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Regulation of RNA Polymerase Sigma Subunit Synthesis in *Escherichia coli*: Intracellular Levels of σ^{70} and σ^{38}

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The intracellular levels of two principal σ subunits, σ^{70} (σ^{D}, the *rpoD* gene product) and σ^{38} (σ^{S}, the *rpoS* gene product), in *Escherichia coli* MC4100 were determined by a quantitative Western immunoblot analysis. Results indicate that the level of σ^{70} is maintained at 50 to 80 fmol per μg of total proteins throughout the transition from the exponential growth phase to the stationary phase, while the level of σ^{38} protein is below the detection level at the exponential growth phase but increases to 30% of the level of σ^{70} when cell growth stops to enter into the stationary phase. Beside the stationary phase, the increase in σ^{38} level was observed in two cases: exposure to heat shock at the exponential phase and osmotic shock at the stationary phase.

Bacterial cells can survive in such diverse environments as the mammalian gut, fresh- and seawater, and soil. In order to adapt to such varied environments, bacteria carry systems that sense changes in nutrient availability, osmolarity, temperature, and other external factors and that respond by turning on and off specific sets of stress response genes (2). For the global changes in gene expression patterns, RNA polymerase is considered to play a key role by rapidly modulating its promoter selectivity. One major mechanism of the promoter selectivity control of RNA polymerase is the replacement of σ subunit (the promoter recognition factor) on core enzyme, which alone is unable to initiate transcription from promoters even though it carries the catalytic function of RNA synthesis. Up to the present time, six different molecular species of σ subunit have been identified for *Escherichia coli* (7). The model of σ replacement relies on changes in the intracellular concentrations of individual σ subunits, because the level of core enzyme stays at a near-constant level (8, 9). However, little is known about the intracellular level of each σ subunit under various conditions.

Transcription of the genes highly expressed in the steady state part of the exponential growth phase is carried out by the RNA polymerase holoenzyme containing σ^{70}, while σ^{38} (one of the alternative σ subunits) is a key factor in the stress response during the transition from the exponential growth phase to the stationary phase (14). σ^{38} synthesis is also induced upon exposure of cells to high osmolarity (12). The mechanism controlling the level of σ^{38} under various growth conditions appears to be complex, involving various factors inducing σ^{38} synthesis. For example, cyclic AMP (a product of adenylcyclase encoded by the *cya* gene) (11), ppGpp (a magic spot synthesized by the *relA* and *spoT* gene products) (1), and homoserine lactone (5) were suggested to be involved as effectors controlling σ^{38} synthesis. A nucleoid protein, H-NS, was also implicated to be involved in posttranscriptional regulation of σ^{38} synthesis (19). In this study, we determined for the first time the intracellular level of σ^{38} in *E. coli* MC4100 growing at various phases by quantitative Western blot (immunoblot) analysis. We also analyzed the level of σ^{38} under various stress conditions.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain used in this study was *E. coli* MC4100 (11). Cells were grown at 30 or 37°C under aeration in Luria broth (LB). Growth was monitored by measuring the turbidity with a Klett-Summerson photometer. The culture conditions were fixed as follows. A few colonies from cultures grown overnight on LB agar plates were inoculated into 5 ml of fresh LB medium. At the cell density of 30 Klett units, the culture was diluted 40-fold by adding 200 ml of fresh LB medium and incubated at 37°C with shaking at a constant rate. For osmotic stress, the culture was divided in half and 5 M NaCl was added to make a 0.5 M solution. For heat shock stress, the culture grown at 30°C was divided in half, to which an equal volume of fresh LB at either 54 (heat shock at 42°C) or 30°C (control) was added.

