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Competition among seven *Escherichia coli* σ subunits: relative binding affinities to the core RNA polymerase

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ABSTRACT

Seven different species of the RNA polymerase σ subunit exist in *Escherichia coli*, each binding to a single species of the core enzyme and thereby directing transcription of a specific set of genes. To test the σ competition model in the global regulation of gene transcription, all seven *E.coli* σ subunits have been purified and compared for their binding affinities to the same core RNA polymerase (E). In the presence of a fixed amount of σ^{70}, the principal σ for growth-related genes, the level of $E\sigma^{70}$ holoenzyme formation increased linearly with the increase in core enzyme level, giving an apparent K_d for the core enzyme of 0.26 nM. Mixed reconstitution experiments in the presence of a fixed amount of core enzyme and increasing amounts of an equimolar mixture of all seven σ subunits indicated that σ^{70} is strongest in terms of core enzyme binding, followed by σ^N, σ^F, σ^E/σ^{FecI}, σ^H and σ^S in decreasing order. The orders of core binding affinity between σ^{70} and σ^N and between σ^{70} and σ^H were confirmed by measuring the replacement of one core-associated σ by another σ subunit. Taken together with the intracellular σ levels, we tried to estimate the number of each holoenzyme form in growing *E.coli* cells.

INTRODUCTION

The bacterial RNA polymerase is composed of the core enzyme (subunit composition $\alpha_2\beta\beta'$) with RNA polymerization catalytic activity and one of multiple species of the σ subunit for promoter recognition (1–6). In *Escherichia coli*, seven different species of the σ subunit, σ^{70}, σ^N (also called σ^{54}), σ^S (σ^{38}), σ^H (σ^{32}), σ^F (σ^{28}), σ^E (σ^{24}) and σ^{FecI}, are known to exist, each directing transcription of a specific set of genes. Most of the growth-related and housekeeping genes expressed in the exponential phase of cell growth are transcribed by the holoenzyme containing σ^{70} (the *rpoD* gene product), while the holoenzyme $E\sigma^S$ is essential for transcription of some stationary phase-specific genes (7,8). The stress response genes are transcribed by RNA polymerase holoenzymes containing the alternative minor σ subunits. The holoenzyme $E\sigma^N$ transcribes genes which are regulated by the availability of nitrogen (9) and some stress response genes (10); the holoenzyme $E\sigma^H$ transcribes the genes for heat shock proteins (4,10); $E\sigma^F$ is needed for expression of the flagella and chemotaxis genes (11); the holoenzyme $E\sigma^E$ is responsible for transcription of the genes for extracytoplasmic functions as well as the heat shock response (12–14); the *fecI* gene product, which was originally identified as a regulatory gene for the ferric citrate transport system (15), is now known as a new member of the extracytoplasmic function (ECF) subfamily of σ factors on the basis of protein sequence (hereafter referred to as σ^{FecI}) and is involved in transcriptional regulation of the genes for extracytoplasmic functions (16–18). We have purified all seven species of the *E.coli* σ subunit and analyzed their recognition specificity for various *E.coli* promoters (18–20). Using specific antibodies raised against the purified σ proteins, we also measured the intracellular concentrations of all seven σ subunits for both exponential and stationary phase cultures of *E.coli* W3110 (A) (20–22).

The global pattern of gene transcription is believed to be determined through competition between available σ subunits (23–25) and, if this is the case, replacement of one core enzyme-associated σ subunit by another should be the major determinant in switching of the global transcription pattern (reviewed in 26,27). In fact, the change in global transcription pattern during the growth phase transition from exponential to stationary phase (21,22) or upon sudden exposure to heat shock (28,29) is accompanied by a change in intracellular levels of the σ subunits. At present, however, it remains unsolved whether changes in σ concentrations alone can explain the change in transcription pattern. To gain an insight into the mechanism of σ switching we have performed a qualitative comparison of the binding affinities of all seven *E.coli* σ subunits for the same core enzyme. On the basis of this first systematic comparison of the binding affinity of each σ subunit for the core enzyme, together with determination of the intracellular concentrations of each σ subunit (20–22), an attempt was made to estimate the total number of each holoenzyme form in *E.coli*. On the basis of these observations, the σ competition model is evaluated.

