In Vitro Functional Characterization of Overproduced *Escherichia coli katF/rpoS* Gene Product†

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ABSTRACT: The *katF/rpoS* gene product (σ^S), a central regulator of stationary-phase gene expression in *Escherichia coli*, has been purified from an overproducing strain. σ^S was used as an immunogen for the production of monoclonal antibodies. Previous sequence analysis of σ^S strongly indicated homology to the σ factor family. We show biochemically in this paper that σ^S is a σ factor. This protein can bind to core RNA polymerase (E), and this binding can be competed effectively by the major *E. coli* transcription initiation factor, σ^{70}. Immunopurified σ^S holoenzyme ($E\sigma^S$) transcribes the promoters of the *bolA*p1 gene and the *xthA* gene. Interestingly, both promoters can also be transcribed by σ^{70} holoenzyme ($E\sigma^{70}$).

Upon entry into stationary phase, *Escherichia coli* cells undergo complex physiological changes. Stationary-phase *E. coli* cells are resistant to high temperature, high concentrations of H_2O_2 (Jenkins et al., 1990b), and very high medium osmolarity (Jenkins et al., 1990a). The stationary-phase cells are also able to survive prolonged periods of starvation (Matin et al., 1989). The *katF/rpoS* gene is induced during transition from exponential to stationary phase and has been identified as a central early regulator of a large number of starvation/stationary-phase genes (Lange & Hengge-Aronis, 1991a). The *xthA* gene, whose gene product is believed to be involved in cellular recovery from oxidative damage, has been shown to be under the control of the *katF/rpoS* gene (Sak et al., 1989). The *xthA* gene encodes exonuclease III. The *bolA* gene has also been shown to be dependent on the *katF/rpoS* gene and appears to code for a small regulatory protein that is involved in the stationary-phase cell morphogenesis process (Lange & Hengge-Aronis, 1991b). Also, transcription of the *bolA*p1 promoter has been shown to be induced upon entry into stationary phase (Aldea et al., 1990).

The *katF/rpoS* gene shows strong amino acid sequence similarity to *E. coli* σ factors such as σ^{70} and σ^{32} (Mulvey & Loewen, 1989). The predicted size of the *katF/rpoS* gene product (σ^S) is 41 500 daltons (Mulvey & Loewen, 1989). Size and sequence heterogeneity of the *E. coli* σ^S gene have been reported (Ivanova et al., 1992). If σ^S is a σ factor like σ^{70}, then it should be able to bind to core RNA polymerase (E) and to confer promoter specificity upon core RNA polymerase. In this report, we present direct biochemical evidence that σ^S binds to core RNA polymerase (E) and that immunopurified σ^S holoenzyme ($E\sigma^S$) directly transcribes the *bolA*p1 and the *xthA* promoters.

MATERIALS AND METHODS

Strains and Plasmid Constructions. To construct a *katF/rpoS*-overproducing plasmid, a blunted *Eco*RI–*Bam*HI DNA fragment from pMM*KATF*2 (Mulvey & Loewen, 1989) was cloned into the blunted *Xba*I and *Bam*HI sites of pET11T (Nguyen et al., 1993). This plasmid, pLHN23, was then transformed into BL21(DE3) pLysS, an overproducing strain based on T7 RNA polymerase (Studier et al., 1990). σ^S from this overproducing strain was used for all functional analyses in this paper. pDEB4 contains a DNA fragment that has the *katF/rpoS* gene also in the pET11T vector. This cloned DNA fragment in pDEB4 was made by a polymerase chain reaction (PCR) with chromosomal DNA from *E. coli* strain C600 as the template. σ^S from the overproducing strain, BL21(DE3) pLysS, pDEB4, was used only for monoclonal antibody production and not for any functional studies. A blunted *Hin*dIII–*Pst*I DNA fragment containing the *xthA* promoter from pRPC156 (Saporito et al., 1988) was cloned into the blunted *Sac*I and *Pst*I sites of pJW1000. This construct was designated pLHN24. pJW1000 had a *Hin*dIII–*Pvu*II DNA fragment containing a T7 transcription terminator from pDH310 (Herendeen et al., 1990) cloned into the *Hin*dIII and *Hin*cII sites of the Bluescript SK– vector of Stratagene (Dr. Jeff Winkelman, personal communication). A DNA fragment containing the *bolA* promoter from +1 to +556 [numbering is according to Aldea et al. (1989)] was made by polymerase chain reaction using Taq DNA polymerase with chromosomal DNA from *E. coli* K12 strain MG1665 as the template. This PCR fragment was cloned into the *Bam*HI and the *Eco*RV restriction sites of the vector pSP72 (Promega). This construct was called pGN66. On the basis of dideoxy DNA sequencing, there were no errors due to Taq DNA polymerase misincorporation in pGN66.

