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The *rpoE* gene encoding the σ^E (σ^{24}) heat shock sigma factor of *Escherichia coli*

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Previous work has established that the transcription factor σ^E (σ^{24}) is necessary for maintaining the induction of the heat shock response of *Escherichia coli* at high temperatures. We have identified the gene encoding σ^E using a genetic screen designed to isolate *trans*-acting mutations that abolish expression from either *htrA* or *rpoH*P3, two promoters recognized uniquely by σ^E-containing RNA polymerase. Such a screen was achieved by transducing strains carrying a single copy of either p*htrA*–*lacZ* or *rpoH*P3–*lacZ* fusions with mutagenized bacteriophage P1 lysates and screening for Lac$^-$ mutant colonies at 22°C. Lac$^-$ mutants were subsequently tested for inability to grow at 43°C (Ts$^-$ phenotype). Only those Lac$^-$ Ts$^-$ mutants that were unable to accumulate heat shock proteins at 50°C were retained for further characterization. In a complementary approach, those genes which when cloned on a multicopy plasmid led to higher constitutive expression of the σ^E regulon were characterized and mapped. Both approaches identified the same gene, *rpoE*, mapping at 55.5 min on the *E.coli* genetic map and encoding a polypeptide of 191 amino acid residues. The wild-type and a mutant *rpoE* gene products were over-expressed and purified. It was found that the purified wild-type σ^E protein, when used in *in vitro* run-off transcription assays in combination with core RNA polymerase, was able to direct transcription from the *htrA* and *rpoH*P3 promoters, but not from known σ^{70}-dependent promoters. *In vivo* and *in vitro* analyses of *rpoE* transcriptional regulation showed that the *rpoE* gene is transcribed from two major promoters, one of which is positively regulated by σ^E itself.

Key words: *cpxA*/*cpxR*/gene regulation/heat shock response/*rpoE*/transcription factor

Introduction

Heat shock, as well as other forms of stress, elicits a physiological response that allows a cell to adjust to its new growth conditions. In *Escherichia coli*, this 'classical' heat shock response appears as a transient acceleration in the synthesis of ~20 proteins (Neidhardt and VanBogelen, 1987). This phenomenon is under the positive regulation of the *rpoH* (*htpR*) gene product, σ^{32}, a sigma subunit of RNA polymerase (RNAP). This set of heat shock genes constitutes the σ^{32} heat shock regulon (reviewed by Gross *et al.*, 1990; Yura *et al.*, 1993; Georgopoulos *et al.*, 1994).

When cells are shifted to temperatures where balanced growth is no longer possible, i.e. above 45°C, the heat shock proteins are essentially the only ones synthesized. Continuous synthesis of σ^{32} is required to achieve this sustained transcription of heat shock genes at elevated temperatures. It has been shown that the *rpoH* gene is transcribed from at least four different promoters: P1, P3, P4 and P5. Out of these, P1, P4 and P5 are transcribed by the Eσ^{70} holoenzyme (RNAP/σ^{70}). At very high temperatures, such as 50°C, only P3 is actively transcribed (Gross *et al.*, 1990). Wang and Kaguni (1989) and Erickson and Gross (1989) were able to isolate from crude RNAP preparations a new 24 kDa sigma subunit, σ^E (σ^{24}), which, when complexed to the RNAP core (E), was able to direct transcription from the P3 promoter. The discovery of another σ^E-dependent promoter, that of the *htrA* (*degP*) gene, encoding a periplasmic protease (Lipinska *et al.*, 1988, 1990; Strauch and Beckwith, 1988), led to the realization that *E.coli* possesses two heat shock regulons, one controlled by σ^{32} and the other by σ^E (Gross *et al.*, 1990).

It is of fundamental importance to *E.coli* physiology to understand the various levels at which the Eσ^{32}- and the Eσ^E-promoted heat shock regulons are controlled. In the case of the *rpoH* (σ^{32}) gene it is known that, besides transcriptional activation via the P3 promoter at high temperatures, translational control also plays an important role (Yura *et al.*, 1993). In addition, the stability of the σ^{32} protein itself is a key regulatory element of this heat shock regulon (Gross *et al.*, 1990; Yura *et al.*, 1993; Georgopoulos *et al.*, 1994).

