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Promoter Selectivity of *Escherichia coli* RNA Polymerase σ^F Holoenzyme Involved in Transcription of Flagellar and Chemotaxis Genes

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The *rpoF* gene of *Escherichia coli* codes for the RNA polymerase σ^F (or σ^{28}) subunit, which is involved in transcription of the flagellar and chemotaxis genes. Both σ^F and σ^{70} (the major σ subunit in growing cells) were overexpressed, purified to homogeneity, and compared with respect to activity and specificity. The affinity of σ^F to core RNA polymerase (E) is higher than that of σ^{70}, as measured by gel filtration high-pressure liquid chromatography. In an in vitro transcription system, the holoenzyme ($E\sigma^F$) containing σ^F selectively transcribed the flagellar and chemotaxis genes, all of which could not be transcribed by $E\sigma^{70}$. This strict promoter recognition property of σ^F is similar to those of other stress response minor σ subunits but different from those of the principal σ subunits, σ^{70} and σ^{38}. σ^{70}-dependent transcription in vitro is inhibited at high concentrations of all salts tested, showing maximum activity at 50 mM. In contrast, σ^F-dependent transcription was maximum at 50 mM KCl and then decreased to negligible level at 300 mM; in the cases of potassium acetate and potassium glutamate, maximum transcription was between 200 and 300 mM. DNase I foot printing of the *fliC* and *fliD* promoters indicated that σ^F alone is unable to bind DNA, but $E\sigma^F$ specifically recognizes −10 and −35 regions of the σ^F-dependent promoters with rather long upstream protection. Alteration of the promoter structure after binding of $E\sigma^F$ was suggested.

The σ subunit of bacterial RNA polymerase confers promoter recognition ability to core RNA polymerase with the subunit structure $\alpha_2\beta\beta'$. Most of the genes expressed in exponentially growing cells of *Escherichia coli* are transcribed by holoenzyme ($E\sigma^{70}$) containing σ^{70} (the *rpoD* gene product). On the other hand, the stress response genes are transcribed by RNA polymerase holoenzymes containing minor σ subunits (8, 9, 14): $E\sigma^{32}$ (or $E\sigma^H$) and $E\sigma^{24}$ (or $E\sigma^E$) transcribe the genes for heat shock proteins; $E\sigma^{54}$ (or $E\sigma^N$) transcribes the genes which are regulated by the availability of nitrogen; $E\sigma^{38}$ (or $E\sigma^S$) is essential for the expression of some stationary-phase-specific and high-osmolarity-induced genes; and $E\sigma^{28}$ is needed for expression of the flagellar and chemotaxis genes. Recently, the *fecI* gene product, which was originally identified as a regulatory gene for the ferric citrate transport system, was identified as σ^{19}, a new member of σ family proteins, and is involved in transcriptional regulation of the genes for extracytoplasmic functions (2).

The flagellar, chemotaxis, and motility genes in *E. coli* and *Salmonella typhimurium* are coordinately expressed (22). Detailed analysis has been carried out for the flagellar genes, which are divided into three coordinately regulated classes, I to III, which together form a regulatory hierarchy (22). Expression of the class III genes, including the *fliC* gene for the major flagellar protein, flagellin, depends on the *fliA* gene organized in the class II gene cluster. The *fliA* gene product, with a molecular mass of 28 kDa, was found to carry σ activity for transcription of the chemotaxis genes such as *tar* and *tsr*, for sensing aspartate and serine, respectively, and the *flaAI* genes, for flagellar formation (3, 27). After DNA sequencing, the *fliA* gene was indeed found to contain a sequence conserved between the σ family proteins (21). Furthermore, the overexpressed and purified FliA protein exhibited σ activity in vitro, using the *tar* gene promoter (21). The *fliA* gene product is similar in structure and specificity to *Bacillus subtilis* σ^D (8). On the basis of these observations, FliA is now recognized as σ^F (or σ^{28}) and *fliA* is renamed *rpoF*.

The formation of bacterial flagella is subject to control depending on environmental conditions (1, 20, 22, 24, 28). The intracellular level of the σ^F subunit, however, stays constant under various growth conditions (15), suggesting that the activity of σ^F may be controlled in response to the demand for flagellar formation. As an initial attempt to understand the control mechanism of σ^F activity, we analyzed factors affecting the activity and specificity of σ^F by using an in vitro mixed transcription system directed by various *E. coli* promoters. This report describes the first systematic analysis of the activity and specificity of σ^F, including (i) the core binding activity as measured by gel filtration high-pressure liquid chromatography (HPLC); (ii) the promoter recognition properties as analyzed by using six different σ^F-dependent promoters; (iii) the effects of species and concentration of salt; and (iv) the promoter binding properties as analyzed by DNase I footprinting.