Buffers and Reagents. All reagents were purchased from Sigma unless otherwise indicated. The Dideoxy Sequenase sequencing kit was purchased from United States Biochemical (USB). Storage buffer was 50 mM NaHepes, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA),[1] 50 mM NaCl, 50% glycerol, and 0.1 mM DTT. TED was 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, and 0.1 mM DTT. TGED was TED with 5% glycerol. TBST was 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, and 0.1% Tween 20. All pH values were determined at 21 °C.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DTT, dithiothreitol; PBS, 10 mM phosphate (pH 7.4) buffered saline (150 mM NaCl); Blotto, non-fat dry milk; IPTG, isopropyl β-D-thiogalactopyranoside; DEAE, diethylaminoethyl; TCA, trichloroacetic acid; mAb, monoclonal antibody; IgG, immunoglobulin; PEG, poly(ethylene glycol).

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σ^s and σ^s Holoenzyme Purification. The σ^s protein that was overproduced here and was used for functional analyses is from the *katF/rpoS* gene isolated by Mulvey and Loewen and not from other σ^s genes (Mulvey & Loewen, 1989; Ivanova et al., 1992). σ^s from the overproducing strain BL21(DE3) pLysS, pLHN23 was found in inclusion bodies following induction with 1 mM IPTG at 37 °C for 4 h. The cells were harvested and lysed with a cell disruption bomb (VWR) at 2000 psi, and the inclusion bodies were isolated by centrifugation at 20000*g* for 15 min. The inclusion bodies were solubilized using Sarkosyl anionic detergent (Sigma) as described previously for ω and for σ^{32} (Gentry & Burgess, 1990; Nguyen et al., 1993). The Sarkosyl detergent was removed by dialysis. The dialysate was then loaded onto a 1.4-mL DEAE MemSep (Millipore) column, and σ^s was partially purified by a 40-mL salt gradient from 50 to 800 mM NaCl in TGED buffer. Individual column fractions were dialyzed and stored in storage buffer at −20 °C. A pool of DEAE fractions containing σ^s was combined with purified core RNA polymerase to form σ^s holoenzyme ($E\sigma^s$). This $E\sigma^s$ fraction was purified using an immunoaffinity NT73–Sepharose column which specifically purifies RNA polymerase as described (Thompson et al., 1992). Core RNA polymerase was purified as described (Hager et al., 1990). The same preparation of core RNA polymerase was used for forming σ^s holoenzyme for immunoaffinity purification and for forming σ^{70} holoenzyme in functional studies. σ^{70} was purified as described (Gribskov & Burgess, 1983).

Isolation of Monoclonal Antibodies. The σ^s that was used as the immunogen was purified as described above from the strain BL21(DE3) pLysS, pDEB4. On day 1, 10 μg of σ^s in Freund's complete adjuvant was injected intraperitoneally and subcutaneously. On day 20 and then day 60, 15 μg of σ^s in Freund's incomplete adjuvant was injected the same way as on day 1. On day 90, 40 μg of σ^s in PBS was injected intraperitoneally. On day 93, the spleen was removed from one mouse, and fusion was performed as described (Strickland et al., 1988). mAb 1RS1 was chosen because, in an immunoblot assay, it showed both strong reactivity to σ^s in *E. coli* whole cell lysates and very low nonspecific reactivity.

Monoclonal Antibody Purification. Monoclonal antibodies that were used in activity inhibition assays were purified as follows. mAb 3D3, an anti-σ^{70} monoclonal antibody (Strickland et al., 1988), was purified to homogeneity by protein A–Sepharose affinity chromatography according to the manufacturer's instructions (Repligen). mAb 3D3 does not cross-react with σ^s, σ^{32}, or the subunits of core RNA polymerase (data not shown). mAb 1RS1, an anti-σ^s monoclonal antibody, has an IgG1 isotype and, therefore, was purified to homogeneity by protein G–Sepharose affinity chromatography according to the manufacturer's instructions (Pierce). mAb 1RS1 does not cross-react with σ^{70}, σ^{32}, or the subunits of core RNA polymerase (data not shown).

Protein Concentration Determination. Protein concentrations were determined using the Coomassie Blue G250 colorimetric assay (Pierce) using BSA as the standard. The level of σ^s in the DEAE pool that was used for the core binding assay was determined by comparison with known amounts of ovalbumin in a Coomassie R250-stained 12% polyacrylamide–SDS gel.

Core Binding Assay. 3.9 μg (93 pmol) of σ^s (DEAE peak fractions pool) alone or with 39 μg (100 pmol) of core RNA polymerase was incubated in 100 μL of TED + 250 mM NaCl (Shaner et al., 1982) for 15 min at 37 °C. In a simultaneous competition experiment, 28 μg (400 pmol) of σ^{70} was added with σ^s. In a postcompetition experiment, 28 μg (400 pmol) of σ^{70} was added after the formation of $E\sigma^s$ as described above, and the reaction was incubated for another 15 min at 37 °C. Each reaction was then loaded onto a 4-mL 15–40% glycerol gradient in TED + 250 mM NaCl. The gradient was centrifuged at 48000*g* for 20 h at 21 °C. Three-drop fractions were collected from a hole at the bottom of the tube. Even-numbered fractions were precipitated with 15% TCA and run on a 12% polyacrylamide–SDS gel.

