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

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The *rpoF* gene of *Escherichia coli* codes for the RNA polymerase $\sigma^F$ (or $\sigma^{28}$) subunit, which is involved in transcription of the flagellar and chemotaxis genes. Both $\sigma^F$ and $\sigma^{28}$ (the major $\sigma$ subunit in growing cells) were overexpressed, purified to homogeneity, and compared with respect to activity and specificity. The affinity of $\sigma^F$ to core RNA polymerase (E) is higher than that of $\sigma^{32}$, as measured by gel filtration high-pressure liquid chromatography. In an in vitro transcription system, the holoenzyme (E$\sigma^F$) containing $\sigma^F$ selectively transcribed the flagellar and chemotaxis genes, all of which could not be transcribed by E$\sigma^{32}$. This strict promoter recognition property of $\sigma^F$ is similar to those of other stress response minor $\sigma$ subunits but different from those of the principal $\sigma$ subunits, $\sigma^{70}$ and $\sigma^{10}$. $\sigma^F$-dependent transcription in vitro is inhibited at high concentrations of all salts tested, showing maximum activity at 50 mM. In contrast, $\sigma^F$-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 footprinting of the flIC and flID promoters indicated that $\sigma^F$ alone is unable to bind DNA, but E$\sigma^F$ specifically recognizes $-10$ and $-35$ regions of the $\sigma^F$-dependent promoters with rather long upstream protection. Alteration of the promoter structure after binding of E$\sigma^F$ was suggested.

The $\sigma$ subunit of bacterial RNA polymerase confers promoter recognition ability to core RNA polymerase with the subunit structure $\alpha_2\beta\beta'$-$\beta$-$\alpha$. Most of the genes expressed in exponentially growing cells of *Escherichia coli* are transcribed by holoenzyme (E$\sigma^{70}$) containing $\sigma^{70}$ (the *rpoD* gene product). On the other hand, the stress response genes are transcribed by RNA polymerase holoenzymes containing minor $\sigma$ subunits ($\sigma^{10}$, $\sigma^{70}$, and $\sigma^{38}$). $\sigma^F$-dependent transcription in vitro is inhibited at high concentrations of all salts tested, showing maximum activity at 50 mM. In contrast, $\sigma^F$-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 footprinting of the flIC and flID promoters indicated that $\sigma^F$ alone is unable to bind DNA, but E$\sigma^F$ specifically recognizes $-10$ and $-35$ regions of the $\sigma^F$-dependent promoters with rather long upstream protection. Alteration of the promoter structure after binding of E$\sigma^F$ was suggested.

The flagellar, chemotaxis, and motility genes in *E. coli* are coordinately expressed (22). Deletion of the *fliA* gene, which was originally identified as a regulatory gene for the ferric citrate transport system, was found to carry the activity and specificity of $\sigma^F$. The overexpression and purified FliA protein exhibited $\sigma$ activity in vitro, using the *tar* gene promoter (21). The *fliA* gene product, which is similar in structure and specificity to *Bacillus subtilis* $\sigma^F$ (8). On the basis of these observations, FliA is now recognized as $\sigma^F$ 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 $\sigma^F$ subunit, however, stays constant under various growth conditions (15), suggesting that the activity of $\sigma^F$ may be controlled in response to the demand for flagellar formation. As an initial attempt to understand the control mechanism of $\sigma^F$ activity, we analyzed factors affecting the activity and specificity of $\sigma^F$ 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 $\sigma^F$, 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 $\sigma^F$-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 $\sigma^F$ and $\sigma^{70}$.** The *E. coli* rpoF gene in pEA20 (a gift from 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(DE3). $\sigma^F$ 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 of 0.01% sodium deoxycholate as in the case of $\sigma^{70}$ purification (12). The overexpressed $\sigma^F$ 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 guanidinium...
hydrochloride. The crude ςF solution was dialyzed against TGED buffer contain-
ing 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 ςF protein identified by polyacrylamide gel electrophoresis (PAGE) on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel were pooled and loaded onto a G5000 gel filtration column. The column was developed with 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM KCl and 6 M urea. ςF fractions were checked by 12% PAGE (12%) gel), pooled, dialyzed against a storage buffer (10 mM Tris-HCl [pH 7.6 at 4°C], 10 mM MgCl2, 0.1 mM EDTA, 0.2 M KCl, 50% glycerol, 1 mM diithiothreitol), and stored at −30°C until use. The ρ0D gene product, ςF, 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 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 mM MgCl2, 0.1 mM EDTA, 0.2 M KCl, 50% by SDS-PAGE (12% gel), pooled, dialyzed against a storage buffer (10 mM Tris-HCl [pH 7.6 at 4°C], 10 mM MgCl2, 0.1 mM EDTA, 0.2 M KCl, 50% glycerol, 1 mM diithiothreitol), and stored at −30°C until use. The ρ0D gene product, ςF, was overexpressed and purified as described by Igarashi and Ishihama (12).

