Promoter Selectivity of Escherichia coli RNA Polymerase $E\sigma^{70}$ and $E\sigma^{38}$ Holoenzymes

EFFECT OF DNA SUPERCOILING*

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The functional specificity was compared between two $\sigma$ factors, $\sigma^{70}$ (the major $\sigma$ at exponentially growing phase) and $\sigma^{38}$ (the essential $\sigma$ at stationary growing phase), of Escherichia coli RNA polymerase. The core enzyme binding affinity of $\sigma^{38}$ was less than half the level of $\sigma^{70}$ as measured by gel filtration column chromatography or by titrating the concentration of $\sigma$ required for the maximum transcription in the presence of a fixed amount of core enzyme. In addition, the holoenzyme concentration required for the maximum transcription of a fixed amount of templates was higher for $E\sigma^{38}$ than $E\sigma^{70}$. The transcription by $E\sigma^{38}$ was, however, enhanced with the use of templates with low superhelical density, in good agreement with the decrease in DNA superhelicity in the stationary growth phase. We thus propose that the selective transcription of stationary-specific genes by $E\sigma^{38}$ holoenzyme requires either a specific reaction condition(s) or a specific factor(s) such as template DNA with low superhelical density.

In Escherichia coli, the total number of RNA polymerase core enzyme is fixed at a level characteristic of the rate of cell growth, which ranges from 1,000 to 3,000 molecules per genome equivalent of DNA (1, 2). On the other hand, the total number of genes on the E. coli genome is estimated to be about 4,000, which is in good agreement with the number estimated from the DNA sequence (up to now, more than 60% has been sequenced). These considerations raise a possibility that the superhelicity of a fixed number of genes are considered to be expressed under various stress conditions that E. coli meets in nature (4–7). For instance, a set of stress-response genes is expressed when cells stop growing at stationary phase (6, 7). Transcription of at least some of these stationary phase-specific genes is catalyzed by RNA polymerase holoenzyme containing $\sigma^{38}$ (the rpoS gene product) (8–11). In addition, the modification of core enzyme is considered to be involved in stationary-specific transcription regulation (12, 13).

Promoters from the stationary-specific genes, however, do not have a single consensus sequence (10, 11, 14, 15). The lack of a consensus could indicate the involvement of a regulatory cascade, in which some genes are directly transcribed by $E\sigma^{38}$ but others are under the control of these gene products. However, we found that the osmo-regulated genes, osmB and osmY, are transcribed preferentially by $E\sigma^{38}$ only in the presence of high concentrations of potassium glutamate (or acetate) (16). This finding raises a possibility that each stationary-specific promoter carries a specific sequence that is recognized by $E\sigma^{38}$ under a specific reaction condition and suggests that the promoter sequences recognized by $E\sigma^{38}$ differ between gene groups with different requirements. Our effort has since been focused to identify specific conditions or factors required for transcription of each stationary-specific gene by $E\sigma^{38}$ holoenzyme. DNA superhelicity is known to change depending on the cell growth conditions. For instance, nutrient downshift and stationary growth phase cause a decrease in the DNA superhelical density (17, 18), while high osmolarity leads to an increase in the superhelicity (19, 20).

In this report, we describe the comparison of two $\sigma$ factors, $\sigma^{70}$ and $\sigma^{38}$, in the following activities: (i) the core binding activity, (ii) the promoter recognition activity, and (iii) the effect of DNA conformation on the promoter recognition patterns. The results show that the selectivity for stationary phase-specific promoters by $E\sigma^{38}$ increases concomitantly with the decrease in DNA superhelicity and that the effects of decreased DNA superhelicity and high potassium glutamate concentrations are additive in enhancing the selectivity for $E\sigma^{38}$.

MATERIALS AND METHODS

Promoters and Templates—The truncated lacUV5 template, 205 bp1 EcoRI-EcoRI fragment, was prepared as described previously (21), while the 156-bp HindIII-EcoRI katE and 796-bp PstI-EcoRI fic fragments were prepared as described by Ding et al. (16) and Tanaka et al. (10), respectively. The BamHI-KpnI fragment of 287 bp in length carrying aAla5 promoter was prepared as described by Nomura et al. (22). These truncated DNA templates produced in vitro transcripts of 63, 69, 257, and 169 nucleotides in length, respectively.