Western Blot Assay. Protein fractions from the glycerol gradients, separated on a 12% polyacrylamide–SDS gel, were electroblotted directly onto nitrocellulose. Immunoblots were then blocked with 1% Blotto for 1 h, and probed for 1 h with 1:2000 dilutions of ascites fluid of mAb 1RS1, an anti-σ^s mAb, and of mAb NT73, an anti-β' mAb (Thompson et al., 1992), in 1% Blotto (Carnation Dry Milk). Blots were rinsed with TBST and then were incubated with a 1:2000 dilution of goat anti-mouse IgG coupled to horseradish peroxidase (HyClone) for 1 h at room temperature. The blots were then washed 5 times with TBST, each time for 5 min. The blots were processed with an enhanced chemiluminescence (ECL) kit as suggested by the manufacturer (Amersham).

Transcription Assay. In vitro transcription assays were performed with supercoiled pLHN24, linearized pGN66, or a 556 base pair DNA fragment containing the *bolA* promoter (made by polymerase chain reaction) as the template. The concentration of the 556 base pair *bolA*-containing DNA fragment was estimated by comparison with known amounts of DNA standard in an ethidium bromide stained agarose gel. Linearization of pGN66 with *Hpa*I should yield an approximately 240-base run-off transcript from the *bolA*p1 promoter. The 556 base pair DNA fragment containing the *bolA*p1 promoter should yield a 176-base run-off transcript from the *bolA*p1 promoter. Transcription reactions were done in a total volume of 25 μL containing 25 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, 3% PEG 8000, 400 μM each of ATP, CTP, and UTP, 20 μM GTP, 2 μCi of [α-^{32}P]GTP, 1 unit/μL RNasin (Promega), and 100 mM NaCl; 1.1 μg of immunopurified σ^s holoenzyme ($E\sigma^s$) and 1.1 μg of core RNA polymerase alone or with 0.28 μg of σ^{70} were or were not preincubated with 18 μg of mAb 3D3 or with 4 μg of mAb 1RS1 for 10 min at 37 °C. mAb 3D3 is an anti-σ^{70} mAb (Strickland et al., 1988), and mAb 1RS1 is an anti-σ^s mAb. One microgram of supercoiled pLHN24, linearized pGN66, or 0.4 μg of the 556 base pair *bolA* promoter-containing DNA fragment in 5 μL was added, and the reactions were continued for 20 min at 37 °C. Each reaction was stopped with 50 μL of 50 mM Tris-HCl, pH 7.9, and 5 mM EDTA solution and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The RNA in the aqueous phase was precipitated with 25 μL of 10 M NH_4-OAc, 10 μg of yeast tRNA, and 250 μL of 100% ethanol. For dinucleotide-primed transcription, 1 μg each of $E\sigma^s$ and pGN66 linearized with *Hpa*I were incubated for 30 min at 37 °C in the transcription buffer described above but lacking nucleoside triphosphates; 12.5 μM each of ATP, GTP, and UTP and 500 μM each of ApA, ApG, or GpG were then added (Kassavetis et al., 1986). In the no-dinucleotide control reaction, 500 μM each of ATP, GTP and UTP was added. After 10 min, for all reactions, the ATP, UTP, and GTP concentrations were brought up to 800 μM; 25 μM CTP, 10 μCi of [α-^{32}P]CTP, and 100 μg/mL heparin were then added. Heparin was added to limit transcription to a single round. The reactions were stopped after 10 min and processed as described above.

