Comparison of the Promoter Selectivity between Two Sigma Factors, σ^{70} and σ^{38}, of *Escherichia coli* RNA Polymerase

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Abbreviations

ATP	adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
CBB	coomassie brilliant blue
CTP	citidine 5'-triphosphate
DEAE	diethylaminoethyl
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diaminetetraacetate
GTP	guanine 5'-triphosphate
HPLC	high perhormance liquid chromatography
IPTG	isopropyl-b-D-thiogalactopyranoside
KCl	potassium chrolide
LB	Luria broth
NaCl	sodium chloride
nt	nucleotide(s)
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
UTP	uridine 5'-triphosphate

Summary

RNA polymerase core enzyme with the subunit structure of $\alpha_2\beta\beta'$ (E) is functionally differentiated into different forms of holoenzyme by interaction with one of multiple molecular species of σ subunit, the promoter recognition subunit. Up to now, six species of σ subunit have been identified in *Escherichia coli*. In order to understand the switching mechanism(s) of transcription by σ replacement in response to changes in growth conditions, such as those during growth phase change or under various stress responses, I compared the functional specificity between two sigma factors, σ^{70} (the major σ at exponentially growing phase) and σ^{38} (the essential σ at stationary growth phase).

At first, I compared the core enzyme-binding affinity and the promoter-binding activity between two σ subunits by gel filtration column chromatography or by titration of the concentration of σ required for the maximum transcription in the presence of a fixed amount of core enzyme. The core enzyme-binding affinity of σ^{38} was found to be less than half the level of σ^{70}. In addition, the holoenzyme concentration required for the maximum transcription of a fixed amount of templates was higher for $E\sigma^{38}$ than for $E\sigma^{70}$. Because the intracellular concentration of σ^{38} is not higher than that of σ^{70} even after prolonged starvation in the stationary phase, these results suggest that the selective transcription of stationary-specific genes by $E\sigma^{38}$ holoenzyme may require either a specific reaction condition(s) or a specific factor(s) which enhances either σ^{38} binding to core enzyme or $E\sigma^{38}$ binding to promoters.

Next, I carried out a systematic analysis of the effect of cellular factors, which vary depending on the cell growth conditions, including salt species, salt concentration, trehalose concentration, and DNA superhelicity, on the promoter

recognition by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes. The effects of potassium acetate and potassium glutamate, the natural solutes which accumulate in *E. coli* in response to the increased extracellular osmolarity, were examined in *in vitro* transcription directed by osmo-regulated promoters (*osmB* and *osmY*). The *osmB* and *osmY* transcription level increased gradually up to 300 to 400 mM of potassium glutamate but only when they were transcribed by $E\sigma^{38}$. In contrast, transcription at these promoters by $E\sigma^{70}$ decreased with increase in potassium glutamate concentration, indicating that $E\sigma^{38}$ RNA polymerase itself monitors the intracellular salt concentration and changes its promoter recognition properties.

In the stationary growth-phase and under high osmolarity stress conditions, the intracellular concentration of trehalose increases. I then examined the effect of trehalose concentration on *in vitro* transcription by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes. The optimum trehalose concentration for maximum transcription by $E\sigma^{38}$ was high, *i.e.*, around 0.7 to 1.2 M. In contrast, the optimum trehalose concentration for maximum transcription by $E\sigma^{70}$ was lower, *i.e.*, around 0.5 to 0.7 M. This enhancement of $E\sigma^{38}$ activity by high concentrations of trehalose was found to be due to the stimulation or stabilization of $E\sigma^{38}$ holoenzyme formation.

The superhelicity of chromosomal DNA in bacterial cells decreases in the stationary growth-phase and/or under the nutrient starvation conditions. I then examined the effect of DNA superhelicity on *in vitro* transcription by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes. The optimum superhelical density for maximum transcription by $E\sigma^{38}$ was low, *i.e.*, around 0 to -0.03, whereas $E\sigma^{70}$ required high levels of the DNA superhelicity. Moreover, the optimum superhelical density for maximum transcription *in vitro* by $E\sigma^{38}$ was almost the same as that of plasmids prepared from stationary-phase *E. coli* cells. The enhancing effects of high potassium glutamate concentration and low DNA superhelicity, and those of high trehalose concentration and low DNA superhelicity were

additive on transcription by $E\sigma^{38}$. However, the effect of potassium glutamate and trehalose were not additive.

Taken all these results together, I propose the switching mechanism of RNA polymerase specificities as follows: i) transcription by $E\sigma^{38}$ is specifically enhanced under high concentrations of potassium glutamate or high concentrations of trehalose; ii) the low superhelicity of chromosomal DNA provides templates suitable for $E\sigma^{38}$. These specific conditions that enhance $E\sigma^{38}$ activities are in good agreement with the intracellular situation in *E. coli* cells growing under high osmolarity or starved conditions.

Introduction

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 (reviewed in Ishihama, 1988; Ishihama, 1991). 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 competition must take place between promoters for binding a small number of RNA polymerase molecules. Among about 4,000 genes on the *E. coli* genome, about 1,000 genes are expressed at various levels in exponentially growing cells under laboratory culture conditions, *i.e.*, at 37 °C and with aeration (reviewed in Ishihama, 1991; Helmann and Chamberlin, 1988). The rest of genes is considered to be expressed under various stress conditions that *E. coli* meets in nature (reviewed in Magasanik, 1982; Neidhardt *et al.*, 1984; Kolter *et al.*, 1993; Hengge-Aronis, 1993). For instance, a set of stress-response genes are expressed when cells stop growing at stationary phase (reviewed in Kolter *et al.*, 1993; Hengge-Aronis, 1993). Transcription of at least some of these stationary phase-specific genes is catalyzed by RNA polymerase holoenzyme containing σ^{38} (the *rpoS* gene product) (Mulvey and Loewen, 1989; Lange and Hengge-Aronis, 1991; Tanaka *et al.*, 1993; Nguyen *et al.*, 1993; reviewed in Loewen and Hengge-Aronis, 1994). In addition, the modification of core enzyme is considered to be involved in stationary-specific transcription regulation (Ozaki *et al.*, 1991; Ozaki *et al.*, 1992).

Promoters from the stationary-specific genes, however, do not have a single consensus sequence (Tanaka *et al.*, 1993; Nguyen *et al.*, 1993; Kolb *et al.*, 1995; Tanaka *et al.*, 1995). 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, from the experimental results herein described,, I propose a hypothesis that each stationary-specific promoter carries a specific sequence which 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. My effort has been focussed to identify specific conditions or factors required for transcription of each stationary-specific gene by $E\sigma^{38}$ holoenzyme. Along this line, I analyzed effects of two cytoplasmic factors, *i.e.*, the species and concentrations of salts and the concentration of trehalose, and one chromosomal factor, *i.e.*, the DNA superhelicity on promoter recognition by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes, because these factors are known to change under stress conditions.

Under high osmolarity conditions, *E. coli* increases its internal solute concentration to avoid plasmolysis. A rapid increase in intracellular potassium ion (K^+) is the primary response of *E. coli* to osmotic stress; the intracellular glutamate concentration also increases to balance the increase in K^+ concentration (Richey *et al.*, 1987). Upon the increase in potassium glutamate, the transcription of *proU*, which encodes glycine betaine transport system (Gowrishankar, 1985), is enhanced *in vitro* (Ueguchi and Mizuno, 1993) and *in vivo* (Sutherland *et al.*, 1986). The increase of glycine betaine and trehalose substitute for the transient increase in potassium glutamate (Cayley *et al.*, 1992).

On the other hand, the stationary-phase response involves drastic changes in cellular physiology and morphology, structural changes in the cell envelope, an altered membrane composition, and alteration in DNA supercoiling and compactness (reviewed in Siegele and Kolter, 1992). Depending on the medium composition, stationary-phase cells produce various storage compounds such as glycogen and polyphosphates (Preiss, 1989) and protective substances such as trehalose (Hengge-Aronis *et al.*, 1991). Trehalose, a non-reducing disaccharide of glucose, is considered to play an important role for protection against environmental stresses such as

desiccation, high osmolarity, frost, and heat in both prokaryotic and eukaryotic organisms (Kaasen *et al.*, 1992; reviewed in Strørm and Kaasen, 1993). The intracellular concentration of trehalose is known to change depending on the cell growth conditions (Welsh *et al.*, 1991; Cayley *et al.*, 1991; reviewed in Strøm and Kaasen, 1993). For instance, high osmolarity and nutrient starvation induce an increase in the intracellular concentration of trehalose in *E. coli* cells (Welsh *et al.*, 1991; Cayley *et al.*, 1991; reviewed in Strørm and Kaasen, 1993).

In addition to changes in the cytoplasmic composition, the state of bacterial chromosome (or nucleoid) also varies depending on the cell growth conditions. DNA superhelicity is one component which changes depending on the cell growth conditions. For instance, nutrient downshift and stationary growth-phase lead to a decrease in the DNA superhelical density (Balke and Gralla, 1987; Jaworski *et al.*, 1991; reviewed in Drlica, 1992) while high osmolarity leads to an increase in the superhelicity (Higgins *et al.*, 1988; Hsieh *et al.*, 1991).

Until now, the relationship between the changes of these intracellular conditions and the changes of gene expression patterns has not been examined. In order to reveal the relationship, I examined in this study possible influence of these factors on the activity and specificity of two sigma factors, σ^{70} and σ^{38}, in particular focussing the following activities: i) the core-binding activity; ii) the promoter recognition activity; iii) the effect of the species and concentrations of salts; iv) the effect of trehalose concentration; and v) the effect of DNA conformation. The results show that the selectivity for osmoregulated and/or stationary-specific gene promoters by $E\sigma^{38}$ increases concomitantly with the increase in potassium glutamate concentration, the increase in trehalose concentration, and the decrease in DNA superhelicity. All these conditions enhancing the $E\sigma^{38}$ activity are close to the intracellular situation in *E. coli* cells observed under high osmolarity and/or in stationary growth phase. These results suggest that the

changes of intracellular conditions play a role in population switching of the functional RNA polymerase.

Materials and Methods

(a) Strains, Plasmids and Media

Luria browth (LB) [1% Bacto-tryptone (Difco Lab.), 0.5% Bacto-yeast extract (Difco Lab.), 0.5% NaCl, pH 7.0] were used as a culture media for *E. coli*. *E. coli* strains, DH5α and XLI-Blue, were used as competent cells for plasmid maintenance and *E. coli* strains BL21(λDE3) was used for over-expression of σ subunits. *E. coli* W3350 was used as a source of purification of core RNA polymerase. Plasmid pKK223-3 (Pharmacia) was used as a source of the fragment containing *rrnB* transcription terminator sequence. pBluescript II SK+ (Stratagene) phagemid was used for cloning of DNA fragment containing promoter sequences.

(b) Chemicals and Enzymes

All chemicals without annotation were purchased from Wako Pure Chemicals. [α-^{32}P]UTP were purchased from Amersham. DEAE-cellulose (DE52), and phosphocellulose (P11) were purchased from Whatman. A prepackaged Mono-Q column, a prepackaged Superose 6 column were purchased from Waters and Pharmacia, respectively. A prepackaged G3000SW gel filtration column and HW-65 were purchased from Tosoh. Protein A-Sepharose 6MB was purchased from Pharmacia. Restriction enzymes, modification enzymes and DNA ligation kit were purchased from Takara. Bio-Rad protein assay kit was purchased from Bio-Rad. Fluorokit PRO-1 protein staining system was purchased from Promega.

(c) Buffers

Buffer A used for purification of RNA polymerase contained 10 mM Tris-HCl (pH 7.8 at 4 °C), 0.1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM DTT. Buffer B used for purification of RNA polymerase was buffer A

containing 5 % glycerol. Buffer C used for purification of RNA polymerase contained 10 mM Tris-HCl (pH 7.8 at 4 °C), 0.1 mM EDTA, 0.1 mM DTT and 5 % glycerol. Lysis buffer used for purification of σ subunits contained 50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM EDTA and 0.1 M NaCl. Triton buffer used for purification of σ subunits contained 50 mM Tris-HCl (pH 8.0 at 4 °C), 10 mM EDTA, 0.1 M NaCl and 0.5 % Triton X-100. TGED buffer contained 50 mM Tris-HCl (pH 7.6 at 4°C), 5 % glycerol, 0.1 mM EDTA and 0.1 mM DTT. Storage buffer for preservation of purified RNA polymerase and σ subunits contained 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.2 M KCl and 50 % glycerol.

(d) Promoters and Templates

Truncated *lac*UV5 template, 205 bp *Eco*RI-*Eco*RI fragment, was prepared as described previously (Kajitani and Ishihama, 1983). *Hin*dIII-*Eco*RI fragment of 156 bp carrying *katE* promoter was prepared from plasmid pKTE31 (Kusano *et al.*, 1996). 796 bp *Pst*I-*Eco*RI *fic* fragment was prepared as described Tanaka *et al.* (1993). *Bam*HI-*Kpn*I fragment of 287 bp in length carrying *alaS* promoter was prepared as described Nomura *et al.* (1986). *Pvu*II fragment of 607 bp carrying *osmB* promoter, *Pst*I-*Cla*I fragment of 646 bp or *Hin*cII fragment of 505 bp carrying *osmY* promoters and *Pst*I-*Hae*I fragment of 834 bp or *Eco*RI-*Hin*dIII fragment of 462 bp carrying *proU* promoters were prepared as described Ding *et al.* (1995). These truncated DNA templates produced *in vitro* transcripts of 63, 53, 257, 169, 270, 242 and 207 nt in length, respectively.

Plasmids pBSOY containing *osmY* promoter and pBSLU containing *lac*UV5 promoter were constructed as described Kusano *et al.*, (1996): plasmid pBluescriptII SK$^+$ was linealized with *Sac*I and blunt-ended using Klenow DNA polymerase; a 783-bp *Hin*cII-digested fragment containing *rrnB* terminator, isolated from pKK223-3, was ligated into pBluescriptII SK$^+$ at the blunt-ended *Sac*I site to yield plasmid pBST. A 261-bp *Hin*cII-*Eco*RV

fragment containing *osmY* promoter, isolated from pDY.1.4 (Yim and Villarejo, 1992), was inserted into pBST between *Hinc*II and *Eco*RV to yield pBSOY. On the other hand, pBSLU was constructed after insertion of the 205 bp *lac*UV5 fragment from pKB252 (Kajitani and Ishihama, 1983) into pBST between *Hinc*II and *Eco*RI. These circular DNA templates produced *in vitro* transcripts of 357 (*lac*UV5 promoter), 346 (*osmY* promoter) and 108 (primer RNA for ColE1) nt, respectively, in length. These template plasmids were prepared from transformed DH5α cells using QIAGEN plasmid kit (QIAGEN).

(e) Purification of Core RNA Polymerase

E. coli W3350 were grown at 37 °C with aeration to late log-phase. Cells was harvested by centrifugation from the culture, suspended in 2 volumes of buffer A containing 0.1 mM PMSF, and disrupted with French Press. Crude extract was obtained after removing particulate materials by centrifugation at 100,000 x g for 90 min at 4 °C. RNA polymerase (RPase) was precipitated by the addition of 6 % volumes of 10 % Polymin P. Precipitates were recovered by centrifugation (15,000 x g for 15 min at 4 °C) and extracted with buffer B containing 0.2 M NaCl. After extraction, precipitates were recovered by centrifugation and extracted with buffer B containing 0.5 M NaCl. Again, precipitation and extraction with buffer B containing 1 M NaCl was done. This eluate with 1 M NaCl was precipitated by the addition of 1.5 volumes of saturated ammonium sulfate (pH 8.0 at 4 °C) followed by centrifugation, and dialyzed against buffer B containing 0.1 M NaCl. The sample was loaded onto a DEAE-cellulose column equilibrated with buffer B containing 0.1 M NaCl. After washing the column with 3 volumes of equilibrated buffer, proteins were eluted with a 5 volumes of linear gradient of NaCl from 0.1 M to 0.5 M in buffer B. Each fraction was analyzed by SDS-7.5 % PAGE. Pooled RPase fraction was precipitated with ammonium sulfate and dialyzed against buffer C containing 55 mM KCl. The sample was loaded onto a phospho-cellulose

column equilibrated with buffer C containing 55 mM KCl. After washing the column with 5 volumes of equilibrated buffer, proteins were eluted with a 5 volumes of linear gradient of KCl from 55 mM to 700 mM in buffer C. Each fraction was analyzed by SDS-7.5 % PAGE. Pooled RPase fraction was precipitated with ammonium sulfate and dialyzed against buffer B containing 50 mM NaCl. The sample was loaded onto a HW-65 gel filtration column equilibrated with buffer B containing 50 mM NaCl and proteins were eluted with the same buffer. Each fraction was analyzed by SDS-PAGE. After gel filtration purification, 3 times of phospho-cellulose chromatography was done for complete removal of holoenzymes. After purification, I estimated the protein concentration of RNA polymerase by Bio-Rad protein assay kit and examined holoenzyme contamination by standard *in vitro* transcription assay except for the use of 10 times more enzyme and templates. After examination of non-contamination with holoenzyme, core RNA polymerase was dialyzed against storage buffer and stored at -80 °C until use.