Plasmids pBSOY containing osmY promoter and pBSLU containing lacUV5 promoter were constructed as follows. Plasmid pBlueScript II (Stratagene) was linearized with SadI and blunt-ended using Klenow DNA polymerase; a 783-bp HindI-digested fragment containing rrnB terminator, purified from pK223-3 (Pharmacia), was ligated into pBlueScript II at the blunt-ended SadI site to yield plasmid pBST. A 261-bp HindI-EcoRI fragment containing osmY promoter, isolated from pDY.1.4 (23), was inserted into pBST between HindI and EcoRI to yield pBSOY. On the other hand, pBSLU was constructed after

1 The abbreviations used are: bp, base pair(s); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.
insertion of the 205-bp lacUV5 fragment from pKB252 (21) into pBST between HindI and EcoRI. These circular DNA templates produced in vitro transcripts of 357 (lacUV5 promoter), 346 (osmY promoter), and 108 (primer RNA for ColE1) nucletides, respectively, in length. These template plasmids were prepared from transformed DH5α cells using QIAGEN plasmid kit (QIAGEN).

RNA Polymerase—RNA polymerase core enzyme was purified by passing RNA polymerase at least three times through phosphocellulose columns (the repeated chromatography is essential for complete removal of minor α factors from core enzyme). α70 (the rpoD gene product) was overexpressed using pGEMD and purified by the method of Igarashi and Ishihama (24), while α38 was overexpressed using pETF and purified as described by Tanaka et al. (10).

In Vitro Single-round Transcription System—Single-round mixed transcription by holoenzyme was carried out under the standard conditions described previously (28). In brief, a mixture of template DNA and RNA polymerase reconstituted from purified core enzyme and either purified α70 or α38 was pre-incubated for 30 min at 37°C to allow open complex formation in the standard reaction mixture, which contained (in 35 μl) 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM magnesium acetate, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 25 μg/ml bovine serum albumin. A 15-μl mixture of substrate and heparin was then added to make the final concentrations of 160 μM each of ATP, GTP, and CTP; 50 μM UTP; 2 μM of (α32P)-UTP; and 200 μM glycerin. After 5 min incubation at 37°C, the reaction was stopped by precipitation with ethanol and subjected to PAGE in the presence of 8 M urea. Gels were dried and exposed to imaging plates. The exposed plates were analyzed with a BAS-2000 image analyzer (Fuji).

Preparation of a Set of Templates with Various Superhelical Densities—Covalently closed plasmids (5 μg each) were treated with 6 units of calf thymus DNA topoisomerase I (Takara Shuzo) in 100 μl of a reaction mixture, which contained 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM MgCl2, 5 mM DTT, 5 mM spermidine, and 0.01% bovine serum albumin. The enzyme reaction was carried out at 37°C for 5 h in the presence of various concentrations of ethidium bromide (0–40 μM). After the incubation, plasmids were purified by two cycles of phenol-chloroform treatment, followed by ethanol precipitation. The average linking number (Δlk) of each DNA molecule was measured by electrophoresis on 0.8% agarose gels containing appropriate concentrations of ethidium bromide, according to the method of Keller (25). The mean superhelical density (n) was calculated by the equation n = 10Δlk/N, where N represents the number of the base pairs of plasmid DNA.

RESULTS

Difference in the Core Enzyme Binding Activity between Two α Factors—Both α70 and α38 were overproduced in E. coli and purified to apparent homogeneity as determined by Coomassie Brilliant Blue staining of the proteins separated by PAGE. On the other hand, core enzyme was purified from exponentially growing E. coli cells by repeated chromatography on phosphocellulose. The affinity of the two species of α subunit to core enzyme was examined by measuring two parameters, i.e., the saturation level of α subunit required for the maximum level of holoenzyme formation from a fixed amount of core enzyme and the saturation level of α subunit to achieve the maximum level of in vitro transcription by a fixed amount of core enzyme.

First, the affinity of the two α subunits to core enzyme was compared by directly measuring the holoenzyme formation. For this purpose, we mixed a fixed amount of core enzyme and various amounts of either α70 or α38 at 30°C, and the mixtures were fractionated by gel filtration-HPLC on a Superose 6 column. The α to core enzyme ratio was measured for the peak fraction of RNA polymerase (Fig. 1). The core enzyme was saturated with α70 at the input molar ratio between 2 and 3, while the saturation of the same amount of core enzyme with α38 required at least 2-fold more α38 protein than α70. The observed difference might be due to a difference in the activity of purified α subunits. To test this possibility, the second-cycle assay was performed using the unassembled α subunits recovered after the first cycle of binding assay. The core binding patterns of both α subunits were essentially the same with those of the first cycle experiments (data not shown).