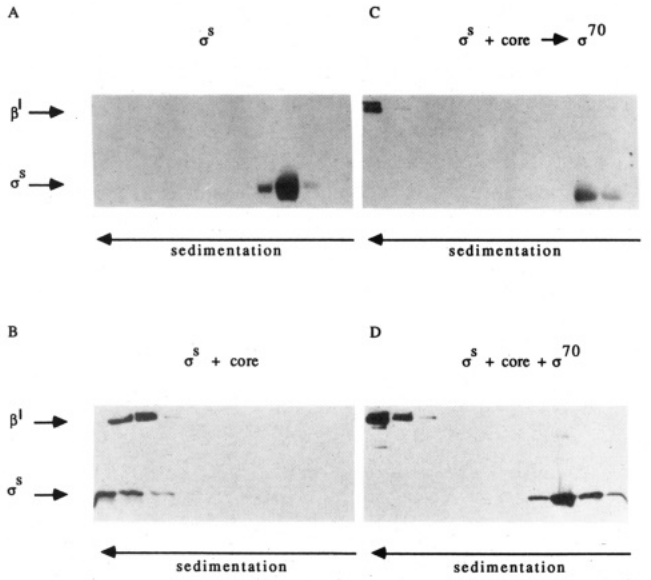

FIGURE 1: *katF/rpoS* gene product (σ^s) binds to core RNA polymerase, and σ^{70} competes with σ^s for core RNA polymerase. The experiment was done as described under Materials and Methods. Panels A–D are Western blots of even-numbered fractions from the glycerol gradient assay. Panel A is σ^s alone, and panel B is σ^s plus core RNA polymerase. Panel C is a Western blot from a postcompetition experiment with σ^{70} as the competitor added after Eσ^s complex had been formed. Panel D is a Western blot from a simultaneous competition experiment between σ^s and σ^{70} for core RNA polymerase. The positions of the β' subunit of core RNA polymerase and σ^s are shown on the left of each panel. The direction of sedimentation is shown at the bottom of each panel. All panels were probed with both mAb 1RS1, an anti-σ^s mAb, and NT73, an anti-β' mAb (Thompson et al., 1992).

Primer Extension Assay. The assay was done as described (Jones et al., 1985) with *in vitro* synthesized RNA or *in vivo* RNA as the substrate. Unlabeled RNA was made *in vitro* as described above except that all nucleoside triphosphate concentrations were 400 μM. The *in vitro* RNA was processed as described (Jones et al., 1985). Total *in vivo* RNA was isolated and quantitated as described (Summers, 1970). *In vivo* RNA was isolated from SK2267 cells that contained pLHN24, pGN66, or no plasmid, except that the RNA was harvested at a cell density (A_{600nm} = 1.5) at which the level of *bolA* mRNA was determined to be maximal (Aldea et al., 1989). The primer for the *xthA* gene was a 5′ end-labeled 22-residue oligonucleotide complementary to nucleotides +100 to +121, with +1 representing the transcriptional start site. Dideoxy DNA sequencing with the same labeled primer was performed with a USB Sequenase sequencing kit according to the manufacturer's protocol. The primer for the *bolA* gene was a 5′ end-labeled 24-residue oligonucleotide complementary to nucleotides +465 to +488, with +380 representing the transcription start site.

RESULTS

σ^s Binds to Core RNA Polymerase. Although the *katF/rpoS* gene product (σ^s) shows strong amino acid sequence similarity to *E. coli* σ factors such as σ^{70} and σ^{32}, there was no direct biochemical evidence that it was a σ factor. We employed a glycerol density gradient assay to determine if σ^s could bind to core RNA polymerase. Free σ^s and core RNA polymerase sediment at different locations in the gradient. When σ^s was mixed with core RNA polymerase, σ^s cosedimented with core RNA polymerase in the glycerol gradient (compare panels A and B of Figure 1). This binding, however, can be competed effectively by σ^{70}, added before or after the formation of σ^s holoenzyme (Eσ^s) (Figure 1, panels C and D). We conclude that σ^s binds to core RNA polymerase, that this binding is disrupted by σ^{70} binding, and that σ^{70} and σ^s binding to core RNA polymerase are mutually exclusive.

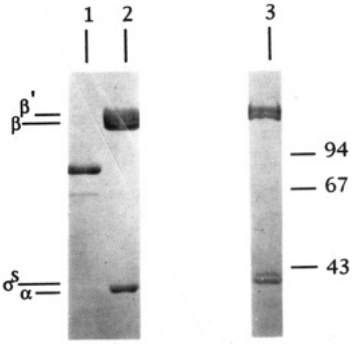

FIGURE 2: Purity of immunopurified Eσ^s, core RNA polymerase, and σ^{70} protein fractions as shown on a 12% polyacrylamide–SDS gel. Lane 1 contains 0.5 μg of σ^{70}. Lane 2 contains 5 μg of core RNA polymerase. Lane 3 contains 3 μg of immunopurified σ^s holoenzyme (Eσ^s). The migration positions of protein markers in kilodalton units are indicated on the right. The migration positions of the various subunits are indicated on the left. β, β', and α are the subunits of core RNA polymerase. The identification of the σ^s band as σ^s was confirmed by its reactivity to mAb 1RS1, an anti-σ^s mAb (data not shown). Proteins were run on a 12% polyacrylamide–SDS gel and stained with Coomassie Blue R250 dye.

The xthA Promoter Is Directly Recognized by Eσ^s and by Eσ^{70}. After discovering that σ^s can bind to core RNA polymerase, we took advantage of this observation in order to purify σ^s holoenzyme (Eσ^s). We combined the overproduced σ^s fraction with purified core RNA polymerase to form Eσ^s. This Eσ^s was fractionated over an NT73–Sepharose column, an immunoaffinity column that specifically purifies RNA polymerase (Thompson et al., 1992). The binding of partially purified σ^s to core RNA polymerase and the immunopurification of σ^s holoenzyme (Eσ^s) by a mAb specific for β', a core RNA polymerase subunit, further confirm the ability of σ^s to bind to core RNA polymerase. Figure 2 shows the purity of the immunopurified σ^s holoenzyme (Eσ^s), the core RNA polymerase, and the σ^{70} fractions that were used in the following functional studies.