(f) Purification of Sigma Subunits

Plasmid pGEMD (Igarashi and Ishihama, 1991) carrying the *rpoD* gene under the T7 promoter was transformed into *E. coli* BL21 (λDE3). At mid-log phase, transformed BL21 (λDE3) grown at 37 °C was induced for over-expression of σ^{70} with 0.5 mM IPTG for 60 min. Cells were harvested by centrifugation from the culture, suspended in 2 volumes of lysis buffer containing 0.1 mM PMSF. Cells were disrupted with sonication in the presence of 100 μg/ml of lysozyme and 0.05 % of sodium deoxycholate (DOC). Inclusion body was obtained by centrifugation at 28,000 x g for 10 min at 4 °C. Inclusion body was washed twice by suspending with Triton buffer, and washed inclusion body was solubilized into TGED buffer containing 6 M guanidine-HCl. Solubilized sample was dialyzed against TGED containing 0.2 M NaCl for renaturation of σ^{70}. After renaturation, the sample was load onto a prepackaged Mono-Q column equilibrated with TGED

containing 0.2 M NaCl. After washing the column with 2 times of equilibration buffer, proteins were eluted with 4 times volumes of gradient of NaCl from 0.1 to 0.5 M in TGED buffer. Each fraction was analyzed by SDS-10 % PAGE and pooled σ^{70} fractions were precipitated with the 1.5 times volumes of saturated ammonium sulfate. Precipitates were collected by centrifugation and dialyzed against TGED containing 0.1 M NaCl. The sample was load onto G3000SW gel-filtration column equilibrated with TGED containing 0.1 M NaCl and proteins were eluted with same buffer. Each fraction was analyzed by SDS-10 % PAGE. After purification, I estimated the protein concentration of σ^{70} by Bio-Rad protein assay kit, and σ^{70} was dialyzed against storage buffer, stored at -80 °C until use. σ^{38} was also over-expressed in *E. coli* BL21(λDE3) carrying pETF (Tanaka *et al.*, 1993) and purified by the use of the same method as for σ^{70}.

(g) Separation of RNA Polymerase using Gel Filtration-HPLC

Core enzyme (50 pmole) and various amounts of σ^{70} and σ^{38} subunit were mixed in 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 50 % glycerol, and incubated for 10 min at 30 °C to form holoenzymes. The mixtures were fractionated by gel filtration-HPLC using a Superose 6 column. Proteins were eluted with 10 mM Tris-HCl (pH 7.8 at 4 °C), 0.1 mM DTT, 0.1 mM EDTA, 5 % glycerol and 0.2 M NaCl. Each fraction was concentrated using 10 % TCA-0.025 % DOC and analyzed by SDS-PAGE. The gels were stained with coomassie brilliant blue and scanned with a Ultroscan-XL laser densitometer (LKB) .

(h) In vitro *Single-round Transcription System*

Single-round mixed transcription by holoenzyme was carried out under the standard conditions described previously (Igarashi and Ishihama, 1991). In brief, a mixture of template DNA and RNA polymerase reconstituted from

purified core enzyme and either purified σ^{70} or σ^{38} 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 Mg-acetate, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT and 25 µg/ml of BSA. A 15 µl mixture of substrate and heparin was then added to make the final concentrations: 160 µM each of ATP, GTP and CTP; 50 µM UTP; 2 µCi [α-^{32}P]UTP; and 200 µg/ml of heparin. After 5 min incubation at 37 °C, transcripts were precipitated 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 BAS2000 Image analyzer (Fuji).

(i) 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) in 100 µl of a reaction mixture, which contained 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine and 0.01% BSA. The enzyme reaction was carried out at 37 °C for 5 h in the presence of various concentrations of ethidium bromide (0 to 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 (1975). The mean superhelical density (σ) was calculated by the equation ($\sigma = 10\Delta Lk/N$, where N represents the number of the base pairs of plasmid DNA).

(j) Immunoprecipitation of RNA polymerase

Core enzyme (20 pmole) and various amounts of σ^{38} subunit were mixed in 50 mM Tris-HCl (pH 7.8 at 37 °C), 3 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 µg/ml of BSA and various concentration of trehalose (Hayashibara), and incubated for 10 min at 30 °C to form

holoenzymes. After the incubation, 1/100 volumes of anti-serum against α subunit of RNA polymerase was added to reaction mixture, and incubated for 2 hr at 4 °C. After the immuno-reaction, 1/5 volumes of 50 % (v/v) of Protein A-Sepharose 6MB containing 0.6 % Triton X-100 was added and incubated for 3 hr at 4 °C. After the incubation, the mixtures were washed at 4 °C for twice by using the reaction buffer containing 0.1 % Triton X-100. After the washing, proteins were subjected by SDS-15 % PAGE. The gels were stained with Fluorokit PRO-1 protein staining system and analyzed with a Fluorimager SI system (Molecular Dynamics).

Results

Part I: Core-binding and Promoter Recognition Activities of σ^{70} and σ^{38}

(a) Difference in the Core Enzyme-Binding Activity between Two σ Factors

Both σ^{70} and σ^{38} were over-produced in *E. coli* and purified to apparent homogeneity as determined by Coomassie brilliant blue staining of the proteins separated by SDS-PAGE (Fig. 1). On the other hand, core enzyme was purified from exponentially growing *E. coli* cells by repeated chromatography on phosphocellulose (Fig. 1). The affinity of the two species of σ subunit to core enzyme was examined by measuring two parameters, *i.e.*, (i) the saturation level of σ subunit required for the maximum level of holoenzyme formation from a fixed amount of core enzyme, and (ii) the saturation level of σ subunit to achieve the maximum level of *in vitro* transcription by a fixed amount of core enzyme.

First, the affinity of the two σ subunits to core enzyme was compared by directly measuring the holoenzyme formation. For this purpose, I mixed a fixed amount of core enzyme and various amounts of either σ^{70} or σ^{38} at 30 °C and the mixtures were fractionated by gel filtration-HPLC on a Superose 6 column. The σ to core enzyme ratio was measured for the peak fraction of RNA polymerase (Fig. 2). The core enzyme was saturated with σ^{70} at the input molar ratio between 2 and 3, while the saturation of the same amount of core enzyme with σ^{38} required at least two-fold more σ^{38} protein than σ^{70}. The observed difference might be due to a difference in the amount of active σ subunit in the σ preparations used. To test this possibility, the second-cycle assay was performed using the unassembled σ subunits recovered after the first-cycle of binding assay. The core-binding patterns of both σ subunits

were essentially the same as those of the first-cycle experiments (data not shown).

(b) Difference in the Promoter Recognition Activity between Two σ Factors

The level of σ subunit required for maximum transcription by a fixed amount of core enzyme was also compared between the two σ subunits. For this purpose, single-round transcription was carried out using a fixed amount of core enzyme and various amounts of σ subunits. The core enzyme was saturated by adding only 2-fold molar excess of σ^{70} subunit, *i.e.*, 2 pmoles σ^{70} per pmole core as measured using *alaS* promoter (Fig. 3), and the σ saturation level was essentially the same for all the σ^{70}-dependent promoters analyzed (data not shown). In contrast, the molar concentration of σ^{38} required for the maximum transcription of *fic*, *katE* and *lac*UV5 was 10, 8 and 4 pmoles, respectively, per pmole core enzyme (Fig. 3). Since the difference in core enzyme-binding activity between the two σ subunits is only two-fold (see above), it can not explain the observed difference in the σ^{38} saturation for maximum transcription. Instead, the transcription assay indicates that the promoter recognition activity is also weaker for σ^{38} than σ^{70}.

(c) Effect of RNA Polymerase Concentration on the Promoter Selectivity

Since both the core enzyme-binding and the promoter recognition activities under our standard assay conditions for *in vitro* transcription were lower for $E\sigma^{38}$ than $E\sigma^{70}$, the promoter selection pattern was analyzed with use of the increasing concentration of RNA polymerase. Figure 4 summarizes the effect of enzyme/promoter ratio on the relative transcription level by two holoenzymes. At low enzyme concentrations, *lac*UV5 was transcribed preferentially by $E\sigma^{70}$ but $E\sigma^{38}$ started to transcribe *lac*UV5 at high enzyme concentrations. Likewise, *fic* was transcribed by both $E\sigma^{70}$ and $E\sigma^{38}$ at low

enzyme concentrations but $E\sigma^{38}$ transcribed better than $E\sigma^{70}$ at high enzyme concentrations.

The *katE* promoter was always transcribed better with $E\sigma^{38}$ than $E\sigma^{70}$, but the $E\sigma^{38}/E\sigma^{70}$ activity ratio increased from 1.3 at 0.5 pmole (per 0.1 pmole promoter; promoter/enzyme ratio of 5) to about 4 at the holoenzyme amounts higher than 1 pmole. For all the promoters examined, the transcription activity by $E\sigma^{38}$ increased at high protein concentrations, supporting the notion that either the affinity of σ^{38} to core enzyme is weaker than that of σ^{70} or the affinity of $E\sigma^{38}$ to promoters is weaker than that of $E\sigma^{70}$. These observations altogether support the concept that the classification of promoters with respect to σ selectivity varies depending on the concentrations of individual holoenzyme species.

Part II: Effect of Potassium Glutamate on Transcription by $E\sigma^{70}$ and $E\sigma^{38}$

(a) Transcription of Osmoregulated Promoters

In order to identify which σ subunit, σ^{70} or σ^{38}, is responsible for transcription initiation from the osmoregulated promoters of *osmB* and *osmY*, an *in vitro* transcription was carried out using reconstituted holoenzymes, $E\sigma^{70}$ and $E\sigma^{38}$, and various truncated DNA templates carrying the test promoters (Fig. 5A). The DNA fragments used carried the regions required *in vivo* for response of these promoters to high osmolarity, *i.e.*, the heptanucleotide sequence located upstream of the *osmB* -35 signal (Jung *et al.*, 1990) and the -36 to +1 *osmY* sequence responsible for both osmoregulation and stationary cell growth (Yim *et al.*, 1994).

Under the standard reaction conditions (50 mM NaCl), all the promoters including *osmB* and *osmY* were transcribed by $E\sigma^{70}$ (Fig. 5B). Judging

from the sizes of transcripts produced by the reference promoters (63 nt *lac*UV5 RNA in Fig. 5B and others described in the legend of Fig. 4), the major products from the test templates were of the sizes expected for RNAs initiated from the *in vivo* start sites of the respective promoters-estimated using *in vivo* RNA for *osmY* (Yim *et al.*, 1994), *osmB* (Jung *et al.*, 1990) and *proU* (Gowrishanker, 1989; May *et al.*, 1989) (illustrated in Fig. 5A). In contrast, when $E\sigma^{38}$ was used the *osmB* and *osmY* promoters were also transcribed but *lac*UV5 and *proU* were not. The transcription levels of *osmB* and *osmY* were higher using $E\sigma^{38}$ even at this salt concentration.

To confirm the transcription start sites, the effect of template cleavage was analyzed on the size of specific transcripts (Fig. 5B). Using pairs of *osmY* and *proU* templates truncated with different restriction enzymes (Fig. 5A), the specific *osmY* and *proU* transcripts migrated on PAGE as expected from the restriction map in this region (Fig. 5B, templates *osmY* and $osmY^{H}$; templates *proU* and $proU^{E}$). The major promoters recognized *in vitro* by reconstituted RNA polymerase holoenzymes were identical to those operating *in vivo*. These observations indicated that $E\sigma^{38}$ can directly recognize both *osmB* and *osmY* promoters in the absence of additional factors and that σ^{38}-dependent transcription may account for their response to the stationary phase signal.

In addition to the expected transcripts (242 nt *osmY* RNA; 270 nt *osmB* RNA; and 207 nt *proU* RNA; Fig. 5B), additional RNA species were observed among the *in vitro* transcription products from these truncated DNA templates. The additional slowly migrating RNA species transcribed from 646 bp *osmY* template (Fig. 5B, template *osmY*, lane 1) was not detected when the *osmY* upstream region was truncated by cutting with *Hin*cII (Fig. 5B, template $osmY^{H}$). This RNA must therefore be transcribed from putative upstream promoter, which occupied at about 240 bp upstream region from major promoter region (Yim and Villarejo, 1992), recognized only by $E\sigma^{70}$ (Fig.

5B, template *osmY*; compare lanes 1 and 2). The extra-slowly migrating RNA was identified, in addition to the expected 270 nt *osmB* RNA, among transcripts formed from the *osmB* template (Fig. 5B, template *osmB*). This RNA was formed more efficiently by $E\sigma^{70}$ than by $E\sigma^{38}$, suggesting that this is due to additional upstream promoter, preferentially recognized by $E\sigma^{70}$. Also, extra-slowly migrating RNA species were preferentially transcribed from 834 bp *proU* templates by $E\sigma^{70}$ and by $E\sigma^{38}$, and these transcripts were not observed when the 462 bp $proU^{E}$ promoter fragment was used. These transcripts were produced from *proU* (P1) promoter identified by Manna and Gowrishankar (1994).

(b) Effect of Salt Concentration on Transcription

High ionic strength is characteristic of the intracellular environment of cells exposed to hyperosmotic stress. The effect of high salt concentration on *in vitro* transcription from the osmoregulated promoters was examined. The standard transcription reaction buffer used in this study contained 50 mM NaCl, that is the optimum concentration for transcription from the promoters for most of the genes expressed under exponential cell growth. This is confirmed by the experiment shown in Fig. 6, in which the concentrations of KCl were varied from 50 to 400 mM. For the test promoters (*osmB* and *osmY*) and the control promoters (*lac*UV5 and *proU*P2), and for both $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes, the level of single-round transcription was maximum between 50 and 100 mM KCl, and then decreased to negligible levels above 300 mM. The effect of NaCl concentration variation was essentially the same as that of KCl (data not shown).

In *E. coli*, the natural solute which accumulates in the cell in response to increased extracellular osmolarity is potassium glutamate. Acetate can substitute for glutamate as a natural counter ion for K^+. In sharp contrast to the KCl effect, the influence of variations in potassium glutamate concentration

differed between the promoters analyzed and depended on the holoenzyme used (Figs. 6 and 7). The transcription level increased gradually up to 300-400 mM for *osmB* and *osmY* but only when $E\sigma^{38}$ was used (Fig. 6). Thus, the relative efficiency of transcription by the two holoenzymes is different depending on the species and concentrations of salts added (Figs. 6 and 7). For example, the ratios of $E\sigma^{38}$:$E\sigma^{70}$ activity for *osmB* and *osmY* are 2.6 and 1.2 at 50 mM potassium glutamate but rise to 18 and 17, respectively, at 400 mM.

In contrast, the pattern of *lac*UV5 transcription in the presence of potassium glutamate *in vitro* was essentially the same as that observed with KCl. The major *proU* promoter (P2) was transcribed by only $E\sigma^{70}$, irrespective of the species or concentrations of salts analyzed. The maximum transcription level was, however, obtained at 200 mM potassium glutamate, higher than the optimum KCl concentration (about 50 mM). The optimum concentration of potassium acetate was between those of chloride and glutamate (Fig. 6). Since these experiments were carried out using the reconstituted holoenzyme free from other contaminating proteins, it appears that RNA polymerase itself is involved in the osmoregulation of transcription, acting as a sensor of the species and concentrations of salts.

Part III: Effect of Trehalose on Transcription by $E\sigma^{70}$ and $E\sigma^{38}$

(a) Effect of Trehalose Concentration on in vitro *Transcription*

σ^{38} plays a major role in transcription of a set of genes essential for survival in stationary-phase (reviewed in Hengge-Aronis, 1993). The intracellular concentration of σ^{38} in the stationary-phase cells of *E. coli* strain MC4100 was, however, only 30% the level of σ^{70} subunit (Jishage and Ishihama, 1995). As an effort to identify factors and conditions affecting

preferential utilization of σ^{38} in stationary-phase cells, I examined differential effect of trehalose on transcription *in vitro* by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes, because the intracellular concentration of trehalose is known to increase sometimes above 1 M in parallel with the increase in σ^{38} (Welsh *et al.*, 1991; Cayley *et al.*, 1991; reviewed in Strøm and Kaasen, 1993).