Difference in the Promoter Recognition Activity between Two α Factors—The level of α subunit required for maximum transcription by a fixed amount of core enzyme was also compared between the two α subunits. For this purpose, single-round transcription was carried out using a fixed amount of core enzyme and various amounts of α subunits. The core enzyme was saturated by adding only 2-fold molar excess of α70 subunit, i.e., 2 pmol of α70 per pmol core as measured using αla5 promoter (Fig. 2), and this level was observed for all the α70-dependent promoters analyzed (data not shown). The molar concentration of α38 required for the maximum transcription of fic, katE, and lacUV5 was 10, 8, and 4 pmol, respectively, per pmol of core enzyme (Fig. 2). Since the difference in core enzyme binding activity between the two α subunits is about 2-fold (see above), the transcription assay indicates that the promoter recognition activity is also weaker for α38 than α70. The promoter recognition activity of α38 is, however, variable depending on the promoter. For instance, only less than 2 pmol of α38 was needed for maximum transcription of the osm-regulated promoters, osmB and osmY, in the presence of 0.3–0.5 mM potassium glutamate (or acetate) (16).

Effect of RNA Polymerase on Promoter Selectivity Control—Since both the core enzyme binding and the promoter recognition activities under our standard assay conditions for in vitro transcription were lower for Erα38 than Erα70, the promoter selection pattern was analyzed with use of the increased concentration of RNA polymerase. Fig. 3 summarizes the effect of enzyme/promoter ratio on the relative transcription level by two holoenzymes. At low enzyme concentrations, lacUV5 was transcribed preferentially by Erα70, but Erα38 started to transcribe lacUV5 at high enzyme concentrations. Likewise, fic was transcribed by both Erα70 and Erα38 at low enzyme concentrations, but Erα38 transcribed better than Erα70 at high enzyme concentrations. These observations altogether lead to the recognition that the classification of promoters with respect to α selectivity varies depending on the concentrations of individual holoenzyme species. The katE promoter was always transcribed better with Erα38 than Erα70, but the Erα38/Erα70 activity ratio increased from 1.3 at 0.5 pmol (per 0.1 pmol promoter; promoter/enzyme ratio of 5) to about 4 at the holoenzyme concentration higher than 1 pmol.
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For all the promoters examined, the transcription activity by E\textsigma{38} increased at high protein concentrations, supporting the notion that either the affinity of E\textsigma{38} to core enzyme is weaker than that of E\textsigma{70} or the affinity of E\textsigma{38} to promoters is weaker than that of E\textsigma{70}.

Effect of the Superhelical Density of DNA on Promoter Selectivity Control—E\textsigma{38} plays a major role in transcription of a set of genes essential for survival in stationary phase and/or nutrient starvation (7). Under such starved growth conditions, the superhelicity of chromosomal DNA in bacterial cells is known to decrease (17, 18). We then examined the effect of DNA superhelicity on transcription in vitro by two RNA polymerase holoenzymes, E\textsigma{70} and E\textsigma{38}. For this purpose, the DNA fragments containing the test promoters were inserted into a single and the same vector plasmid containing rrnB transcription termination sequence so as to produce in vitro transcripts of defined sizes (for construction see “Materials and Methods”). The resulting plasmids were treated with calf thymus DNA topoisomerase I in the presence of various concentrations of ethidium bromide. The superhelicity of each template thus obtained was measured by gel electrophoresis (see “Materials and Methods”). Using these circular template DNAs with different degrees of superhelicity, an in vitro transcription assay was carried out under the standard conditions. The gel patterns, shown in Fig. 4, indicated that the effect of DNA superhelicity on transcription was different between E\textsigma{38} and E\textsigma{70}.

The quantitative analysis data, shown in Fig. 5, indicated that the optimum superhelical density for maximum transcription by E\textsigma{38} was low for all the promoters (lacUV5, osmY, and RNA-I) tested, i.e., the superhelical density of around 0–0.03. In general, E\textsigma{38} required lower levels of DNA superhelicity for maximum transcription than E\textsigma{70}. In particular, the activity of E\textsigma{38} is enhanced with the decrease in DNA superhelicity in transcription of stationary-specific promoters.

In contrast, E\textsigma{70} required high levels of DNA superhelicity for maximum activity. The optimum DNA superhelicity for the maximum transcription of osmY promoter by E\textsigma{70} was high (above 0.1), and upon decrease in DNA superhelicity, E\textsigma{70} becomes inactive in transcription of the osmY promoter. Transcription of lacUV5 and RNA-I by E\textsigma{70} was rather insensitive to the change in DNA superhelicity (these promoters are classified into a group of promoters that are recognized by both E\textsigma{70} and E\textsigma{38} holoenzymes (10, 15)).