MATERIALS AND METHODS

Overexpression and purification of σ subunits

Overexpression of σ^{70}, σ^N, σ^S, σ^F, σ^H, σ^E and σ^{FecI} was performed using the expression plasmids pGEMD (30), pKES259 (31),

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pETF (18), pETSF (19), pET21H (S.Kusano and A.Ishihama, unpublished results), pRPOE (32) and pETFecI (20), respectively. *Escherichia coli* strain BL21(DE3) was transformed with the respective σ expression plasmid and grown in Luria–Bertani (LB) medium containing ampicillin (0.2 mg/ml). After induction with isopropyl β-D-thiogalactopyranoside (IPTG), cells were harvested, washed with 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 150 mM NaCl and stored at –80°C until use. Cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.1 mM NaCl) and treated with phenylmethylsulphonyl floride (PMSF), lysozyme and sodium deoxycholate. The lysate was homogenized by gentle sonication and insoluble materials were recovered by centrifugation. All seven σ proteins were extracted from the inclusion bodies with elution buffer (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM DTT and 5% glycerol) containing 0.5% Triton X-100 or 0.5 M KCl. Purification of the solubilized σ subunits was performed without using protein denaturants essentially as described previously (30).

The protein concentration was determined with the Bradford protein assay kit (Bio-Rad) using bovine serum albumin as a standard. The relative content of each σ subunit in purified σ preparations was estimated by measuring the staining intensity with Coomassie brilliant blue (CBB) of the σ band after separation of contaminating proteins by SDS–PAGE. The purified σ subunits were stored frozen at –80°C in storage buffer [50 mM Tris–HCl (pH 7.6 at 4°C), 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.2 M KCl and 50% (v/v) glycerol] until use.

RNA polymerase core enzyme

RNA polymerase was purified from *E.coli* W3350 and the core enzyme was purified by passing the purified RNA polymerase three times through a phosphocellulose column. Repetition of the phosphocellulose column chromatography at least three times was needed to completely remove minor σ subunits from the core enzyme (33,34). The level of remaining σ subunits was double checked by testing for *in vitro* transcription activity directed by specific promoters and by immunostaining with antibodies raised against each σ subunit. The purified core enzyme was stored frozen at –80°C in storage buffer [50 mM Tris–HCl (pH 7.6 at 4°C), 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.2 M KCl and 50% (v/v) glycerol] until use.

Reconstitution of holoenzymes

The core enzyme and a single species or combinations of various σ subunits were mixed in 50 µl of reconstitution buffer [10 mM Tris–HCl (pH 7.6 at 4°C), 0.1 mM DTT, EDTA, 0.1 mM, 200 mM NaCl and 5% glycerol] and incubated for 10 min at 30°C. Aliquots of 40 µl were subjected to gel filtration through a Superdex 200 column PC3.2/300 (bed volume 0.9 ml) with a SMART system (Pharmacia, Sweden). Elution with reconstitution buffer was performed at a flow rate of 40 µl/min at 20°C. Both holoenzyme and core enzyme were recovered in the void volume (elution time 22–23 min). Aliquots of each elution fraction were analyzed by SDS–PAGE. Gels were stained with a SYPRO Orange staining kit (Molecular Probes, USA). The protein concentration was determined by scanning the gels with a FluorImager (Vistra, USA). The concentrations of RNA polymerase proteins determined with the SYPRO Orange staining kit paralleled those stained with CBB, although the sensitivity of detection is much higher for SYPRO staining. The level of holoenzyme formation was determined by measuring the ratio between the α (the representative subunit of the core enzyme) and σ subunits in the RNA polymerase peak (holoenzyme plus core enzyme).

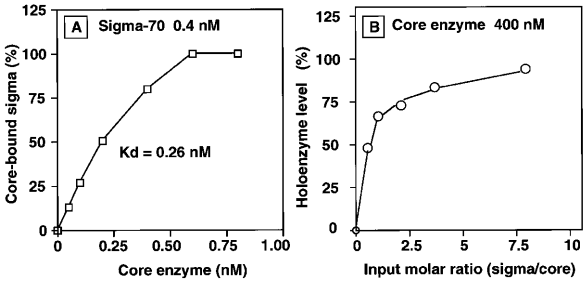

Figure 1. Determination of the affinity between the σ^{70} subunit and the core enzyme. (**A**) Core enzyme saturation. A fixed amount (0.4 nM) of the σ^{70} subunit and increasing amounts of the core enzyme were mixed and the holoenzyme ($E\sigma^{70}$) formed was isolated by Superdex-200 chromatography using a SMART system. The amount of core enzyme-bound σ^{70} was measured by SDS–PAGE followed by staining with SYPRO Orange. The K_d was estimated using Sigma Plot Software. (**B**) σ^{70} saturation. A fixed amount (20 pmol/50 µl or 400 nM) of the core enzyme was mixed with increasing amounts of the σ^{70} subunit and the amount of $E\sigma^{70}$ holoenzyme was measured as in (A).