Preparation of cell lysates. Cells were collected by centrifugation and resuspended in 40 mM Tris-HCl (pH 8.1 at 4°C) containing 25% sucrose. After treatment with 1 mM EDTA and 500 μg of lysozyme per ml at 0°C for 10 min, cells were lysed by adding 0.5% Brij 58. The Brij lysate was supplemented with 0.01 M $MgCl_2$ and 0.2 M KCl, digested at 37°C for 10 min with 20 μg of RNase A per ml and 100 μg of DNase I per ml in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated for 1 min with a Cosmo Bio Bioruptor. The supernatant after centrifugation for 30 min at 15,000 (Tomy mrX-150) rpm was used as the cell lysate for all the experiments. The protein concentrations of cell lysates were determined with a Bio-Rad Protein Assay Kit (Bio-Rad).

Purification of σ subunits and preparation of antibodies. σ^{70} subunit was overexpressed with pGEMD and purified as described by Igarashi and Ishihama (6), while σ^{38} was expressed with pETF and purified as described by Tanaka et al. (17). Antibodies against each σ subunit were produced in rabbits by injecting the purified σ subunits. Anti-σ^{70} and anti-σ^{38} antibodies used in this study did not cross-react with each other, as analyzed by Western blotting.

Immunological methods. For the measurement of σ subunits, a quantitative Western blot analysis was employed with the monospecific anti-σ antibodies. In brief, cell lysates were treated with a sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue) and separated on SDS–7.5 or 10% polyacrylamide gels. Proteins in the gels were directly electroblotted onto polyvinylidene difluoride membranes (Nippon Genetics). Blots were blocked overnight at 4°C in 3% bovine serum albumin in phosphate-buffered saline (PBS), probed with the monospecific antibodies against each σ subunit, washed with 0.5% Tween 20 in PBS, and incubated with goat anti-rabbit immunoglobulin G conjugated with hydroxyperoxidase (Cappel). The blots were developed with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo). Staining intensity was measured with a PDI image analyzer system equipped with a white light scanner.

RESULTS

Measurement of σ subunits by quantitative Western blotting. For measurement of σ subunit levels in cell extracts, a quantitative Western blot system was developed by using monospecific polyclonal antibodies against each σ subunit. In each determination, we first prepared a standard curve for each of