MATERIALS AND METHODS

Overexpression and purification of σ^F and σ^{70}. The *E. coli rpoF* gene in pEA20 (a gift of K. Ohnishi, Waseda University) was subcloned into the vector pET21 under the control of the T7 promoter. The GTG initiation codon in *rpoF* was changed to ATG for better expression. The newly constructed plasmid, pETSF, was transformed into *E. coli* BL21(λDE3). σ^F was overexpressed by adding isopropylthiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. Cells were treated with lysozyme and lysed by sonication after addition 0.01% sodium deoxycholate as in the case of σ^{70} purification (12). The overexpressed σ^F protein recovered in inclusion bodies was washed with a Triton buffer (50 mM Tris-HCl) [pH 8 at 4°C], 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100) and then dissolved in TGED buffer (10 mM Tris-HCl [pH 7.6 at 4°C], 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) containing 6 M guanidium

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hydrochloride. The crude σ^F solution was dialyzed against TGED buffer containing 6 M urea and then subjected to DEAE ion-exchange chromatography. The bound fraction was eluted with a linear gradient of 0.1 to 0.5 M NaCl in TGED buffer containing 6 M urea. Peak fractions of 28-kDa σ^F protein identified by polyacrylamide gel electrophoresis (PAGE) on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel were pooled and loaded onto a G3000 gel filtration column. The column was developed with 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM KCl and 6 M urea. σ^F fractions were checked by SDS-PAGE (12% gel), pooled, dialyzed against a storage buffer (10 mM Tris-HCl [pH 7.6 at 4°C], 10 mM $MgCl_2$ 0.1 mM EDTA, 0.2 M KCl, 50% glycerol, 1 mM dithiothreitol), and stored at −30°C until use.

The *rpoD* gene product, σ^{70}, was overexpressed and purified as described by Igarashi and Ishihama (12).

RNA polymerase core enzyme. RNA polymerase was purified from strain W3350, and the core enzyme was purified by passing purified RNA polymerase at least three times through phosphocellulose columns (18). Repetition of the phosphocellulose column chromatography at least three times was needed to completely remove minor σ subunits from the core enzyme.

In vitro transcription. Single-round in vitro transcription was carried out under the standard conditions described previously (12, 16). Briefly, a mixture of σ subunit and the core enzyme was incubated at 30°C for 10 min to form the holoenzyme and then mixed with template DNA to make a final 35-μl solution. After incubation for 30 min at 37°C, the substrate solution (15 μl) was added to make final concentrations of 160 μM each ATP, GTP, and CTP, 50 μM UTP, 4 μCi of [α-^{32}P]UTP per ml, and 200 μg of heparin per ml, and then the elongation reaction was continued for 5 min. Transcripts were precipitated with ethanol and analyzed on a 6% polyacrylamide gel in the presence of 6 M urea. Gels were exposed to imaging plates, and the exposed plates were analyzed with a BAS2000 image analyzer (Fuji).

Promoters and templates. Truncated 205-bp *lacUV5* fragment was prepared as described previously (16). A 287-bp *Bam*HI-*Kpn*I fragment carrying the *alaS* promoter was prepared as described previously (26).

As the representative promoters belonging to the flagellar operons, we used the *flgK*, *fliC*, and *fliD* genes from *S. typhimurium*. The promoter core sequences of *S. typhimurium fliC* (13) and *fliD* (10) genes are identical with those of the respective *E. coli* genes (23, 30), but the promoter −10 sequence of *flgK* is different at two less essential positions between *E. coli* (4) and *S. typhimurium* (10). A 400-bp fragment carrying the *flgK* promoter was prepared by digestion of plasmid pKK1490 (11) (a gift from K. Kutsukake, Hiroshima University) with enzymes *Bcn*I and *Mlu*I. Plasmid pKK1012 (13) (a gift from K. Kutsukake) was digested with *Hinc*II to isolate a 350-bp *fliDC* promoter fragment. This fragment was further digested with *Hha*I or *Bna*I to separate the *fliC* and *fliD* promoters. Fragments carrying the *fliC* (*Hha*I large fragment) or *fliD* (*Bna*I large fragment) promoter were blunt ended by Klenow enzyme and subcloned into pBluescript-IISK$^+$ at the *Eco*RV site. Truncated templates containing the *fliD* or *fliC* promoter were then prepared by *Pst*I-*Xho*I digestion.