We wanted to find a starvation/stationary-phase-specific gene promoter that could be transcribed directly by Eσ^s. The two *katF*-dependent genes available to us are the *xthA* and *bolA* genes. For both genes, *in vivo* +1 transcription start sites have been mapped (Aldea et al., 1989; Saporito et al., 1988). We used a primer extension assay with *in vitro* synthesized RNA and *in vivo* RNA to determine if any +1 transcript had been synthesized *in vitro*. Using this assay, we discovered that the *xthA* +1 transcript (at T110) could be generated with Eσ^s (Figure 3A, lane 1) but not with core RNA polymerase (E) alone (Figure 3B, lane 3). This Eσ^s transcription of the *xthA* promoter was inhibited by the addition of mAb 1RS1, an anti-σ^s mAb (Figure 3A, lane 3), but was not inhibited by the addition of mAb 3D3, an anti-σ^{70} mAb (Figure 3A, lane 2), confirming that transcription of the *xthA* promoter is σ^s-dependent.

As shown in Figure 3B, interestingly, we were also able to obtain the +1 transcript with Eσ^{70} (lane 4). This σ^{70}-specific signal was inhibited by the addition of mAb 3D3, an anti-σ^{70} mAb (lane 5), but was not inhibited by the presence of mAb 1RS1, an anti-σ^s mAb (lane 6), confirming that this signal is σ^{70}-dependent. We conclude that Eσ^{70} and Eσ^s each can transcribe the *xthA* promoter directly.

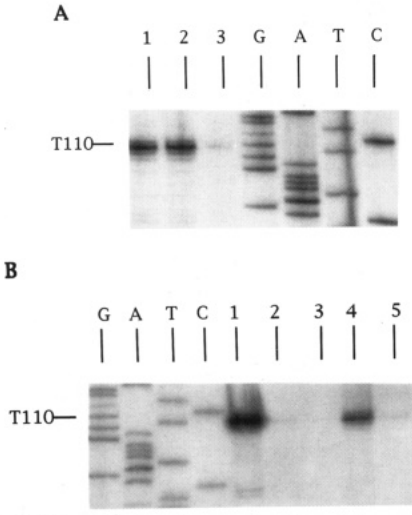

FIGURE 3: $E\sigma^{70}$ and $E\sigma^s$ can transcribe the *xthA* promoter as shown by a primer extension assay. The transcription reactions were done using a supercoiled pLHN24 plasmid that contains the *xthA* promoter. Lanes G, A, T, and C represent dideoxy sequencing reactions of pLHN24 in both panels A and B. Using the numbering system of Saporito et al. (1988), T110 is the transcriptional start site as indicated on the left of the panels A and B. (A) Lane 1 is $E\sigma^s$-synthesized RNA. Lane 2 is identical to lane 1 except that 18 μg of mAb 3D3, an anti-σ^{70} mAb, was added to the reaction before the addition of pLHN24. Lane 3 is identical to lane 1 except that 4 μg of mAb 1RS1, an anti-σ^s mAb, was added to the reaction before the addition of pLHN24. (B) Lane 1 is 34 μg of total *in vivo* RNA isolated from SK2267 cells that contained the pLHN24 plasmid. Lane 2 is 34 μg of total *in vivo* RNA isolated from SK2267 cells that did not contain pLHN24. Lane 3 is *in vitro* RNA synthesized by core RNA polymerase alone. Lane 4 is $E\sigma^{70}$-synthesized RNA. Lane 5 is identical to lane 4 except that 18 μg of mAb 3D3, an anti-σ^{70} mAb, was added to the reaction before the addition of pLHN24. Lane 6 is identical to lane 4 except that 4 μg of mAb 1RS1, an anti-σ^s mAb, was added to the reaction before the addition of pLHN24.

$E\sigma^s$ and $E\sigma^{70}$ Transcribe the bolAp1 Promoter Directly. We employed a transcription assay to determine if $E\sigma^s$ could transcribe the *bol*Ap1 promoter. The *bolA* gene contains two promoters, p1 and p2. The *bol*Ap1 promoter has been shown to be stronger and to be a growth-phase-dependent promoter (Aldea et al., 1989). Since the *bolA* promoter, like the *xthA* promoter, may be recognized by both $E\sigma^s$ and $E\sigma^{70}$, we performed a transcription assay using just a 556 base pair DNA fragment containing the *bolA* promoter in order to eliminate high background due to $E\sigma^{70}$ transcription of the plasmid promoters. With this promoter construct, transcription from the *bolAp1* promoter should give a 176-base run-off transcript. As shown in Figure 4, both $E\sigma^s$ and $E\sigma^{70}$ can give the expected 176-base run-off transcript (lanes 2 and 4). σ^s-specific transcription could not be inhibited by the presence of mAb 3D3, an anti-σ^{70} mAb (lane 5), while $E\sigma^{70}$ transcription was inhibited by the addition of mAb 3D3 (lane 3). Figure 4 also shows that the sizes of transcripts and relative amounts vary in $E\sigma^s$ versus $E\sigma^{70}$ reactions, indicating that the two holoenzymes do not have the exact same promoter specificity.