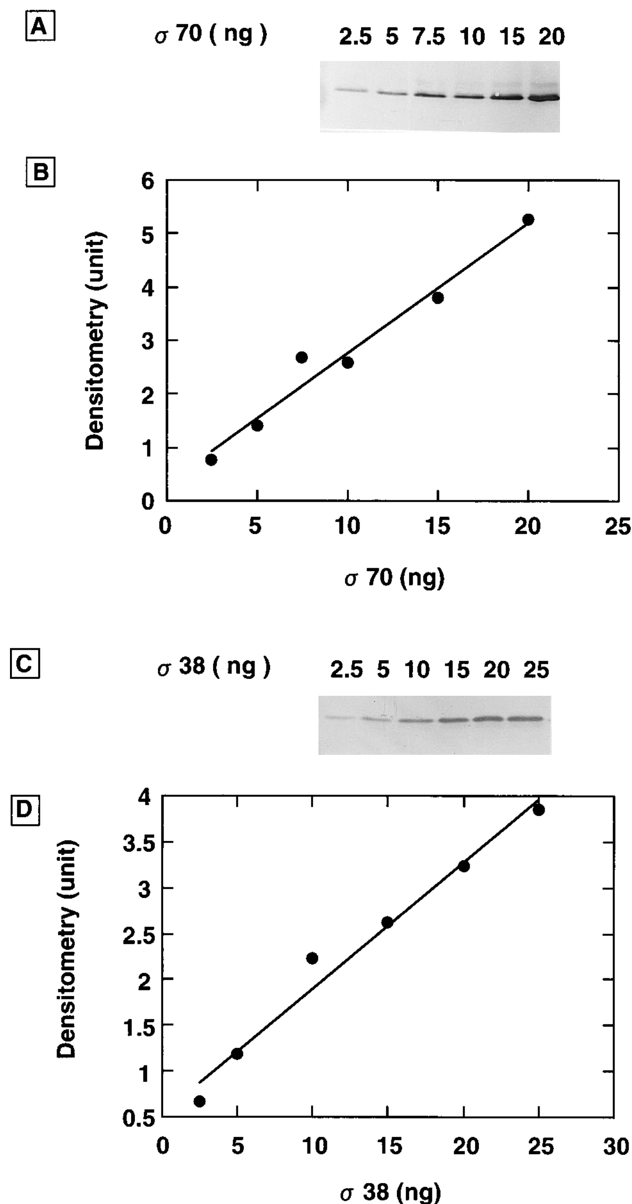

FIG. 1. Standard curves for the determination of σ^{70} and σ^{38} subunits. (A and C) Various concentrations of purified σ^{70} (A) or σ^{38} (C) subunits were analyzed by the quantitative Western blot system. The blot was probed with anti-σ^{70} (A) or anti-σ^{38} (C) antibodies. (B and D) The intensities of σ^{70} (B) and σ^{38} (D) bands after immunostaining were quantitated with a PDI image analyzer system equipped with a white light scanner.

the purified σ proteins and determined the range for which there is linearity between the protein concentration and the intensity of immunostaining. In this study, we measured the intracellular levels of two σ subunits, σ^{70} and σ^{38}, at various phases of cell growth. For both σ^{70} and σ^{38} subunits and under the Western blot conditions employed (see Materials and Methods), linearity was detected over a 10-fold range at least between 2 and 20 ng (Fig. 1).

For accurate determination of σ subunits in test samples, we analyzed several different volumes of the cell lysates and estimated the volumes that include 2 to 20 ng of each σ subunit. Using the optimum volumes of cell lysates thus estimated, we finally repeated the determination of individual σ subunits at least three times, always in parallel with the determination of six different concentrations of the purified σ subunits as the assay standards. The maximum fluctuation between different measurements described in this report was ±20%.

Levels of σ subunits during the steady-state growth. Cells of *E. coli* MC4100 cells were grown in LB medium at 37°C, and samples were taken periodically during the growth transition from the exponential growth phase (20 Klett units) to the stationary phase (160 Klett units) (Fig. 2).

The concentration of σ^{70} is maintained at a constant level, ranging from 50 to 80 fmol per μg of total proteins throughout the change from the logarithmic growth phase to the stationary phase (Fig. 2) (10). In contrast, the level of σ^{38} started to increase when the cells stopped growing and reached the maximum level of almost 30 fmol per μg of total proteins in the stationary phase. Since the level of σ^{38} was below the detection level at the early exponential phase, the molar ratio of σ^{38} to σ^{70} increases from a negligible level to about 0.3 in the stationary phase.

Variation of σ levels under various stress conditions. The above experiments confirmed the concept that the entry of cell growth into stationary phase is accompanied by the increase in the intracellular concentration of σ^{38} (12, 17). Mutations in *rpoS* give pleiotropic phenotypes and often result in a rapid loss of cell viability under various stress conditions, including exposure to near-UV radiation, elevated temperatures, high salt levels, hydrogen peroxide, and prolonged starvation (14). These observations indicate the involvement of σ^{38} in response to certain stress conditions. In order to reveal the stress-induced variation in σ subunit levels, we exposed both the exponentially growing and stationary-phase cells to various stress conditions, including exposure to temperature upshift (from 30 to 42°C), high salt level (0.5 M NaCl), and hydrogen peroxide (between 0.02 and 20 mM), and measured the levels of σ^{70} and σ^{38}. Of all the stress conditions tested, a significant increase in σ^{38} level was observed for heat shock treatment at the exponentially growth phase and osmotic shock at the stationary phase.

Following the temperature upshift, for instance, the concentration of σ^{38} increased to 13 fmol per μg of total proteins by 20 min at 42°C, and at 60 min, it dropped to the steady-state level at 30°C (Fig. 3A). The results support the notion that beside σ^{32} (σ^{H}, the *rpoH* gene product), σ^{38} plays a role in heat shock protection, although σ^{38} reached the maximum level later than σ^{32} did.