As the representative promoters from the chemotaxis genes, we used three promoters from the *E. coli tar* gene for aspartate sensor (plasmid pLAN931), the *E. coli tsr* gene for serine sensor (plasmid pLAN1031), and the *S. typhimurium tcp* gene for citrate sensor (plasmid pAS101). Plasmid pLAN 931 (33) (a gift from I. Kawagishi, Nagoya University) and pLAN1031 (33) (a gift from I. Kawagishi) were digested with *Eco*RI-*Eco*RV and *Eco*RI-*Mlu*I, respectively, to generate the truncated *tar* (1,100-bp) and *tsr* (600-bp) promoter fragments. pAS101 plasmid (34) (a gift from I. Kawagishi) was subjected to PCR amplification for preparation of a 350-bp fragment containing the *tcp* promoter. The promoter-containing fragment was subcloned into pBluescript, and the fragment was confirmed by sequencing using a Pharmacia LKB A.L.F. sequencer (see Fig. 2 for the structures of truncated templates).

DNase I footprinting. The 350-bp DNA templates carrying the *fliC* and *fliD* promoters were prepared by PCR amplification. One of the primers was labeled at the 5′ end with [γ-^{32}P]ATP, using T4 polynucleotide kinase. The labeled promoter fragment was mixed with either holoenzyme ($E\sigma^F$) or σ^F for 30 min at 37°C under the conditions used for in vitro transcription assays. The reaction mixture was incubated at 25°C for 5 min in the presence of 5 mM $CaCl_2$ and then subjected to DNase I digestion for 45 s. Digested DNA was extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis on a 6% polyacrylamide sequencing gel.

RESULTS

Overexpression and purification of σ^F. σ^F was overexpressed by using the T7 promoter/polymerase system and purified by using ion-exchange chromatography on a DEAE-cellulose column followed by gel filtration in HPLC. The yield of σ^F subunit was approximately 2 to 3 mg per 100 ml of culture, and the purity was more than 98% as analyzed by SDS-PAGE (12% gel) and visualization by Coomassie brilliant blue staining. Although σ^F is a protein of 28 kDa, calculated from its amino acid sequence (21), it migrated along with a 32-kDa marker protein band on PAGE (Fig. 1A). The purified σ^F was electrophoresed together with σ^{70}, σ^N, σ^S, and σ^H purified essentially by the same procedure (6, 12, 15, 31). The anomalous low mobility is associated with all the σ subunits (σ^{70}, σ^N, σ^S, σ^H, and σ^F) examined because of their acidic nature (Fig. 1B).

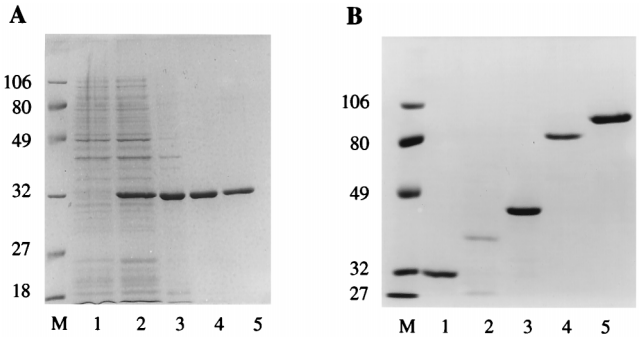

FIG. 1. Overexpression and purification of σ^F protein. (A) σ^F was purified from *E. coli* cells expressing pETSF carrying the *rpoF* gene. Aliquots from each step of the purification were analyzed on an SDS–12.5% polyacrylamide gel. Lane 1, cell extract before addition of IPTG; lane 2, cell extract after IPTG induction; lane 3, guanidine solution of inclusion bodies; lane 4, combined pool of DEAE column fractions; lane 5, combined pool of G3000 column fractions (purified σ^F); lane M, mixture of M_r marker proteins. (B) Purified σ factors were analyzed on an SDS–10% polyacrylamide gel. Lane 1, σ^F; lane 2, σ^H; lane 3, σ^S; lane 4, σ^{54}; lane 5, σ^{70}; lane M, mixture of M_r marker proteins. Positions of markers are indicated in kilodaltons.

