JOURNAL OF BACTERIOLOGY, Feb. 2000, p. 1181–1184 Vol. 182, No. 4
0021-9193/00/$04.00+0

Two Extracytoplasmic Function Sigma Subunits, σ^E and σ^{FecI}, of *Escherichia coli*: Promoter Selectivity and Intracellular Levels

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Received 30 August 1999/Accepted 17 November 1999

The promoter selectivity of two extracytoplasmic function (ECF) subfamily σ subunits, σ^E (σ^{24}) and σ^{FecI} (σ^{18}), of *Escherichia coli* RNA polymerase was analyzed by using an in vitro transcription system and various promoters. The $E\sigma^E$ holoenzyme recognized only the known cognate promoters, *rpoE*P2, *rpo*HP3, and *degP*, and the $E\sigma^{FecI}$ recognized only one known cognate promoter, *fecA*. The strict promoter recognition properties of σ^E and σ^{FecI} are similar to those of other minor σ subunits. Transcription by $E\sigma^E$ and $E\sigma^{FecI}$ was enhanced by high concentrations of glutamate, as in the case of other minor σ subunits. The optimum temperature for transcription by $E\sigma^{FecI}$ was low, around 25°C, apparently in agreement with the high rate of iron sequestration by *E. coli* at low temperatures. By quantitative Western blot analysis, the intracellular levels of σ^E and σ^{FecI} in the uninduced steady-state culture of *E. coli* W3110 (type A) were determined to be 0.7 to 2.0 and 0.1 to 0.2 fmol per μg of total proteins (or 3 to 9 and 0.4 to 0.9 molecules per cell), respectively, and less than 1% of the level of the major σ^{70} subunit.

The DNA-dependent RNA polymerase of *Escherichia coli* is composed of the core enzyme with the subunit structure $\alpha_2\beta\beta'$ and one of seven molecular species of the σ subunit, σ^{70}, σ^N, σ^S, σ^H, σ^F, σ^E, or σ^{FecI} (11, 13). Molecular properties and functional specificity have been studied in detail for all of these σ subunits except for two, σ^E and σ^{FecI}. The σ^E subunit encoded by the *rpoE* gene controls transcription of the genes for extracytoplasmic stress response (3–5, 8, 25, 26, 31). The synthesis of σ^E is induced upon exposure to heat shock or ethanol stress or following accumulation of unfolded proteins in the periplasm (8, 26, 29, 33). The holoenzyme $E\sigma^E$ is responsible for transcription of at least 10 genes (32), of which 4 have been identified, including *rpoH*, which encodes σ^H for transcription of the heat shock response genes (9, 31); *degP*, which encodes a periplasmic protease for degradation of misfolded proteins (14, 23, 31, 34); *fkpA*, which encodes a periplasmic peptidyl-prolyl isomerase (4); and *rpoE* itself (31). On the other hand, the *fecI* gene was originally identified as a regulatory gene for the ferric dicitrate transport system (30), but after sequencing, the FecI protein was recognized as a member of the extracytoplasmic function (ECF) subfamily of σ factor (hereafter referred to as σ^{FecI} in this report) (1, 24). Transcription of the ferric dicitrate transport system of *E. coli* is repressed by Fe^{2+}-Fur and activated by ferric dicitrate (2, 7, 12). Ferric dicitrate does not have to enter into the cytoplasm for transcription activation, but it initiates a signal transduction pathway by binding to the outer membrane receptor FecA (2, 12). The signal is then transmitted through the inner membrane-associated FecR, which ultimately activates the σ^{FecI} subunit. The *fecA* promoter is the only one identified to date that is transcribed specifically by the $E\sigma^{FecI}$ holoenzyme.

Here we performed the first systematic analysis of the promoter of σ^E and σ^{FecI} by using in vitro transcription assay systems. In addition, we determined the intracellular concentrations of these two ECF subfamily σ subunits in *E. coli* W3110 (A) growing at various phases.

