Stimulatory Effect of Trehalose on Formation and Activity of Escherichia coli RNA Polymerase $\sigma^{38}$ Holoenzyme

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The intracellular concentration of trehalose increases in the stationary-phase cells of Escherichia coli. The effects of trehalose on transcription in vitro by E. coli RNA polymerase were compared for two holoenzymes, $\sigma^{70}$ and $\sigma^{38}$, which were reconstituted from purified core enzyme and either $\sigma^{70}$ (the major $\sigma$ at the exponential growth phase) or $\sigma^{38}$ (the essential $\sigma$ at the stationary growth phase), respectively. The optimum trehalose concentration giving maximum transcription by $\sigma^{38}$ was higher than that by $\sigma^{70}$. Transcription activation by trehalose was attributed to both increased formation of $\sigma^{38}$ holoenzyme and increased transcription initiation by $\sigma^{38}$ from $\sigma^{38}$-dependent promoters. The activation of $\sigma^{38}$ by trehalose was additive with the transcription enhancement by decreased superhelicity of template DNA prepared from stationary-phase cells. We thus propose that the selective activation of transcription by $\sigma^{38}$ holoenzyme takes place in the presence of specific conditions and factors present under stress conditions.

The total number of genes on the Escherichia coli genome is estimated to be about 4,000, but the maximum number of genes expressed in rapidly growing E. coli cells under laboratory culture conditions (i.e., at 37°C and with aeration) is about 1,000, as estimated from the species of protein products fractionated by two-dimensional gel electrophoresis (29). The rest of the genes are considered to be expressed under the various stress conditions that E. coli meets in nature (reviewed in references 6, 16, 19, and 21). For instance, a set of stress-response genes begins to be expressed when cells stop growing at the stationary phase (6, 16), while most of the genes highly expressed in exponentially growing cells are turned off or repressed.

The total amount of RNA polymerase core enzyme in E. coli 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 (reviewed in references 9 and 10). The core enzyme is functionally differentiated by binding one of multiple species of $\sigma$ subunits (reviewed in reference 5), and each core enzyme-$\sigma$ subunit complex (holoenzyme) recognizes a specific set of promoters. The replacement of promoter recognition subunit $\sigma$ on RNA polymerase is the most efficient method for alteration of the promoter recognition property of RNA polymerase (5, 9). The concentration of each form of holoenzyme should be a factor for determination of the species and level of transcription of the genes under a given growth condition. During the growth phase transition of E. coli from the exponential to stationary phases, a novel species of $\sigma$ factor ($\sigma^{38}$ [the rpoS gene product]) appears (18, 20, 22, 27). The intracellular concentration of $\sigma^{38}$ increases in stationary-phase E. coli cells, but the maximum level is only 30% of the level of $\sigma^{70}$ (11, 12). Since the level of $\sigma^{70}$ (the rpoD gene product) stays constant without being degraded, the increase in the level of $\sigma^{38}$ alone cannot explain the transcriptional switching in stationary-phase cells, and therefore an as-yet-unidentified factor(s) or condition(s) might be involved in efficient and preferential utilization of $\sigma^{38}$ holoenzyme. The modification of core enzyme may be a factor involved in the specificity modulation of RNA polymerase (24, 25).

Promoters from the stationary-phase-specific genes so far analyzed do not match a consensus sequence (15, 27, 28). We have previously found that transcription of some osmoregulated genes such as osmB and osmY by $\sigma^{38}$ takes place only in the presence of high concentrations of potassium glutamate (or acetate) (3). Transcription by $\sigma^{38}$ is also enhanced when directed by templates with low superhelical density, in good agreement with the decrease in DNA superhelicity in the stationary-phase cells (17). This finding raises the possibility that each stationary-phase-specific promoter carries a specific sequence which is recognized by $\sigma^{38}$ under a specific reaction condition or in the presence of a specific factor. If this is the case, the promoter sequences recognized by $\sigma^{38}$ must be different between groups which require different conditions or factors for function. As an extension of this line of studies, we have analyzed the effect of trehalose concentration on promoter recognition by $\sigma^{70}$ and $\sigma^{38}$ holoenzymes. In microorganisms, trehalose, a nonreducing disaccharide of glucose, plays an important role in protection against environmental stresses such as desiccation, high osmolarity, frost, and heat (13, 23, 26). The intracellular concentration of trehalose in E. coli is known to increase under high-osmolarity or starved-culture conditions (2, 23, 26, 30).