High osmolality stress at the stationary phase also induced the production of σ^{38} from 10 to 50 fmol per μg of total proteins by 30 min, i.e., ca. fivefold increase over that of the control culture without osmotic stress (Fig. 3B). In both cases, the level of σ^{70} remained constant, at least within the time ranges examined. Temperature upshift at the stationary phase and osmotic upshift at the exponential phase had no effect on the levels of both σ subunits (data not shown).

DISCUSSION

Previously, Kawakami et al. (10) showed that the intracellular concentration of σ^{70} subunit is maintained at a constant level throughout the transition from the exponential growth phase to the stationary phase, although the levels of core enzyme subunits decrease concomitantly with the arrest of cell growth. In this study, we confirmed this conclusion with a quantitative Western blot assay. The concentration of σ^{70} in strain MC4100 grown in LB is maintained at a constant level ranging from 50 to 80 fmol per μg of total proteins throughout the change from the exponential growth phase to the stationary

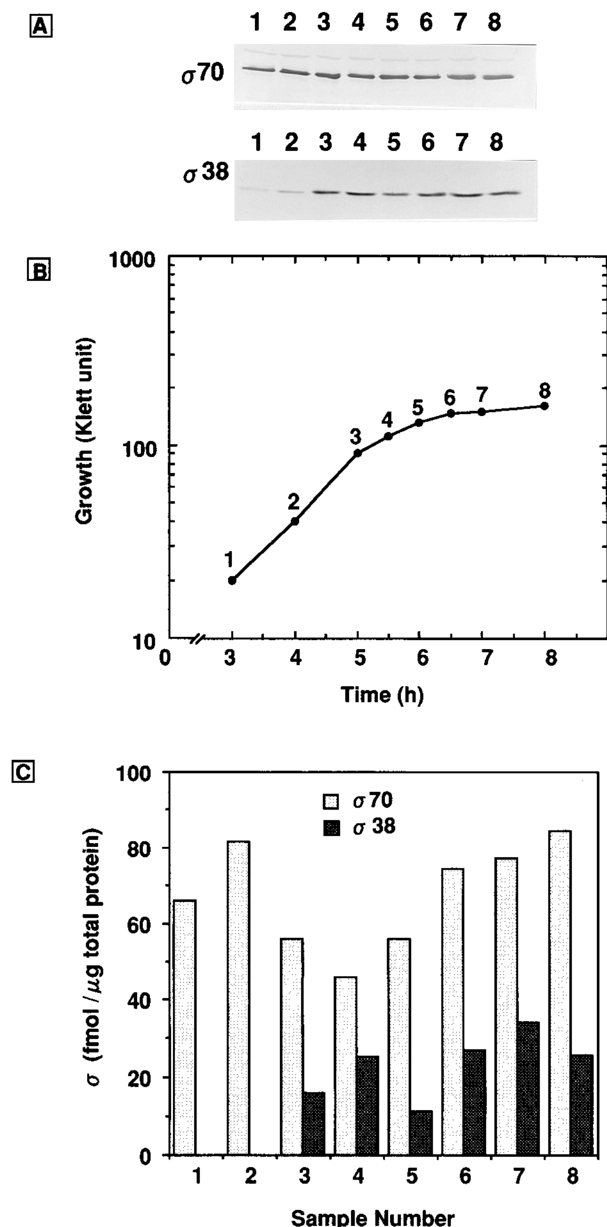

FIG. 2. Growth phase-dependent variation in the intracellular levels of σ^{70} and σ^{38} proteins. (A) Either 5 μg (for σ^{70}) or 10 μg (for σ^{38}) of total proteins from cell lysates prepared at various times of the cell culture (see panel B for the growth curve) was subjected to SDS-polyacrylamide gel electrophoresis, and the blots were probed with anti-σ^{70} or anti-σ^{38} antibodies. (B) Cells of *E. coli* MC4100 were grown in LB medium at 37°C, and growth was monitored by measuring the turbidity with a Klett-Summerson photometer. At various time points, aliquots of the culture were taken and cell lysates were prepared for Western blot analysis as described in Materials and Methods (see panel A for the Western blot pattern). The numbers along the growth curve correspond to the sample numbers shown in panels A and C. The first sample (sample number 1) was taken 3 h after the culture dilution. (C) The immunostained blot was analyzed with a PDI image analyzer system equipped with a white light scanner to determine the intensities of σ^{70} and σ^{38} proteins. For each sample, the measurement was repeated at least three times and the values shown are the averages of three independent determinations.

phase. In contrast, the level of σ^{38} increases when the cell growth enters the stationary phase and reaches the maximum level of approximately 30 fmol per μg of total proteins. These results indicate that the molar ratio of σ^{38} to σ^{70} reaches about 30% in the stationary phase.