For this purpose, I used a σ-limiting condition where I could detect activity changes including both σ-binding to core enzyme and holoenzyme-binding to promoters. From the results of σ saturation curve under our standard reaction condition (see Part I; Kusano *et al.*, 1996), the core-to σ ratio added in the in vitro transcription assay was fixed at 1:0.5 and 1:5 for $E\sigma^{70}$ and $E\sigma^{38}$, respectively, which allow approximately 50 % level transcription compared with the maximum level obtained in the presence of excess amounts of σ subunits. At first, *in vitro* single-round transcription assay was carried out using three promoters, *lac*UV5, *fic* and *osmY*, which are all transcribed by both $E\sigma^{70}$ and $E\sigma^{38}$, and in the presence of 0 to 1.2 M trehalose. The gel patterns of transcripts, shown in Fig. 8, and the quantitative analysis data, shown in Fig. 9, indicated that the effect of trehalose concentration on transcription was indeed different between $E\sigma^{70}$ and $E\sigma^{38}$; the optimum trehalose concentration for the maximum transcription by $E\sigma^{38}$ was high for all promoters tested, *i.e.*, trehalose concentration of above 1.2 (*lac*UV5), 1.0 (*fic*) or 0.7 (*osmY*) M, respectively (Fig. 9D-F), while the optimum trehalose concentration for the maximum transcription of these promoters by $E\sigma^{70}$ was lower, *i.e.*, around at 0.5 to 0.7 M (Fig. 9A-C). The levels of transcription by $E\sigma^{38}$ in the presence of 1.2 M trehalose were 2-4 fold higher than those in its absence (Fig. 9D-F). Thus, I concluded that transcription by $E\sigma^{38}$ was specifically enhanced by high concentrations of trehalose.

These results indicated that either the affinity of σ^{38} subunit to core enzyme or the affinity of holoenzyme (Eσ) increases in the presence of trehalose. To confirm this prediction, I analyzed the σ saturation curve for the

maximum transcription by a fixed amount of core enzyme in the absence or presence (1 M) of trehalose. The amount of σ^{70} subunit required for the maximum transcription of *lac*UV5 at 1 M trehalose was about half the amount in the absence of trehalose (Fig. 10A) [this stimulation agrees with the slight increment of σ^{70}-dependent transcription of *lac*UV5 by trehalose (Fig. 9A)]. On the other hand, the molar concentrations of σ^{38} required for the maximum transcription of *fic* and *osmY* at 1 M trehalose were about 5-times less than those in its absence (Fig. 10B and 10C). The results indicate that the enhancing effect of trehalose on transcription is more profound with σ^{38} than σ^{70}. The observed enhancement of transcription by trehalose might be due to either increased affinity of σ^{38} subunit to core enzyme or increased binding of $E\sigma^{38}$ holoenzyme to stationary-specific gene promoters.

(b) Effect of trehalose on the core-binding activity of σ^{38}

As an attempt to identify the step(s) of transcription which was affected by the high concentration of trehalose, the core-binding activity of σ^{38} was directly measured in the presence of various concentrations of trehalose. For this purpose, I mixed a fixed amount of core enzyme and various amounts of σ^{38} at 30 °C in the presence of increased concentrations of trehalose, and RNA polymerase was immunoprecipitated with use of monospecific anti-α subunit polyclonal antibodies, which do not interfere with RNA polymerase assembly. The amount of σ^{38} coprecipitated was measured after SDS-PAGE of antigen-antibody complexes.

At the input molar ratio of σ^{38}/core polymerase of 1 or 2, the holoenzyme level increased concomitantly with the increase in trehalose concentration (Fig. 11), and the level of $E\sigma^{38}$ holoenzyme formed was about 2-fold higher at 1 M trehalose than that in its absence. In the presence of excess amount of σ^{38} (σ^{38}/core ratio of 4), however, the core enzyme was

saturated with σ^{38} even in the absence of trehalose and thus the holoenzyme level stayed constant upon the increase in trehalose concentration (Fig. 11). In contrast to $E\sigma^{38}$ formation, the level of $E\sigma^{70}$ holoenzyme was virtually unaffected upon increase in the trehalose concentration and even at the low level of σ^{70} addition (σ^{70}/core ratio of 1). The results indicated that the high concentration of trehalose stimulates $E\sigma^{38}$ holoenzyme formation and this effect is more pronounced at the low concentration of σ^{38}.

(c) Effect of trehalose on $E\sigma^{38}$ holoenzyme-binding to promoters

Next I analyzed the effect of trehalose on the holoenzyme binding to promoters. For this purpose, I prepared holoenzymes, $E\sigma^{70}$ and $E\sigma^{38}$, by adding excess amount of each σ subunit [thus I could eliminate the effect of trehalose on σ–binding to core enzyme] and measured the level of transcription using increased concentrations of each holoenzyme in the presence and absence of 1 M trehalose. If trehalose enhances holoenzyme binding to promoters, I should observe stimulation of transcription under enzyme limiting conditions. Figure 12 shows the results of *lac*UV5 transcription by $E\sigma^{70}$, and *fic*, *osmY* transcription by $E\sigma^{38}$. The transcription level of *lac*UV5 by $E\sigma^{70}$ was roughly the same between in the presence and absence of 1 M trehalose and using various enzyme concentrations (Fig. 12A). In contrast, the transcription levels of *fic* and *osmY* by $E\sigma^{38}$ were about 2-fold higher in the presence of 1 M trehalose than in its absence (Fig. 12B and 12C). These results indicate that the high concentration of trehalose enhances both the formation of holoenzyme between σ^{38} and core enzyme (2-3 folds) and the formation of promoter complex between $E\sigma^{38}$ holoenzyme and σ^{38}-dependent promoters (2-3 folds), altogether leading to several fold stimulation of transcription.

(d) Non-Additive Effect of Potassium Glutamate and Trehalose on Transcription by $E\sigma^{38}$

Under certain stress conditions such as at high osmolarity, the intracellular concentration of trehalose and potassium glutamate increases simultaneously. The transcription level of high osmolarity-response *osmY* and *osmB* genes by $E\sigma^{38}$ holoenzyme (and the level of *proU* transcription by $E\sigma^{70}$) increases with the increase in potassium glutamate concentration (Ding *et al.*, 1995; and Part II) and trehalose concentration (see Figs. 8 and 9). I then examined the combined effect of trehalose and potassium glutamate on transcription. In the experiments shown in Fig. 13, the trehalose concentration was increased in the presence of low (50 mM) or high (300 mM) concentrations of potassium glutamate. The *lac*UV5 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 pattern was essentially the same between the two promoters, both showing maximum transcription at 0.7 M trehalose]. However, trehalose did not enhance transcription in the presence of 300 mM potassium glutamate, the concentration inhibitory to transcription by $E\sigma^{70}$ (Fig. 13A and 13C).

On the other hand, transcription by $E\sigma^{38}$ was enhanced by trehalose at both low and high concentrations of potassium glutamate (Fig. 13B and 13D). Transcription of *lac*UV5 by $E\sigma^{38}$ is inhibited by 300 mM potassium glutamate but trehalose suppressed this inhibition, giving a significant level of transcription (Fig. 13B). Transcription of *osmY* by $E\sigma^{38}$ is enhanced by adding a high concentration (300 mM) of potassium glutamate (Ding *et al.*, 1995; and Part II). Upon addition of trehalose above 0.5 M, the level of *osmY* transcription at a low concentration (50 mM) of potassium glutamate increased and reached to the maximum level observed in the presence of 300 mM potassium glutamate and increased concentrations of trehalose (Fig. 13D). This level of transcription was achieved by adding 300 mM potassium

glutamate without trehalose (Ding *et al.*, 1995; and Part II). The results suggest that the requirement for high concentrations of potassium glutamate on transcription of *osmY* can be replaced by the increase in trehalose concentration. However, once the maximum level transcription is achieved by the addition of one of the two stimulatory factors, the other factor does not give additive effect.

Part IV: Effect of DNA Superhelicity on Promoter Selectivities of $E\sigma^{70}$ and $E\sigma^{38}$

(a) Effect of DNA Superhelicity on Transcription

σ^{38} plays a major role in transcription of a set of genes essential for survival in stationary-phase and/or nutrient starvation (reviewed in Hengge-Aronis, 1993). Under such starved growth conditions, the superhelicity of chromosomal DNA in bacterial cells is known to decrease (Balke and Gralla, 1987; Jaworski, *et al.*, 1991). I then examined the effect of DNA superhelicity on transcription *in vitro* by two RNA polymerase holoenzymes, $E\sigma^{70}$ and $E\sigma^{38}$. For this purpose, 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 Figure 14, indicated that the effect of DNA superhelicity on transcription was different between $E\sigma^{38}$ and $E\sigma^{70}$. The quantitative analysis data, shown in Figure 15, indicated that the optimum

superhelical density for maximum transcription by $E\sigma^{38}$ was low for all the promoters (*lac*UV5, *osmY* and RNA-I) tested, *i.e.*, the superhelical density of around 0 to -0.03. In general, $E\sigma^{38}$ required lower levels of the DNA superhelicity for maximum transcription than $E\sigma^{70}$. In particular, the activity of $E\sigma^{38}$ is enhanced with the decrease in DNA superhelicity in transcription of stationary-specific promoters.

In contrast, $E\sigma^{70}$ required high-levels of DNA superhelicity for maximum activity. The optimum DNA superhelicity for the maximum transcription of *osmY* promoter by $E\sigma^{70}$ was high (above -0.1) and upon decrease in DNA superhelicity, $E\sigma^{70}$ becomes inactive in transcription of the *osmY* promoter. Transcription of *lac*UV5 and RNA-I by $E\sigma^{70}$ was rather insensitive to the change in DNA superhelicity [these promoters are classified into a group of promoters which are recognized by both $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes (Tanaka *et al.*, 1993; Tanaka *et al.*, 1995)].

(b) Effect of Growth-Phase on DNA Superhelicity

In order to correlate these *in vitro* observations with *in vivo* situations, I next examined the levels of DNA superhelicity for the plasmids that were purified from transformed DH5α cells at various phases grown in LB at 37 °C. The results, shown in Figure 16, 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 which gave the maximum transcription *in vitro* by $E\sigma^{38}$ (see Fig. 15). To confirm this relationship, I examined the template activity in *in vitro* transcription by $E\sigma^{70}$ and $E\sigma^{38}$ for all the templates prepared at various phases of the cell growth. The level of transcription by $E\sigma^{38}$ 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 $E\sigma^{70}$ stayed at constant levels (*lac*UV5 and RNA-I) or rather decreased (*osmY*) with the decrease in DNA superhelicity (data not shown). Thus, I concluded that $E\sigma^{38}$ preferentially transcribes template DNA with the low superhelicity.

(c) Transcription Step Affected by DNA Superhelicity

In order to identify the step(s) of transcription that was affected by the DNA superhelicity, I examined the effect of $E\sigma^{38}$ 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. 17). The results suggest that the affinity of $E\sigma^{38}$ is higher for DNA with low superhelical density than for DNA with high superhelical density.

To confirm this prediction, I measured the rate of open complex formation. The time course of open complex formation by $E\sigma^{38}$ 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 was different, 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).

(d) Additive Effect of Potassium Glutamate and DNA Superhelicity

The level of *osmY* transcription by $E\sigma^{38}$ holoenzyme increases with the increase in potassium glutamate concentration (see Part II). I then examined the effect of potassium glutamate concentration on transcription directed by circular DNA templates with different superhelicity. The patterns of *lac*UV5

and *osmY* transcription by $E\sigma^{70}$ were essentially the same between DNA templates prepared from the exponentially growing and stationary-phase cells, both showing decreased activity concomitantly with the increase in potassium glutamate concentration (Fig. 18, 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. 18, 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 (see Part II). The maximum transcription of DNA with low superhelicity was observed between 200 to 300 mM concentrations of potassium glutamate, while using DNA with high superhelicity the maximum transcription was observed above 300 mM potassium glutamate (Fig. 18D). Thus, the requirement for high concentrations of potassium glutamate is partly replaced by the decrease in DNA superhelicity.

(e) Additive Effect of DNA Superhelicity and Trehalose on Transcription

$E\sigma^{38}$ holoenzyme prefers template DNA with low superhelicity, in agreement with the decrease in DNA superhelicity in stationary-phase cells (Kusano *et al.*, 1996; and this study). I then examined the effect of trehalose concentration on transcription directed by circular DNA templates with different superhelicity, prepared from the stationary-phase cells. The patterns of *lac*UV5 and *osmY* transcription level by both holoenzymes, $E\sigma^{70}$ and $E\sigma^{38}$, at various concentrations of trehalose were essentially the same between template DNAs from the exponentially growing- and stationary-phase cells (Fig. 19). The optimum trehalose concentration on transcription of circular DNA templates by $E\sigma^{70}$ was around 0.5 to 0.7 M (Fig. 19A and 19C) and that by $E\sigma^{38}$ was 1.0 M (Fig. 19B and 19D). These optimum concentrations of trehalose were nearly the same as those observed using the truncated

promoter templates (see Fig. 9). The results indicated that transcription enhancement by the high concentration of trehalose is independent of the stimulatory effect of DNA superhelicity. I then conclude that the high concentration of trehalose and the low DNA superhelicity in stationary-phase *E. coli* cells in combination lead to the preferential utilization of σ^{38}.

Discussion

(a) Comparison of Core-binding and Promoter Recognition Activities between σ^{70} and σ^{38}

The σ saturation experiments of *in vitro* transcription indicate that the affinity of σ^{38} to core enzyme is lower than that of σ^{70} (see Fig. 3). Direct measurement of the core enzyme-bound σ subunits by gel filtration-HPLC on Superose 6 column indeed showed that the binding affinity of σ^{38} to core enzyme is at least two-fold weaker than that of σ^{70} (see Fig. 2). This was rather unexpected because the intracellular concentration of σ^{38} is not higher than that of σ^{70} even after prolonged starvation in the stationary phase (Jishage and Ishihama, 1995). Growth-coupled replacement of core enzyme-associated σ subunit from σ^{70} to σ^{38} may therefore require an additional factor(s) or an as yet unidentified condition(s). For example, the modification of core enzyme in stationary-phase cells (Ozaki *et al.*, 1991; Ozaki *et al.*, 1992) may increase selective binding of σ^{38}.

The single-round *in vitro* transcription assay also supported this conclusion that the level of $E\sigma^{38}$ holoenzyme required for maximum transcription of a fixed amount of template was higher than that of $E\sigma^{70}$ holoenzyme (see Figs. 3 and 4). 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 (Levin *et al.*, 1987). 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 my preliminary gel retardation assay indicated that $E\sigma^{38}$-*lac*UV5 initiation complexes were partly dissociated at 200 μg/ml heparin while $E\sigma^{70}$-*lac*UV5 complexes stayed constant (data not shown).

Another unexpected observation is that the affinity of $E\sigma^{38}$ holoenzyme to σ^{38}-dependent promoters was rather weaker than that of $E\sigma^{70}$ to σ^{70}-dependent promoters at least under the standard transcription assay conditions employed. Thus, in mixed transcription assays, σ^{70}-dependent promoters are preferentially transcribed at low enzyme concentrations, but upon the increase in enzyme concentration, the σ^{38}-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-$E\sigma^{38}$ interaction may also be influenced by a specific factor(s) and/or condition(s) present in stationary-phase *E. coli* cells.

(b) Effect of Salt Species and Concentrations on Promoter Selectivity of RNA Polymerase

Using the reconstituted holoenzyme $E\sigma^{38}$ and a collection of *E. coli* promoters, Tanaka *et al.* (1993) found that a number of *E. coli* promoters are recognized by both $E\sigma^{70}$ and $E\sigma^{38}$. The results described in this report indicate that the classification of promoters made on the basis of *in vitro* transcription patterns varies depending on the reaction conditions such as the species and concentrations of salts. For example, the *lac*UV5 promoter was classified as one of the promoters recognized both $E\sigma^{70}$ and $E\sigma^{38}$ under the conditions employed in the previous study (Tanaka *et al.*, 1993), but I have now found that it is more preferentially transcribed by $E\sigma^{70}$ than by $E\sigma^{38}$ upon increase in salt concentration (see Fig. 6, A and E).

