCl to 0.3 M. Samples were removed for enzyme assays at the times indicated in the figures.

Enzyme assays. Strains containing single-copy promoter-*lacZ* fusions were lysed with sodium dodecyl sulfate and chloroform and assayed for β-galactosidase activity according to the method described by Miller (46). Measurements of activity are given in Miller units.

Chloramphenicol acetyltransferase activity in cells containing pAW411 or pAW412 was assayed according to the spectrophotometric method described by Shaw (51). Five-milliliter samples were removed at the end of the 3rd h of stationary phase after growth in LB. The cells were washed and resuspended in TDTT (50 mM Tris [pH 7.8], 30 μM dithiothreitol) and subjected to sonication. Broken cells were microcentrifuged to remove cell wall debris. Diluted cell extract was added to a reaction mixture consisting of 1 μM 5,5′-dithiobis-2-

TABLE 2. Nucleotide sequences of 11 *rpoS*-dependent gene promoters

σ^S-dependent promoter	Sequence[a]			References
	−35	16–18 bp	−10	
σ^D consensus sequence	TTGACA		TATAAT	20
osmY	TATC CCGAGC	GGTTTCAAAATTGTGATC	TATATT	TAACAAA 64
osmB (P1)	TCAT CCGCTC	TAAGATGATTCCTGGTT	GATAAT	TAAGA 30
fic	CTCT CCGGCG	TAACCCGATTTGCCGCT	TATACT	TGTGG 58
proP (P2)	TTAA CCGGAG	GGTGTAAGCAAACCCGC	TACGCT	TGTTACA 11, 45
aldB	ACTG GCGAAG	ATTTCGCCAGTCACGTC	TACCCT	TGTTATA 62
bolA (P1)	TAAG CTGCAA	TGGAAACGGTAAAAGCG	GCTAGT	ATTTA 1, 35
xthA	CAGG CGGTAA	GCAACGCGAAATTCTGC	TACCAT	CCACGCA 50
glgS (P2)	ATTT ACGCAC	GTTATGTTTAAAGGCAC	TACACT	GATTGGGG 23
poxB	CATC CCTTCC	CCCTCCGTCAGATGAAC	TAAACT	TGTTACCG 9
cfa (P2)	GATT TCTCAC	AAAGCCCAAAAAGCGTC	TACGCT	GTTTTA 60
pexB (*dps*)	CAGA ATAGCG	GAACACATAGCCGGTGC	TATACT	TAATCTCG 2, 38

[a] The σ^D consensus promoter sequence is shown for comparison. Sequences that appear to be conserved in *rpoS*-dependent promoters are underlined. The last nucleotide shown for each promoter sequence has been identified as the start site of transcription.

nitrobenzoic acid (Sigma) and 0.1 mM acetyl coenzyme A (Sigma) in 0.1 M Tris (pH 7.8). After the background rate of acetylation was measured, 20 μl of chloramphenicol was added to start the reaction. The rate of acetylation was obtained by measuring the increase in A_{412}. The amount of protein in cell extracts was measured by the Lowry method (40). Units of chloramphenicol acetylation are expressed as nanomoles per minute per milligram of protein.

Primer extension analysis. Whole-cell RNA was prepared as previously described (45) from MC4100 and RH90 cells which contained one of the following plasmids: pJM409, pAW409, pRB10, or pRB11. Cells were grown as for enzyme assays in either LB, M9, or M9 with 0.3 M NaCl. Primer extension of the *proU* and $proU_{CCTATAAT}$ promoters was performed with an oligonucleotide complementary to nucleotides 736 to 755 of the *proU* sequence (18). The oligonucleotide used for primer extension of the *osmY* and $osmY_{TT}$ promoters is complementary to +102 to +121 of the *osmY* sequence (64).

RESULTS

We began our promoter analysis by searching for homologies among 11 *rpoS*-dependent promoters for which transcription initiation sites have been identified (Table 2). Promoter recognition by $E\sigma^S$ has been demonstrated through in vitro assays for at least 5 of the 11 promoters listed in Table 1 (13, 48, 57).

In line with observations by others (9, 38, 57, 60, 64), we noted that each *rpoS*-dependent promoter contained a sequence with three to five matches to the σ^D consensus −10 hexamer (TATAAT). Spacing between these putative σ^S −10 hexamers and transcription start sites varied from four to seven nucleotides, which is close to that found for σ^D promoters (20).

Another region of possible sequence conservation was noted in the region which corresponds spatially to the −35 hexamer of σ^D promoters. In this area, 10 of the 11 promoters contained one or two cytosine nucleotides at the 5′ end usually followed by guanidine. This suggested that the CCG sequence in the −35 region might be a recognition motif for σ^S-dependent transcription. In this study, we have used site-directed mutagenesis to test the importance of sequences in the −35 region to σ^S promoter recognition in vivo. First, we attempted to create a σ^S-dependent promoter by substituting CC for TT in the −35 region of the σ^D-dependent promoter of the *proU* operon. In the reciprocal experiment, we replaced the CC at the −35 region of the σ^S-dependent *osmY* promoter with TT or AA.