RESULTS

Dissociation constant of $E\sigma^{70}$ holoenzyme formation

The RNA polymerase holoenzyme can be formed *in vitro* by mixing purified core enzyme and σ subunit. For comparison of the binding affinities of different σ subunits for the core enzyme, we purified the σ-free core enzyme by passing the purified RNA polymerase three times through phosphocellulose columns (33,34). All seven σ subunits were purified from *E.coli* cells, which were induced for transient expression of each σ subunit, without using protein denaturants as in the case of σ^{70} purification (30). The formation of holoenzyme was measured by mixing the purified core enzyme and the purified σ subunit(s) under various conditions. The amount of core enzyme-bound σ subunit(s) was determined by measuring the molar ratio of σ to α subunits in the RNA polymerase peak (holoenzyme plus core enzyme) after gel filtration column chromatography.

First we determined the equilibrium dissociation constant of $E\sigma^{70}$ holoenzyme formation (E represents the core enzyme). A constant amount (0.02 pmol/50 µl or 0.4 nM) of the σ^{70} subunit and increasing amounts of the core enzyme were mixed at 0°C and, after 10 min incubation at 30°C to establish equilibrium, the reconstituted $E\sigma^{70}$ holoenzyme was quickly separated, within 22–23 min, from unbound σ^{70} by gel filtration through a Superdex 200 column using a SMART system (Pharmacia, Sweden). Figure 1A shows the level of core-bound σ^{70} as determined by measuring the molar ratio between the σ^{70} and α subunits in the RNA polymerase peak. In the presence of excess amounts of the core enzyme virtually all the purified σ^{70} subunits formed the $E\sigma^{70}$ holoenzyme (see below), indicating

that the σ^{70} preparation used was fully active in binding to the core enzyme. Since dissociation of preformed $E\sigma^{70}$ is virtually negligible within this time range, the apparent dissociation constant (K_d) of σ^{70} from the core enzyme was estimated to be ~0.26 nM (Fig. 1A). To confirm the high level activity of σ^{70} in core enzyme binding, we performed holoenzyme reconstitution with increasing amounts of the σ^{70} subunit in the presence of an excess (20 pmol in 50 µl or 400 nM) of the core enzyme. The input σ^{70} was quantitatively bound to the core enzyme up to 10 pmol and almost 75% of the input core enzyme was converted to the holoenzyme ($E\sigma^{70}$) by adding 20 pmol of σ^{70} (an input molar ratio of 1) (Fig. 1B). On adding a 10-fold molar excess of the σ^{70} subunit all the input core enzyme was converted to the holoenzyme form. The amount of σ^{70} required for saturation of the core enzyme was slightly more than that expected from the K_d value, presumably because some of σ^{70} and/or core enzyme formed aggregates under such high protein concentrations.

The finding that almost all the input σ^{70} subunit was reconstituted to holoenzyme by adding excess amounts of the core enzyme (see Fig. 1A) indicates that virtually all the σ molecules in the σ^{70} preparation used are active, at least in binding to the core enzyme. The holoenzyme formed from the σ^{70} molecules was, however, not fully active in transcription as measured using a single round *in vitro* transcription assay directed by the *lac*UV5 promoter, implying that some of the inactive σ^{70} subunit with respect to overall transcription is still active in binding to the core enzyme. Thus the dissociation constant (K_d) of $E\sigma^{70}$ formation should be <0.26 nM.

Competition in core enzyme binding among seven σ subunits

For comparison of the core enzyme binding affinities among seven *E.coli* σ subunits we purified all other six σ subunits from overexpressed *E.coli* cells to apparent homogeneity (Fig. 2A). To recover all seven σ subunits in active forms, care was taken to purify the σ subunits by the same procedure from overexpressed cell extracts, i.e. extraction of the σ proteins from the pellet fraction of overexpressed cell extracts using a solubilization buffer containing a non-ionic detergent, without using ionic detergents. The concentration of each σ subunit was determined after correction for purity (90–98%), as estimated by SDS–PAGE followed by CBB staining. Since all these purified σ subunits were completely converted into the respective holoenzymes by adding excess amounts of the core enzyme (data not shown), the σ preparations used were fully active, at least in binding to the core enzyme.