During our previous studies aimed at characterizing elements involved in the regulation of the heat shock response, we identified two heat shock genes, *htrC* (Raina and Georgopoulos, 1990) and *htpY* (Missiakas *et al.*, 1993a), whose products modulate this response. The *htpY* gene is located immediately upstream of the *dnaK dnaJ* operon. Over-expression of HtpY leads to a global increase in the levels of expression of both the σ^{32}- and σ^E-dependent heat shock regulons. In agreement with this finding, mutations in *htpY* lead to a decline in the levels of heat shock proteins. However, it is intriguing that over-expression of HtpY induces only the *htrA* promoter, but not *rpoH*P3, implying that some additional factors might be involved in the regulation of the σ^E regulon, particularly *htrA*. While HtpY seems to positively modulate the heat shock response, the *htrC* gene product appears to be a negative regulator. This conclusion is based on the fact that mutations in *htrC* lead to constitutively elevated levels of all heat shock proteins. The HtrC mutant phenotype is very complex, since in addition to the known heat shock proteins, certain other proteins are also induced, whereas

the synthesis of certain others is noticeably diminished (Raina and Georgopoulos, 1990).

The presence of a second heat shock regulon in *E.coli*, whose transcription depends on σ^E, suggests a physiological role complementary to that of the σ^{32} regulon in sustaining bacterial growth under extreme stress conditions. Recently it has been shown that σ^E activity can be specifically induced by the increased expression of outer membrane proteins and, alternatively, is reduced by their decreased expression (Mecsas *et al.*, 1993). In order to attain a better understanding of the σ^E regulon and undertake a detailed genetic and biochemical analysis of the σ^E protein itself, it was first necessary to clone the *rpoE* gene. Taking advantage of the known promoter sequences of the *htrA* and *rpoH* genes, we constructed two transcriptional gene fusions using these two σ^E-dependent promoters and a promoterless *lacZ* gene. These *lacZ* transcriptional fusions were used in a screen for chromosomally encoded *trans*-acting mutations that abolish or reduce the expression of the *lacZ* gene and simultaneously confer a temperature sensitive (Ts^-) bacterial growth phenotype. Here, we describe the isolation of such mutations and the subsequent cloning of the corresponding *rpoE*$^+$ wild-type gene by complementation.

Molecular characterizations of the *rpoE* mutations, as well as biochemical studies performed with the purified σ^E protein, prove conclusively that the *rpoE* gene encodes the sigma factor responsible for transcription of the *rpoH*P3 and *htrA* promoters. Interestingly, the *rpoE* gene was found to be under its own transcriptional regulation, with one of its promoters being quite similar in sequence to that of the *htrA* gene and the P3 promoter of the *rpoH* gene.

Results

Isolation of mutations in the rpoE gene

To identify the *rpoE* gene, we first isolated point mutants marked with a mini-Tn*10* (Kan^R or Tet^R), which conferred a Lac^- phenotype to bacterial strains carrying single copy *htrA*−*lacZ* or *rpoH*P3−*lacZ* transcriptional gene fusions. Various Lac^- mutants obtained following different strategies (as outlined in Materials and methods and listed in Table I) were subsequently tested for colony-forming ability at 43°C. This step was included because our previous work with *htrA* mutant bacteria strongly suggested that the σ^E polypeptide is essential for bacterial growth, at least at 43°C (Lipinska *et al.* 1989). Since the penetrance of mutations of the *htrA* gene is known to be strain-specific, we first transduced the mutations, causing the Lac^- phenotype by virtue of their linkage to the Tn*10* marker, into the wild-type strain W3110. About 300 mutant candidates which exhibited a similar Lac^- phenotype in strains carrying either the *htrA*−*lacZ* or *rpoH*P3−*lacZ* fusions and a Ts^- growth phenotype at 43°C were retained.

Mutations in genes affecting overall macromolecular syntheses were eliminated by transducing them, along with their linked drug resistance markers, into strains carrying a single copy *lacZ* fusion to the known $E\sigma^{70}$-transcribed promoter of either the *htrP* (SR940; Raina *et al.*, 1991) or *htrL* (SR1588; Missiakas *et al.*, 1993a) genes (Table II). Out of the 300 original Lac^- mutant candidates, only 64 were retained, those that were affected uniquely in transcription from $E\sigma^E$-transcribed promoters and not from $E\sigma^{70}$-trancribed promoters.

By using such strategies, we avoided isolating: (i) mutations in the chromosomally encoded *lac* operon, since the *lacZ* gene is deleted in the MC4100 host strain; (ii) *cis*-acting mutations in the promoter regions of the *htrA* and *rpoH* genes that lower or abolish gene transcription; and (iii) multiple unlinked mutations, since all the putative point mutations detected in our screen were co-transducible with a nearby Tn*10* insertion conferring either Kan^R or Tet^R, thus providing a convenient way for further transfer into different genetic backgrounds.