Creating a σ^S-dependent promoter. The *proU* promoter encodes an active transport system for the osmoprotectant glycine betaine (6, 43). After hyperosmotic shock, transcription from the *proU* promoter increases dramatically, from 60- to more than 100-fold (17, 43, 44). It has been demonstrated with a temperature-sensitive *rpoD* strain and immunoprecipitation experiments that induction of *proU* transcription is dependent on the presence of σ^D (29, 64).

A 0.4-kb DNA fragment containing the *proU* promoter was used as the template for promoter modifications. This DNA segment excludes a 3′ region implicated as a primary site for negative regulation of *proU* expression (12, 41, 44) but retains sequences required for osmotic induction (42).

The *proU* promoter (Fig. 1) is typical of σ^D promoters in that it contains thymidine residues in the first two positions of the −35 hexamer. PCR-directed mutagenesis was used to change these thymidine residues to cytosine. This alteration changed the *proU* −35 hexamer (TTGCCT) from a fairly good match to the σ^D consensus to a sequence similar to the −35 region of several σ^S-dependent promoters (CCGCCT). We designated the mutant promoter $proU_{CC}$ (Fig. 1).

$proU_{CC}$ was cloned in front of a promoterless *lacZ* gene and transferred in single copy to the *E. coli* chromosome. We analyzed the sigma factor dependence of transcription from $proU_{CC}$ by assaying β-galactosidase activity in strains RB110 (*trpDC*::$proU_{CC}$-*lacZ*) and RB120 (*trpDC*::$proU_{CC}$-*lacZ rpoS*::Tn*10*). Figure 2 compares sigma factor dependence at the $proU_{CC}$ promoter with that at the wild-type *proU* promoters.

		−35		−10	
σ^D consensus		TTGACA	16–18 bp	TATAAT	
proU	GGGG	TTGCCT	CAGATTCTCAGTATGT	TAGGGT	AGAAAA
$proU_{CC}$		CCGCCT		TAGGGT	
*proU*446		TTGCCT	CAGATTCTCAGTAGGT	TAGGGT	
$proU_{CC}$446		CCGCCT	CAGATTCTCAGTAGGT	TAGGGT	
$proU_{CCTATAAT}$		CCGCCT		TATAAT	
$proU_{TATAAT}$		TTGCCT		TATAAT	
	*	*			
osmY	TATC	CCGAGC	GGTTTCAAAATTGTGATC	TATATT	TAACA
$osmY_{TT}$		TTGAGC		TATATT	
*osmY*AA		AAGAGC		TATATT	

FIG. 1. Test promoters. The wild-type *proU* and *osmY* promoter sequences, along with the σ^D consensus promoter sequence, are shown. Underlined nucleotides in test promoters indicate changes incorporated into mutant promoter sequences. Nucleotides marked with an asterisk in the *osmY* promoter represent single nucleotide changes (C→G or G→A).

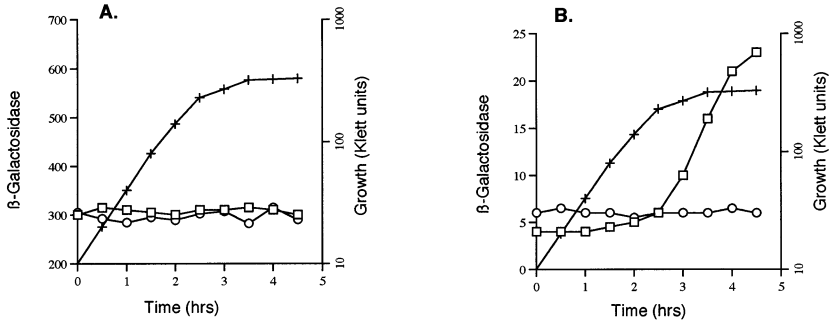

FIG. 2. Comparison of stationary-phase induction and *rpoS* dependence of transcription at the wild-type *proU* and $proU_{CC}$ promoters after growth in LB. (A) Squares, activity in JM409 (*proU-lacZ*); circles, activity in RB409 (*proU-lacZ rpoS*::Tn*10*). (B) Squares, RB110 ($proU_{CC}$-*lacZ*); circles, activity in RB120 ($proU_{CC}$-*lacZ rpoS*::Tn*10*); crosses, cell growth.

A null mutation in *rpoS* had no obvious effect on transcriptional activity at the wild-type *proU* promoter. Additionally, transcription from that promoter showed no growth phase dependence. In contrast to the wild-type promoter, transcription from $proU_{CC}$ was clearly induced during stationary phase, and this induction required an intact *rpoS* gene. However, the most obvious effect of substituting cytosine for the thymidine residues at *proU*'s −35 hexamer was the severe reduction of transcription by $E\sigma^D$.