Promoter-specific transcription by $E\sigma^F$ holoenzyme. The promoter selectivity of $E\sigma^F$ holoenzyme was analyzed in a single-round in vitro transcription assay using different promoters for the *S. typhimurium* and *E. coli* genes involved in flagellar formation and chemotaxis (Fig. 2A). Holoenzyme $E\sigma^F$ was able to initiate transcription from the promoters associated with the *Salmonella fliD*, *fliC*, and *flgK* genes coding for filament cap protein, flagellin, and hook filament junction protein, respectively (Fig. 2B, *fliDC*, *flgK*, *fliD*, and *fliC* lanes). Among the three flagellar regulon promoters tested, the *fliC* promoter was the strongest under the reaction conditions used (Fig. 2B). On the other hand, the holoenzyme containing σ^{70} was virtually inactive in transcription of these flagellar genes (Fig. 2B, *fliDC* and *flgK* lanes).

We also examined the promoters from three chemotaxis genes, i.e., the *Salmonella tcp* gene for sensing citrate, *E. coli tar* for sensing aspartate, and *E. coli tsr* for sensing serine. All of these promoters were efficiently transcribed by $E\sigma^F$ (Fig. 2B, lanes 2) but not by $E\sigma^{70}$ (lanes 1) (however, $E\sigma^{70}$ gave transcripts initiated from as yet unidentified promoters on the *tsr*, *tar*, and *tcp* promoter fragments). Some σ^{70}-specific promoters were also tested for comparison. Holoenzyme containing σ^F was unable to transcribe the σ^{70}-specific promoters such as *alaS* and *lacUV5*. These results indicate that the holoenzyme containing σ^F specifically transcribes only the genes involved in flagellar formation and chemotaxis. Such strict promoter selectivity has been identified for the holoenzymes containing the minor σ subunits such as σ^N and σ^H but not for those containing the principal σ subunits, σ^{70} and σ^S (17, 31, 32).

Affinity of σ^F to core RNA polymerase. Affinity of each σ factor to core RNA polymerase should be a factor affecting the rate and level of σ replacement upon changes in the physiological conditions. The affinities of σ^{70} and σ^F to core RNA polymerase were compared by measuring two different assays as used for the comparison between σ^{70} and σ^S (6, 18): (i) the saturation level of σ factor required for maximum level of

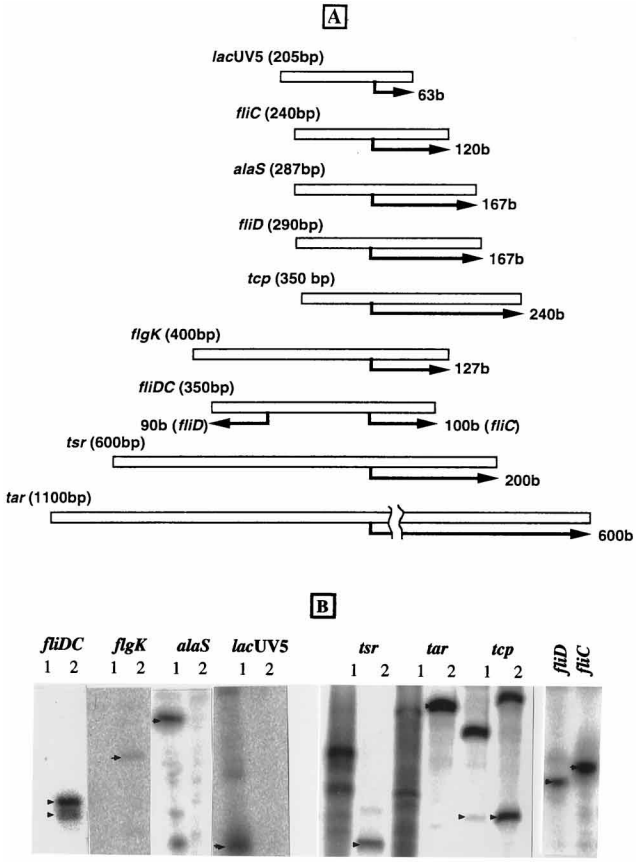

FIG. 2. Transcription in vitro by RNA polymerase $E\sigma^F$ holoenzyme. (A) Structures of the truncated DNA templates used in this study and transcription maps expected for these templates. (B) Single-round in vitro transcription was carried out under the standard reaction conditions, using 1 pmol each of two different forms of the reconstituted holoenzyme, $E\sigma^{70}$ (lane 1) and $E\sigma^F$ (lane 2). Promoters tested are (0.1 pmol each) *S. typhimurium fliDC* and *flgK* (the flagellar class III genes), *E. coli alaS* and *lacUV5* (σ^{70}-dependent genes), *E. coli tsr* and *tar* (the chemotaxis genes sensing serine and aspartate), and *S. typhimurium tcp* (the chemotaxis gene sensing citrate). The arrowheads indicate transcripts from the test promoters.

holoenzyme formation from a fixed amount of core enzyme and (ii) the amount of σ subunit required for maximum transcription by a fixed amount of core enzyme.