In this report, we describe the effect of trehalose on transcription in vitro by two RNA polymerase holoenzymes, $\sigma^{70}$ and $\sigma^{38}$. The results show that both the formation and the activity of $\sigma^{38}$ holoenzyme increase concomitantly with the increase in trehalose concentration. The enhancing effect of trehalose on transcription by $\sigma^{38}$ is additive with that of decreased superhelicity of template DNA, indicating that the changes in cytoplasmic composition and nucleoid conformation together affect growth-dependent changes in gene transcription.

MATERIALS AND METHODS

Promoters and templates. A 438-bp NcoI-EcoRI fragment containing the fic promoter was prepared from pFC2 plasmid (27), while a 205-bp EcoRI-EcoRI lacUV5 fragment and 646-bp PstI-ClaI osmY fragment were prepared as de-
scribed by Kajitani and Ishihama (14) and Ding et al. (3), respectively. These truncated DNA templates produced in vitro transcripts 257, 63, and 242 nucleotides (nt) in length, respectively.

As closed circular templates, plasmids pBSOY containing the osmY promoter and pbSLU containing the lacUV5 promoter were used as described by Kusano et al. (17). These circular templates produced 346-nt osmY RNA and 357-nt lacUV5 RNA, respectively, and 108-nt RNA-I (primer RNA for ColE1 replication).

**RNA polymerase.** RNA polymerase core enzyme was purified by passing RNA polymerase at least three times through phosphocellulose columns (repeated chromatography being essential for complete removal of minor σ factors from core enzymes). σ70 (the rpoD gene product) was overexpressed with pGEMD and purified by the method of Igarashi and Ishihama (8), while σ38 was overexpressed with pETF and purified as described previously (3, 27).

**In vitro single-round transcription system.** Single-round mixed transcription by holoenzyme was carried out under the standard conditions described previously (8). In brief, a mixture of template DNA and RNA polymerase reconstituted from purified core enzyme and either purified σ70 or σ38 was preincubated for 30 min at 37°C to allow open complex formation in the standard reaction mixture, which contained the following in 35 μl: 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM MgCl2, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25 μg of bovine serum albumin (BSA) per ml. A 15-μl mixture of substrate and heparin was then added to make the final concentrations: 160 μM (each) ATP, GTP, and CTP; 50 μM UTP; 2 μCi of [α-32P]GTP; and 200 μg of heparin per ml. After 5 min of incubation at 37°C, transcripts were precipitated with ethanol and subjected to polyacrylamide gel electrophoresis (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-II Bio-Image analyzer (Fuji).

**Immunoprecipitation of RNA polymerase.** Core enzyme and various amounts of σ38 subunit were mixed in 0.1 ml of reaction buffer, which contained 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM MgCl2, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25 μg of BSA per ml, and various concentrations of trehalose (a gift from Hayashibara Biochemistry Laboratory). After 30°C to form holoenzymes. After the incubation, 1/100 volumes of antiserum mixture, and this mixture was then incubated for 2 h at 4°C. After the immunoprecipitation, 1/5 volumes of 50% (vol/vol) protein A-Sepharose 6MB (Pharmacia) containing 0.6% Triton X-100 and 1% BSA were added, and this mixture was then incubated for 3 h at 4°C. After the incubation, the mixtures were washed twice at 4°C with the reaction buffer containing 0.1% Triton X-100. Subsequently, proteins were subjected to sodium dodecyl sulfate (SDS)-PAGE (15% polyacrylamide). Gels were stained with the Fluorokit PRO-1 protein staining system (Promega) and analyzed with a Fluorimaguer SI system (Molecular Dynamics).