These mutant bacteria were further characterized by measuring the levels of HtrA protein, *htrA* transcripts and the accumulation of heat shock proteins following a temperature upshift from 30 to 50°C (see below).

Characterization of rpoE mutants

rpoE mutants express reduced levels of heat shock proteins at 50°C. Since the transcription of the *rpoH* gene from the P3 promoter at temperatures such as 50°C is exclusively dependent on $E\sigma^E$, we rationalized that a mutation in the *rpoE* gene should prevent the induction of all heat shock proteins at such extreme temperatures. Thus, we assayed the induction of heat shock proteins in all 64 Lac^- Ts^- mutants following a temperature shift from 30 to 50°C. As can be seen in Figure 1, the only proteins synthesized at 50°C in a wild-type background are the heat shock proteins, such as DnaK and GroEL. Among the 64 Lac^- Ts^- mutant candidates, only 28 showed a lack of or very low heat shock protein synthesis at 50°C. As an example, labeled extracts from wild-type bacteria, SR1576 and SR1723 (carrying a L25P or a S172P mutation in the *rpoE* gene respectively; see below) are shown in Figure 1.

rpoE mutants exhibit specifically lower levels of htrA mRNA and HtrA protein. We tested the levels of *htrA* transcripts present in the various *rpoE* mutant candidates at 30 and 50°C. The fact that the *htrA* gene possesses a unique $E\sigma^E$ regulated promoter made the interpretation of these results unambiguous. The autoradiogram of a Northern blot, using as a probe a DNA fragment internal to *htrA*, is shown in Figure 2A for the mutant bacteria SR1576 and the isogenic wild-type bacteria. Mutants which accumulated fewer *htrA* transcripts at 30 and 50°C (presumably affected in transcription from the *htrA* promoter) were further analyzed by the Western blot technique, using polyclonal antibodies raised against the HtrA protein. The results, shown in Figure 2B, confirm that expression from the *htrA* promoter is greatly reduced in such mutants.

Altogether, 28 mutations causing Lac^- Ts^- which possessed all of the three predicted phenotypes were retained for further characterization.

Mapping and cloning of the rpoE gene

As a first step, we used the linked Tn*10* Kan^R or Tet^R drug resistance markers to assign the 28 Lac^- Ts^- mutants to different complementation groups. Cosmid clones able to recombine the linked Tn*10* markers were selected. Nick-translated probes prepared from such cosmids were hybridized to the Kohara library (Kohara *et al.*, 1987). One major complementation group comprising 12 mutants

Table I. The bacterial strains and plasmids used in this study

	Relevant characteristics	Reference or source
Strains		
MC4100	F⁻ *araD139* Δ(*argF*−*lac*)U169	Casadaban (1976)
SR1576	MC4100 *rpoE*25 (L25P)	This work
SR1502	MC4100 *rpoE*178 (R178G)	This work
SR1723	MC4100 *rpoE*172 (S172P)	This work
SR2393	CA8000 Δ*rpoE*(*Hin*dIII−*Hpa*I)::ΩTet^R	This work
SR1421	MC4100 *lon*::λ*plac*Mu53	This work
SR1458	MC4100 ϕ (*phtrA*−*lacZ*)	This work
SR1710	MC4100 ϕ (P3*rpoH*−*lacZ*)	This work
SR2141	MC4100 ϕ (P3P4P5*rpoH*−*lacZ*)	This work
SR2195	MC4100 ϕ (p*rpoE*−*lacZ*)	This work
SR940	MC4100 ϕ (*phtrP-lacZ*)	Raina *et al.* (1991)
SR1588	MC4100 ϕ (*phtrL*−*lacZ*)	Missiakas *et al.* (1993a)
SR381	CA8000 Δ*htrC*(21–575)::ΩTet^R	Raina and Georgopoulos (1990)
SR1748	CA8000 *trxB*::Tn*10*	Missiakas *et al.* (1993b)
DM547	CA8000 *dsbA*::Tn*10*	Missiakas *et al.* (1993b)
DM995	CA8000 *dsbC*::Tn*10*	Missiakas *et al.* (1994)
Plasmids		
pSR1451	pRS415 (p*htrA*−*lacZ*) carrying the *htrA* promoter	This work
pSR1709	pRS550 (P3*rpoH*−*lacZ*) carrying the P3 *rpoH* promoter	This work
pSR2138	pRS550 (*rpoH*−*lacZ*) carrying the P3, P4, P5 *rpoH* promoters	This work
pSR2177	pRS528 (p*rpoE*−*lacZ*) carrying 360 nt of the *rpoE* promoter	This work
pSR2189	pKS (p*rpoE*–*lacZ*) carrying 360 nt of the *rpoE* promoter	This work
pSR2180	pRS550 (p*rpoE*–*lacZ*) carrying 360 nt of the *rpoE* promoter	This work
pSR1546	pOK12 carrying a 3.3 kbp *rpoE*$^+$ partial *Sau*3A fragment cloned into a *Bam*HI site	This work
pSR1628	pOK12 carrying a 1.4 kbp *rpoE*$^+$ partial *Sau*3A fragment cloned into a *Bam*HI site	This work
pDM616	pOK12 carrying 2.6 kbp *Kpn*I−*Eco*RI *rpoE*$^+$ fragment	This work
pDM619	pOK12 carrying 1.6 kbp *Ssp*I−*Eco*RI *rpoE*$^+$ fragment	This work
pDM1055	pAED-4 carrying a minimal 573 bp *rpoE*$^+$ *Nde*I−*Bam*HI fragment	This work
pDM1056	pAED-4 carrying the mutant *rpoE* allele R178G	This work
pSR2370	pBIP carrying truncated *rpoE* gene with ΩTet^R	This work