The relatively weak transcriptional activity by $E\sigma^S$ at the $proU_{CC}$ promoter could be explained if our −35 mutation merely unmasked a low level of $E\sigma^S$ activity that was innate to the $proU_{CC}$ promoter fragment. The σ^S dependence of $proU_{CC}$ might be due to σ^S-dependent transcription from a weak upstream promoter (12, 41). On the other hand, σ^S dependence could be an inherent aspect of the unmutated promoter, but one that is normally obscured by high levels of σ^D-dependent transcription.

The mutant $proU_{446}$ (44) promoter was used to reduce recognition by $E\sigma^D$. This promoter is wild type, except for a T-to-G transversion at position −15 within the spacer region separating the −10 and −35 hexamers (Fig. 2). This mutation within *proU*'s so-called extended −10 region nearly eliminates transcription by $E\sigma^D$ (44). The data in Fig. 3 show that no σ^S-dependent transcription was unmasked when transcription by $E\sigma^D$ was reduced because of the 446 mutation. The low baseline of transcriptional activity at $proU_{446}$ was unaffected by a mutation in *rpoS*. The 446 mutation did not prevent transcription by $E\sigma^S$ when an appropriate −35 sequence was available.

Replacement of the thymidine residues at the −35 site of $proU_{446}$ with cytosine created the $proU_{CC446}$ promoter (Fig. 1). The cytosine nucleotides in the −35 hexamer of $proU_{CC446}$ did lead to a modest increase in transcription at stationary phase (Fig. 3). This stationary-phase induction required an intact *rpoS* gene. We concluded that the cytosine nucleotides were, in fact, a positive effector of promoter recognition by $E\sigma^S$.

The inefficient transcriptional activity at $proU_{CC}$ and $proU_{CC446}$ suggested that $E\sigma^S$ might not be able to interact effectively with *proU*'s −10 region. *proU*'s −10 hexamer (TAGGGT), although a three-of-six match to the σ^D consensus, is unusual in containing 3 consecutive guanidine-cytosine bp. The comparatively high level of free energy of G-C bonds compared with that of the more common A-T bonds might negatively affect the efficiency of $E\sigma^S$ transcription. Because the −10 regions of *rpoS*-dependent promoters resemble the σ^D consensus −10 hexamer, we reasoned that sequences in this region were likely to contribute to promoter strength but would not favor σ^S-dependent transcription over that by σ^D holoenzyme. We tested the ability of $E\sigma^S$ to recognize the σ^D consensus −10 hexamer by creating the $proU_{CCTATAAT}$ promoter.

The $proU_{CCTATAAT}$ promoter (Fig. 1) was constructed by replacing the wild-type −10 sequence of $proU_{CC}$ with the σ^D consensus −10 hexamer, TATAAT. The consensus −10 region increased transcription during stationary phase to a level approximately 6-fold higher than that from the $proU_{CC}$ promoter (compare Fig. 4A and 2B). Stationary-phase induction of transcription remained *rpoS* dependent, but the *rpoS*-independent baseline transcription was also increased. $E\sigma^D$ is the only form of *E. coli* polymerase (other than $E\sigma^S$) known to recognize this −10 hexamer. Thus, we assume that the constitutive expression in the *rpoS* mutant strain VR120 depended on strengthening of $E\sigma^D$'s interaction with the promoter.

We also examined the effect of osmotic shock on transcription directed by $proU_{CCTATAAT}$ (Fig. 4B). A baseline of *rpoS*-independent transcription was again observed, but full osmotic induction required the presence of σ^S. Thus, the sigma factor dependence of the *proU* promoter was changed from σ^D to σ^S by replacing the −35 thymidine residues with cytosine while the inclusion of a consensus σ^D −10 hexamer strengthened transcription by both $E\sigma^S$ and $E\sigma^D$.

The $proU_{TATAAT}$ promoter. In *E. coli*, the −10 promoter sequence TATAAT has been commonly associated, through statistical and mutagenic analyses, with transcription by $E\sigma^D$.

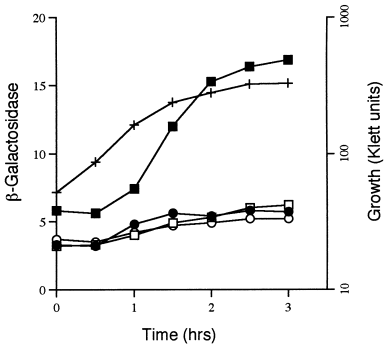

FIG. 3. Stationary-phase transcription at the $proU_{446}$ and $proU_{CC446}$ promoters after growth in LB. Filled squares, activity in AW448 ($proU_{CC446}$-*lacZ*); filled circles, activity in AW449 ($proU_{CC446}$-*lacZ rpoS*::Tn*10*); empty squares, activity in AW446 ($proU_{446}$-*lacZ*); empty circles, activity in AW447 ($proU_{446}$-*lacZ rpoS*::Tn*10*); crosses, cell growth.