One group of the osmoregulated genes, including *osmB* and *osmY*, responds to dual signals: hyperosmolarity and stationary growth-phase. Both $E\sigma^{70}$ and $E\sigma^{38}$ recognize the *osmB* and *osmY* promoters at low salt concentrations *in vitro*. This is consistent with the *in vivo* results which show that the basal level of *osmB* and *osmY* expression is reduced at low

osmolarity, and this based transcription is not eliminated by mutations reducing σ^{38} activity (Weichart *et al.*, 1993). Upon the addition of potassium glutamate at concentrations comparable to those produced *in vivo* in response to osmotic stress (Cayley *et al.*, 1991; Welsh *et al.*, 1991), transcription *in vitro* from the *osmB* and *osmY* promoters increased considerably, but only when $E\sigma^{38}$ was used. In contrast, transcription initiation at these promoters by $E\sigma^{70}$ decreased with the increase in potassium glutamate concentration. Among various salts tested, the physiologically relevant salt, potassium glutamate, showed the strongest effect in increasing the preference for $E\sigma^{38}$ transcription.

In addition to RNA polymerase activation, potassium glutamate stimulates the repression of $E\sigma^{70}$ transcription caused by binding of H-NS (Ueguchi and Mizuno, 1993). The activation of $E\sigma^{38}$ transcription by potassium glutamate and the simultaneous inhibition of $E\sigma^{70}$ activity altogether explain why osmotic induction is dependent on σ^{38}. Thus, I propose that $E\sigma^{38}$ itself is involved in the osmoregulation of transcription by directly sensing the increase in intracellular concentrations of specific salts.

(c) Effect of Trehalose on Promoter Selectivity of RNA Polymerase

The intracellular trehalose concentration increases under high osmolarity stress (Welsh *et al.*, 1991; Cayley *et al.*, 1991) and nutrient starved conditions such as carbon or nitrogen limitation (Welsh *et al.*, 1991). The σ^{38}-dependent *otsA* and *otsB* genes involved in trehalose synthesis indeed play important roles in osmotic tolerance (Giæver *et al.*, 1988) and thermotolerance (Hengge-Aronis *et al.*, 1991) in *E. coli*. In *otsA* mutants defective in the synthesis of trehalose, the expression of σ^{38}-dependent osmoregulated genes such as *osmY* is not induced even under high osmolarity conditions (Böhringer *et al.*, 1995). These observations altogether indicated that transcription of a set of σ^{38}-dependent genes by

$E\sigma^{38}$ might increase in the presence of trehalose. In this study, I indeed demonstrated that trehalose enhances transcription *in vitro* by purified RNA polymerase and that the optimum concentration of trehalose for the maximum transcription by $E\sigma^{70}$ and $E\sigma^{38}$ are around 0.5 and 1.0 M, respectively (see Fig. 9). This optimum trehalose concentration for $E\sigma^{38}$ transcription *in vitro* is in good agreement with the intracellular concentration of trehalose under nutrient starved or high osmolarity conditions (Welsh *et al.*, 1991; Cayley *et al.*, 1991).

In the presence of high concentrations of trehalose, the minimum concentration of σ^{38} for maximum transcription by a fixed amount of core enzyme was approximately one fifth the level in its absence (see Fig. 10), indicating that trehalose gave more than 5-fold enhancement of transcription under σ^{38}-limiting conditions. The high concentrations of trehalose enhance the formation of $E\sigma^{38}$ holoenzymes (or the reduction in dissociation of $E\sigma^{38}$ holoenzyme) at least by 2-fold (see Fig. 12). This effect is significant only when the amount of σ^{38} is less than that of core enzyme (see Fig. 12). Since the molar ratio of σ^{38} to core polymerase is about 0.3 in stationary-phase *E. coli* cells (Jishage and Ishihama, 1995), the formation of $E\sigma^{38}$ holoenzyme must be stimulated *in vivo* upon the increase of trehalose concentration. Likewise, $E\sigma^{38}$ holoenzyme-binding to DNA promoters was also enhanced more than 2-fold (see Fig. 13). Thus, I conclude that trehalose stimulates both steps of transcription initiation, overall leading to more than 5-fold stimulation of transcription. At present, however, it is not yet determined how trehalose enhances the σ^{38}-binding to core enzyme and how trehalose enhances the formation of $E\sigma^{38}$-promoter complex. Trehalose may stabilize the conformation of either σ^{38} or core polymerase for effective assembly and/or the conformation of either holoenzyme or DNA promoters in transcription competent states.

I showed in this study that the selective transcription of *osmY* promoter by $E\sigma^{38}$ increased by adding high concentrations of potassium glutamate

(see Part II; and Ding *et al.*, 1995). Under certain stress conditions, a number of cytoplasmic components are induced for adaptation to the stresses, including potassium glutamate and trehalose (Richey *et al.*, 1987; Welsh *et al.*, 1991; Cayley *et al.*, 1991; reviewed in Strøm and Kaasen, 1993). In this study, I examined the combined effect of trehalose and potassium glutamate, which are major cytoplasmic factors for osmotic tolerance (Sutherland *et al.*, 1986; Richey *et al.*, 1987; Giæver *et al.*, 1988; Welsh *et al.*, 1991; Cayley *et al.*, 1991; reviewed in Strøm and Kaasen, 1993). The maximum transcription of σ^{38}-dependent *osmY* by $E\sigma^{38}$ was achieved by adding high concentrations of either potassium glutamate or trehalose. Essentially the same level of transcription was observed by adding combinations of these two components but at lower concentrations. These results indicated that potassium glutamate and trehalose, two of the cytoplasmic factors showing increased levels in high osmolarity and stationary-phase *E. coli* cells (Welsh *et al.*, 1991; Cayley *et al.*, 1991), affect the same step(s) of transcription by $E\sigma^{38}$ and that the stimulatory effects by these two factors are additive at low factor concentrations, but are not additive under saturated activation by one of these factors.

(d) Effect of DNA Superhelicity on Promoter Selectivity of RNA Polymerase

The superhelicity of chromosomal DNA in bacterial cells is known to decrease under nutrient starvation and/or in stationary growth-phase (Balke and Gralla, 1987; Jaworski *et al.*, 1991). Since the DNA gyrase requires ATP to generate negative supercoils in DNA, cellular energetics such as the ratio of ATP to ADP, may play a role in the change in DNA superhelicity under such starved conditions (reviewed in Drlica, 1992). In fact, both the [ATP]/[ADP] ratio and DNA supercoiling decrease in concert when *E. coli* cells are shifted from aerobic to anaerobic conditions. Furthermore, a close relationship was observed between the extent of DNA relaxation and the decrease in [ATP]/[ADP] using purified DNA gyrase and plasmid DNA

(Hsieh *et al.*, 1991; reviewed in Drlica, 1992). In this study, I found that $E\sigma^{38}$ preferentially transcribes promoters on low superhelical density DNA (see Figs. 14 and 15) and the optimum superhelical density for the maximum transcription by $E\sigma^{38}$ is almost the same as that of plasmids prepared from stationary-phase *E. coli* (see Fig. 16). Preferential transcription of the low superhelical density templates by $E\sigma^{38}$ was due to the high affinity of $E\sigma^{38}$ binding to promoter on the low superhelical density of DNA (see Fig. 17). Transcription *in vivo* of *osmY* in cells growing in nutrient-rich media such as LB 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 (Yim and Villarejo, 1992; Weichart *et al.*, 1993). In good agreement with these observations, the superhelical density of plasmids prepared from cells grown in minimal media such as M9-glucose is about half of that of plasmids prepared from exponentially growing cells in LB (data not shown). In addition, thermotolerance at 48 °C, which is known to be controlled by σ^{38}, requires the DNA relaxation (Lange and Hengge-Aronis, 1991; reviewed in Loewen and Hengge-Aronis, 1994; Friedman *et al.*, 1995). These observations indicated that the low superhelicity of DNA provides templates suitable for $E\sigma^{38}$.

The stimulatory effects of the two cytoplasmic factors, potassium glutamate and trehalose, are additive at low concentrations of these factors but not additive when transcription is fully activated at saturated concentrations of a single factor (see Part III). However, the stimulatory effects are always additive between the DNA superhelicity (a nuclear factor) and either potassium glutamate or trehalose (cytoplasmic factors). The selective transcription of *osmY* by $E\sigma^{38}$ increases by adding high concentrations of potassium glutamate (see part II). However, the optimum concentration of potassium glutamate for the maximum transcription of *osmY* is different: it is 200 to 300 mM on the template with low superhelical density, whereas it is above 300 mM on the DNA with high superhelical

density (see Fig. 18). Likewise, enhancement of $E\sigma^{38}$ transcription by high concentrations of trehalose and that by low DNA superhelicity of template is additive (see Fig. 19). This additive effect of trehalose and DNA superhelicity is in good agreement with the observations that the enhancing mechanisms of transcription by trehalose and DNA superhelicity are different, *i.e.*, trehalose stimulates the core-binding activity of σ^{38} (see Fig. 12), while the low DNA superhelicity of templates increases the promoter affinity of $E\sigma^{38}$ holoenzyme (see Fig. 17).

These results altogether raise such a model of global regulation as that transcription by $E\sigma^{38}$ is enhanced by the decrease in DNA superhelical density under starved conditions and, independently, by the increase in potassium glutamate and trehalose concentrations. 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 σ^{38}-dependent promoters do not carry a consensus promoter sequence (Tanaka *et al.*, 1993; Nguyen *et al.*, 1993; Kolb *et al.*, 1995; Tanaka *et al.*, 1995).

H-NS (or H1a), which is one of the most abundant protein in the bacterial nucleoid, accumulates in the stationary growth-phase to a level about 10-fold higher than that in the logarithmic growth-phase. H-NS is known to constrain negative supercoiling of DNA and to repress σ^{70}-dependent transcription *in vitro* and *in vivo* (Spassky *et al.*, 1984; Hinton *et al.*, 1992; Ueguchi and Mizuno, 1993; Arnquist *et al.*, 1994; Tupper *et al.*, 1994). This constraining effect of H-NS on DNA supercoiling, however, is not observed *in vitro* when the concentration of potassium glutamate is above 100 mM or when H-NS is present in saturating concentrations against DNA (Tupper *et al.*, 1994). The intracellular concentration of potassium glutamate is known to increase to above 200 mM under high osmolarity or nutrient starved conditions (Welsh *et al.*, 1991; Cayley *et al.*, 1991), and H-NS is present at about 20000 copies per cell, sufficiently abundant to cover a

significant proportion of chromosomal DNA (Lammi *et al.*, 1984). These results altogether suggest that the constraining of DNA supercoiling by H-NS may decrease at the stationary growth-phase and that the repression of σ^{70}-dependent transcription at the stationary growth-phase by H-NS may be independent of the H-NS derived DNA superhelicity differences. Likewise, Dps, which was recently identified as a nucleoid-protein, accumulates in the stationary growth-phase cells and may play dual roles in DNA protection and repressing transcriptional repression of a set of genes (Almiron *et al.*, 1992). These observations suggest that the DNA-binding proteins can cause differential repression of $E\sigma^{70}$- versus $E\sigma^{38}$-dependent transcription independently of DNA superhelicity and that such a mechanism may lead to selective transcription of σ^{38}-dependent genes in the stationary phase.

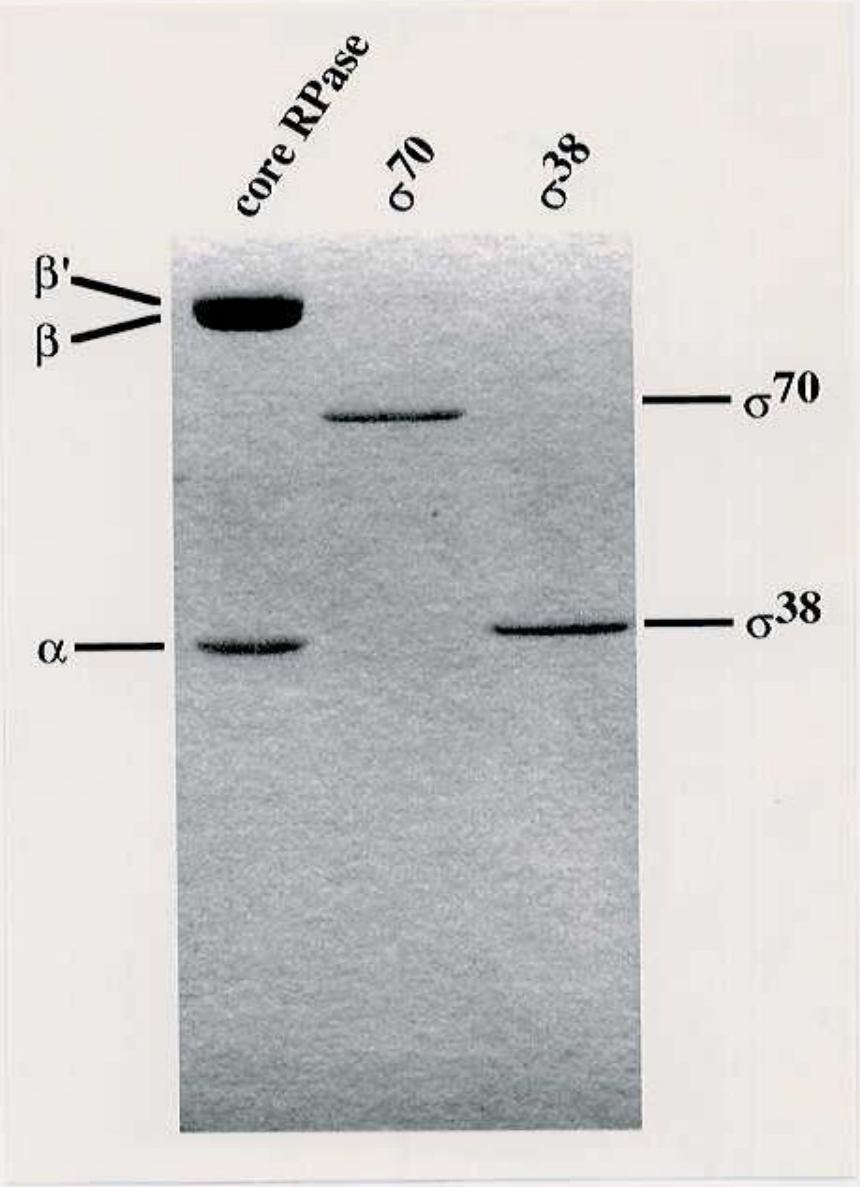

Fig.1. **SDS polyacrylamide gel electrophoresis of purified core RNA polymerase and σ subunits.** Core RNA polymerase was purified from *E. coli* W3350 as described in Materials and Methods. σ^{70} and σ^{38} were over-expressed and purified as described in Materials and Methods. Five μg of core polymerase and 1 μg each of σ subunits were subjected to SDS-12.5 % PAGE. Gels were stained with CBB.

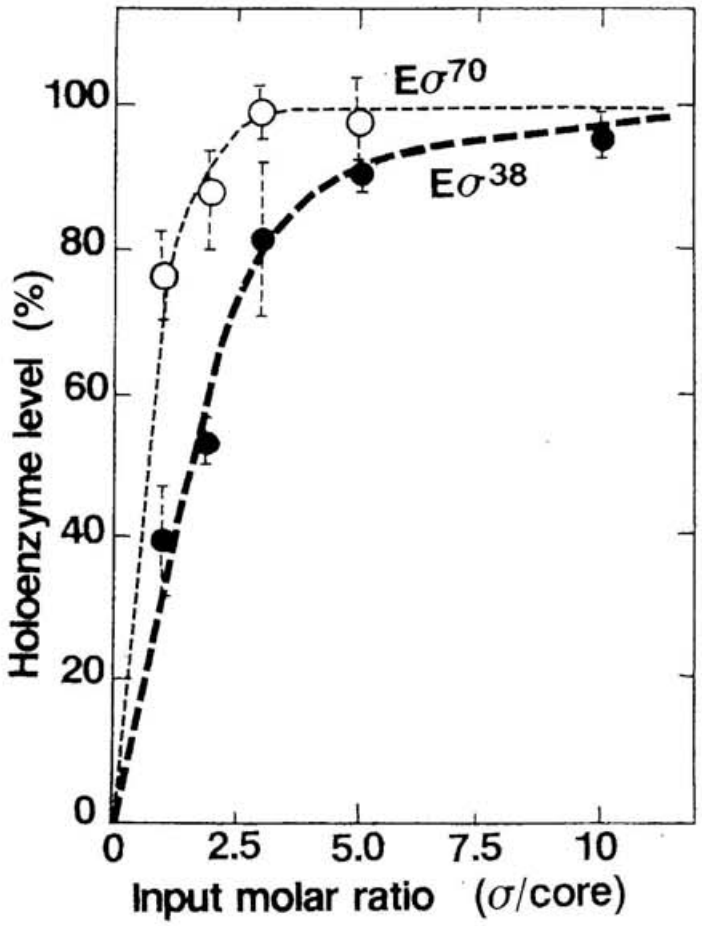

Fig. 2. **σ saturation curve for holoenzyme formation.** Core enzyme (50 pmole) and various amounts of σ^{70} or σ^{38} subunit were mixed in 10 mM Tris-HCl (pH 7.8 at 4 °C), 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.2 M KCl and 50% glycerol, and incubated for 10 min at 30 °C to form holoenzymes. The mixtures were fractionated by gel filtration-HPLC using a Superose 6 (Pharmacia) column. Proteins were eluted with 10 mM Tris-HCl (pH 7.8 at 4 °C), 200 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA and 5 % glycerol. Each fraction was analyzed by SDS-PAGE and gels were stained with Commassie brilliant blue and scanned with a Ultroscan-XL laser densitometer (LKB). The molar ratio of α and σ was calculated for the peak fractions. The dotted lines represent the standard deviations of 4 or 5 independent measurements.