First, the level of holoenzyme formation was directly measured by mixing a fixed amount of core enzyme with various amounts of σ^{70} or σ^F at 30°C followed by separation of holoenzymes from unbound σ subunits by gel filtration HPLC on a Superdex-200 column. The amount of each σ subunit bound to core enzyme was measured for the peak fractions of RNA polymerase, and the results are summarized in Fig. 3A. The core enzyme was saturated with σ^F at the input molar ratio of about 1, while twofold more σ^{70}, i.e., input molar ratio of about 2, was required to saturate the same amount of core enzyme. Under the experimental conditions used, the level of σ^{70} required for saturation of the core enzyme is essentially the same between the overexpressed and renatured σ^{70} as used in this and previous experiments (6, 18) and the native σ^{70} dissociated from purified holoenzyme (data not shown), indicating that the renatured σ^{70} is as active as the native σ^{70}. These observations suggest that the affinity of σ^F to core enzyme is about two times higher than that of σ^{70} under the conditions used.

To rule out the possibility that the fractions of active σ

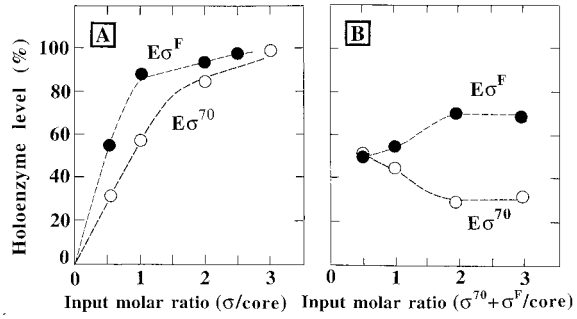

FIG. 3. Binding of σ subunit to core RNA polymerase. (A) Core enzyme (50 pmol) and various amounts of σ^{70} or σ^F were mixed and incubated for 10 min at 30°C. The mixtures were fractionated by gel filtration HPLC using a Superdex-200 (Pharmacia) column. Each fraction was analyzed on an SDS–10% polyacrylamide gel, and the gels were stained with ALLPRO fluorescence dye and scanned with a scanning Fluor Imager SI (Molecular Dynamics). The molar ratio of α, ββ′, and σ was calculated for the peak fractions. (B) Core enzyme (50 pmol) and various amounts of a mixture of σ^{70} and σ^F (molar ratio = 1:1) were mixed and analyzed as for panel A. The relative amounts of core-bound σ^{70} and σ^F are plotted.

molecules differ between the σ^F and σ^{70} preparations used, we repeated the experiment, using the unbound σ subunits after one cycle of the core binding assay at an input σ-to-core ratio of 2.5. The results were essentially the same as for the first experiment (data not shown). Furthermore, a competition binding assay was carried out in the presence of both σ^{70} and σ^F. Upon an increase of input σ subunits, keeping the σ^{70} to σ^F ratio at a constant level of 1, the amount of core-bound σ was always higher for σ^F (Fig. 3B). We concluded from these results that the core binding activity is about twofold higher for σ^F than σ^{70}.

Next, the σ saturation curves for maximum transcription by a fixed amount (1 pmol per assay) of core enzyme were compared between two test σ subunits in an in vitro single-round transcription assay using one σ^{70}-specific (*alaS*) and two σ^F-specific (*fliC* and *fliD*) promoters (Fig. 4). In transcription of the *alaS* promoter, the core RNA polymerase was saturated by adding about 1.5-fold molar excess of σ^{70} subunit (Fig. 4A). The requirement of σ^{70} subunit for the maximum transcription was virtually independent of the promoters used (18). Interestingly, the amount of σ^F required for the maximum transcription of both the *fliC* (Fig. 4B) and *fliD* (Fig. 4C) promoters

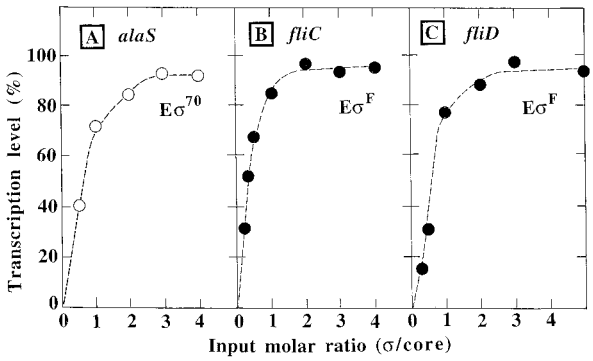

FIG. 4. Saturation curve of σ subunit for maximum transcription. Core enzyme (1 pmol) and various amounts of σ^{70} (A) and σ^F (B and C) were mixed and used for single-round transcription with 0.1 pmol each of the test promoters, alaS (A), fliC (B), and fliD (C). The maximum level of transcription in each assay was set at 100%.