On the basis of these measurements, we can estimate the intracellular state of RNA polymerase in *E. coli*. The steady-state cells at the exponential growth phase contain 1,500 to 2,000 molecules of RNA polymerase core enzyme and 500 to 700 molecules of σ^{70} per cell (8, 9). Hence, we can calculate the number of σ^{38} to be 170 to 230 molecules per cell in the stationary phase. It should be noted, however, that the strain MC4100 used in this study does not carry the functional *flhD* gene and thus lacks σ^{28} (or σ^{F}) (the *rpoF* gene encoding σ^{28} is under the control of the FlhD-FlhC complex, a class I activator-interacting RNA polymerase α subunit [13]). Any possible influence of the absence of σ^{28} on the concentrations of other σ subunits remains to be determined.

A set of about 1,000 different genes are transcribed in exponentially growing cells (8), while more than 20 genes or operons have been identified as being expressed in the stationary-phase cells under the direct or indirect control of σ^{38} (3). The overall rate of transcription decreases to less than 10% of the level in the exponential growth phase (8). Since most, if not all, of the genes constitutively expressed during the exponential growth phase are shut off in the stationary phase, it is supposed that most σ^{70} molecules in the stationary phase are not involved in transcription and are stored in an inactive form. Otherwise, $E\sigma^{70}$ in the stationary-phase cells are all used for transcription of some constitutively expressed genes which carry weak promoters.

Upon temperature upshift, the intracellular level of σ^{38} was found to increase even in the exponential growth phase, as in the case of σ^{32}, the alternative sigma factor involved in the heat shock response (15). Subunit σ^{32} is a highly unstable protein in vivo, but following temperature shift to 42°C, the level of σ^{32} transiently increases. Two possibilities for this increase have been proposed: an increase in the translation efficiency of *rpoH* mRNA at 42°C and transient stabilization of σ^{32} protein (20). Both chaperones and proteases are involved in control of σ^{32} stability (16), including a membrane-bound, ATP-dependent protease (18). The level of σ^{38} is also controlled by a complex mechanism involving not only transcriptional and translational regulation but also control of protein stability (12). However, the key signal(s) or factor(s) which leads to an increase in the level of σ^{38} or stabilization of the σ^{38} protein at the stationary phase have not yet been identified. Like σ^{32}, the σ^{38} protein is also highly unstable in exponentially growing cells. The secondary structure of σ^{38} mRNA is also similar to that of σ^{32} mRNA (12). From these considerations, the lifetime of σ^{38} protein may be under the control of a chaperone(s) or protease(s). Otherwise, the translation of *rpoS* mRNA may increase at 42°C. In any case, σ^{38} seems to play a role, even in exponentially growing cells under certain stress conditions, even though we have not determined whether σ^{38} induced under the heat shock conditions is functional.

Osmotic shock at the stationary phase also induced the production of σ^{38}. The concentration reaches the same level of σ^{70} at 30 min after osmotic shock. Upon osmotic shock at least 18 proteins, as identified on two-dimensional gels, are induced in an *rpoS*-dependent manner (4). The increase in the intracellular concentration of σ^{38} by osmotic shock may lead to production of these proteins.

In this study, we demonstrated that the intracellular level of σ^{38} increased in the stationary phase and under the various stress conditions. However, it is not clear yet how many σ^{38} molecules are bound to RNA polymerase core enzyme and involved in transcription cycle. The distribution of σ^{38} between core enzyme-bound and dissociated free forms remains to be determined.