Promoter selectivity of the $E\sigma^E$ and $E\sigma^{FecI}$ holoenzymes. For analysis of the promoter selectivity of RNA polymerase holoenzymes containing σ^E or σ^{FecI}, two ECF family σ subunits were overexpressed in *E. coli* M15 by using plasmid pRPOE (14) or *E. coli* BL21(DE3) by using plasmid pETFecI. The plasmid pETFecI for the expression of σ^{FecI} protein with a hexahistidine tag (His_6) at the C terminus was constructed by insertion of PCR-amplified FecI-coding sequence into pET-21b (Novagen) between the *Nde*I and *Hin*dIII sites. Both σ^E and σ^{FecI} were extracted from the inclusion bodies with an extraction buffer containing 0.5% Triton X-100 and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography. For reconstitution of the holoenzymes, we purified the σ-free core enzyme by chromatography of the purified RNA polymerase of *E. coli* W3350 (10) at least three times through phosphocellulose columns (6, 22). The repeated chromatography is essential for complete removal of traces of minor σ subunits. To detect the activity in vitro of purified σ^E, we used truncated DNA templates, each containing one of the three known promoters, i.e., the 210-bp *Eco*RI-*Sph*I *rpoE* promoter fragment, the 220-bp *Eco*RI-*Sph*I *rpoH* fragment, or the 214-bp *Eco*RI-*Sph*I *degP* fragment, each producing specific transcripts of 71 (*rpoE*), 81 (*rpoH*), and 74 (*degP*) nucleotides in length, respectively. For detection of the σ^{FecI} activity, we used the only known FecI-dependent promoter, *fecA*, which produces RNA of 62 (*fecA*) nucleotides in length.

Under the standard transcription assay conditions for the $E\sigma^{70}$ holoenzyme (E represents the core enzyme) (20), the reconstituted holoenzymes containing σ^E and σ^{FecI} produced specific transcripts directed by the respective cognate promoters (Fig. 1). None of the other holoenzymes containing σ^{70}, σ^N, σ^S, σ^H, or σ^F, however, produced significant levels of transcripts from the σ^E-dependent *rpoE*, *rpoH*, and *degP* promoters or from the σ^{FecI}-dependent *fecA* promoter, even though all of these holoenzymes gave similar levels of the template-sized end-to-end transcripts, which migrated near the top of the gels (Fig. 1). On the other hand, both $E\sigma^E$ and $E\sigma^{FecI}$ holoenzymes were unable to transcribe the σ^{70}-dependent *lacUV5* (Fig. 1), *trp*, and *rpsA* promoters. Thus, we concluded that the ECF

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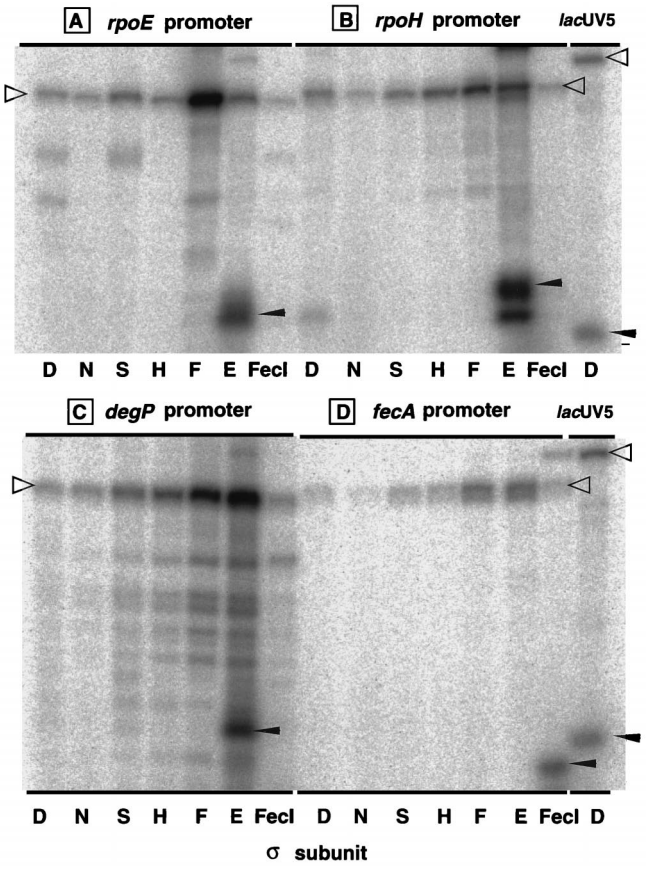