**RESULTS**

**Effect of trehalose on in vitro transcription by Er38.** The relative rate of utilization of two principal σ subunits, σ70 and σ38, in the transcription cycle in vitro is affected by the components in reaction mixtures. For instance, the activity of Er38 is specifically enhanced upon increases in potassium glutamate (3) and decreases in DNA superhelicity (17). As an extension of this line of studies, we examined in this study the effects of trehalose on transcription in vitro by Er70 and Er38 holoenzymes because the intracellular concentration of trehalose sometimes increases above 1 M under stress conditions (2, 26, 30). In order to examine the effect of trehalose on various steps of transcription, including both the formation of Er holoenzyme and the transcription initiation by the holoenzyme, we used a r-limiting condition in which a linearity exists between the amount of σ subunit added and the level of holoenzyme formed. From the results of σ saturation experiments of transcription in vitro under our standard reaction conditions with the lacUV5 promoter (7), the core/σ ratios in the transcription assay were fixed at 1:0.5 and 1:5 for Er70 and Er38, respectively. At these input molar ratios, the level of transcription by both holoenzymes was approximately 50% of the maximum level of transcription obtained in the presence of saturating amounts of σ subunits.

First, an in vitro single-round transcription assay was carried out in the presence of 0 to 1.2 M trehalose with three promoters, lacUV5, fic, and osmY, all of which are recognized by both Er70 and Er38 (3, 15, 27, 28). The gel pattern of transcripts shown in Fig. 1A clearly demonstrates that the amounts of transcripts produced by Er38 increase concomitantly with the increase in trehalose concentration. The quantitative analysis of data shown in Fig. 1B indicates that the effects of trehalose concentration on transcription are indeed different for Er70 and Er38. The optimal trehalose concentrations to achieve maximum transcription by Er38 were high for all of the promoters tested (i.e., trehalose concentrations of above 1.2 [lacUV5], 1.0 [fic], or 0.7 [osmY] M, respectively, in Fig. 1B, panels D to F), while the optimum trehalose concentration for

**FIG. 1.** Effect of trehalose on in vitro transcription. (A) Single-round in vitro transcription was carried out with 0.1 pmol each of lacUV5, fic, and osmY promoter and 1 pmol of either reconstituted Er70 or Er38 holoenzyme under the standard reaction conditions, except that trehalose was added at the indicated concentrations. Transcripts were subjected to 8 M urea-PAGE. The gel was exposed to an imaging film, and the film was analyzed with a BAS-2000-II Bio-Image analyzer (Fuji). (B) The gel pattern of the single-round in vitro transcription shown in panel A was analyzed with a BAS-2000-II Bio-Image analyzer (Fuji). lacUV5 (A and D), fic (B and E), and osmY (C and F) were the templates used. Er70 (A to C) and Er38 (D to F) holoenzymes were the reconstituted RNA polymerases used. The maximum level of transcription for each template was set at 100% (lacUV5 [A and D], Er70, 1.0 M trehalose, 995 arbitrary units measured with Bio-Image Analyzer; fic [B and E]-Er38, 1.0 M trehalose, 5,151 arbitrary units; osmY [C and F]-Er38, 0.7 M trehalose, 5,151 arbitrary units). The data represent the averages of three independent experiments (the gel pattern shown in panel A is the result of one experiment).
was set at 100% (lacUV5 Bio-Image analyzer (Fuji). The maximum level of transcription for each template analyzed by 8 M urea–PAGE, and gels were analyzed with a BAS-2000-II except that trehalose was added at the indicated concentrations. Transcripts were in vitro transcription was carried out under the standard reaction conditions, except that trehalose was added at the indicated concentrations. Transcripts were analyzed by 8 M urea–PAGE and gels were analyzed with a BAS-2000-II Bio-Image analyzer (Fuji). The maximum level of transcription for each template was set at 100% (lacUV5 [A], 1 M trehalose, σ38/core polymerase ratio = 5, 3,232 arbitrary units measured with Bio-Image analyzer; fic [B], 1 M trehalose, σ38/core polymerase ratio = 32, 12,438 arbitrary units; osmY [C], 1 M trehalose, σ38/core polymerase ratio = 40, 3,202 arbitrary units). The data represent the averages of three independent experiments.