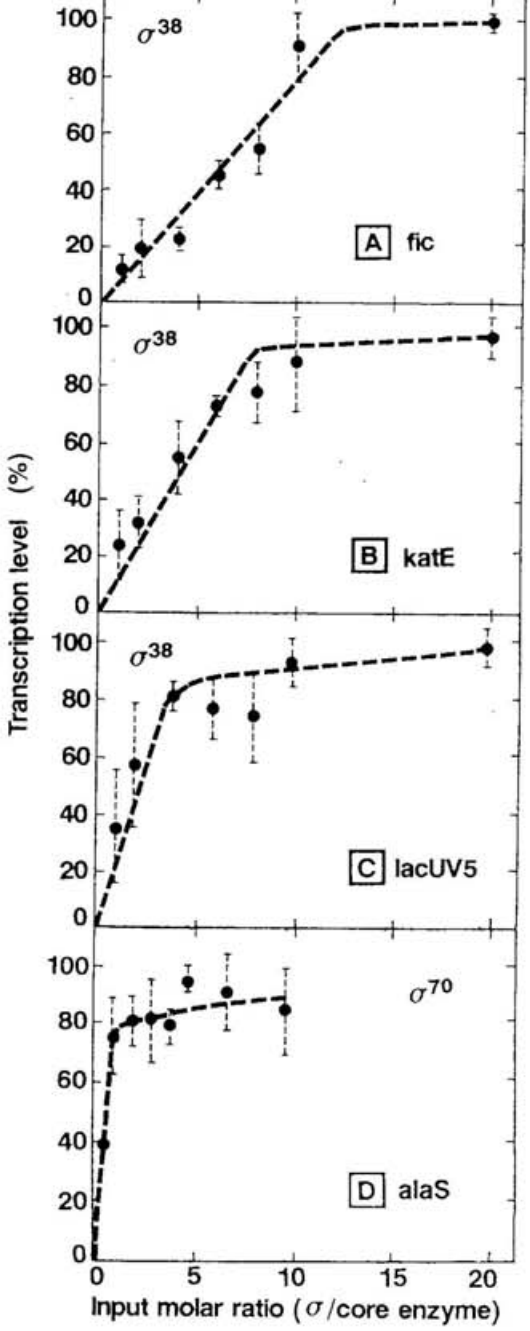

Fig. 3. **σ saturation curve for maximum transcription.** Core enzyme (1 pmole) and various amounts of σ^{38} [A-C] or σ^{70} [D] subunit were mixed in 10 μl as in Fig. 2. To the RNA polymerase mixtures, one of the truncated DNA templates (0.1 pmole) carrying *fic* [A], *katE* [B], *lac*UV5 [C] or *alaS* [D] promoter was added and incubated for 30 min at 37 °C to form open complexes. After addition of a substrate mixture containing [α-^{32}P]UTP as a labeled substrate, RNA synthesis was carried out for 5 min at 37 °C. RNA was analyzed by electrophoresis on 8 M urea-PAGE. Gels were analyzed with a BAS-2000 Bio-Imaging Analyzer (Fuji). The transcription levels represent the average values of 4 or 5 independent assays for each template and the standard deviations. The 100 % level corresponds to about 0.05 (about 0.5 molecule RNA per molecule DNA template), 0.03, 0.015 and 0.015 pmol transcript for *alaS*, lacUV5, *katE* and *fic* promoter, respectively.

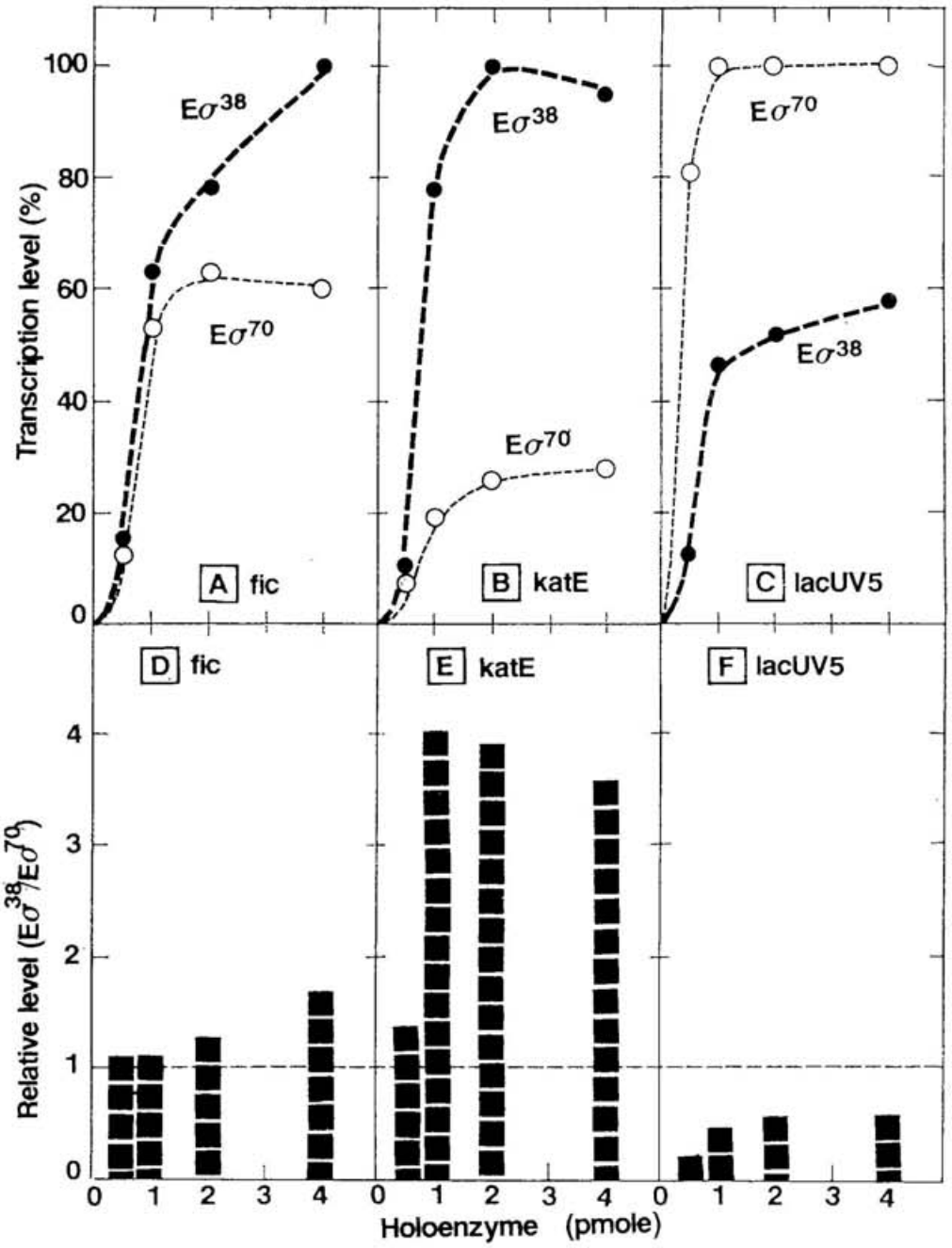

Fig. 4. **Effect of the holoenzyme concentration on transcription.** Truncated DNA templates (0.1 pmole each) carrying *fic* [A], *katE* [B] or *lac*UV5 [C] were transcribed *in vitro* by various amounts of either $E\sigma^{70}$ or $E\sigma^{38}$ holoenzyme under the standard single-round assay conditions. Transcripts were analyzed by 8 M urea-6 % PAGE, and gels were analyzed with a BAS-2000 Bio-Imaging Analyzer (Fuji). The maximum levels of transcription for each template were set as 100% value, measured using 4 pmole $E\sigma^{38}$ for *fic*, 2 pmole $E\sigma^{38}$ for *katE* and 1-4 pmole $E\sigma^{70}$ for *lac*UV5, respectively. The 100 % value corresponds to about 0.015, 0.015 and 0.5 pmol transcript for *fic*, *katE* and *lac*UV5, respectively. The ratios of transcription levels between two holoenzymes are plotted in lower panels ([D] *fic*, [E] *katE* and [F] *lac*UV5).

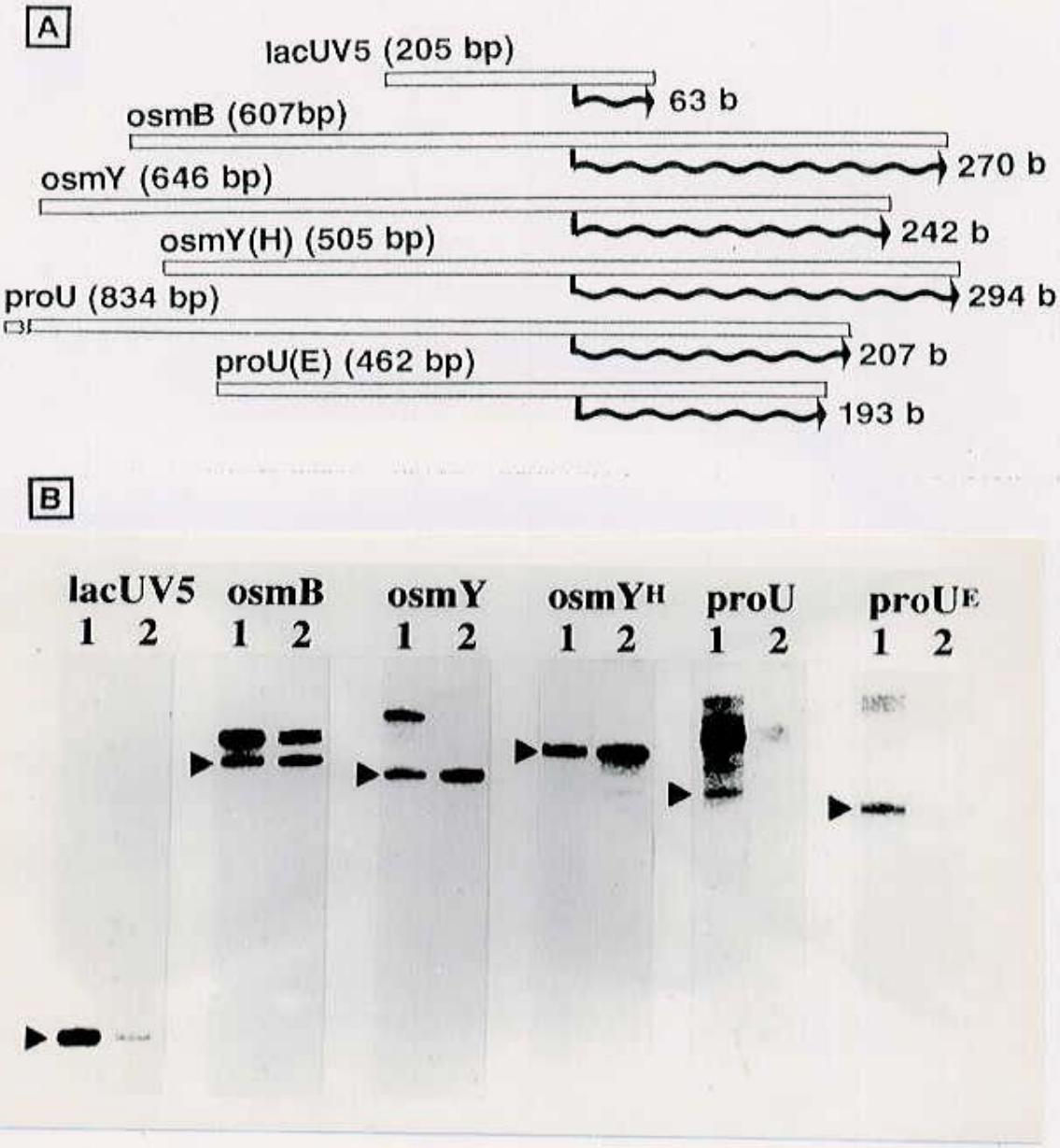

Fig. 5. **Transcription of osmoregulated promoters by two forms of RNA polymerase.** [A] Structure of truncated promoter fragments. Truncated DNA templates carrying *lac*UV5, *osmB*, and *osmY* promoters were prepared as described in Materials and Methods. RNA transcripts from these templates are shown as wavy lines. [B] Transcription *in vitro* was carried out under the standard reaction conditions using 1 pmole each two different forms of the reconstituted holoenzyme, $E\sigma^{70}$ (lane 1) and $E\sigma^{38}$ (lane 2), and 0.1 pmole each of a reference promoter (*lac*UV5) and five test promoters, *osmB*, *osmY*, $osmY^{H}$, *proU*, and $proU^{E}$. Transcripts were analyzed by 8 M urea-7 % PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The arrow head indicates the expected transcripts from the tested promoters.

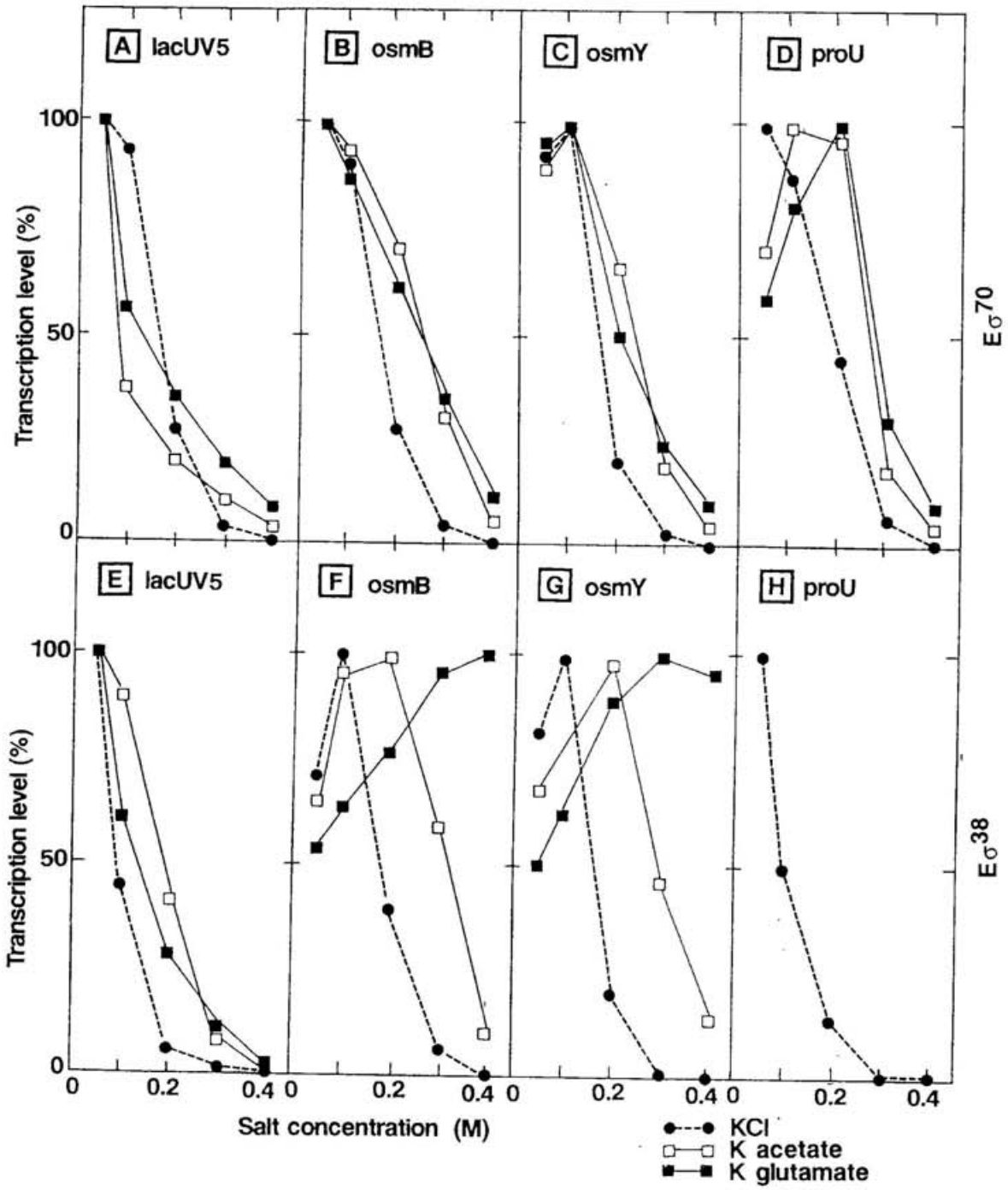

Fig. 6. **Effect of salt concentrations on *in vitro* transcription of the osmoregulated genes.** Single-round transcription *in vitro* was carried out using 0.05 pmole *lac*UV5 or 0.1 pmole *osmB*, *osmY* and *proU* templates and 1 pmole of either reconstituted $E\sigma^{70}$ and $E\sigma^{38}$ holoenzyme under the standard reaction conditions except that 50 mM NaCl was replaced with the indicated concentration of KCl, K acetate, or K glutamate. Transcripts were analyzed by 8 M urea-PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum levels of transcription for each templates was set as 100 % value.