FIG. 1. Transcription in vitro of truncated DNA templates by RNA polymerase holoenzymes $E\sigma^E$ and $E\sigma^{FecI}$. RNA polymerase holoenzymes were reconstituted by mixing the core enzyme with each σ subunit in a core-to-σ molar ratio of 1:4. Single-round transcription was carried out under the standard assay conditions (20) with 1 pmol each of seven different holoenzymes containing σ^{70}, σ^N, σ^S, σ^H, σ^H, σ^F, σ^H, and σ^{FecI} (the species of σ subunit used is shown at the bottom of each gel lane) and 0.1 pmol each of three σ^E-dependent (*rpoE* [A], *rpoH* [B], and *degP* [C]) and one σ^{FecI}-dependent (*fecA* [D]) promoter. RNA products were separated by 8% PAGE in the presence of 8 M urea, and gels were analyzed with a Bio-Imaging Analyzer BAS-2000 (Fuji). Arrowheads indicate the specific transcripts from the test promoters, while open triangles indicate the template-sized nonspecific transcripts.

family σ subunits carry high selectivity for a specific set of the cognate promoters, as in the case of other minor σ subunits, σ^N, σ^H, and σ^F. In contrast to the strict promoter selectivity characteristic of the minor σ subunits, the σ^{70} subunit recognizes in vitro most σ^S-dependent promoters, and the σ^S subunit recognizes some σ^{70}-dependent promoters (21, 35).

Stimulation of σ^E- and σ^{FecI}-dependent transcription by potassium glutamate. The reaction conditions such as DNA superhelicity, the species and concentrations of salts, trehalose, and polyphosphate, and the reaction temperature affect in vitro transcription in different ways for the different holoenzymes containing different σ subunits, presumably reflecting the difference in physiological conditions under which each σ subunit works (reviewed in references 15 and 16).

The standard reaction mixture to give maximum-level transcription of *lacUV5* by the $E\sigma^{70}$ holoenzyme contains 50 mM NaCl (Fig. 2C) (see also reference 20). The optimum concentrations of NaCl to give maximum transcription activity on *trp*, *recA*, and *rpsA* templates were between 50 and 100 nM (data not shown). The activity of $E\sigma^{70}$ holoenzyme is, however, negligible at NaCl concentrations above 200 mM (Fig. 2C) (see also references 8 and 28). In contrast, transcription of *rpoE* by $E\sigma^E$ (Fig. 2A) and of *fecA* by $E\sigma^{FecI}$ (Fig. 2B) stays almost at the same level, between 50 and 300 mM NaCl, indicating that transcription by these two holoenzymes is relatively resistant to inhibition by high NaCl concentrations. Previously, we found that high concentrations of glutamate enhance transcription by the $E\sigma^S$ and $E\sigma^F$ holoenzymes (6, 22). Here we also examined the effect of increasing concentrations of potassium glutamate. As shown in Fig. 2A and B, transcription by both $E\sigma^E$ and $E\sigma^{FecI}$ holoenzymes was significantly enhanced upon increasing the potassium glutamate concentration up to at least 400 mM. The molecular mechanism underlying the activation of minor σ-dependent transcription by high concentrations of glutamate remains to be solved.