Results

Overexpression and purification of ςF. ςF was overexpressed by using the T7 promoter/polymerase system and purified by using ion-exchange chromatography on a DEAE column. The column was followed by gel filtration in HPLC. The yield of ςF 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 Coomasie brilliant blue staining. Although ςF 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 ςF was electrophoresed together with ςH, ςJ, and ςI† purified essentially by the same procedure (6, 12, 15, 31). The anomalous low mobility is associated with all the σ subunits (ςH, ςJ, ςI, and ςI) examined because of their acidic nature (Fig. 1B).

Promoter-specific transcription by ςF holoenzyme. The promoter selectivity of ςF 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 ςF was able to initiate transcription from the promoters associated with the Salmonella flbD, flc, and flgK genes coding for filament cap protein, flagellin, and hook filament junction protein, respectively (Fig. 2B, flbD, flc, and flgK lanes). Among the three flagellar regulon promoters tested, the flc promoter was the strongest under the reaction conditions used (Fig. 2B). On the other hand, the holoenzyme containing ςF was virtually inactive in transcription of these flagellar genes (Fig. 2B, flbD, flc, 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 ςF (Fig. 2B, lanes 2) but not by ςH (lanes 1) (however, ςF gave transcripts initiated from as yet unidentified promoters on the tsr, tar, and tcp promoter fragments). Some ςH-specific promoters were also tested for comparison. Holoenzyme containing ςF was unable to transcribe the ςH-specific promoters such as alaS and lacUV5. These results indicate that the holoenzyme containing ςF 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 ςH and ςJ but not for those containing the principal σ subunits, ςF and ςI (17, 31, 32).

Affinity of ςF to core RNA polymerase. Affinity of each σ 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 ςH and ςF to core RNA polymerase were compared by measuring two different assays as used for the comparison between ςF and ςI (6, 18): (i) the saturation level of σ factor required for maximum level of
between the overexpressed and renatured required for saturation of the core enzyme is essentially the same suggest that the affinity of (the chemotaxis genes sensing serine and aspartate), and S. typhimurium tcp

Promoters tested are (0.1 pmol each) S. typhimurium fliDC

The requirement of (i) the amount of σ70 subunit required for maximum transcription by 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 σ70 or σF 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 σF at the input molar ratio of about 1, while twofold more σ70, 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 σ70 required for saturation of the core enzyme is essentially the same between the overexpressed and renatured σ70 as used in this and previous experiments (6, 18) and the native σ70 dissociated from purified holoenzyme (data not shown), indicating that the renatured σ70 is as active as the native σ70. These observations suggest that the affinity of σF to core enzyme is about two times higher than that of σ70 under the conditions used.

To rule out the possibility that the fractions of active σ molecules differ between the σF and σ70 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 σ70 and σF. Upon an increase of input σ subunits, keeping the σ70 to σF ratio at a constant level of 1, the amount of core-bound σ was always higher for σF (Fig. 3B). We concluded from these results that the core binding activity is about twofold higher for σF than σ70.

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 σ70-specific (alaS) and two σF-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 σ70 subunit for the maximum transcription (Fig. 4A). The requirement of σ70 subunit for the maximum transcription was virtually independent of the promoters used (18). Interestingly, the amount of σF 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 σ70 (A) and σF (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 σ70 for transcription of σ70-dependent promoters. The molar ratios of σF 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 σ70 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 σF than for σ70. 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 σ70- and σF-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 σF-dependent transcription of the flagellin gene (fliC). Since the level of σF 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 σF, we examined effects of changing the reaction conditions for in vitro transcription.