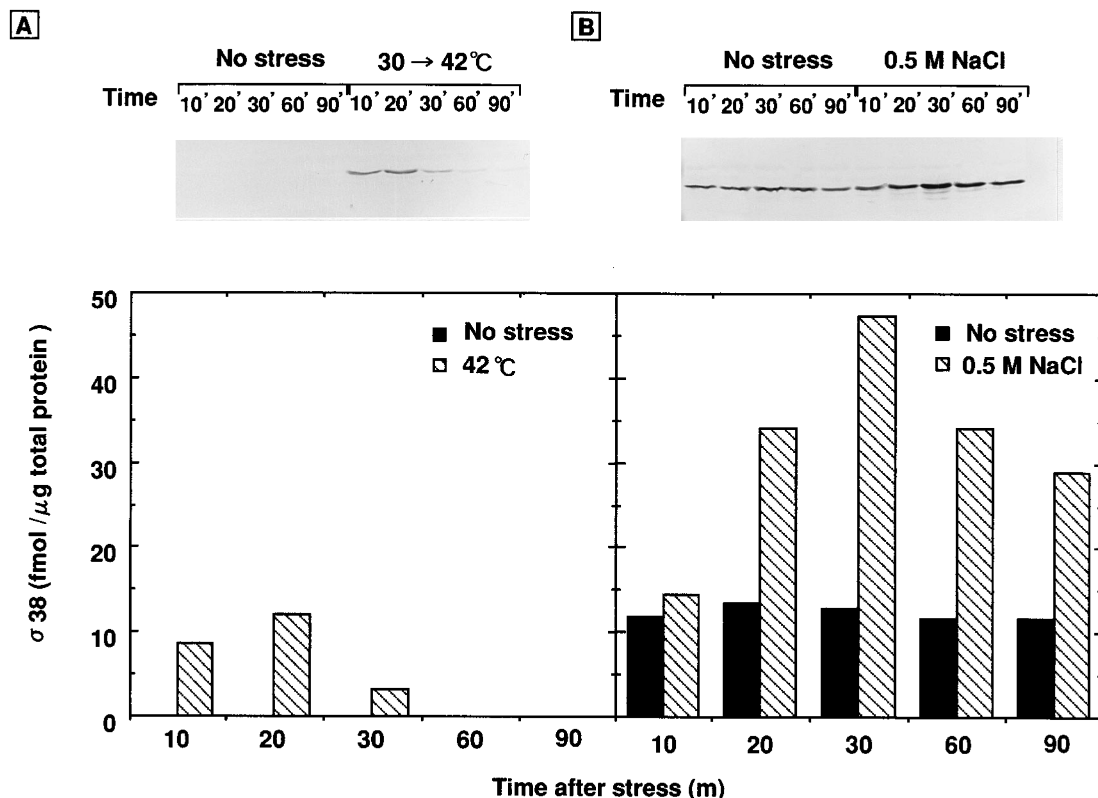

FIG. 3. Variation in the σ^{38} protein level under stress conditions. (A) Cells of *E. coli* MC4100 were grown to the exponential phase in LB medium at 30°C. At the cell density of 15 Klett units, the culture temperature was raised to 42°C by adding an equal volume of LB warmed at 54°C, and samples were taken at 10, 20, 30, 60, and 90 min (minutes indicated by apostrophes) after the temperature upshift. (B) Cells were grown in LB medium at 37°C, and at 5 h after the culture dilution (about 2 h after growth arrest), 1/10 volume of fresh medium containing 5 M NaCl was added to make a final 0.5 M solution. Samples were taken at 10, 20, 30, 60, and 90 min (m) after the addition of NaCl. Aliquots of the cell lysates containing 20 μg of total proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The blots were probed with anti-σ^{70} and anti-σ^{38} antibodies. The immunostained σ bands were quantitated by a PDI image analyzer system equipped with a white light scanner. For each sample, the measurement was repeated at least three times. The values shown are the averages of three independent determinations.

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