Preference for low temperatures of σ^{FecI}-dependent transcription. The optimum temperature for maximum-level transcription by the regular holoenzyme $E\sigma^{70}$ is about 37°C, whereas the optimum temperature of transcription of certain promoters by $E\sigma^H$ and $E\sigma^F$ significantly deviates from this optimum temperature for the $E\sigma^{70}$ holoenzyme (22, 36). We then examined the effect of reaction temperature on *rpoE* promoter-directed transcription by $E\sigma^E$ and *fecA* promoter-

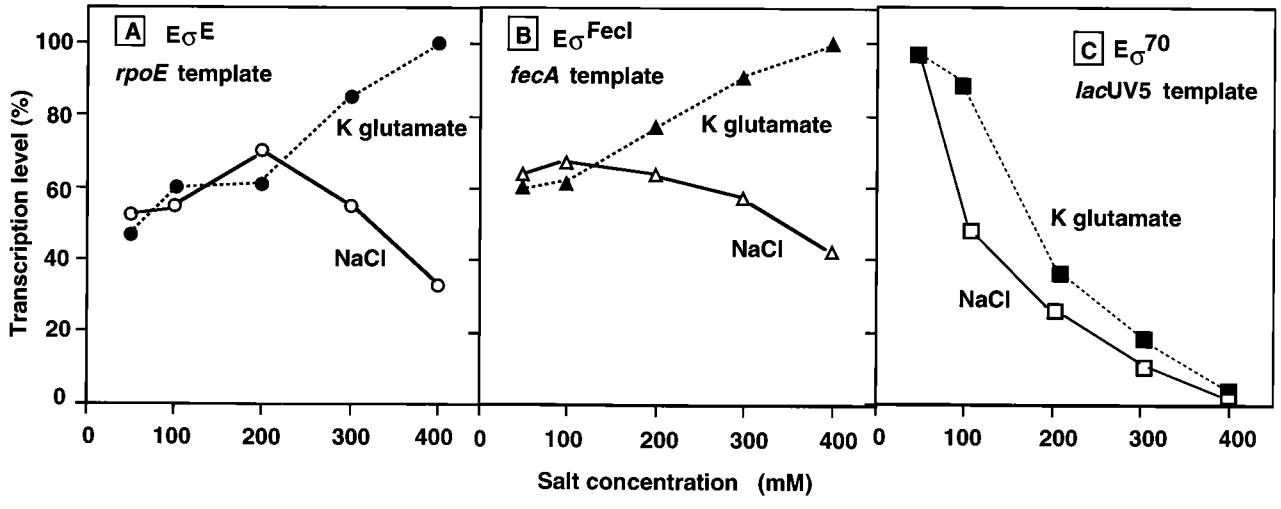


FIG. 2. Effects of salt concentrations on in vitro transcription by RNA polymerase $E\sigma^H$ and $E\sigma^{FecI}$ holoenzymes. Single-round transcription was carried out by using 0.1 pmol of each of three test promoters, *rpoE* (A), *fecA* (B), and *lac*UV5 (C), and 1 pmol each of three different forms of the reconstituted holoenzyme, $E\sigma^E$ (A), $E\sigma^{FecI}$ (B), and $E\sigma^{70}$ (C), under the standard reaction conditions except that 50 mM NaCl was replaced by the indicated concentrations of either NaCl or K glutamate. Transcripts were fractionated by 8% PAGE, and gels were examined with a Bio-Imaging Analyzer BAS2000 (Fuji). The maximum levels of transcription observed with each template are set at 100%.