Using the purified σ subunits, we first analyzed the saturation curve of each σ subunit to convert a fixed amount (20 pmol) of the core enzyme to the respective holoenzyme. At an input molar ratio of 1 more than 80% of the input core enzyme was converted to the holoenzyme for five σ subunits, σ^{70}, σ^{N}, σ^{F}, σ^{E} and σ^{FecI}, and 60–65% of the input core was converted to the holoenzyme for σ^{S} and σ^{H} (data not shown). For σ saturation of the input core enzyme, higher protein concentrations were required for both σ^{S} and σ^{H}. The input core enzyme was saturated with addition of a 2-fold molar excess of σ even for σ^{S} and σ^{H}, with weak binding affinities. A similar order of σ activity was obtained when the level of functional holoenzyme was determined by measuring the σ saturation curve using *in vitro* transcription assays directed by specific promoters for each σ subunit (data not shown). In the presence of single σ additions,

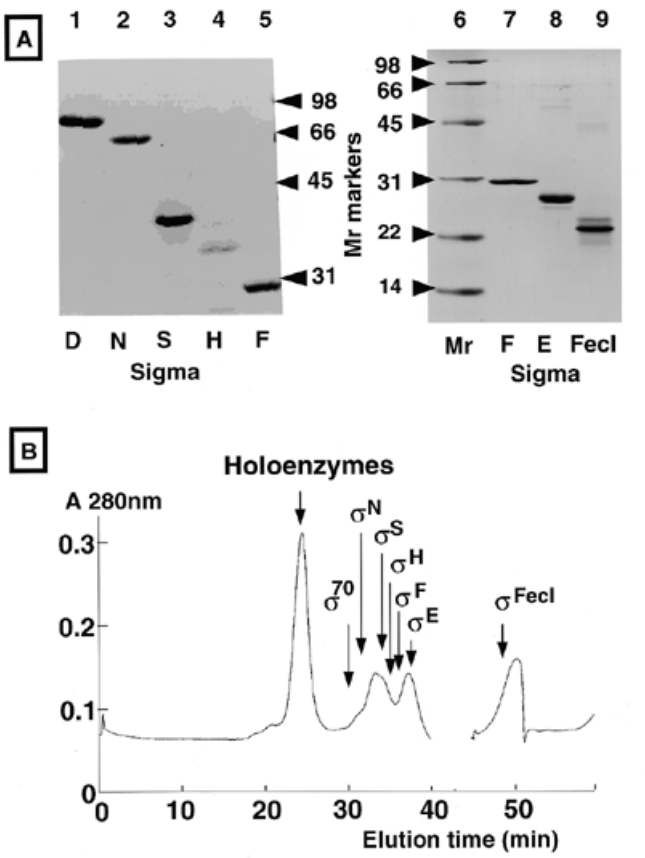

Figure 2. Purification of various σ subunits and fractionation of core enzyme-bound and unbound σ subunits. (**A**) SDS–PAGE of purified σ subunits. Seven species of the σ subunit were purified as described in Materials and Methods and analyzed by SDS–PAGE (9% gel for lanes 1–5, 15% gel for lanes 6–9). Gels were stained with Coomassie brilliant blue. M_r markers used were rabbit muscle phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa). (**B**) Mixtures of seven σ subunits and the core enzyme were fractionated by gel filtration through a Superdex 200 column (0.25 × 30 cm) with a SMART system (Pharmacia, Sweden). The elution positions of holoenzymes and unbound σ subunits are indicated by arrows.

however, it was difficult to determine the slight difference in core binding affinity among the seven σ subunits.