First, we analyzed effects of the species and concentrations of salt on σF-dependent transcription in vitro, using the fliC and fliD promoters. For comparison, the salt effect was also analyzed for σ70-dependent transcription of the aalS promoter. Our standard reaction buffer contains NaCl at 50 mM, which is the optimum concentration for transcription from σ70-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 aalS promoter by ErF70 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 σF-dependent fliD and fliC promoters was essentially the same as that of σ70-dependent aalS transcription (Fig. 5B and C). The effects of potassium acetate and potassium glutamate on σF-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 σF-dependent test promoters and thereafter decreased to 85 to 95% at 400 mM. Stimulation by potassium glutamate was observed previously for σ70-dependent single-round transcription (6). Possible links between the stimulation in vitro of ErF70 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 σF function at high temperatures. Our preliminary studies indicated that the optimum temperature of transcription by ErF70 holoenzyme was 37°C for both aalS and lacUV5 promoters, whereas the maximum level of σF-dependent transcription was observed at 30°C for both fliC and fliD promoters (data not shown).

Identification of the DNA binding regions by ErF70. Results of the holoenzyme formation and in vitro transcription assays clearly demonstrated that ErF70 carries a very strict promoter selectivity. To determine the promoter sequence recognition properties of ErF70, we performed DNase I footprinting experiments of the fliC and fliD promoters by either σF alone or ErF70 holoenzyme. Under the experimental conditions used, σF 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 σF showed clear footprints on both the fliC and fliD promoters (Fig. 6). Some unique features were found for the footprint by ErF70 holoenzyme. The background is less than that by other holoenzymes, and the upstream protection is longer than that by ErF70 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.
DISCUSSION

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

One of the most interesting findings of this investigation is the higher affinity of \( \sigma^F \) than of \( \sigma^{70} \) to the core RNA polymerase. The number of genes under the control of \( \sigma^{70} \) is not less than 1,000 (9, 14), whereas only 18 to 20 genes are transcribed by \( \sigma^F \) (22). However, the intracellular concentration of \( \sigma^F \) in both exponentially growing and stationary-phase cells is almost half the level of \( \sigma^{70} \) (15). Consequently, the question arises as to why \( E. coli \) cells need such a high level of \( \sigma^F \), even though it has a higher affinity to the core enzyme than \( \sigma^{70} \), it recognizes only a unique promoter sequence associated with the \( \sigma^F \)-dependent genes, and it is used for transcription of only 18 to 20 genes. One possible explanation is that \( \sigma^F \) is stored in an inactive form by forming complexes with the anti-\( \sigma \) factor, FlgM protein (19). The level of \( \sigma^F \)-anti-\( \sigma \) complexes may vary in response to the environmental conditions. The activity of \( \sigma^F \) 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 \( \sigma^F \) 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 \( \sigma^F \) molecules and the number the genes under the control of this \( \sigma \) subunit is that \( \sigma^F \) 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 \( \sigma^F \)-dependent transcription is activated at up to 300 mM potassium acetate and potassium glutamate. The response of \( \sigma^F \) to KCl, potassium acetate, and potassium glutamate is essentially the same as that of \( \sigma^B \), the stationary-phase-specific \( \sigma \) factor (6). The activation of \( \sigma^F \) 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 \( \sigma^B \), however, the activation by the transient increase in potassium acetate or potassium glutamate concentrations is antagonized by the induction of synthesis of FlgM (anti-\( \sigma^F \) factor), as measured by immunoblot analysis (17a).

The \( B. subtilis \) \( \sigma^D \) subunit, a homolog of \( E. coli \) \( \sigma^F \), 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). \( S. typhimurium \) \( \sigma^F \) alone has also been reported to bind to DNA (7). In both cases, however, the binding affinity of \( \sigma \) alone seems to be very weak. Under our gel shift assay conditions, the DNA binding activity was not detected for \( E. coli \) \( \sigma^F \). In DNase I footprinting experiments, the holoenzyme containing \( \sigma^F \) protected the promoter region of both \( fliC \) and \( fliD \), but the region protected seems to be longer than that protected by \( \sigma^{70} \). The wide-range protection by \( \sigma^F \) is, however, observed for a group of promoters carrying the UP (upstream) element which is recognized and protected by \( \alpha \) subunit (29). The clear background in the protected region by \( E\sigma^F \) holoenzyme may suggest a difference in the mode of \( \sigma \)-promoter contact between \( \sigma^F \) and \( \sigma^{70} \). As in the case of \( \sigma^{70} \), \( E\sigma^F \) 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 \( B. subtilis \) \( \sigma^D \), a homolog of \( E. coli \) \( \sigma^F \), and holoenzyme \( E\sigma^F \) induce structural alteration in the flagellin gene promoters.

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