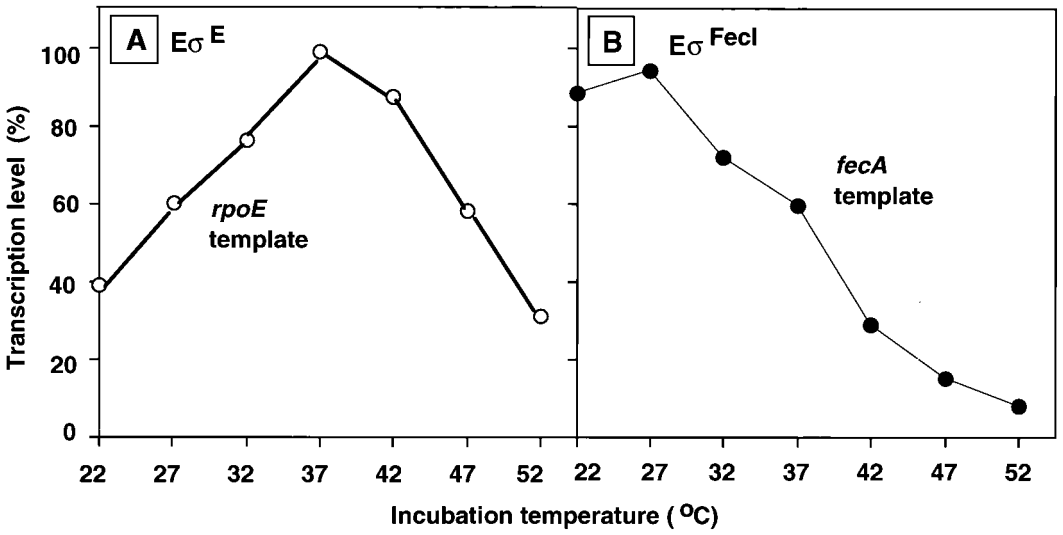
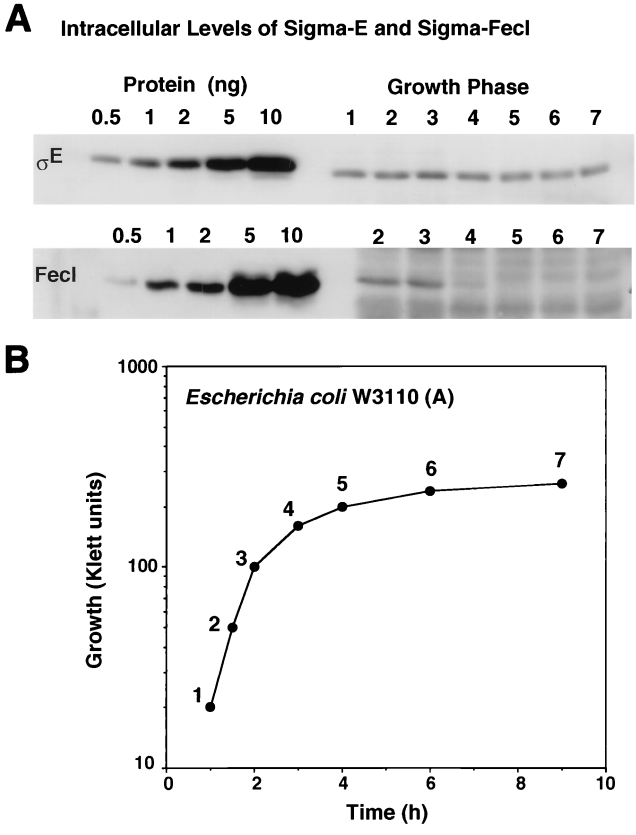


FIG. 3. Effect of temperature on in vitro transcription by RNA polymerase $E\sigma^E$ and $E\sigma^{FecI}$ holoenzymes. Single-round transcription was carried out with 0.1 pmol each of two test promoters, *rpoE* (A) and *fecA* (B), and 1 pmol each of either $E\sigma^E$ (A) or $E\sigma^{FecI}$ (B) holoenzyme under the standard reaction conditions in the presence of 400 mM K glutamate. Both preincubation for RNA polymerase-promoter complex formation and incubation for transcription were carried out at the various temperatures indicated. The maximum levels of transcription observed with each template are set at 100%.

directed transcription by $E\sigma^{FecI}$. As shown in Fig. 3A, the optimum temperature for maximum transcription by $E\sigma^E$ was 37°C, but about half the maximum-level activity was retained above 50°C, indicating that transcription by $E\sigma^E$ predominates at high temperatures, in good agreement with the expected role of σ^E in response to extremely high temperatures (8).