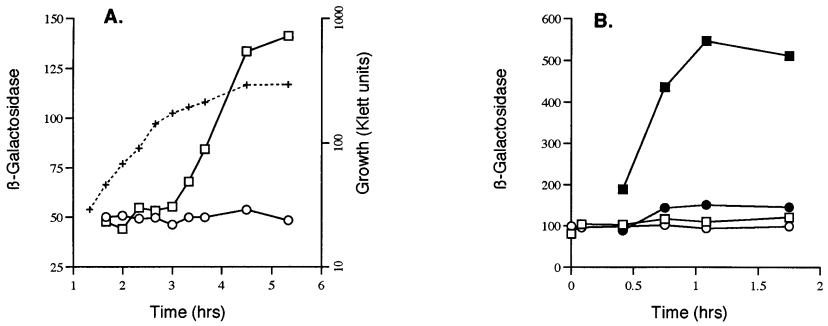

FIG. 4. Stationary-phase (A) and hyperosmotic (B) induction of transcription at the $proU_{CCTATAAT}$ promoter. Squares, activity in VR110 ($proU_{CCTATAAT}$-*lacZ*); circles, activity in VR120 ($proU_{CCTATAAT}$-*lacZ rpoS*::Tn*10*); crosses, growth in LB; filled symbols, activity after the addition of 0.3 M NaCl to M9 medium at 0 h.

For the most part, these analyses were done prior to identification of σ^S and did not take alternate sigma factors into consideration. For that reason, it was important to verify that the consensus −10 hexamer merely strengthened $E\sigma^S$'s interaction with $proU_{CCTATAAT}$ and did not itself cause a preference for σ^S-dependent transcription. Toward that end, we created the $proU_{TATAAT}$ promoter. $proU_{TATAAT}$ includes the σ^D consensus −10 hexamer but retains wild-type sequences at the −35 site (Fig. 1).

$proU_{TATAAT}$ was an exceptionally strong promoter. We were, in fact, unable to isolate $proU_{TATAAT}$ in front of the *lacZ* gene of pRS551. As an alternative, we cloned $proU_{TATAAT}$ in front of the poorly translated *cat* gene carried by pKK232-8, a multicopy promoter assay vector. Chloramphenicol acetyltransferase assays provided a measure of transcription derived from $proU_{TATAAT}$ and $proU_{CCTATAAT}$ during stationary phase, in the presence and absence of the σ^S protein (Table 3).

In agreement with the results depicted in Fig. 4A, transcriptional activity driven by the $proU_{CCTATAAT}$ promoter was about threefold higher when σ^S was available. The $proU_{TATAAT}$ promoter, with the wild-type −35 region TTGCCT, behaved differently. The level of activity was consistently very high, but even higher when σ^S was unavailable. The twofold-higher activity in the absence of σ^S may be due to an increased availability of core polymerase which leads to a higher concentration of $E\sigma^D$. Alternatively, $E\sigma^S$ may bind to the promoter but may be unable to efficiently initiate transcription. Thus, in the presence of σ^S, recognition and transcription by $E\sigma^D$ may be somewhat obstructed. We conclude that the −10 consensus sequence contributed considerably to the strength of $proU_{CCTATAAT}$'s promoter function for both σ^S and σ^D holoenzymes but did not bias transcription in favor of *rpoS* dependence.

The $osmY_{TT}$ promoter. Replacement of thymidine with cytosine at the −35 site of the σ^D-dependent *proU* promoter gave rise to σ^S-dependent transcription. The reciprocal experiment analyzed changes in sigma factor dependence after modification of the −35 site of the σ^S-dependent *osmY* promoter. The *osmY* gene encodes a periplasmic protein (65). Transcription directed by its promoter (Fig. 1) is induced at stationary phase or after hyperosmotic shock (34, 61, 64).

The $osmY_{TT}$ promoter was created by replacing two cytosine residues in the −35 region of the *osmY* promoter with thymidine (Fig. 1). The nucleotide sequence of $osmY_{TT}$ resembles a typical σ^D promoter in that it contains a hexamer with a four-of-six match to the σ^D consensus −35 hexamer. This upstream site is separated by 18 bp from a −10 hexamer that is a five-of-six match to the σ^D consensus −10 sequence. Figure 5 compares the sigma factor dependence of transcription at $osmY_{TT}$ with that at the wild-type *osmY* promoter.