In addition, we employed a primer extension assay to further determine if the +1 *bolA* transcript is synthesized *in vitro* with either $E\sigma^s$ or $E\sigma^{70}$. As shown in Figure 5A, using isolated *in vivo* RNA as the positive control in the primer extension assay, we obtained the same primer-extension signal with *in vivo* RNA as with *in vitro* RNA synthesized by immunopurified $E\sigma^s$ (lanes 2 and 3). The σ^s-specific signal was not inhibited by the presence of mAb 3D3, an anti-σ^{70} mAb (lane 5), but was inhibited by the addition of mAb 1RS1, an anti-σ^s mAb (lane 4). As shown in Figure 5B, $E\sigma^{70}$ can also synthesize the +1 transcript from the *bolA*p1 promoter (lane 1). This

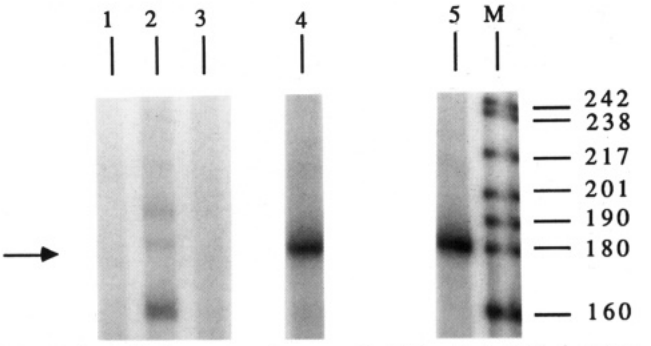

FIGURE 4: *bolA*p1 promoter is transcribed directly by $E\sigma^s$ and $E\sigma^{70}$ in a transcription assay. The assay was done as described under Materials and Methods with a linear PCR DNA fragment containing the *bolA* promoter. The expected run-off transcript from the *bolA*p1 promoter is 176 bases long. Lane 1 is the reaction with core RNA polymerase alone. Lane 2 is the reaction with $E\sigma^{70}$. Lane 3 is the same as lane 2 except that 18 μg of mAb 3D3, an anti-σ^{70} mAb, was preincubated with $E\sigma^{70}$ prior to the addition of the DNA template. Lane 4 is the reaction with $E\sigma^s$. The reaction in lane 5 was identical to that of lane 4 reaction except that $E\sigma^s$ was preincubated with 18 μg of mAb 3D3, an anti-σ^{70} mAb, before the addition of DNA template. Lane M is the DNA marker lane (pBR322 digested with *Msp*I). On the right are the DNA marker sizes in nucleotides. The arrow on the left indicates the expected transcript size from the *bolA*p1 promoter.

signal is σ^{70}-specific because it cannot be detected in the presence of mAb 3D3, an anti-σ^{70} mAb (lane 2). In contrast to $E\sigma^s$ transcription, $E\sigma^{70}$ transcription not only gives the *bolAp1* +1 transcript but also gives a transcript that is 10 bases longer, both in a transcription assay (Figure 4, lane 2) and in a primer extension assay (Figure 5B, lane 1). We conclude that the *bolA*p1 promoter is transcribed directly by $E\sigma^s$ and $E\sigma^{70}$.

Determining the Preferred in Vitro Transcription Start Site of the bolAp1 Promoter by $E\sigma^s$. Using an S1 nuclease protection assay, Aldea et al. (1989) determined the start site to be at A378 or at A379, using their numbering system. Our primer extension data indicated that transcription tends to start at a trio of G's located at 381–383, which is only a few bases different from the start site determined by Aldea et al. (1989). This slight discrepancy is probably due to the fact that we used a primer extension assay while Aldea et al. used an S1 protection assay.

In order to determine the preferred *in vitro* start site by $E\sigma^s$ and to eliminate the possibility that in our primer extension assay the reverse transcriptase pauses at the trio of G's and thereby does not extend to the 5′ end of the mRNA, we employed dinucleotide-primed transcription assays (Kassavetis et al., 1986). We reasoned that if transcription by $E\sigma^s$ started at the trio of G's and not at the A's, then we should be able to obtain transcripts by using GpG and not by using ApA dinucleotide. As shown in Figure 6A, we obtained the appropriate-sized *bolA* transcript in the presence of GpG dinucleotide and not in the presence of ApA (lanes 2 and 3). We were also able to obtain a weaker signal using ApG dinucleotide compared with that of GpG (lanes 1 and 2). Our data indicate that the trio of G's at 381–383 are the preferred transcription initiation sites for $E\sigma^s$.