In order to measure the relative affinity of core enzyme binding among seven σ subunits we next carried out a mixed reconstitution experiment in the presence of all seven σ subunits in the same reaction mixture. To 20 pmol of the core enzyme were added increasing amounts of an equimolar mixture of all seven σ subunits and the σ subunits were fractionated into core enzyme-bound and unbound fractions by gel filtration column chromatography (Fig. 2B). The core enzyme-associated σ subunits were determined after measuring the molar ratios between the α subunit and each σ subunit in the holoenzyme peak. Almost 68% of the core enzyme was converted into the holoenzymes at an input molar ratio of 0.25 for each σ subunit (or 1.75 for the combined σ subunits) (Fig. 3A) and >90% of the input core enzyme was bound with one of the seven σ subunits

Table 1. Intracellular concentrations of RNA polymerase holoenzymes

σ subunit	K_d (nM)[a]	Core binding affinity	Intracellular concentration[b]		Holoenzyme ratio[c] (%)
			(fmol/mg protein)	(molecules/cell)	
σ^{70} (σ^{D})	0.26	1.0	160	700	78
σ^{54} (σ^{N})	0.30	1.55	25	110	8
σ^{38} (σ^{S})	4.26	16.4	<1	<1	0
σ^{32} (σ^{H})	1.24	4.75	2.1	<10	0
σ^{28} (σ^{F})	0.74	2.85	85	370	14
σ^{24} (σ^{E})	2.43	9.35	1.4	<10	0
σ^{18} (σ^{FecI})	1.73	6.65	<1	<1	0

[a]K_d values of the minor σ subunits were estimated from the relative levels of core enzyme binding in the mixed reconstitution experiments (Fig. 4) and the K_d value (0.26 nM) for σ^{70} (Fig. 1).
[b]Intracellular concentrations of all seven σ subunits in *E.coli* W3110 (A) were described previously (20–22).
[c]The relative level of each holoenzyme was estimated from the K_d value and the intracellular concentration.

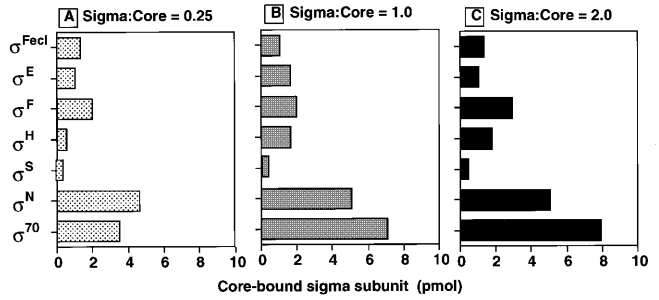

Figure 3. Mixed reconstitution of various holoenzymes. A fixed amount (20 pmol) of the core enzyme was mixed with increasing amounts of an equimolar mixture of seven σ subunits (σ^{70}, σ^{S}, σ^{N}, σ^{F}, σ^{H}, σ^{E} and σ^{FecI}) and, after incubation for 10 min at 30°C, fractionated at 20°C by gel filtration chromatography through a Superdex 200 column (0.25 × 30 cm) using a SMART system as shown in Figure 2B. The molar ratio of combined σ subunits to the core enzyme was 1.75 (**A**), 7.0 (**B**) and 14 (**C**). The amounts of core enzyme-bound σ subunits were determined as described in Figure 1.

at an input molar ratio of 1.0 for each σ subunit (or 7.0 for the combined σ subunits) (Fig. 3B). The input core enzyme was saturated with one of the σ subunits at an input molar ratio of 2.0 for each σ subunit (or 14 for the combined σ subunits) (Fig. 3C). The core enzyme-associated fraction was, however, significantly different among the seven σ subunits. At low σ concentration (or 60–70% saturation level) σ^{N} showed an affinity as high as that of the σ^{70} subunit and the combined $E\sigma^{N}$ and $E\sigma^{70}$ level was as high as 60% of the total holoenzymes. The holoenzyme level was almost the same among $E\sigma^{F}$, $E\sigma^{FecI}$ and $E\sigma^{E}$, each constituting 14, 10 and 8%, respectively, while the amount of $E\sigma^{H}$ was <1% of total holoenzymes. The level of $E\sigma^{S}$ formation was lowest under the reconstitution conditions employed.

The core enzyme-bound fractions increased in parallel for all seven σ subunits concomitantly with the increase in input σ subunits to a fixed amount of the core enzyme (Fig. 3B and C). At saturation the order of core enzyme-associated σ subunits was $\sigma^{70} > \sigma^{N} > \sigma^{F} > \sigma^{H}/\sigma^{FecI} > \sigma^{E} > \sigma^{S}$. Under σ saturation conditions the fraction of $E\sigma^{70}$ holoenzyme was ~39% of the total holoenzymes. We then conclude that under competitive binding conditions in the presence of all seven σ subunits the affinity of σ^{70} is strongest and that of σ^{S} is weakest. Judging from the affinity difference (>16-fold) in core enzyme binding between σ^{70} and σ^{S} and the K_d value of 0.26 nM for σ^{70} (see above), we estimate that the apparent K_d value of σ^{S} for the core enzyme is ~4.3 nM (Table 1).