Transcription from the wild-type *osmY* promoter (Fig. 5A) was induced about 10-fold by the 2nd h of stationary phase. As previously observed (34, 61, 64), expression of the *osmY-lacZ* fusion was almost entirely σ^S dependent. Replacement of the CC sequence at the −35 site with TT (RB12) did not eliminate σ^S-dependent growth-phase induction (Fig. 5B). However, the rate and magnitude of induction were reduced, and in contrast to the wild-type promoter, activity at $osmY_{TT}$ reached a level just 3- to 4-fold higher than the preinduced level.

The most conspicuous change in sigma factor usage was demonstrated by the increase in *rpoS*-independent baseline transcription. No growth-phase regulation of $osmY_{TT}$ was apparent in the absence of σ^S, but β-galactosidase activity increased from the 8 to 9 units produced by the wild-type promoter in RB11 to approximately 100 U in RB13 (*rpoS*::Tn*10*). Presumably, the 10-fold increase in σ^S-independent transcription is due to increased recognition of the mutant promoter by $E\sigma^D$.

Thymidine nucleotides at the −35 site of $osmY_{TT}$ also caused a large increase in σ^S-independent transcription in minimal medium. Osmotically induced transcription remained substantially *rpoS* dependent; however, β-galactosidase activity reached a level less than one-half of that seen with the wild-type *osmY* promoter (compare Fig. 5C and D). Thus, the cytosine nucleotides at the −35 site of the *osmY* promoter favor σ^S-dependent transcription and act, in part, by excluding recognition by the σ^D holoenzyme. The residual *rpoS*-dependent transcriptional induction from $osmY_{TT}$ suggests that additional determinants of $E\sigma^S$ recognition remain to be identified.

The $osmY_{AA}$ promoter. Our alignment of *rpoS*-dependent promoters shows that cytosine nucleotides frequently occur in the −35 region in positions normally occupied by thymidine in a σ^D promoter. Promoter recognition by $E\sigma^S$ might be specifically favored by cytosine nucleotides at the −35 site. On the other hand, σ^S-dependent gene transcription might require

TABLE 3. Stationary-phase induction of chloramphenicol acetyltransferase

Strain/plasmid	Promoter	Activity[a]
MC4100/pAW411	$proU_{CCTATAAT}$	9.0
RH90 (*rpoS*::Tn*10*)/pAW411	$proU_{CCTATAAT}$	2.8
MC4100/pAW412	$proU_{TATAAT}$	390
RH90 (*rpoS*::Tn*10*)/pAW412	$proU_{TATAAT}$	870

[a] Units are nanomoles of chloramphenicol acetylated per minute per milligram of protein.

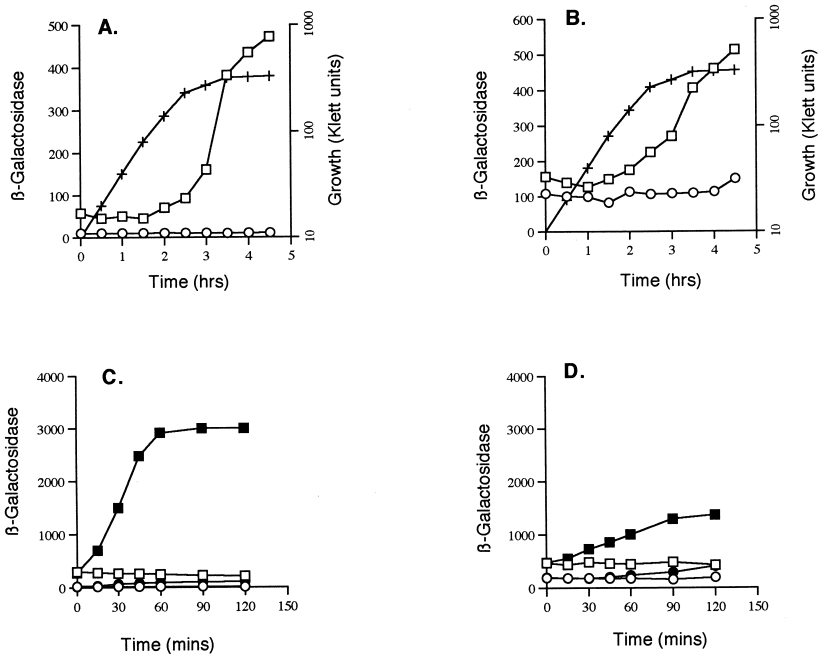

FIG. 5. *rpoS* dependence at the *osmY* (A and C) and $osmY_{TT}$ (B and D) promoters during stationary phase (A and B) and after hyperosmotic shock (C and D). Squares, activity in RB10 (*osmY-lacZ*) and RB12 ($osmY_{TT}$-*lacZ*); circles, activity in RB11 (*osmY-lacZ rpoS*::Tn*10*) and RB13 ($osmY_{TT}$-*lacZ rpoS*::Tn*10*). For stationary-phase induction, cells were grown in LB. For osmotic induction, cells were grown in M9 and 0.3 M NaCl was added at 0 h. Activity after the addition of NaCl is represented by filled symbols.

only a −35 region that excludes or reduces transcription by $E\sigma^D$ coupled with a usable −10 region.