DISCUSSION

The *katF*/*rpoS* gene is a central regulator of a large number of starvation/stationary-phase-dependent genes. In this paper, we have presented direct biochemical evidence that the *katF*/*rpoS* gene product (σ^s) is a σ factor. It was shown prior to our work that the *katF*/*rpoS* gene has strong amino acid sequence similarity to σ^{70} and σ^{32}. In our work, we provide

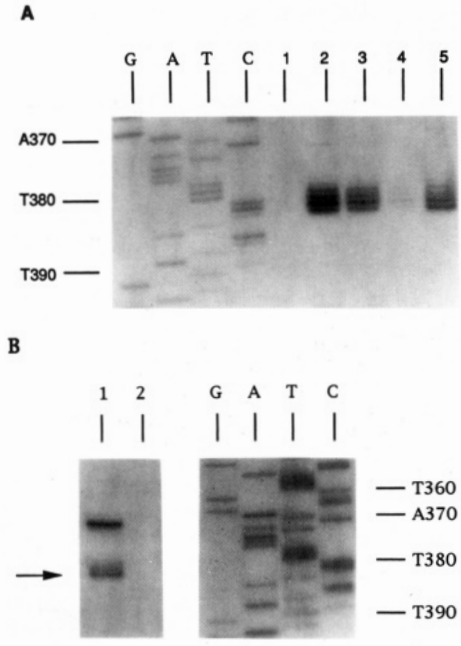

FIGURE 5: Primer extension assay with *in vitro* RNA made from *bolA* promoter plasmid (pGN66) linearized with *Hpa*I. The assay was done as described under Materials and Methods. Lanes G, A, T, and C represent dideoxy sequencing reactions of pGN66 with the same labeled primer in both panels A and B. (A) Lane 1 is *in vitro* RNA synthesized with core RNA polymerase alone. Lane 2 is 20 μg of total *in vivo* RNA isolated from SK2267 cells that have the pGN66 plasmid. Lane 3 is *in vitro* RNA that was synthesized with $E\sigma^s$. Lane 4 is identical to lane 3 except that 4 μg of mAb 1RS1, an anti-σ^s mAb, was added to the reaction before addition of the promoter template. Lane 5 is identical to lane 3 except that 18 μg of mAb 3D3, an anti-σ^{70} mAb, was added to the reaction before addition of the promoter template. Nucleotide positions of the complementary DNA strand are shown on the left with the numbering according to Aldea et al. (1989). (B) Lane 1 is *in vitro* RNA synthesized by $E\sigma^{70}$ using the *bolA* promoter plasmid, pGN66, linearized with *Hpa*I. Lane 2 is identical to lane 1 except that 18 μg of mAb 3D3, an anti-σ^{70} mAb, was added to the reaction before the addition of the promoter template. The arrow on the left indicates the expected primer-extension signal from the *bolA*p1 promoter. Nucleotide positions of the complementary DNA strand are shown on the right with the numbering according to Aldea et al. (1989).

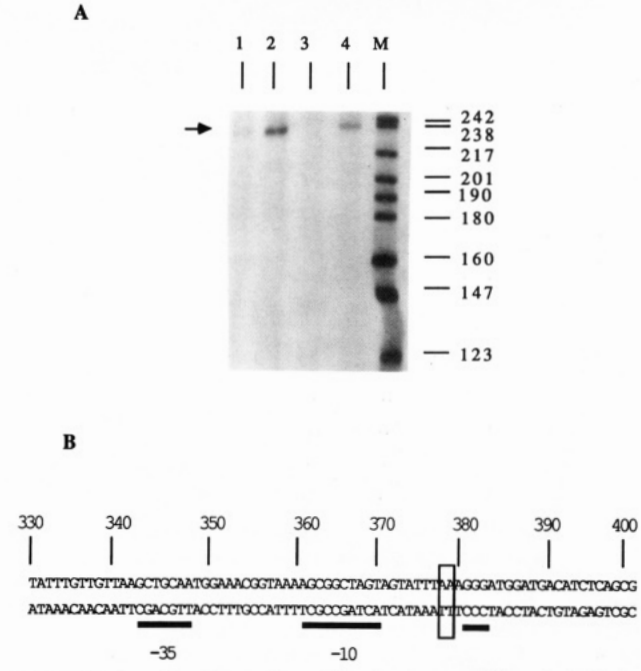

FIGURE 6: Locating the preferred *in vitro* transcription start site by $E\sigma^s$. (A) Dinucleotide-primed single-round transcription assays were performed as described under Materials and Methods with pGN66 linearized with *Hpa*I. The expected *bolA*p1 transcript is 240 bases long. Lane 1 is the reaction with ApG dinucleotide. Lane 2 is the reaction with GpG, and Lane 3 is with ApA. Lane 4 is the transcription reaction not primed with any type of dinucleotide and has regular nucleoside triphosphates. Lane M is the DNA marker (pBR322 digested with *Msp*I) with sizes in nucleotides indicated on the right. The arrow on the left indicates the expected transcript size from the *bolA*p1 promoter. (B) Schematic of the DNA sequence of the *bolA*p1 promoter. Putative –35 and –10 regions are underlined and marked accordingly (Aldea et al., 1989). The transcription start point determined by Aldea et al. is boxed. The *in vitro* transcription start point as determined by (A) is underlined.