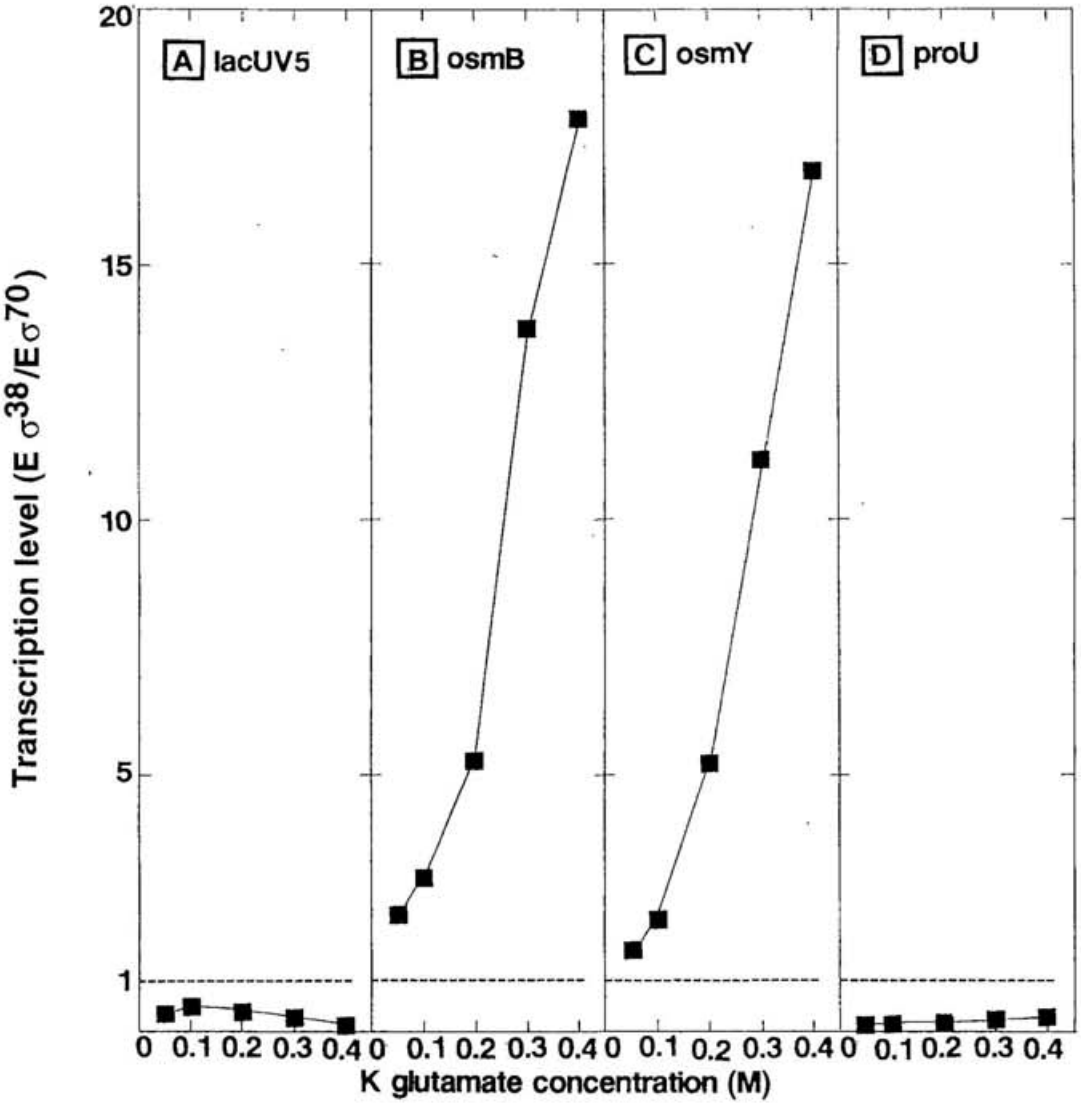

Fig. 7. **Ratio of transcription level by $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes.** *In vitro* transcription was carried out at various concentrations of K glutamate (see Fig. 6). The ratio of the transcription levels of various promoters by $E\sigma^{70}$ and $E\sigma^{38}$ is shown at various concentrations of K glutamate.

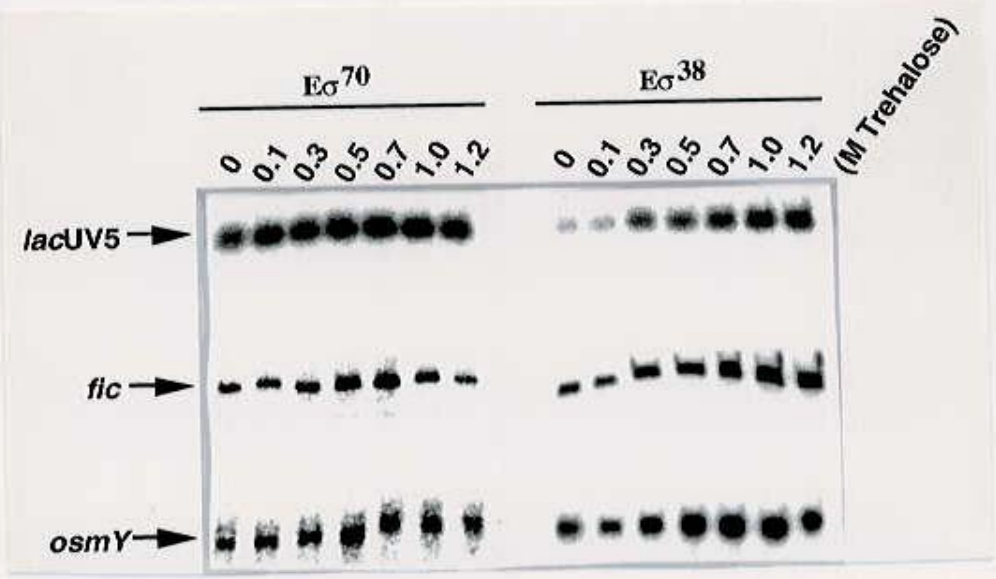

Fig. 8. **Effect of trehalose concentrations on *in vitro* transcription.** Single-round *in vitro* transcription was carried out using 0.1 pmole *lac*UV5, *fic* or *osmY* and 1 pmole each of either reconstituted $E\sigma^{70}$ or $E\sigma^{38}$ holoenzyme under the standard reaction conditions except that trehalose was added at the indicated concentrations. Transcripts were subjected to 8 M urea-PAGE and the gel was analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji).

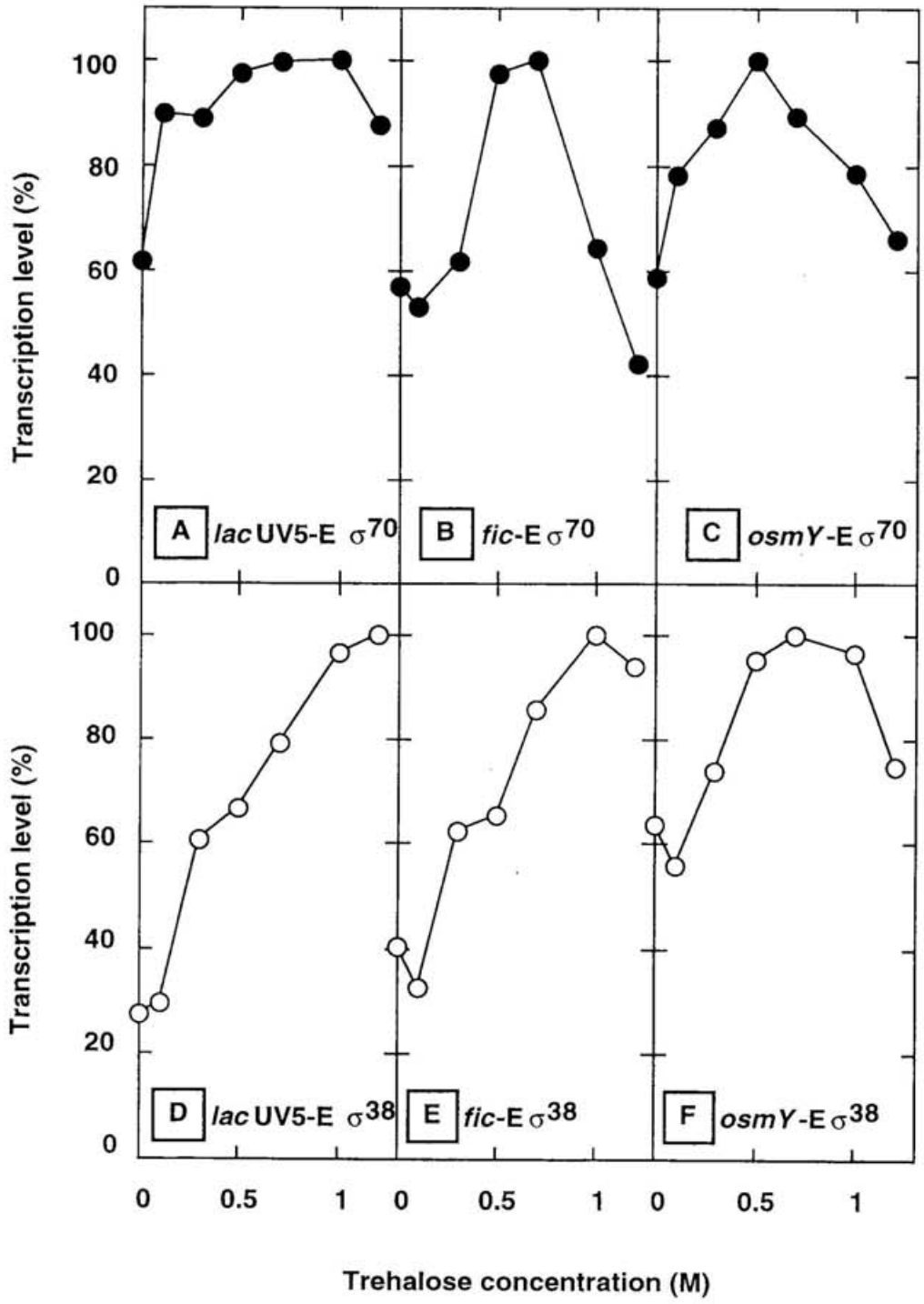

Fig. 9. **Effect of trehalose concentrations on *in vitro* transcription.** Single-round *in vitro* transcription was carried out using 0.1 pmole *lac*UV5 [A and D], *fic* [B and E] or *osmY* [C and F] and 1 pmole of either reconstituted $E\sigma^{70}$ [A - C] or $E\sigma^{38}$ [D - F] holoenzyme under the standard reaction conditions except that trehalose was added at indicated concentrations. Transcripts were analyzed by 8 M urea-PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template was set as 100 % value. These data represent the averages of 3 independent experiments.

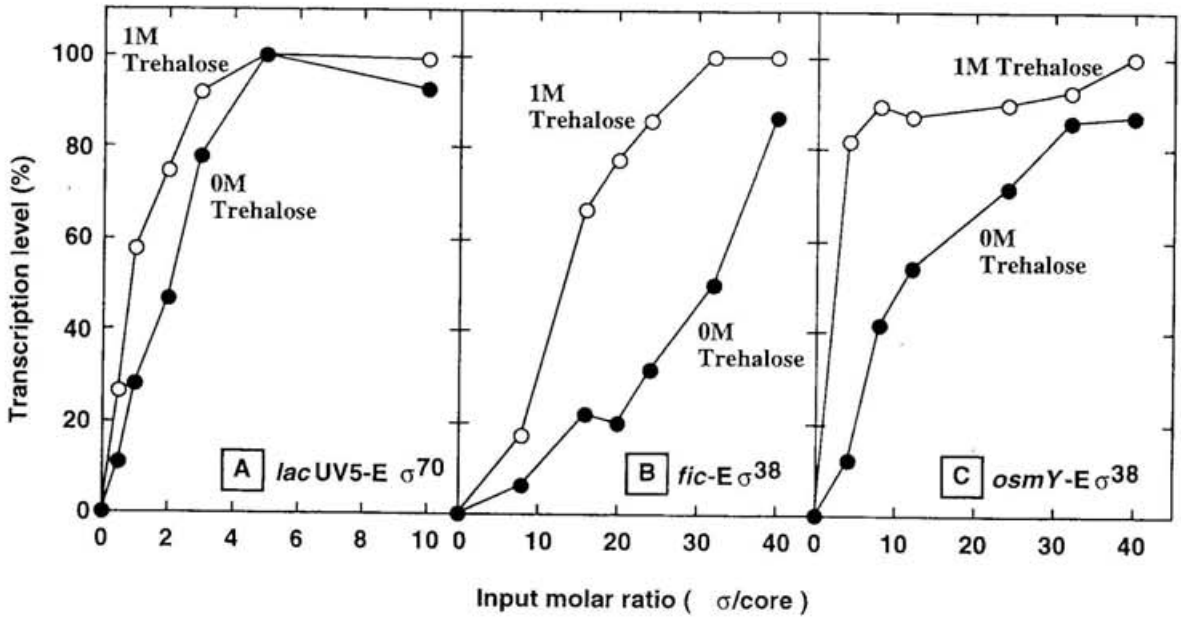

Fig. 10. **σ saturation curve for maximum transcription in the absence and presence of trehalose.** Core enzyme (1 pmole) and various amounts of σ^{70} [A] or σ^{38} [B and C] subunit were mixed as in Fig. 3. To the RNA polymerase mixtures, one of the truncated DNA templates (0.1 pmole) carrying *lac*UV5 [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 BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template was set as 100 % value. These data represent the averages of 3 independent experiments.