Effect of trehalose on the core enzyme-binding activity of σ38.

The above results indicated that either the formation of Ec38 holoenzyme or the activity of Ec38 holoenzyme is enhanced in the presence of trehalose. In an attempt to identify the step(s) of transcription which was affected by the high concentration of trehalose, we examined the stimulatory effect of trehalose on holoenzymes carrying different levels of σ subunits. Figure 2 shows the σ saturation curve that yields maximal transcription by a fixed amount of core enzyme in the absence or presence of 1 M trehalose. The core concentrations of σ38 required for half-maximal transcription of fic and osmY at 1 M trehalose were about five times lower than those in its absence (Fig. 2B and C). This fivefold increase in σ38 utilization by trehalose might be due to either enhancement in the σ38 binding to the core enzyme and/or the increase in the stability of the core enzyme-σ38 complex, thereby leading to the recruitment of small amounts of σ38 to transcription cycle, or activation of the transcription initiation by Ec38 holoenzyme. On the other hand, the amount of σ70 subunit required for the half-maximal transcription of lacUV5 at 1 M trehalose was more than half the amount required in the absence of trehalose (Fig. 2A). This less-than-twofold increase in the efficiency of σ70 utilization agrees with the slight increment of σ70-dependent transcription of lacUV5 by trehalose (Fig. 1B, panel A).

In order to identify the step of Ec38 activation by trehalose, we measured the formation of Ec38 in the presence of various concentrations of trehalose. For this purpose, a fixed amount of core enzyme was mixed with various amounts of σ38 at 30°C in the presence of increasing concentrations of trehalose, and the RNA polymerase was immunoprecipitated with monospecific anti-α subunit polyclonal antibodies. The anti-α subunit antibodies used did not interfere with RNA polymerase assem-

FIG. 2. σ saturation curve for maximal transcription in the absence and presence of trehalose. Core enzyme (1 pmol) and various amounts of σ38 (A and C) subunit were mixed as described in the legend to Fig. 1A. To the RNA polymerase mixtures, one of the truncated DNA templates (0.1 pmol) carrying lacUV5 (A), fic (B), or osmY (C) promoter was added, and single-round in vitro transcription was carried out under the standard reaction conditions, except that trehalose was added at the indicated concentrations. Transcripts were analyzed by 8 M urea–PAGE, and gels were analyzed with a BAS-2000-II Bio-Image analyzer (Fuji). The maximum level of transcription for each template was set at 100% (lacUV5 [A], 1 M trehalose, σ38/core polymerase ratio = 5, 3,232 arbitrary units measured with Bio-Image analyzer; fic [B], 1 M trehalose, σ38/core polymerase ratio = 32, 12,438 arbitrary units; osmY [C], 1 M trehalose, σ38/core polymerase ratio = 40, 3,202 arbitrary units). The data represent the averages of three independent experiments.

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FIG. 3. Effect of trehalose concentration on holoenzyme formation. Core enzyme (E [20 pmol]) and various amounts of σ38 were mixed in 0.1 ml of 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM MgCl2, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25 μg of BSA per ml, and various concentrations of trehalose. After incubation for 10 min at 30°C, RNA polymerase was immunoprecipitated with monospecific polyclonal antibodies specific for α subunit and protein A-Sepharose 6MB. Antigen-antibody complexes formed were subjected to SDS-PAGE (15% polyacrylamide). The gels were stained with the Fluorokit PRO-1 protein staining system and analyzed with a Fluorimag R1 system. The molar ratio of α subunit and σ38 was calculated for each sample.
centration of trehalose enhances both the step of holoenzyme formation between $\sigma^{38}$ and core enzyme (two- to threefold) and the step leading to open complex formation between E$\sigma^{38}$ holoenzyme and $\sigma^{38}$-dependent promoters (two- to threefold), altogether leading to about fivefold stimulation of transcription.