by the same amount (1 pmol) of core enzyme was roughly 1 pmol, significantly lower than that of σ^{70} for transcription of σ^{70}-dependent promoters. The molar ratios of σ^F to core required for 50% saturation in *fliC* and *fliD* transcription were 0.4 to 0.5 and 0.5 to 0.6, respectively, whereas the ratio of σ^{70} to core was 0.7 to 0.8 at 50% saturation. The in vitro transcription results are in good agreement with those of the holoenzyme formation assay, and thus we conclude that the affinity to core enzyme is about twofold higher for σ^F than for σ^{70}. However, the absolute levels of σ subunits required for holoenzyme formation (Fig. 3) were slightly higher than those of the transcription assay (Fig. 4), presumably because σ subunits are partially dissociated during the gel filtration due to decreased protein concentration.

Effect of salts and temperature on σ^{70}- and σ^F-dependent transcription. The formation of flagella in *E. coli* changes markedly in response to environmental stimuli (1, 20, 22, 24, 28). The mechanism underlying the regulation of flagellar formation may involve the inhibition of σ^F-dependent transcription of the flagellin gene (*fliC*). Since the level of σ^F stays at a constant level at various growth phases and under various stress conditions (15), its activity must be controlled in response to the bacterial demand for flagellar formation. In an effort to understand the activity control of σ^F, we examined effects of changing the reaction conditions for in vitro transcription.

First, we analyzed effects of the species and concentrations of salt on σ^F-dependent transcription in vitro, using the *fliC* and *fliD* promoters. For comparison, the salt effect was also analyzed for σ^{70}-dependent transcription of the *alaS* promoter. Our standard reaction buffer contains NaCl at 50 mM, which is the optimum concentration for transcription from σ^{70}-dependent promoters associated with most of the genes highly expressed under the steady state of cell growth. In this study, we examined various concentrations, from 50 to 400 mM, of three different potassium salts, KCl, potassium acetate, and potassium glutamate.

The level of single-round transcription initiated at the *alaS* promoter by $E\sigma^{70}$ was maximum at 50 mM all three potassium salts examined and then decreased to negligible levels between 150 and 200 mM (Fig. 5A). The effect of KCl on transcription level of the σ^F-dependent *fliD* and *fliC* promoters was essentially the same as that of σ^{70}-dependent *alaS* transcription (Fig. 5B and C). The effects of potassium acetate and potassium glutamate on σ^F-dependent transcription were, however, quite contrasting: the level of single-round transcription increased gradually up to 300 mM potassium acetate for the *fliC* promoter and 200 mM for the *fliD* promoter and then decreased to 10 to 20% at 400 mM; in the case of potassium glutamate, the maximum level of transcription was obtained at 350 mM for both σ^F-dependent test promoters and thereafter decreased to 85 to 95% at 400 mM. Stimulation by potassium glutamate was observed previously for σ^S-dependent transcription (6). Possible links between the stimulation in vitro of $E\sigma^F$ holoenzyme by high concentrations of potassium glutamate and potassium acetate and the inhibition of flagellar formation under prolonged exposure to high salt concentrations are discussed below.

Flagellar synthesis in bacteria is known to be inhibited at high temperatures (24). For instance, the relative rate of synthesis of flagellin at 42°C decreases to around 6% of the level at 37°C, and as a result, the amount of flagella decreases to a negligible level (20, 28). One of the possible mechanisms leading to reduction in flagellar formation could be inactivation of σ^F function at high temperatures. Our preliminary studies indicated that the optimum temperature of transcription by $E\sigma^{70}$ holoenzyme was 37°C for both *alaS* and *lacUV5* promoters, whereas the maximum level of σ^F-dependent transcription was observed at 30°C for both *fliC* and *fliD* promoters (data not shown).