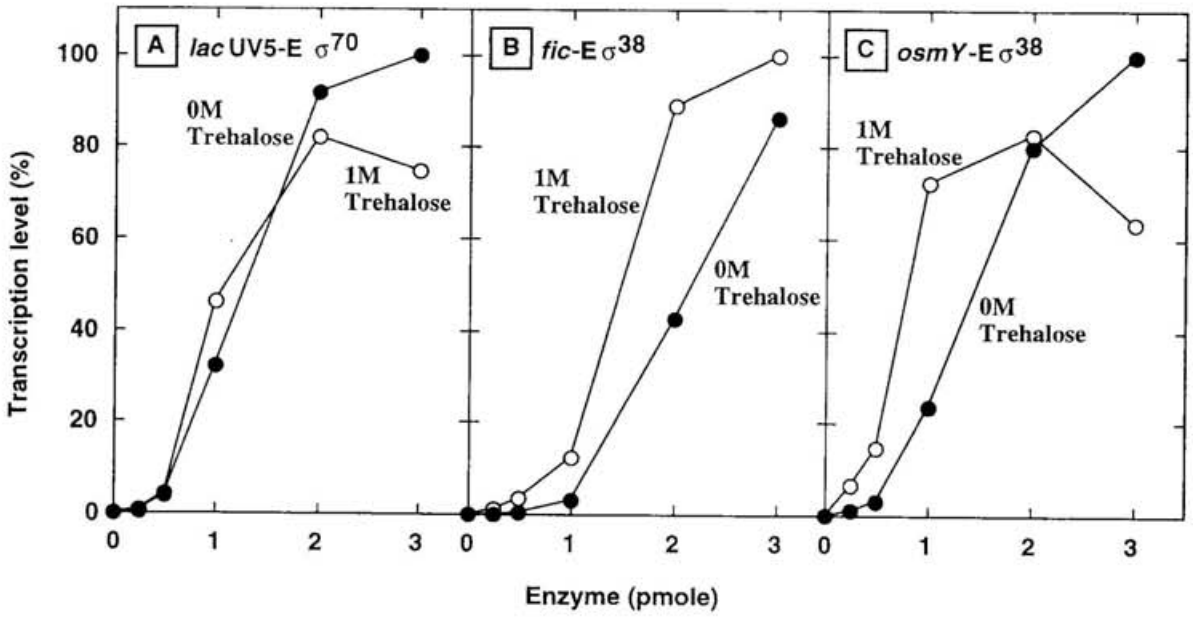

Fig. 11. **Effect of the holoenzyme concentration on transcription under the absence and presence of trehalose conditions.** Truncated DNA templates (0.1 pmole each) carrying *lac*UV5 [A], *fic* [B], or *osmY* [C] were transcribed *in vitro* by various amounts of either reconstituted $E\sigma^{70}$ or $E\sigma^{38}$ holoenzyme under the standard single-round 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 BAS2000 Bio-Imaging Analyzer (Fuji). The maximum levels of transcription for each template were set as 100 % value. These data represent the averages of 3 independent experiments.

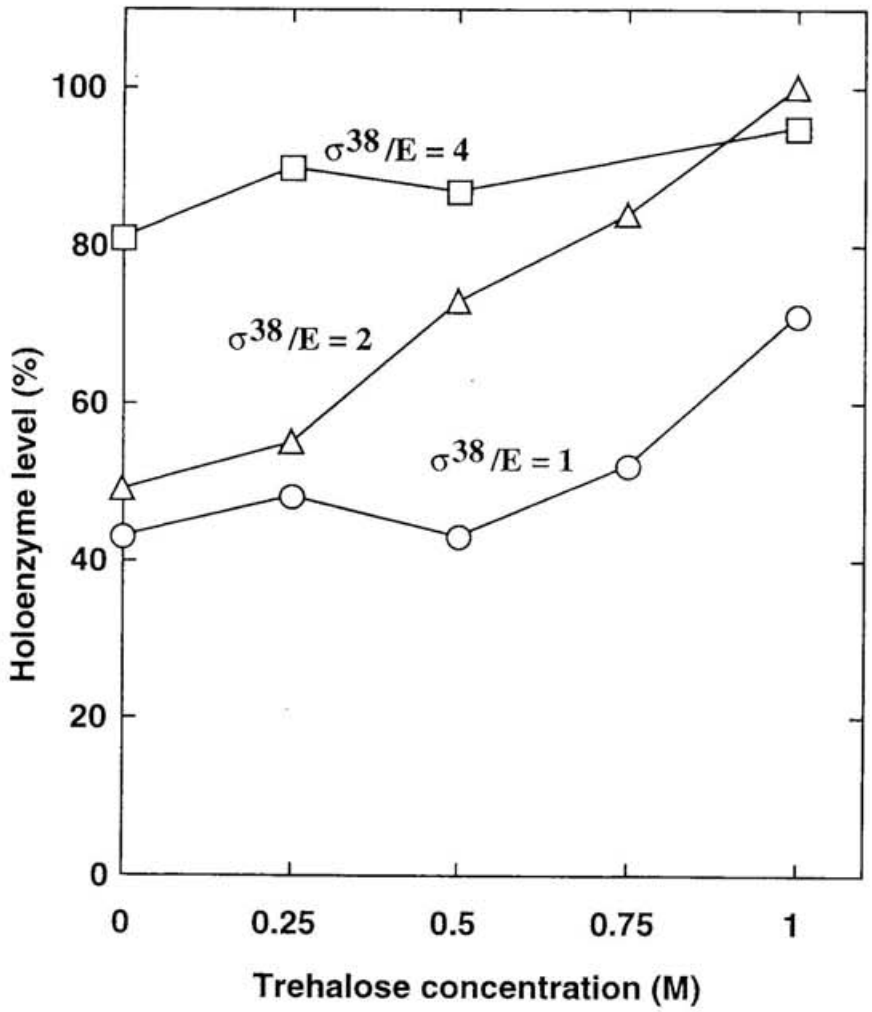

Fig. 12. **Effect of trehalose concentrations for holoenzyme formation.** Core enzyme (E, 20 pmole) and various amounts of σ^{38} were mixed in 50 mM Tris-HCl (pH 7.8 at 37 °C), 3 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 μg/ml of BSA and various concentrations of trehalose, and incubated for 10 min at 30 °C to form holoenzymes. After the incubation, RNA polymerase was isolated by using monospecific polyclonal antibodies against α subunit and Protein A-Sepharose 6MB. Proteins were subjected to SDS-15 % PAGE. The gels were stained with Fluorokit PRO-1 protein staining system and analyzed with a Fluorimager SI system (Molecular Dynamics). The molar ratio of α and σ^{38} was calculated for each sample.

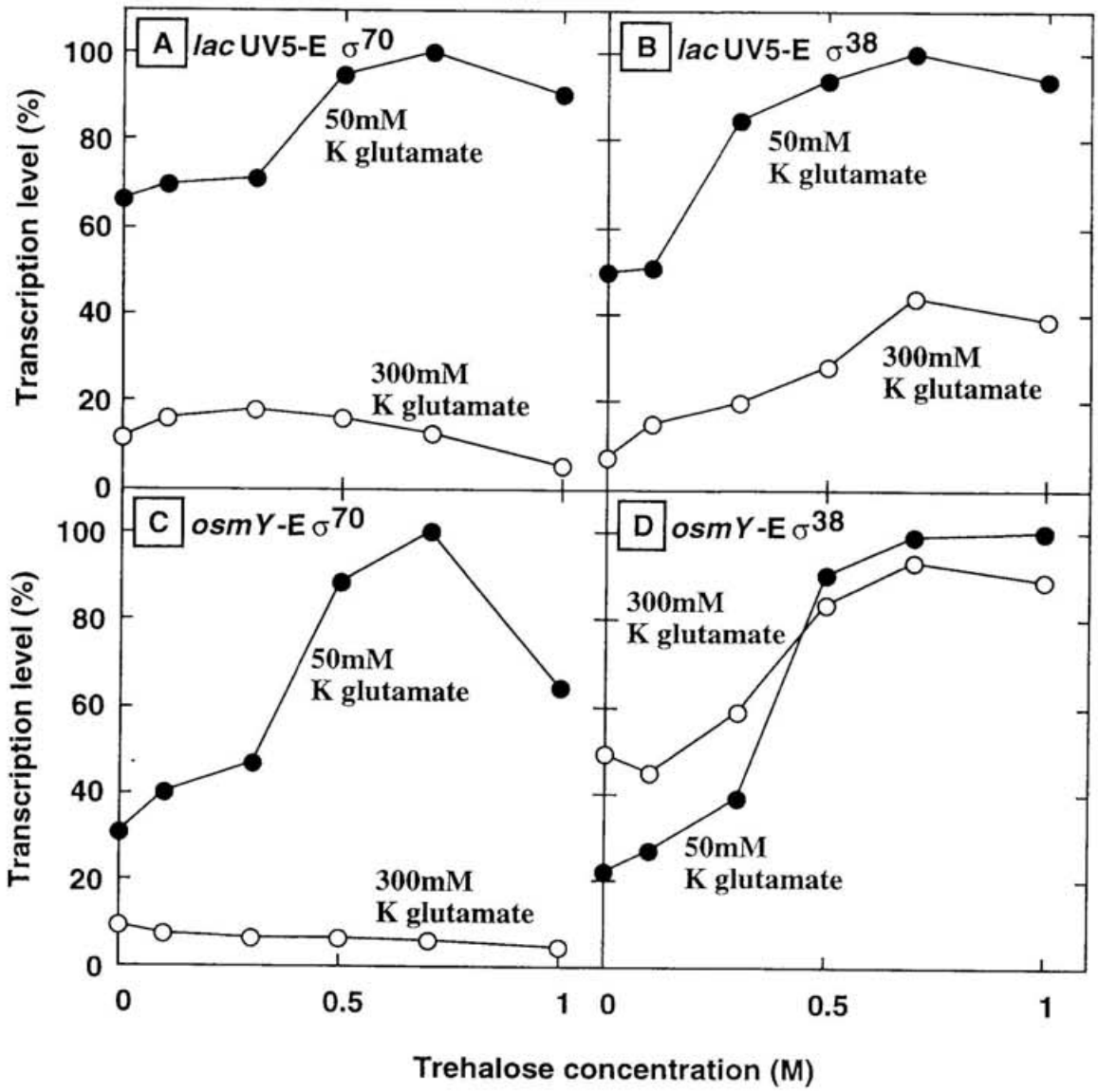

Fig. 13. **Effect of trehalose concentrations on *in vitro* transcription under the low and high concentration of K glutamate.** Single-round *in vitro* transcription was carried out using 0.1 pmole truncated template carrying *lac*UV5 [A and B] or *osmY* [C and D] promoter and 1 pmole of either reconstituted $E\sigma^{70}$ [A and C] or $E\sigma^{38}$ [B and D] holoenzyme under the standard reaction conditions, except that the 50 mM NaCl was replaced by the indicated concentrations of K glutamate, and trehalose was addedat the indicating concentrations. Transcripts were analyzed by 8 M urea-PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template was set as 100 % value. These data represent the averages of 3 independent experiments.

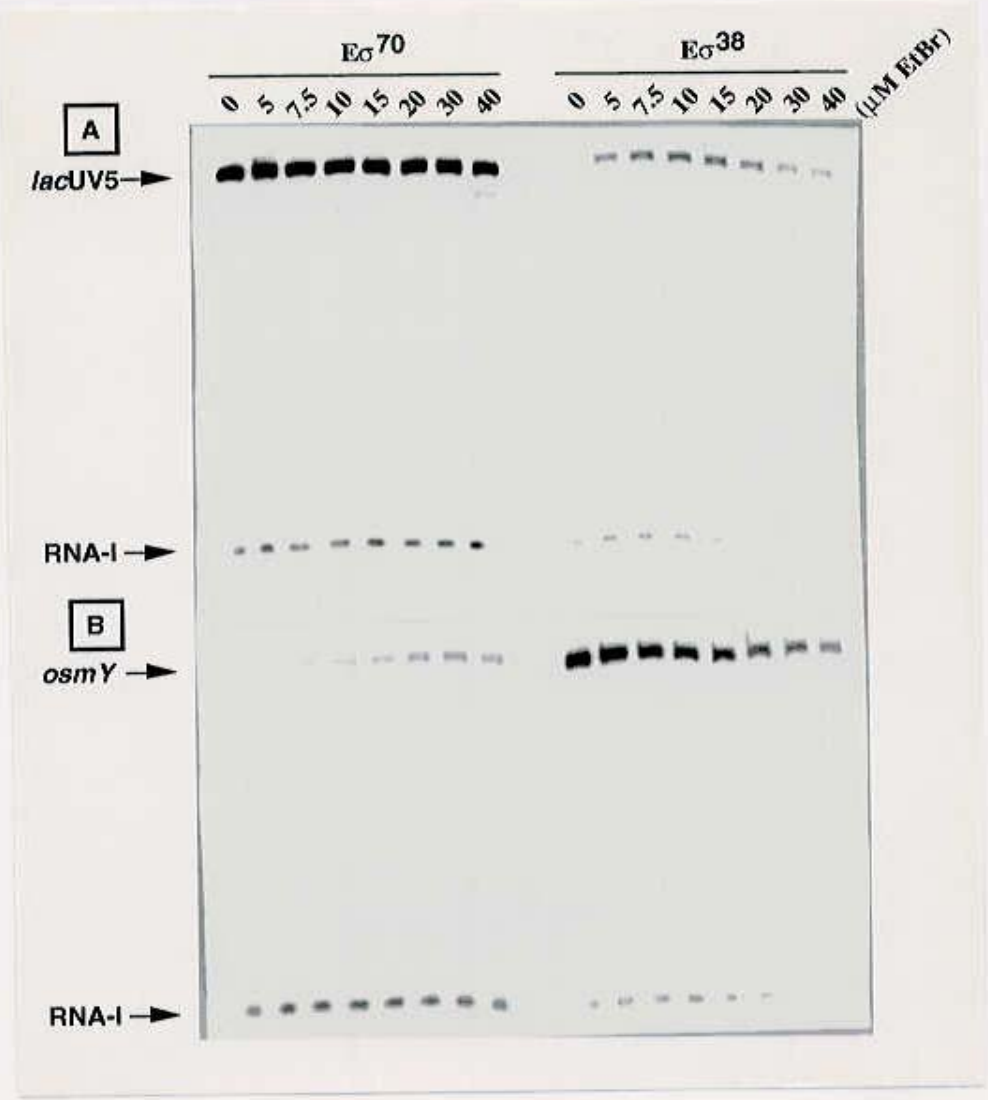

Fig. 14. **Effect of the superhelical density of template DNA on transcription.** A set of circular DNA templates, pBSLU [A] and pBSOY [B] (0.1 pmole each), with various superhelical densities was transcribed *in vitro* by reconstituted $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes under the standard single-round assay conditions. Transcripts were subjected to 8 M urea-4 % PAGE and the gel was analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji).

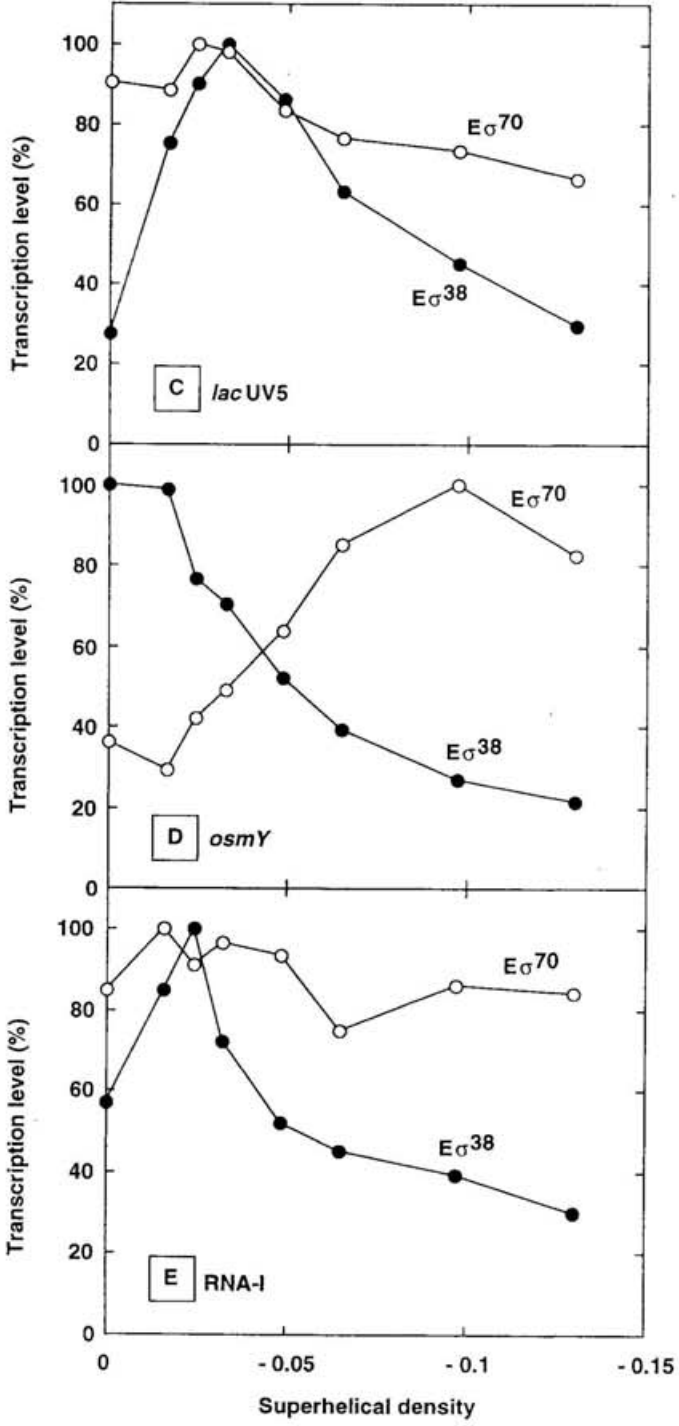

Fig. 15. **Effect of the superhelical density of template DNA on transcription.** The *in vitro* transcription reaction was carried out as described in Figure 8. Transcripts, *lac*UV5 RNA derived from pBSLU [A], *osmY* RNA derived from pBSOY [B] and RNA-I derived from the ColE1 origin promoter from both plasmids [C] were measured with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template was set as 100 % value. These data represent the averages of 3 independent experiments for *lac*UV5 and *osmY*, and 6 independent experiments for RNA-I.