**Combined effect of potassium glutamate and trehalose on transcription by E$\sigma^{38}$**. Under certain stress conditions, such as at high osmolarity, the intracellular concentrations of trehalose and potassium glutamate increase simultaneously. We then examined the combined effect of trehalose and potassium glutamate on transcription. In the experiments shown in Fig. 5, the trehalose concentration was increased in the presence of low (50 mM) or high (300 mM) concentrations of potassium glutamate. The lacUV5 and osmY transcription by E$\sigma^{70}$ at a low concentration (50 mM) of potassium glutamate was stimulated by the addition of trehalose. The trehalose concentration-dependent variation patterns were essentially the same for the two promoters, both showing maximal transcription at 0.7 M trehalose. However, trehalose did not enhance transcription at a potassium glutamate concentration (300 mM) inhibitory for transcription by E$\sigma^{70}$ (Fig. 5A and C).

On the other hand, transcription by E$\sigma^{38}$ was enhanced by trehalose at both low and high concentrations of potassium glutamate (Fig. 5B and D). Transcription of lacUV5 by E$\sigma^{38}$ is inhibited by 300 mM potassium glutamate, but trehalose partially overcame this inhibition, giving a significant level of transcription (Fig. 5B). Transcription of osmY by E$\sigma^{38}$ is enhanced at a high concentration (300 mM) of potassium glutamate (reference 3 and this paper). Upon addition of trehalose above 0.5 M, the level of osmY transcription at a low concentration (50 mM) of potassium glutamate increased to a level as high as that observed in the presence of 300 mM potassium glutamate (Fig. 5D). This level of transcription was achieved by adding 0.5 M potassium glutamate without trehalose (3). The results suggest that the requirement for high concentrations of potassium glutamate upon transcription of osmY can be replaced by the high concentration of trehalose. Once the maximum transcription is achieved by one of these factors, however, no additive enhancement takes place with the addition of the second factor (Fig. 5D).

**DISCUSSION**

The intracellular trehalose concentration in *E. coli* increases under high-osmolarity-stress (2, 30) and nutrient-starved conditions such as carbon or nitrogen limitation (30). The *otsA* and *otsB* genes for trehalose synthesis are required for osmotic
tolerance (4) and thermotolerance (7). These observations indicate that trehalose plays a role in bacterial survival under certain stress conditions. Furthermore, the expression of genes under the control of rpoS, encoding the RNA polymerase σ^{38} subunit, is reduced in otsA mutants of E. coli defective in the synthesis of trehalose (1). One possible explanation is that trehalose activates transcription by Er^{38} RNA polymerase. In this study, we have demonstrated that trehalose enhances transcription in vitro by purified RNA polymerase and the optimal concentrations of trehalose for the maximal transcription by Er^{38} and σ^{38} are around 0.5 and 1.0 M, respectively (Fig. 1B). This optimal trehalose concentration for Er^{38} transcription is in good agreement with the intracellular concentration of trehalose under nutrient-starved or high-osmolarity conditions (2, 30).

In the presence of high concentrations of trehalose, the minimal concentration of σ^{38} for maximal transcription by a fixed amount of core enzyme was approximately one-fifth the level in the absence of trehalose (Fig. 2), indicating that trehalose activates σ^{38}-dependent gene transcription by more than fivefold. Since more than a twofold enhancement was observed for both the formation of Er^{38} (Fig. 3) and transcription initiation by Er^{38} holoenzyme (Fig. 4), we conclude that trehalose stimulates both the formation and activity of Er^{38} holoenzyme, leading to more than fivefold stimulation of overall transcription.