A test of the specificity of the cytosine effect involved analyzing the sigma factor dependence of transcription from the $osmY_{AA}$ promoter. The $osmY_{AA}$ promoter replaces *osmY*'s −35 cytosine residues with adenine (Fig. 1). The effect of the adenine nucleotides differed significantly from that of the thymidine residues at the −35 site of the $osmY_{TT}$ promoter (compare Fig. 5B and 6). Adenine nucleotides effectively destroyed transcription by $E\sigma^S$ without creating recognition by another holoenzyme. Therefore, cytosines are specific positive effectors of $E\sigma^S$'s recognition of the *osmY* promoter, suggesting that their role is not merely the exclusion of recognition by $E\sigma^D$. Furthermore, the precision of the interaction between $E\sigma^S$ and the first two positions of *osmY*'s −35 site is such that thymidine nucleotides allow partial function, but adenine nucleotides do not.

Two additional single-nucleotide modifications of the *osmY* −35 region were made. Mutation of the cytosine and guanidine marked by asterisks in Fig. 1 reduced stationary-phase expression of *lacZ* fusions to the mutant promoters by 50 and 25%, respectively (data not shown). These mutations weakened transcription by $E\sigma^S$ but did not transmute sigma dependence.

Transcription start sites of the $proU_{CCTATAAT}$ and $osmY_{TT}$ promoters. The interpretation of the data from our modified promoters assumes that the mutations have not altered transcriptional start sites. Primer extension analysis compared the transcription initiation sites used at $proU_{CCTATAAT}$ and $osmY_{TT}$ with the wild-type promoters. As shown in Fig. 7, the transcription initiation site determined for $proU_{CCTATAAT}$ (lanes 2, 3, and 4) is identical to that of the wild-type *proU* promoter (lane 1). The same start site of transcription was used during both hyperosmotic (lane 2) and stationary-phase induction (lane 3). The fainter bands seen in the RH90 background (lane 4) are likely due to a constitutive baseline of transcription by σ^D holoenzyme. Replacement of *proU*'s −10 hexamer with the consensus sequence did not alter the multiple bands which are characteristic of *proU* 5′ RNA analysis (18, 42). The main start site of *proU* transcription is, generally, considered to be within a stretch of six adenine residues (18, 42, 44).

The transcription initiation sites of the *osmY* and $osmY_{TT}$

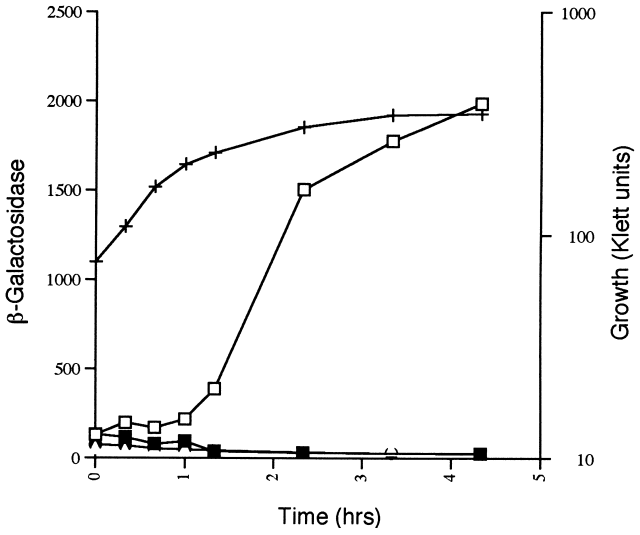

FIG. 6. Comparison of stationary-phase induction and *rpoS* dependence of the wild-type *osmY* and $osmY_{AA}$ promoters. Empty squares, activity in RB10 (*osmY-lacZ*); filled squares, activity in MG12 ($osmY_{AA}$-*lacZ*); filled circles, activity in MG13 ($osmY_{AA}$-*lacZ rpoS*::Tn*10*) (this is obscured by the symbols representing activity in MG12); crosses, growth.