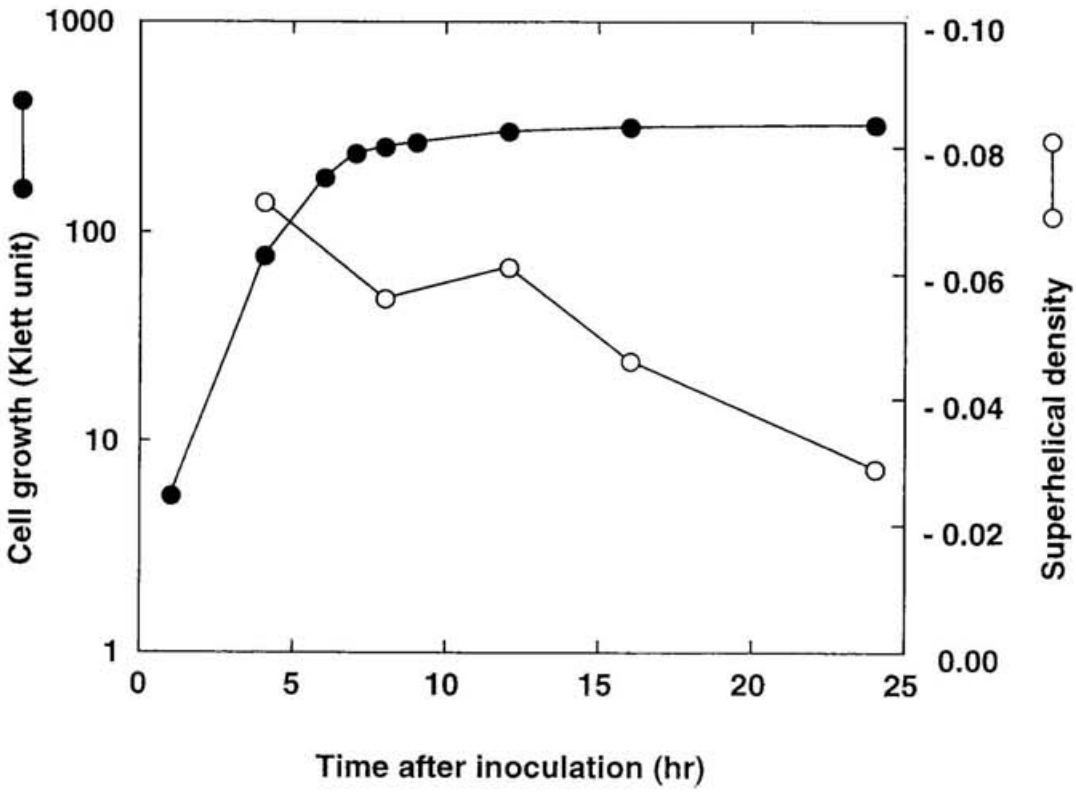

Fig. 16. **Effect of cell growth-phase on the superhelical density of plasmid DNA.** *E. coli* DH5α cells carrying a plasmid, pBSOY, was grown in LB. At the times indicated, the plasmid was purified using QIAGEN plasmid kit, and the superhelical density was determined by electrophoresis on 0.8 % agarose gels and in the presence of appropriate concentrations of ethidium bromide as described in the Materials and Methods.

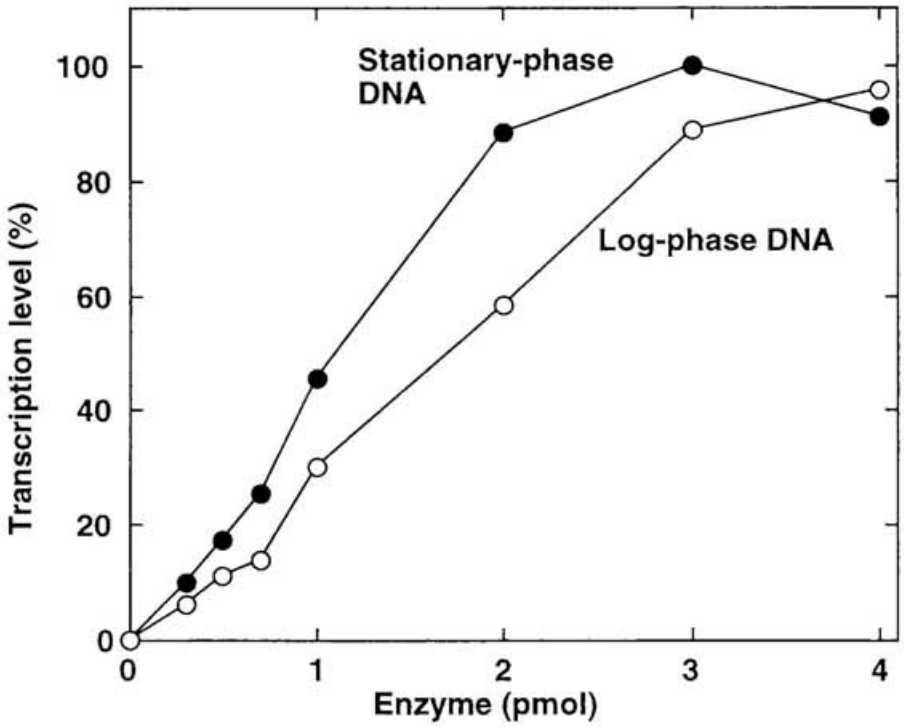

Fig. 17. **Promoter activity of plasmid DNA isolated from exponentially growing and stationary-phase cells.** Low- and high-superhelical density DNA templates (0.1 pmole each) carrying the *osmY* promoter were prepared from transformed DH5α cells at 4 and 24 h, respectively, after inoculation and transcribed *in vitro* by various amounts of the reconstituted $E\sigma^{38}$ holoenzyme under the standard single-round assay conditions. Transcripts were analyzed by 8 M urea-4 % PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for the low-superhelical-density template was set as 100 % value.

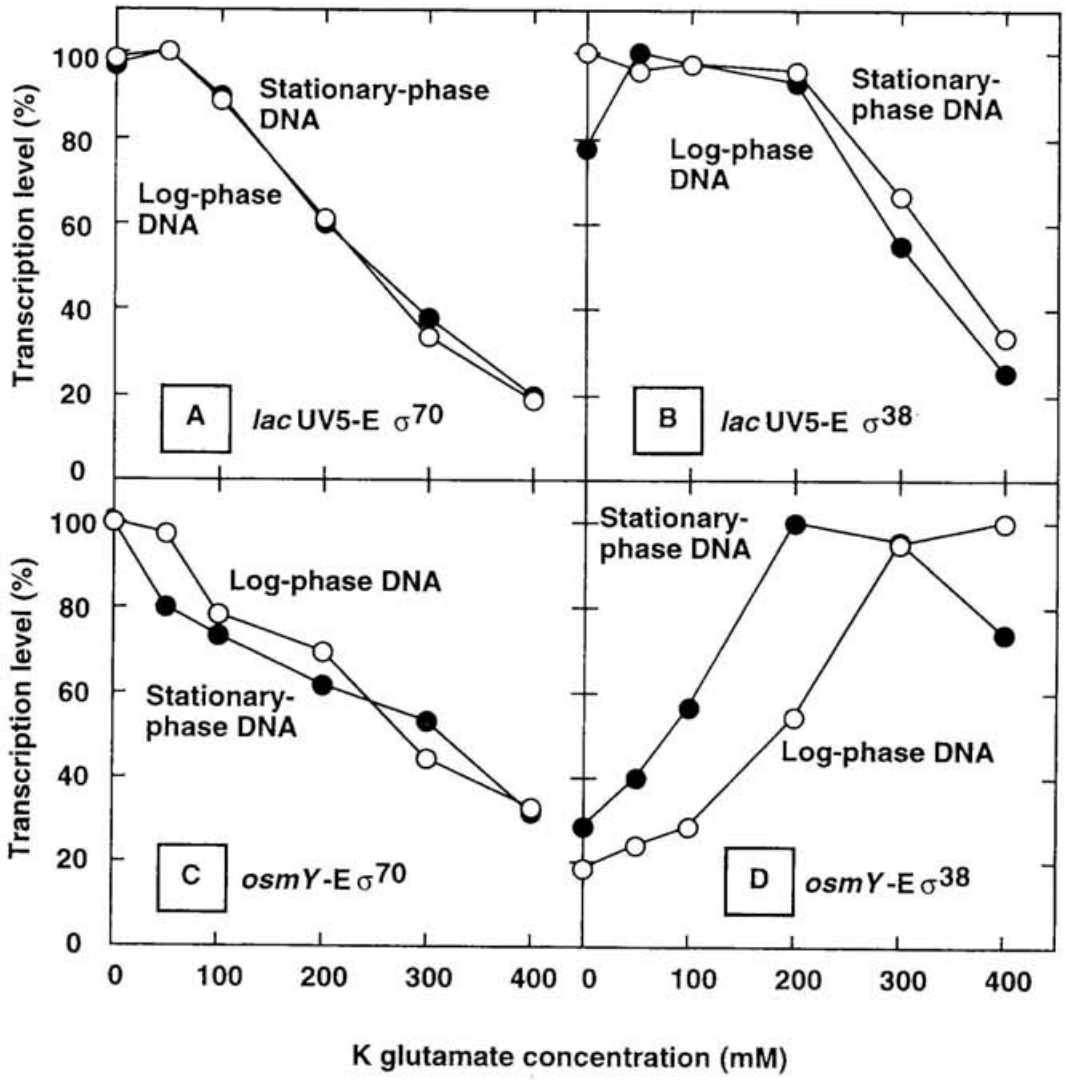

Fig. 18. **Effect of K glutamate concentrations on *in vitro* transcription of templates with different superhelical densities.** [A and B] Low- and high-superhelical density DNA templates (0.1 pmole each) carrying *lac*UV5 promoter, prepared from 4 and 24 h, respectively, after inoculation were transcribed *in vitro* by various amounts of reconstituted $E\sigma^{70}$ [A] and $E\sigma^{38}$ [B] holoenzymes under the standard single-round assay conditions, except that 50 mM NaCl was replaced by the indicated concentrations of K glutamate. Transcripts were analyzed by 8 M urea-4 % PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template is set as 100 % value. [C and D] Low- and high-superhelical density DNA templates (0.1 pmole each) carrying the *osmY* promoter, prepared from 4 and 24 h, respectively, were transcribed *in vitro* by various amounts of reconstituted $E\sigma^{70}$ [C] and $E\sigma^{38}$ [D] holoenzymes under the same conditions as in panel A and B. Analysis of transcripts were carried out as in panel A and B. These data represent the averages of 3 independent experiments.

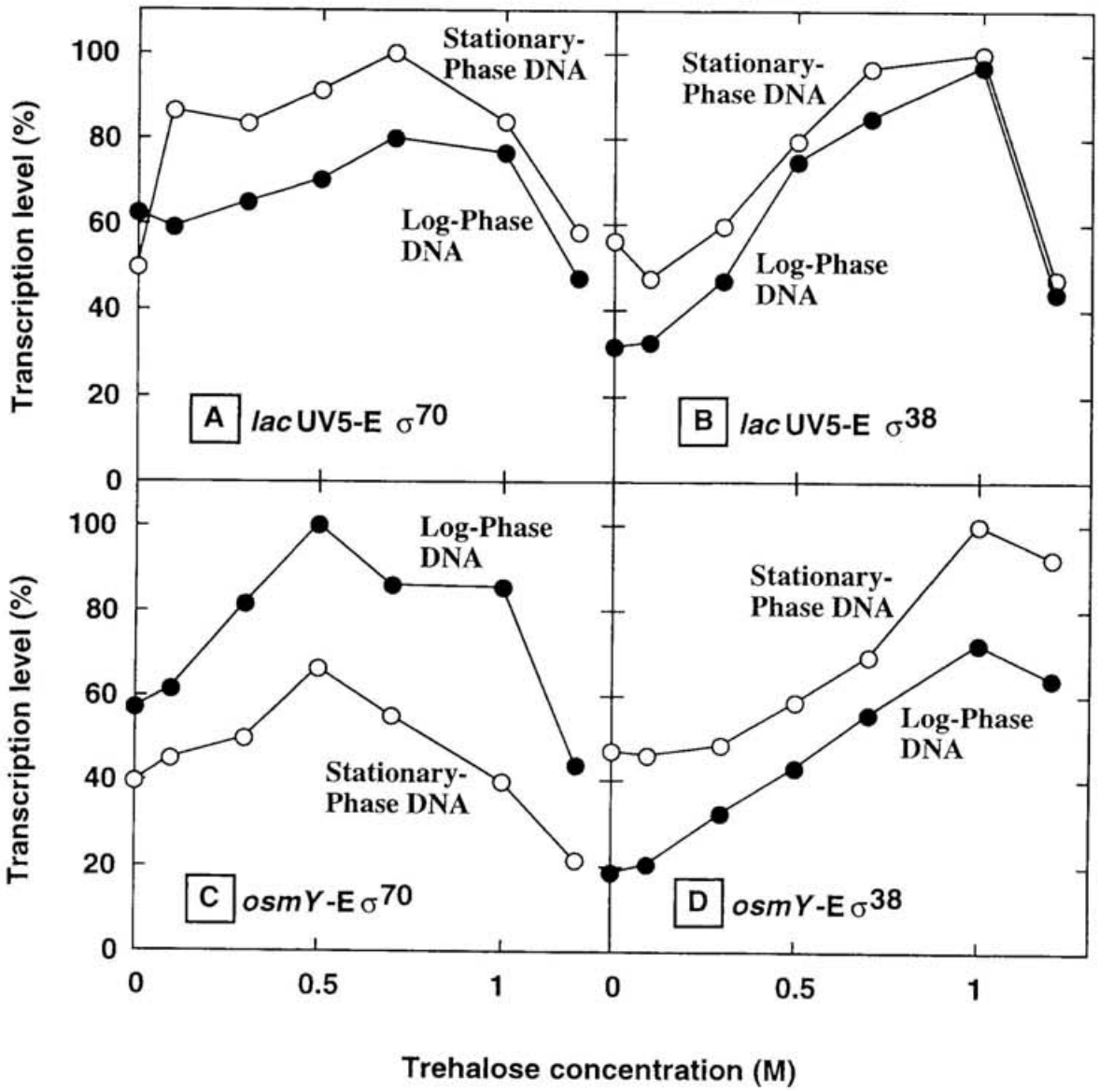

Fig. 19. **Effect of trehalose concentrations on *in vitro* transcription of templates with different superhelical densities.** [A and B] Low- and high-superhelical density DNA templates (0.1 pmole each) carrying *lac*UV5 promoter, prepared from 4 and 24 h, respectively, after inoculation were transcribed *in vitro* by various amounts of reconstituted $E\sigma^{70}$ [A] and $E\sigma^{38}$ [B] holoenzymes under the standard single-round assay conditions, except that trehalose was added at the indicated concentrations. Transcripts were analyzed by 8 M urea-4 % PAGE and gels were analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji). The maximum level of transcription for each template is set as 100 % value. [C and D] Low- and high-superhelical density DNA templates (0.1 pmole each) carrying the *osmY* promoter, prepared from 4 and 24 h, respectively, were transcribed *in vitro* by various amounts of reconstituted $E\sigma^{70}$ [C] and $E\sigma^{38}$ [D] holoenzymes under the same conditions as in panel A and B. Analysis of transcripts were carried out as in panel A and B. These data represent the averages of 3 independent experiments.

Acknowledgement

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