Effect of Growth Phase on DNA Superhelicity and Promoter Activity—To correlate these in vitro observations with in vivo
situations, we next examined the levels of DNA superhelicity for the plasmids that were purified from transformed DH5α cells at various phases grown in Luria broth at 37°C. The results, shown in Fig. 6, indicated that the superhelicity of plasmids, prepared from the stationary phase cells, was approximately half of the level of plasmids from the exponentially growing cells. Furthermore, the plasmids from the stationary phase cells showed nearly the same extent of superhelicity as the plasmids that gave the maximum transcription in vitro by E\(\text{S}^{38}\) (see Fig. 5). To confirm this relationship, we examined the template activity in vitro transcription by E\(\text{S}^{70}\) and E\(\text{S}^{38}\) for all the templates prepared at various phases of the cell growth. The level of transcription by E\(\text{S}^{38}\) was maximum for the promoters prepared at the stationary phase, i.e. about 2-fold higher than the levels of DNA from the log phase cells (data not shown). Again, the transcription levels by E\(\text{S}^{70}\) stayed at constant levels (lacUV5 and RNA-I) or rather decreased (osmY) with the decrease in DNA superhelicity (data not shown). Thus, we concluded that E\(\text{S}^{38}\) preferentially transcribes template DNA with the low superhelicity.

Transcription Step Affected by DNA Superhelicity—To identify the step(s) of transcription that were affected by the DNA superhelicity, we examined the effect of E\(\text{S}^{38}\) concentration on the relative level of transcription from osmY templates with different superhelicity. At low enzyme concentrations, the maximum transcription level on the template with low superhelical density was about 2-fold higher than that on the high helical density template, but at high enzyme concentrations, the maximum transcription level was almost the same between the two templates (Fig. 7). The results suggest that the affinity of E\(\text{S}^{38}\) is higher for DNA with low superhelical density than for DNA with high superhelical density. To confirm this prediction, we measured the rate of open complex formation. The time course of open complex formation by E\(\text{S}^{38}\) is roughly the same between the two templates (the formation of open complexes was achieved within 15 min of preincubation at 37°C). However, the maximum level of open complexes formed differed, indicating that the DNA superhelicity difference does not affect the rate of open complex formation but influences the promoter recognition activity by RNA polymerase (see “Discussion”).

Additive Effect of Potassium Glutamate and DNA Superhelicity on Transcription—The level of osmY transcription by E\(\text{S}^{38}\) holoenzyme increases with the increase in potassium glutamate concentration (16). We then examined the effect of potassium glutamate concentration on transcription directed by circular DNA templates with different superhelicity. The patterns of lacUV5 and osmY transcription level by E\(\text{S}^{70}\) were essentially the same between DNA from the exponentially growing and stationary phase cells, both showing decreased activity concomitantly with the increase in potassium glutamate concentration (Fig. 8, A and C). On the other hand, the
maximum transcription level by \( E_\sigma^{38} \) holoenzyme was observed at high concentrations of potassium glutamate (Fig. 8, B and D). In particular, \( E_\sigma^{38} \) preferentially transcribed the osmY promoter only at high potassium glutamate concentrations, and this result agreed with the previous result by using truncated osmo-regulated gene promoters (16). The maximum transcription of DNA with low superhelicity was observed between 200 and 300 mM concentrations of potassium glutamate, while using DNA with high superhelicity the maximum transcription was observed above 300 mM potassium glutamate (Fig. 8D).

Thus, the requirement for high concentrations of potassium glutamate is partly replaced by the decrease in DNA superhelicity.

**DISCUSSION**

The \( \sigma \) saturation experiments of in vitro transcription indicate that the affinity of \( \sigma^{38} \) to core enzyme is lower than that of \( \sigma^{70} \) (see Fig. 2). Direct measurement of the core enzyme-bound \( \sigma \) subunits by gel filtration-HPLC on Superose 6 column indeed showed that the binding affinity of \( \sigma^{38} \) to core enzyme is at least 2-fold weaker than that of \( \sigma^{70} \) (see Fig. 1). This was rather unexpected because the intracellular concentration of \( \sigma^{38} \) is not higher than that of \( \sigma^{70} \) even after prolonged starvation in the stationary phase (26). Growth-coupled replacement of core enzyme-associated \( \sigma \) subunit from \( \sigma^{70} \) to \( \sigma^{38} \) may therefore re-
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Fig. 8. Effect of potassium glutamate concentrations on in vitro transcription of templates with different superhelical densities. A and B, low and high superhelical density DNA templates (0.1 pmol each) carrying lacUV5 promoter, were prepared from 4 and 24 h, respectively, after inoculation and were transcribed in vitro by various amounts of reconstituted Eσ38 (A) and Eσ70 (B) holoenzymes under the standard single-round assay conditions, except that 50 mM NaCl was replaced with the indicated concentrations of potassium glutamate. Transcripts were analyzed with a BAS-2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template was set as 100% value. C and D, low and high superhelical density DNA templates (0.1 pmol each) carrying the osmY promoter, prepared from 4 and 24 h, respectively, were transcribed in vitro by various amounts of reconstituted Eσ38 (C) and Eσ70 (D) holoenzymes under the same conditions as in panels A and B. Analyses of transcripts were carried out as in panels A and B. These data represent the averages of three independent experiments.