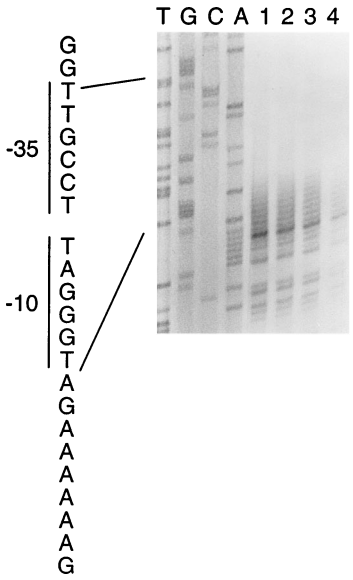

FIG. 7. Transcription initiation sites of the *proU* (lane 1) and the $proU_{CCTATAAT}$ (lanes 2 to 4) promoters. For primer extension assays, ^{32}P-labeled oligonucleotide probe complementary to the *proU* noncoding strand was annealed to total RNA from plasmid-containing cells. The DNA-sequencing ladder was prepared with the same oligonucleotide probe and pBP33U. For RNA preparation, cells were subject to osmotic shock in M9 medium (lanes 1 and 2) or were grown through the 3rd h of stationary phase in LB (lanes 3 and 4). Lanes: 1, (MC4100/pJM409); 2 and 3, MC4100/pAW409; 4, RH90/pAW409.

promoters are compared in Fig. 8. In agreement with previous analysis (65), an adenine residue 7 bases downstream from the −10 hexamer was confirmed as the transcription initiation site for the wild-type *osmY* promoter during stationary phase (Fig. 8A, lane 1) and after hyperosmotic stress (lane 2). The identical start site was identified at the $osmY_{TT}$ promoter (Fig. 8B) after hyperosmotic (lane 1) and stationary-phase (lane 2) induction. Excess product was loaded in lane 3 to allow visualization of the stationary-phase start site of transcription at $osmY_{TT}$ in the absence of σ^S. We concluded that the change in sigma factor usage caused by modifications to the *osmY* and *proU* promoters did not alter the start sites of transcription.

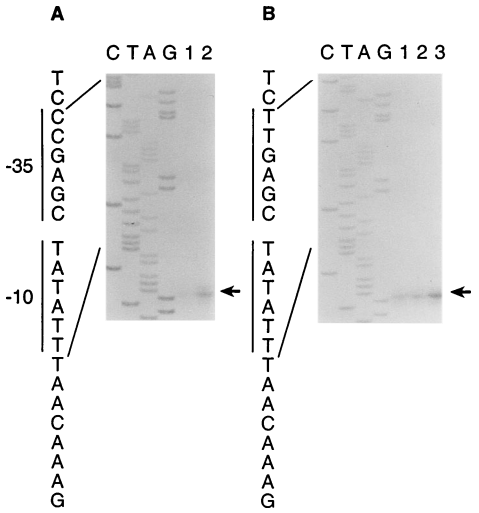

FIG. 8. Transcription initiation sites of the *osmY* (A) and $osmY_{TT}$ (B) promoters. A ^{32}P-labeled oligonucleotide probe complementary to the *osmY* noncoding strand was annealed to total RNA isolated from plasmid-containing cells. DNA sequencing ladders were prepared with the same oligonucleotide probe and plasmids containing the *osmY* (A) or $osmY_{TT}$ (B) promoters. (A) RNA isolated from MC4100/pRB10 during exponential growth in M9 (lane 1) and after hyperosmotic shock with 0.3 M NaCl (lane 2); (B) RNA isolated from MC4100/pRB11 after hyperosmotic shock in M9 medium (lane 1) or at the end of the 3rd h of stationary phase after growth in LB (lane 2). For lane 3, RNA was isolated from RH90/pRB11 at the end of the 3rd h of stationary phase in LB.

DISCUSSION

$E\sigma^S$ transcribes a number of genes whose products help the cell cope with various environmental stresses. Previously, identification of a σ^S-dependent gene has included the observation that the promoter contains a region resembling the −10 hexamer recognized by $E\sigma^D$, with no recognizable sequence motif in the region corresponding to the σ^D −35 hexamer. Although our alignment of *rpoS*-dependent promoters comprised a relatively small population, we noted a possible conservation of cytosine nucleotides in the region spatially corresponding to the −35 thymidines of σ^D promoters. We show here that cytosine residues in this region promote transcription by $E\sigma^S$.

The analysis of mutant versions of the *proU* and *osmY* promoters was directed toward discovering a region of promoter sequence, upstream from a putative −10 hexamer, which would act as a stimulus for transcription by $E\sigma^S$, while excluding or reducing σ^D-dependent transcription. Our approach was based, in part, on the fact that thymidines at the −35 site of σ^D promoters are important effectors of promoter binding and transcription by $E\sigma^D$ (32, 55). Additionally, similarities in the amino acid sequences of the σ^S and σ^D proteins (39) suggested that $E\sigma^S$ might also interact with promoter DNA at approximately 35 nucleotides upstream from the start of transcription. The abolition of transcription at $osmY_{AA}$ indicates that cytosines in this area may function in a manner similar to that of the thymidine residues of σ^D promoters by promoting recognition and transcription by $E\sigma^S$.