Switching of one core enzyme-bound σ for another σ subunit

From the binding affinity of each σ subunit determined as above we could predict the efficiency of replacement of one core enzyme-bound σ subunit by another σ subunit. Here we tested two extreme combinations: (i) σ^{70} and σ^{H} which has a 10-fold lower binding affinity for the core enzyme than σ^{70}; and (ii) σ^{70} and σ^{N} which has a core enzyme binding affinity as high as σ^{70}. An equimolar mixture of core enzyme (20 pmol) and one σ subunit (20 pmol) was incubated to equilibrium and then an equimolar amount (20 pmol) of the second σ subunit was added. At various times after addition of the second σ the core enzyme-associated σ subunits were measured. Results of σ^{70}/σ^{H} competition experiments indicated that: there was little replacement of the core enzyme-associated σ^{70} by the added σ^{H} even after 600 min incubation (Fig. 4A); core-bound σ^{H} was rapidly replaced by σ^{70} within 10 min (Fig. 4B). On the other hand, in the competition experiment between σ^{70} and σ^{N} ~30% of the core enzyme-associated σ^{70} was replaced by the added σ^{N} (Fig. 4C) while at least half of the core-bound σ^{N} remained associated even after prolonged incubation for 600 min following addition of σ^{70} (Fig. 4D). All these results are in good agreement with the differences in core enzyme binding affinity between σ^{70} and σ^{H} and between σ^{70} and σ^{N} (Fig. 3; see also Table 1). Moreover, the slight difference in the rate of σ replacement between the two combinations, $E\sigma^{70}/E\sigma^{N}$ (30% in Fig. 4C) and $E\sigma^{N}/E\sigma^{70}$ (50% in Fig. 4D), reflects the slight difference in the core enzyme binding affinity between σ^{70} and σ^{N} (see Fig. 3).

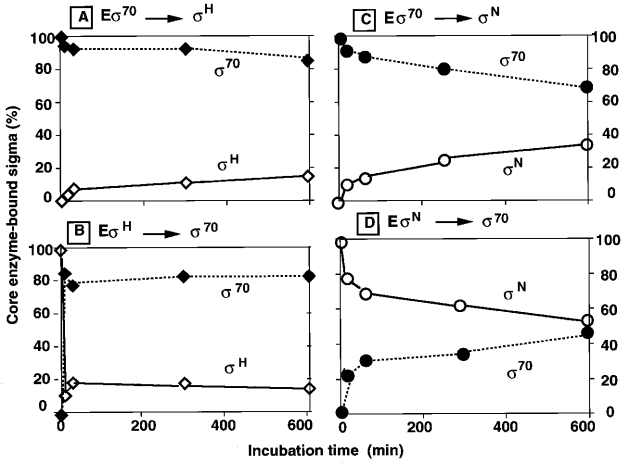

Figure 4. Replacement of the core enzyme-bound σ subunit. An equimolar mixture of the core enzyme (20 pmol) and the first σ subunit (20 pmol) was incubated for 10 min at 30°C and then 20 pmol of the second σ subunit was added. After incubation for various times as indicated, the core enzyme-bound σ subunits were measured as in Figure 1. The combined amount of core enzyme-bound σ subunits was the same as the core enzyme within the sensitivity range of protein determination. The first and second σ subunits were (**A**) σ^{70} and σ^{H}, (**B**) σ^{H} and σ^{70}, (**C**) σ^{70} and σ^{N} and (**D**) σ^{N} and σ^{70}.