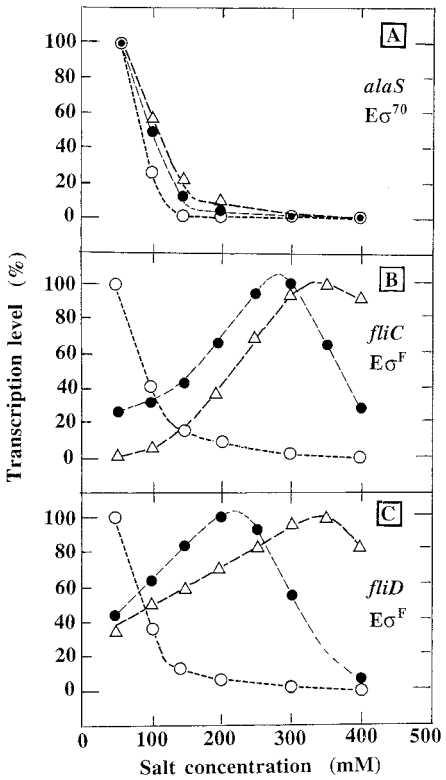

FIG. 5. Effects of species and concentration of salts on in vitro transcription. Single-round in vitro transcription was carried out with 0.1 pmol of the truncated *alaS* (A), *fliC* (B), or *fliD* (C) template and 1 pmol of either $E\sigma^{70}$ (A) or $E\sigma^F$ (B and C) holoenzyme under the standard reaction conditions except that 50 mM NaCl was replaced with the indicated concentrations of KCl (○), potassium acetate (●), or potassium glutamate (△). Transcripts were analyzed by electrophoresis on a 6% denaturing urea-acrylamide gels, and the gels were examined with a BAS2000 image analyzer (Fuji).

Identification of the DNA binding regions by $E\sigma^F$. Results of the holoenzyme formation and in vitro transcription assays clearly demonstrated that $E\sigma^F$ carries a very strict promoter selectivity. To determine the promoter sequence recognition properties of $E\sigma^F$, we performed DNase I footprinting experiments of the *fliC* and *fliD* promoters by either σ^F alone or $E\sigma^F$ holoenzyme. Under the experimental conditions used, σ^F alone was unable to bind to DNA, as revealed by a gel mobility shift assay (data not shown) and DNase I footprinting. The holoenzyme containing σ^F showed clear footprints on both the *fliC* and *fliD* promoters (Fig. 6). Some unique features were found for the footprint by $E\sigma^F$ holoenzyme. The background is less than that by other holoenzymes, and the upstream protection is longer than that by $E\sigma^{70}$ holoenzyme. Although the overall protection patterns are the same between these two promoters, some minor differences were observed. In the case of the *fliD* promoter, the protection extended from +23 to −58 (Fig. 6A), while the protection of the *fliC* promoter extended from +14 to −62 (Fig. 6B). Thus, the downstream protection varies by about 9 bp but the upstream protections are similar in the two promoters. A distinct characteristic feature of the *fliC* promoter was the generation of two hypersensitive sites at −37 and −39 relative to the transcription start site.

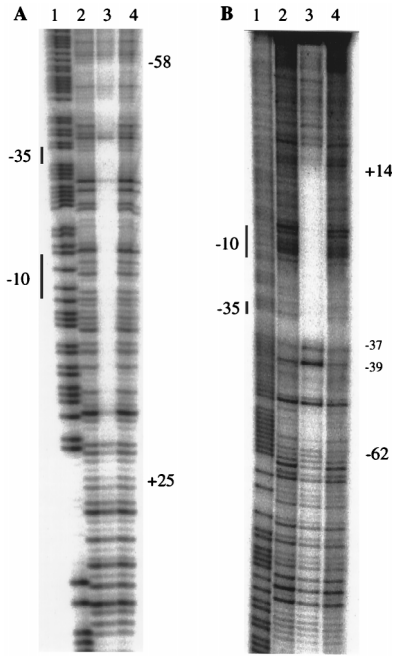

FIG. 6. DNase I footprinting analysis. DNase I footprinting analysis was carried out as described in Materials and Methods, using $E\sigma^F$ holoenzyme and the *fliD* (A) or *fliC* (B) promoter. Lane 1, chemical cleavage at A and G; lane 2, control without addition of RNA polymerase proteins; lane 3, $E\sigma^F$ holoenzyme; lane 4, σ^F alone.