The strongest effect of the cytosine nucleotides in the $proU_{CC}$ promoter was to severely decrease transcription by $E\sigma^D$. For this reason, we asked whether the σ^S dependence of the $proU_{CC}$ promoter fragment might be due to a weak σ^S-dependent upstream promoter (12, 41) or to the unmasking of an intrinsically low level of σ^S-directed transcription at the unmutated promoter. The σ^S dependence of $proU_{CC446}$, as well as the increase in σ^S-dependent transcription after inclusion of the consensus −10 hexamer in $proU_{CCTATAAT}$, confirms that recognition by $E\sigma^S$ originates at the modified promoter and is due to the inclusion of cytosine at the −35 site.

The changed sigma factor dependence effectuated by the mutations creating $proU_{CC}$ and $osmY_{TT}$ suggests that nucleotides in the first two positions of the −35 region may act as a mechanism to shift transcription between $E\sigma^D$ and $E\sigma^S$. Sigma usage of mutant and wild-type promoters was, in part, determined by which nucleotides (cytosines or thymidines) were present at the −35 site.

However, not all σ^S-dependent promoters include cytosine residues at the 5′ end of the −35 region. The *pexB* (*dps*) promoter is in this category (2, 38). Considering the multiple factors that are known to influence the strength of σ^D promoters (DNA sequence, spacing between the −35 and −10 hexamers, regulatory proteins, and DNA secondary structure), it is not very remarkable to find sequence variation at the −35 site of σ^S promoters. We note that the *pexB* promoter is recognized by both $E\sigma^S$ and $E\sigma^D$ and that its stationary-phase σ^S-dependent transcription requires the histone-like IHF protein (2). Perhaps this rather complex regulation accounts for the absence of cytosines in the −35 site of *pexB*.

The mixed sigma factor dependence of $osmY_{TT}$ and $proU_{CCTATAAT}$ indicates the existence of additional determinants of promoter recognition by $E\sigma^D$ and $E\sigma^S$. The residual σ^D dependence of the $proU_{CCTATAAT}$ promoter may be due to the interaction of $E\sigma^D$ with certain nucleotides in the spacer region between the −10 and −35 hexamers. This so-called extended −10 sequence is essential for $E\sigma^D$'s transcriptional activity at the *proU* promoter and likely assists the holoenzyme in using (or tolerating) its G-C rich −10 sequence (44). Similar extended −10 regions are present in other promoters (8, 31), where they apparently augment transcription in the absence of a functional −35 sequence. The extremely high level of activity of the $proU_{TATAAT}$ promoter is probably due to the promoter strengthening effect of the extended −10 sequence, which, together with the consensus −10 hexamer and the wild-type −35 sequence, comprises a third area of sequence known to support transcription by $E\sigma^D$.

The replacement of cytosine residues with thymidine at the −35 site of the *osmY* promoter caused a 10-fold increase in σ^D-dependent transcription, further demonstrating the discriminatory nature of sequences at this site. The persistence of σ^S-dependent transcriptional activity at the $osmY_{TT}$ promoter indicates that elements other than the −35 site cytosine residues contribute to transcription by σ^S holoenzyme. Our unpublished experiments with a fully σ^S-dependent 108-bp *osmY* promoter (beginning at position −42 relative to the start site of transcription) lead us to believe that additional determinants of σ^S-dependent transcription are likely to be found in the region separating the −10 and −35 sites. Determinants of σ^S dependence in the spacer region are in line with the in vitro findings of Tanaka and coworkers (56).

$E\sigma^S$'s ability to interact effectively with σ^D's consensus −10 hexamer was demonstrated by the increase in σ^S-dependent transcription caused by the inclusion of the hexamer in the $proU_{CCTATAAT}$ promoter. However, there is insufficient evidence to conclude that TATAAT is the optimal −10 sequence for σ^S holoenzyme. We note that a threonine at position 440 of the σ^D protein which has been implicated in interaction with the −10 sequence of σ^D promoters (52) is not conserved in the σ^S protein (39).

The ability of $E\sigma^D$ and $E\sigma^S$ to interact with similar −10 regions may be responsible for the observation that some promoters can be recognized in vitro by both $E\sigma^S$ and $E\sigma^D$ (48, 57). For example, under certain in vitro conditions, both *bolA* P1 and the *osmY* promoter can be effectively utilized by $E\sigma^D$ (13, 48). However, in vivo transcription from both promoters is nearly abolished by a mutation in *rpoS* (35, 64). Thus, the in vitro studies of σ^S-dependent transcription do not necessarily reflect the situation inside the cell. One would expect divergent forms of polymerase to be less promiscuous in vivo, particularly since the cell uses alternate sigma factors to limit the expression of genes whose products are required only under specific conditions. We suggest that DNA sequences at the −35 site of σ^S promoters form part of a discriminatory mechanism which limits transcription to conditions that increase the availability of $E\sigma^S$.

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