DISCUSSION

Difference in the core enzyme binding affinity among seven *E.coli* σ subunits

The comparison of core binding affinity among seven σ subunits from *E.coli* indicates that the σ^{70} subunit for transcription of growth-related and housekeeping genes has the highest affinity for the core enzyme. The affinity difference was 16-fold between σ^{70} (0.26 nM) and σ^{S}, with the weakest binding activity (4.28 nM) (see Table 1). This estimation relied on the observation that the content of functional σ molecules with respect to core enzyme binding was the same between the seven σ subunit preparations. Some of the σ molecules with core enzyme binding activity are, however, inactive in carrying out the complete cycle of transcription. In the case of the σ^{70} subunit, for instance, the content of transcriptionally competent molecules was ~50% as measured by the synthesis level of template sized *lac*UV5 RNA in a single round transcription assay. This value of fully functional σ^{70} subunit represents the minimum, because: (i) a certain fraction of initiation complexes with $E\sigma^{70}$ become abortive prior to promoter escape (the level of abortive initiation depends on the nature of the promoter) (see for instance 35); (ii) some transcriptionally inactive $E\sigma^{70}$, as determined using the *lac*UV5 promoter-directed transcription assay, could be active with promoters other than *lac*UV5. The precise estimation of functional molecules is more difficult for other σ subunits because available promoters recognized by these σ subunits are limited and because we do not know the ratio of productive versus abortive initiation of *in vitro* transcription catalyzed by each holoenzyme. However, we took maximum care to obtain the other six σ subunits in as active a form as the σ^{70} subunit, by employing the same procedures for protein expression and purification as used for the σ^{70} subunit. As a result, all the σ preparations used were fully active in binding to the core enzyme.

The purity of the seven σ subunits ranged from 90 to 98%, as detected by SYPRO Orange staining, which is as sensitive as silver staining. Contamination of σ preparations by the anti-σ factors Rsd (36), FlgM (37) and Rse (38,39) would result in a decrease in the concentration of functional σ molecules or interference with σ–core enzyme interactions. However, an immunoblot analysis of the σ preparations used in this study did not give any signal against anti-Rsd and anti-FlgM antibodies (data not shown).

The K_d value (0.26 nM) for σ^{70} herein determined is slightly lower than a previous estimation (0.5–1.0 nM) using HPLC gel filtration and fluorescence techniques (40). The difference might be due to a difference in content of the functional σ^{70} subunit and/or core enzyme or the assay conditions. Joo *et al.* (41) estimated a K_d value of 1 nM for σ^{H} from a σ saturation of *in vitro* transcription experiment, but of 100 nM by glycerol gradient centrifugation. The binding constants obtained by both gel filtration and glycerol gradient centrifugation should be lower than that obtained in transcription assays, because of the dilution effect during separation of core enzyme-bound σ from unbound free σ. Thus, further technical improvements are needed for accurate determination of the binding affinity of each σ subunit for the core enzyme, but the present study provides the relative order of core enzyme binding affinity among seven *E.coli* σ subunits. The present study clearly shows that the affinity of the σ^{N} subunit for the core enzyme is as high as those of other σ subunits. Thus the ATP-dependent reaction in formation of the $E\sigma^{N}$–promoter open complex may be at a step after binding of the $E\sigma^{N}$ holoenzyme to the promoter.

Structural basis of the difference in core enzyme binding affinity between σ subunits

The σ^{70} family of proteins have a common structural organization, except for σ^{54}, consisting of four conserved domains (4,5). The role of each domain in promoter recognition has been extensively studied employing genetic, chemical and physical approaches (for a review see 4). On the other hand, our knowledge of the role of σ structural domains in protein–protein interactions with the core enzyme is limited. We have identified the σ^{70}, σ^{N} and σ^{S} subunit contact surfaces on the core enzyme subunits after mapping contact-dependent cleavage sites with iron(*S*)-1-(*p*-bromoacetamidobenzyl)ethylenediamine tetraacetate (FeBABE) (42) tethered at various positions on the σ subunits (43–45). The results indicated that multiple sites along these σ polypeptides are involved in contact with the β and β′ subunits of the core enzyme, most of which are located in the conserved domains among the three σ subunits. Mutant studies also indicated that the σ^{70} subunit contains multiple contact interfaces with the core enzyme (46) and, moreover, the same regions, and even equivalent amino acid residues, in both σ^{70} and σ^{H} are involved in core enzyme binding. If the major interfaces for protein–protein contacts with the core enzyme are the same within the σ family of proteins, the differences in core enzyme binding affinity observed in this study may be attributed to differences in the number of contact surfaces or contact amino acid residues or in the affinity of minor interfaces characteristic of each σ subunit. The contact-dependent protein cleavage experiments with FeBABE indeed indicated the

presence of small numbers of unique core subunit contact sites characteristic of each σ subunit (43–45).