DISCUSSION

The promoter specificity of *E. coli* σ^F was first reported by Arnosti and Chamberlin (3), who used a purified RNA polymerase fraction containing a 28-kDa protein with σ-like function. This protein was later identified as σ^F encoded by the *rpoF* gene after gene cloning and DNA sequencing (21, 27) and was found to be a homolog of *B. subtilis* σ^D (8). In this study, we carried out the first systematic analysis of the promoter recognition properties of σ^F by using various *E. coli* promoters. All of the flagellum cascade gene promoters tested and the promoters from the *tar* and *tsr* chemotaxis genes were transcribed only by $E\sigma^F$. One exception is the *tcp* promoter, which could be transcribed by $E\sigma^{70}$ but only very weakly. On the other hand, all of the σ^{70}-dependent promoters so far tested were not transcribed by $E\sigma^F$. The strict promoter selectivity of σ^F is probably attributable to the conserved nature of σ^F-dependent promoters: the −10 and −35 sequences and the spacer length between these two signals are highly conserved between σ^F-specific promoters (8).

One of the most interesting findings of this investigation is the higher affinity of σ^F than of σ^{70} to the core RNA polymerase. The number of genes under the control of σ^{70} is not less than 1,000 (9, 14), whereas only 18 to 20 genes are transcribed by σ^F (22). However, the intracellular concentration of σ^F in both exponentially growing and stationary-phase cells is almost half the level of σ^{70} (15). Consequently, the question arises as to why *E. coli* cells need such a high level of σ^F, even though it has a higher affinity to the core enzyme than σ^{70}, it recognizes only a unique promoter sequence associated with the σ^F-dependent genes, and it is used for transcription of only 18 to 20 genes. One possible explanation is that σ^F is stored in an inactive form by forming complexes with the anti-σ, factor, FlgM protein (19). The level of σ^F–anti-σ complexes may vary in response to the environmental conditions. The activity of σ^F was also suggested to be under the control of FliZ and FliY, encoded by genes organized in the same operon with *rpoF* (25). The storage of σ^F in an inactive form enables bacteria to respond quickly to changes in the environment, by forming flagella or sensing environmental chemical stimuli. The other explanation for the difference in the number of σ^F molecules and the number the genes under the control of this σ subunit is that σ^F requires a specific intracellular condition(s) for function. In fact, it is known that bacterial cells stop the formation of flagella at high temperatures (1, 24), in the presence of high concentrations of salts, carbohydrates, and low-molecular-weight alcohol, or in the presence of DNA gyrase inhibitors (20). For instance, under prolonged exposure of *E. coli* cells to high-salt environments such as at 500 mM NaCl, the formation of flagella is reduced markedly, as measured by enzyme-linked immunosorbent assay, because the relative rate of flagellin synthesis decreases to as low as 6% (28). In the single-round in vitro transcription system, the levels of transcription from both *fliC* and *fliD* promoters are negligible at 300 mM KCl, but σ^F-dependent transcription is activated at up to 300 mM potassium acetate and potassium glutamate. The response of σ^F to KCl, potassium acetate, and potassium glutamate is essentially the same as that of σ^S, the stationary-phase-specific σ factor (6). The activation of σ^S by high concentrations of potassium acetate or potassium glutamate is needed for selective transcription of the genes for osmotic stress response. In the case of σ^F, however, the activation by the transient increase in potassium acetate or potassium glutamate concentrations is antagonized by the induction of synthesis of FlgM (anti-σ^F factor), as measured by immunoblot analysis (17a).

The *B. subtilis* σ^D subunit, a homolog of *E. coli* σ^F, is known to bind to DNA in the absence of core enzyme and to form a discrete complex as demonstrated in a gel mobility shift assay (5). *S. typhimurium* σ^F alone has also been reported to bind to DNA (7). In both cases, however, the binding affinity of σ alone seems to be very weak. Under our gel shift assay conditions, the DNA binding activity was not detected for *E. coli* σ^F. In DNase I footprinting experiments, the holoenzyme containing σ^F protected the promoter region of both *fliC* and *fliD*, but the region protected seems to be longer than that protected by $E\sigma^{70}$. The wide-range protection by $E\sigma^{70}$ is, however, observed for a group of promoters carrying the UP (upstream) element which is recognized and protected by α subunit (29). The clear background in the protected region by $E\sigma^F$ holoenzyme may suggest a difference in the mode of σ-promoter contact between σ^F and σ^{70}. As in the case of σ^{70}, $E\sigma^F$ generates two hypersensitive sites at −37 and −39 of the *fliC* promoter, suggesting that $E\sigma^F$ alters the local promoter structure, but the locations of hypersensitive sites generated by $E\sigma^F$ are different from those generally observed by $E\sigma^{70}$. Chen and Helmann (5) have also reported that both *B. subtilis* σ^D, a homolog of *E. coli* σ^F, and holoenzyme $E\sigma^D$ induce structural alteration in the flagellin gene promoters.

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