In contrast, *fecA* promoter-directed transcription by $E\sigma^{FecI}$ was at the maximum at around 25°C and linearly decreased thereafter up to 52°C, where the activity was almost negligible (Fig. 3B). Among seven species of the holoenzyme examined under the same conditions, the $E\sigma^{FecI}$ species required the lowest temperature to give the maximum activity of transcription. The acquisition of iron by bacteria is known to be more efficient at low temperatures (37). The in vivo activation of σ^{FecI}-dependent transcription at low temperatures awaits further analysis.

Intracellular levels of the σ^E and σ^{FecI} proteins. Previously, we determined the intracellular concentrations of σ^{70}, σ^N, σ^S, σ^H, and σ^F in *E. coli* W3110 (type A lineage) by the quantitative Western blot method (17, 19). Here we determined the concentration of two ECF family subunits, σ^E and σ^{FecI}, in the same cell extracts of *E. coli* W3110 (A) used in our previous determination. In the exponential growth phase, the concentrations of σ^E and σ^{FecI} were 0.7 to 2.0 and 0.1 to 0.2 fmol per μg of total proteins, respectively (Fig. 4). In the same extract, the concentration of the major σ subunit, σ^{70}, is 150 to 170 fmol/μg of total proteins (17). The number of σ^{70} molecules per cell is estimated to be around 700 (19). The numbers of σ^E

FIG. 4. Intracellular concentrations of σ^E and σ^{FecI} in *E. coli* W3110 (type A). *E. coli* W3110 (type A) was grown with shaking in Luria-Bertani medium at 37°C. Cell extract was prepared by the method of Jishage et al. (17, 19). The protein concentration of cell lysates was determined by using the Bio-Rad protein assay kit. Polyclonal antibodies against σ^E and σ^{FecI} were raised in rabbits by injecting the overexpressed and purified σ proteins. The quantitative Western blot analysis was employed for the measurement of σ subunits exactly as described in previous reports (17, 19). The immunostained blots were developed with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo). Staining intensity was measured with a PDI image analyzer system equipped with a white light scanner. (A) Aliquots containing 10 μg of total proteins from cell lysates of *E. coli* W3110 (A) prepared at various times of the cell culture (see B for the growth curve) were subjected to quantitative Western blot analysis using anti-σ^E and anti-σ^{FecI} antibodies. (B) *E. coli* W3100 (type A) was grown in Luria-Bertani medium at 37°C under the same conditions employed in the determination of other σ subunits (17, 19), and growth was monitored by measuring the turbidity with a Klett-Summerson photometer. At the indicated time points labeled 1 to 7, aliquots were taken for preparation of the cell lysates.

and σ^{FecI} molecules per cell can then be calculated to be 3 to 9 and 0.4 to 0.9, respectively. The level of σ^E stayed constant throughout the growth phase examined, but the σ^{FecI} level further decreased in the stationary phase (Fig. 4). The critical factors leading to induction of the synthesis or activation of the ECF family σ subunits, however, remain unclear. Taken together with the previous determinations (17, 19), we conclude that under the steady state of cell growth, the uninduced levels of the minor subunits σ^S, σ^H, σ^E, and σ^{FecI} are all lower than 1% of the level of the major σ^{70} subunit.

In addition to the synthesis control, the activity is negatively regulated, at least in the case of σ^E subunit, by a membrane-bound anti-σ factor, RseA (5, 27). Such an activity control of the σ subunit has been found for both σ^F (28) and σ^{70} (18). However, the factor affecting the σ^{FecI} activity has not yet been identified.

We thank S. Kusano and T. S. Kundu for preparation of σ^S, σ^H, and σ^F and A. Iwata and S. Ueda for preparation of anti-σ^E and anti-σ^{FecI} antibodies.

This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and from CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation.

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