Table II. Effect of different *rpoE* mutant alleles on β-galactosidase activity as judged by *lacZ* fusions to σ^E- or σ^{70}-dependent promoters

Temperature (°C)	β-Galactosidase activity (Miller units)		
	30	42	50
SR1458 = MC4100 ϕ (*htrA*−*lacZ*)			
SR1458 *rpoE* wild-type	271 ± 20	339 ± 27	365 ± 29
SR1458 *rpoE* R178G	73 ± 7	62 ± 6	64 ± 7
SR1458 *rpoE* S172P	83 ± 6	71 ± 7	63 ± 7
SR1458 *rpoE* L25P	112 ± 9	89 ± 8	92 ± 11
SR1710 = MC4100 ϕ (*rpoH*P3−*lacZ*)			
SR1710 *rpoE* wild-type	149 ± 12	172 ± 14	201 ± 21
SR1710 *rpoE* R178G	49 ± 7	46 ± 7	37 ± 6
SR1710 *rpoE* S172P	52 ± 6	47 ± 8	42 ± 7
SR1710 *rpoE* L25P	63 ± 10	58 ± 6	42 ± 9
SR940 = MC4100 ϕ (*htrP*−*lacZ*)			
SR940 *rpoE* wild-type	1527 ± 85	942 ± 65	742 ± 62
SR940 *rpoE* R178G	1470 ± 83	951 ± 68	714 ± 57
SR1588 = MC4100 ϕ (*htrL*−*lacZ*)			
SR1588 *rpoE* wild-type	1524 ± 72	1467 ± 82	1259 ± 77
SR1588 *rpoE* R178G	1503 ± 77	1436 ± 70	1194 ± 66

Assays were performed after a 15 min incubation following the temperature shift.

corresponded to cosmids hybridizing to Kohara's bacteriophage λ transducing clones 432 (8E12), 433 (6H2), 434 (7G4) and 435 (4A12). These clones cover the region from 54.5 to 55.5 min of the *E.coli* physical map. Among the other mutants so far characterized, six mutations were found to map at 40 min [λ transducing clones 335 (5D5) and 336 (19H3)] and four other mutations were shown to map in the *cpxA/cpxR* operon (Weber and Silverman, 1988; Dong *et al.*, 1993). However, both the Ts⁻ phenotype and decrease in β-galactosidase activity from *htrA*−*lacZ* associated with mutations in *cpxA/cpxR* loci were not as severe as compared with those associated with mutations defining the *bona fide rpoE* gene. In addition, the affect on *rpoH*P3−*lacZ* activity in *cpxA/cpxR* mutations was much smaller than observed with *htrA*−*lacZ* fusions.

In a complementary approach, we used a chromosomal DNA library prepared from wild-type *E.coli* MC4100 and cloned on a p15A-based vector (Missiakas *et al.*, 1993b). DNA prepared from those clones able to complement the Lac⁻ Ts⁻ phenotypes of the original 28 mutants were nick-translated and 12 were found to hybridize to Kohara bacteriophages λ 434 and 435. The restriction digestion patterns of complementary *rpoE*$^+$ clones (pDM616) predicted that the *rpoE* gene lies between the 2719 and 2720 kbp of the *E.coli* genome. We also used known nearby markers, such as *nadB*::Tn*10* (CAG18481 and CAG18480), to confirm the mapping of the mutations. The mutations causing Lac⁻ Ts⁻ were found to be ~90% linked to the *nadB*::Tn*10* marker.