direct evidence that σ^s has the following σ factor functions: it can bind to core RNA polymerase (Figure 1) and can confer upon core RNA polymerase the ability to directly transcribe the *bolA*p1 and the *xthA* promoters (Figures 3A, 4 and 5A).

Interestingly, however, both the *bolA*p1 and the *xthA* promoters can also be recognized by $E\sigma^{70}$ (Figures 3B, 4, and 5B) (Tanaka et al., 1993). The σ^s-dependent transcription of both promoters cannot be due to cross-contamination of σ^{70} in the $E\sigma^s$ fraction because σ^s-specific transcription is not inhibited by mAb 3D3, an anti-σ^{70} mAb, but is inhibited in the presence of mAb 1RS1, an anti-σ^s mAb. The same reasoning can also be applied to determine that the specific +1 transcript generated by $E\sigma^{70}$ is σ^{70}-dependent. In addition, using the Western blot assay, we cannot detect any σ^{70} in the $E\sigma^s$ preparation or any σ^s in the σ^{70} preparation that was used for functional analysis (data not shown). Having non-cross-reactive mAbs that can serve as specific functional inhibitors of particular σ factor-dependent activity helped to verify that transcription activity was dependent on that particular σ factor and not on another contaminating σ factor. Such antibody inhibition approaches were used to determine the σ-dependence of various promoters in an *in vitro E. coli* S-30 extract transcription–translation system (Jovanovich et al. 1989).

Since the proposed promoter-recognizing regions of σ^s are not very different from those of σ^{70}, σ^{70} and σ^s may have similar promoter recognition specificities (Hengge-Aronis, 1993; Tanaka et al., 1993). However, the two different holoenzymes may have differing promoter affinities for the *bolA*p1 and the *xthA* promoters. In the case of the *bolA*p1 promoter, the data in Figure 4 suggest that $E\sigma^s$ transcription is stronger than that of $E\sigma^{70}$. Figure 4 also shows that the sizes of transcripts and relative amounts vary in $E\sigma^s$ versus $E\sigma^{70}$ reactions, indicating that the two holoenzymes do not have the exact same promoter specificity. Different promoter affinities by the two different holoenzymes might be due to a cis-acting promoter component. Such differing affinities may reflect the cell's varying need for the gene products during the exponential and stationary growth phases. It is presently unclear why the *bolA*p1 promoter is not expressed well in exponential cells if it can be recognized by $E\sigma^{70}$. It is possible that trans-acting factors *in vivo* prevent such transcription.

Both the *xthA* and *bolA*p1 promoters have been shown to be regulated by the *katF*/*rpoS* gene (Sak et al., 1989; Lange & Hengge-Aronis, 1991b), and their expression increases as cells enter stationary phase (Sak et al., 1989; Lange & Hengge-Aronis, 1991; Aldea et al., 1989). As cells enter stationary phase, there is also an increase in *katF*/*rpoS* gene expression (Mulvey et al., 1990) and in the σ^s protein level (our unpublished observations). Our data showing that $E\sigma^s$ transcribes the *bolA*p1 promoter preferentially are consistent with a model in which expression of the σ^s gene increases as cells enter stationary phase. The increase in the level of σ^s in the cell leads to increased transcription of the *bolA* gene.

In the case of the *xthA* gene, there may or may not be a strong difference in promoter affinity between $E\sigma^s$ and $E\sigma^{70}$.

The *xthA* gene product, exonuclease III, is the major apurinic (AP) endonuclease in *E. coli* (Saporito et al., 1988). It plays a key role in repairing AP sites which arise in *E. coli* either spontaneously or by the action of glycosylases which recognize damaged or incorrect bases that are formed under oxidative stress conditions (Saporito et al., 1988). We postulate that the exonuclease III is important in most, if not all, growth conditions. Therefore, at least in the exponential growth phase, its expression is probably dependent on $E\sigma^{70}$ to some extent because σ^s expression is low in exponential growth phase (Mulvey et al., 1990; unpublished observations).

ADDED IN PROOF

While this paper was being reviewed and revised, Tanaka et al. (1993) showed that σ^s holoenzyme can transcribe a number of typical σ^{70}-type promoters, some as efficiently as σ^{70} holoenzyme. These data are consistent with our data that σ^{70} and σ^s may have similar promoter recognition specificities.

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