The high concentration of trehalose may stabilize the conformation of either σ^{38} or core enzyme in assembly-competent states, thereby leading to enhancement of the formation of Er^{38} holoenzyme. Trehalose may also stabilize the conformation of assembled Er^{38} holoenzyme, resulting in reduction of the dissociation of Er^{38} holoenzyme. The increased level of Er^{38} becomes significant when the concentration of σ^{38} is less than that of core enzyme (Fig. 3). Since the maximal molar ratio of σ^{38} to core polymerase is about 0.3 in stationary-phase E. coli cells (11, 12), trehalose may stimulate the formation in vivo of Er^{38} holoenzyme. In addition, transcription initiation by Er^{38} holoenzyme increases in the presence of trehalose by enhancing either the formation of closed promotor complexes or the isomerization of closed to open complexes (Fig. 4).

We previously showed that selective transcription of osmY promoter by Er^{38} increased by adding high concentrations of potassium glutamate (3) and by using template DNA with low superhelical density (17). Under stress conditions, the intracellular concentration of cytoplasmic components changes markedly, including those of potassium glutamate and trehalose. In parallel, the configuration of the nucleoid changes as DNA superhelicity decreases (17). In this study, we examined the combined effects of trehalose and either potassium glutamate (a cytoplasmic factor) or DNA superhelicity (a nuclear factor). Maximal transcription of σ^{38}-dependent osmY by Er^{38} was achieved by adding high concentrations of either potassium glutamate or trehalose. The additive effect of trehalose and potassium glutamate was observed only at low concentrations. In contrast, the optimal trehalose concentration for maximal transcription was virtually independent of the superhelical density of template DNA (Fig. 6). Furthermore, even at the trehalose concentrations giving maximal transcription, additional transcription enhancement was observed with DNA with low superhelicity. We then conclude that the effects of trehalose and low DNA superhelicity are additive with respect to the enhancement of transcription by Er^{38} (Fig. 6). Taking all of the previous and present results together, we propose that selective transcription of stress-response and stationary-growth-phase-specific genes by Er^{38} holoenzyme takes place even in the presence of constant levels of σ^{38}, provided the intracellular environment changes favorable for utilization of σ^{38}, such as high concentrations of potassium glutamate (3) or trehalose (this report) or low superhelicity of templated DNA (17), are present.

From the systematic attempt to set up an in vitro transcription system of stress-response and stationary-phase-specific genes, it becomes clear that care should be taken to use reaction mixtures mimicking the cytoplasmic compositions and nucleotide structure present in bacterial cells under stress conditions or in the stationary phase.

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FIG. 6. Effect of trehalose on in vitro transcription directed by templates with different superhelical densities. In vitro transcription was carried out with 0.1 pmol each of closed circular lacUV5 (A and B) or osmY (C and D) template with low or high superhelical densities, which were prepared from Escherichia coli cells at 4 and 24 h, respectively, after inoculation of the overnight cultures into fresh media and with various amounts of reconstituted Er^{70} (B and D) holoenzymes under the standard single-round assay conditions, except that trehalose was added at the indicated concentrations. Transcripts were analyzed by 8 M urea–PAGE (4% polyacrylamide), and gels were analyzed with a BAS-2000-II Bio-Image analyzer (Fuji). The maximum level of transcription for each template is set at 100% (lacUV5-Er^{70} [A], stationary-phase DNA, 0.7 M trehalose, 2,161 arbitrary units measured with Bio-Image analyzer; lacUV5-Er^{38} [B], stationary-phase DNA, 1.0 M trehalose, 539 arbitrary units; osmY-Er^{70} [C], log-phase DNA, 0.5 M trehalose, 762 arbitrary units; osmY-Er^{38} [D], stationary-phase DNA, 1.0 M trehalose, 3,696 arbitrary units). The data represent the averages of three independent experiments.