An additional strategy was employed to eliminate those genes which may only indirectly affect the expression of the two $E\sigma^E$-transcribed *lacZ* fusions. To achieve this, we directly searched for those genes which when cloned on high copy plasmids were able to positively affect

Fig. 1. *E. coli rpoE* mutants exhibit very low levels of heat shock proteins at 50°C. Isogenic *rpoE*$^+$ (MC4100, lanes 1 and 2) and *rpoE* mutant (SR1576 *rpoE* mutant L25P, lanes 3 and 4; SR1723 *rpoE* mutant S172P, lanes 5 and 6) bacterial cultures were grown at 30°C in M9 minimal medium. Equivalent amounts of exponentially growing cultures were labeled for 5 min with [^{35}S]methionine (50 μCi/ml) at 30°C (lanes 1, 3 and 5). Lanes 2, 4 and 6 correspond to cultures shifted to 50°C for 5 min. Protein samples were separated by 12.5% SDS−PAGE. An autoradiogram of the dried gel is shown.

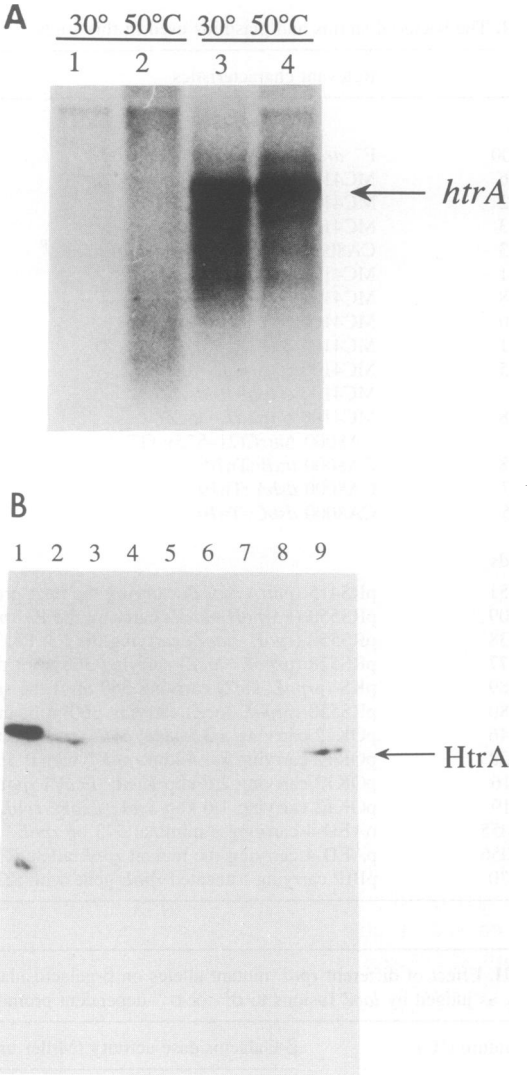


Fig. 2. Analysis of *htrA* mRNA and HtrA protein antigen in different mutant strains. (**A**) Northern analysis of *htrA* transcripts. RNA was extracted from bacterial cultures grown at 30°C or shifted to 50°C for 15 min before extraction. Lanes 1 and 2, SR1576 (*rpoE* L25P mutant); lanes 3 and 4, SR1458 isogenic wild-type (*rpoE*$^+$). Approximately 10 μg total RNA/lane was analyzed by the Northern blot technique and probed with ~50 ng ^{32}P-labeled probe (1.3 kb *Pst*I fragment carrying the *htrA* gene). (**B**) The HtrA protein levels are reduced in *rpoE* mutant bacteria. Crude extracts prepared from various strains were separated by SDS−PAGE (12.5%). Lane 1, SR1576 transformed with pSR1628 (*rpoE*$^+$); lanes 2–8, seven different *rpoE* mutant alleles, SR1723, SR1672, SR1895, SR1576, SR1577, SR1502 and SR1602; lane 9, MC4100 (isogenic wild-type). Proteins were transferred to a nitrocellulose filter and probed with antibodies raised against purified HtrA protein (Lipinska *et al.*, 1990).

transcription from the *htrA* promoter or the P3 promoter of *rpoH* even at 30°C, using β-galactosidase as the reporter. We obtained two major classes, which were mapped at either 55 or 40 min on the *E.coli* chromosome, and four minor classes, which mapped to 15, 27, 69 and 95 min respectively. The 55 min clone was able to complement the *rpoE* mutations and was shown to indeed carry the wild-type *rpoE*$^+$ gene (see below).