require an additional factor(s) or a yet unidentified condition(s). For example, the modification of core enzyme in stationary phase cells (12, 13) may increase selective binding of σ38.

The single-round in vitro transcription assays also indicated that the level of Eσ38 holoenzyme required for maximum transcription of a fixed amount of template was higher than that of Eσ70 holoenzyme (see Figs. 2 and 3). However, the maximum yields of transcription in the presence of saturated amounts of enzyme were often lower than the template levels and were different between the promoters. The difference in abortive initiation between promoters provides a part of the explanation as has been experimentally demonstrated (27, 28). In addition, the role of sensitivity difference of various initiation complexes to heparin cannot be excluded as a possible cause for the differing yields because our preliminary gel retardation assay indicated that Eσ38 lacUV5 initiation complexes were partly dissociated at 200 μg/ml heparin, while Eσ70 lacUV5 complexes stayed unchanged.2

Another unexpected observation is that the affinity of Eσ70 holoenzyme to σ28-dependent promoters was rather weaker than that of Eσ70 to σ38-dependent promoters at least under the standard transcription assay conditions employed. Thus, in mixed transcription assays, σ28-dependent promoters are preferentially transcribed at low enzyme concentrations, but upon the increase in enzyme concentration, the σ38-dependent promoters become predominantly transcribed. Again, this is apparently in conflict with the intracellular concentrations of the two holoenzymes during the transition from exponentially growing to stationary growth phase. Thus, promoter-Eσ38 interaction may also be influenced by a specific factor(s) and/or condition(s) present in stationary phase E. coli cells. Previously, we found that addition of high concentrations of potassium glutamate selectively enhances transcription by Eσ38, although Eσ70 activity is inhibited at the high salt concentrations (16). In addition, we found in this study that Eσ38 preferentially transcribed the promoters on low superhelical density DNA (see Figs. 4 and 5). Moreover, the optimum superhelical density for maximum transcription by Eσ38 was almost the same as that of plasmids prepared from stationary phase E. coli cells (see Fig. 6). Preferential transcription of the low superhelical density templates by Eσ38 was due to the high affinity of Eσ38 binding to promoter on the low superhelical density DNA (see Fig. 7).

The selective transcription of osmY by Eσ38 increases by adding high concentrations of potassium glutamate (16). The optimum potassium glutamate concentrations for the maximum transcription of osmY on the stationary phase plasmid template with low superhelical density by Eσ38 was 200–300 mM, whereas as that on the DNA with high superhelical density prepared from log phase E. coli cells was above 300 mM (see Fig. 8). Transcription in vivo of osmY in cells growing in nutrient-rich media such as Luria-broth is observed only at the stationary phase, whereas in cells growing in poor media such as M9-glucose, osmY transcription occurs at both log and stationary phases (23, 29). In good agreement with these observations, the superhelical density of plasmids prepared from cells grown in M9-glucose is about half the density of plasmids prepared from exponentially growing cells in Luria-broth.2 These results altogether raise such a model of global regulation that transcription by Eσ38 is enhanced with the decrease in DNA superhelical density under starved conditions. In addition, the increase in intracellular potassium glutamate concentration may lead to enhanced transcription of at least osm-regulated genes. As an extension of this consideration, each of the stationary phase-specific gene promoters may require a specific transcription factor(s) or condition(s) for efficient transcription. Such predictions are in good agreement with the observations that the σ38-dependent promoters do not carry a consensus promoter sequence (10, 11, 14–16).

In addition to selective activation of Eσ38, a nucleoid-associated protein, H-NS (or H1a), which accumulates in the stationary growth phase, represses σ70-dependent transcription in vitro and in vivo (30–32). Likewise, Dps accumulating in the stationary growth phase cells may play dual roles in not only DNA protection but also repressing gene transcription (33). The differential repression by these DNA binding proteins on transcription between Eσ70 and Eσ38 may also lead to apparently selective transcription of σ38-dependent genes in the stationary phase.

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