Intracellular levels of various holoenzyme forms of *E.coli* RNA polymerase

The intracellular concentration in *E.coli* W3110 (A) is highest for the σ^{70} subunit among the seven *E.coli* σ subunits in both the exponential and stationary phases and under various stress conditions (20–22). From the dissociation constants of core enzyme binding and the intracellular concentrations we are now able to estimate the intracellular concentration of each holoenzyme (see Table 1). The concentration of RNA polymerase in *E.coli* W3350 is ~2000 molecules/cell in the exponential phase, of which about one-third (~700 molecules) stays in the cytosol (3,6). Since the total number of combined σ subunits (~1200 molecules/cell) is more than that of RNA polymerase not engaged in the transcription cycle (~700 molecules/cell), the majority of the RNA polymerase in the cytosol must be in the holoenzyme form associated with one of the σ subunits. For instance, in exponential phase *E.coli* W3110 cells the numbers of each holoenzyme can be calculated to be 545 molecules for $E\sigma^{70}$, 100 molecules for $E\sigma^{F}$ and 55 molecules for $E\sigma^{N}$.

However, some of the σ^{70}, σ^{F} and σ^{N} subunits are considered to exist as complexes with anti-σ factors (24,36). Thus, the actual concentrations of the $E\sigma^{70}$, $E\sigma^{F}$ and $E\sigma^{E}$ holoenzymes in *E.coli* should be lower than the levels estimated above. For instance, a considerable fraction of the σ^{F} subunit stays as a complex with FlgM, the anti-σ^{F} factor, under the same culture conditions as employed in this study (36,37). σ^{E} activity is regulated by RseA (regulator of σ E or anti-σ^{E} factor), which is associated with the inner membrane and inhibits the activity of σ^{E} by directly interacting with σ^{E} (38,39). Recently we identified Rsd (regulator for σ D), the putative anti-σ^{70} subunit, which is produced in *E.coli* during the transition from the exponential to the stationary phase (25,36). The control of functional forms of the σ subunits by anti-σ factors or σ switching factors may contribute, at least to a certain extent, in regulation of the relative levels of various forms of the holoenzyme (6,27,47).

Factors affecting the core enzyme binding affinity of σ subunits

σ competition has been shown to take place between the *E.coli* σ^{70} subunit and the phage T4 σ subunit (48–50). In uninfected cells, however, the model has been accepted without any quantitative measurements of the core enzyme binding affinity for each σ subunit and of the intracellular concentrations of the seven σ subunits. The observation that overexpression of one σ subunit affects transcription of genes under the control of other σ subunits (24,25) supports the σ competition model. Judging from the intracellular concentrations of all seven σ subunits (20–22) and the binding affinity of each σ subunit for the core enzyme (this paper), it became clear that the σ subunits compete for a limited number of core enzyme molecules. However, both the intracellular level and the binding affinity for the core RNA polymerase are highest for σ^{70}, the major σ subunit for transcription of growth-related genes, at least under the experimental conditions employed, indicating that σ competition alone cannot explain efficient replacement of the σ^{70} subunit by the alternative σ subunits.

Previously we proposed that, to explain the growth-coupled change in transcription pattern, some specific intracellular conditions or additional factors are involved in efficient replacement of the core enzyme-associated σ subunit (26,27). For instance, a growth-dependent change in cytosol composition such as an increase in glutamate (51), trehalose (52) or polyphosphate (53) can promote preferential utilization of σ^{S} over σ^{70}. Regulatory nucleotides such as cAMP, ppGpp and AppppA may also affect transcription by different holoenzymes in different ways (54). Along these lines, the binding affinity of each σ subunit to the core RNA polymerase should be examined under various conditions mimicking the intracellular conditions for each σ subunit function. The major factor(s) affecting σ replacement awaits further studies.

In addition to σ switching control at the step of core enzyme binding, the recruitment frequency of various holoenzymes into the transcription cycle is also subject to control. For instance, a growth-coupled change in DNA superhelicity affects the utilization of $E\sigma^{70}$ and $E\sigma^{38}$ in different ways (34,55). The protein composition of the nucleoid also changes markedly depending on cell growth conditions (56,57). Most of the nucleoid proteins are known to be global regulators of gene transcription, influencing transcription by various holoenzymes in different manners.

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