Sequencing of the rpoE gene

The *rpoE* gene and its adjacent DNA regions were sequenced using template DNA from plasmid pDM616 or its derivative subclones. Sequencing of the 2.6 kb *Kpn*I−*Eco*RI fragment revealed an open reading frame (ORF) of 573 nucleotides. The direction of transcription of this putative ORF is divergent to that of the *nadB* gene, i.e. in the direction of the *Eco*RI sites, and hence counter-clockwise with respect to the conventional *E.coli* map (Figure 3).

The predicted ORF encodes a 191 amino acid polypeptide with an estimated pI of 5.3 at pH 7. These predictions were further verified by two-dimensional equilibrium polyacrylamide gel electrophoresis (PAGE) analysis of the purified RpoE protein and an extract in which RpoE was exclusively labeled using the T7 expression system (see below). Downstream of the putative *rpoE* ORF is another ORF, whose amino acid sequence shows a high degree of homology with the MucA protein of *Pseudomonas aeruginosa* (Martin *et al.*, 1993b).

The evidence that the *rpoE* ORF indeed encodes a sigma factor is based on the following observations: (i) the predicted amino acid sequence shows significant homology with various other sigma factors; (ii) the purified RpoE protein is active in the run-off transcription assay (see below); and (iii) among the 12 putative point mutations which confer the Lac$^-$ Ts$^-$ phenotypes, we cloned three of them by recombining them onto the *rpoE*-carrying cosmid and sequenced them. One mutation was found to

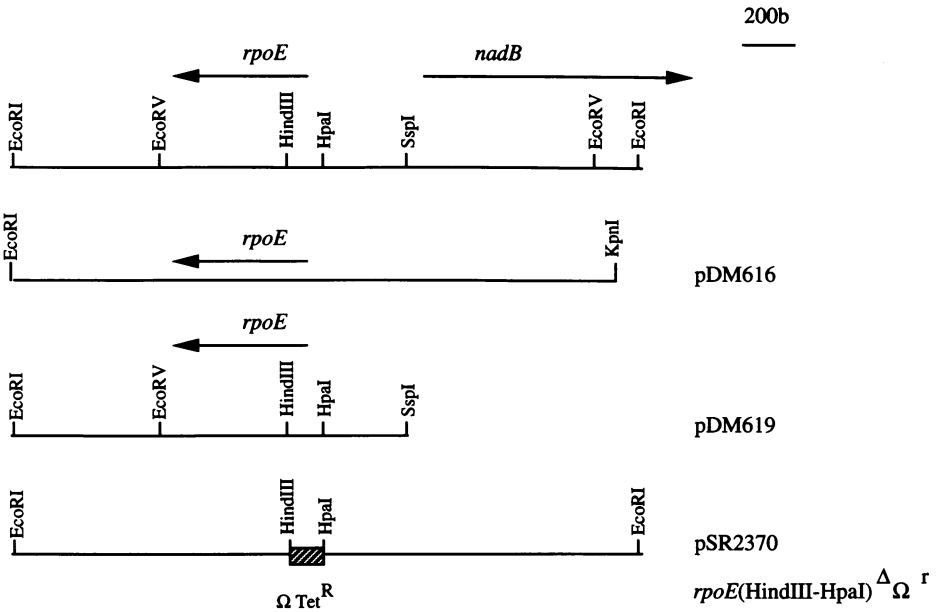


Fig. 3. Restriction map of the *rpoE* gene and *in vitro* construction of the *rpoE*::Ω Tet^R allele.

cause a change at amino acid position 25; L25 (CCG)→P (CTG). This L residue is located in the region 2.1, which is conserved in the σ^{70} and group 3 superfamilies (Lonetto *et al.*, 1992). The second mutation caused the change S172 (TCA)→P (CCA). This amino acid is located in the predicted helix−turn−helix domain of region 4.2 characteristic of all major sigma factors. The last mutation also caused a change in the same helix−turn−helix domain and was due to the replacement of R178 (AGG) by G (GGG).

Expression of the rpoE gene

The *rpoE* gene product was over-expressed and labeled with [^{35}S]methionine using BL21(DE3) cells carrying either the vector alone (as a control) or plasmid pDM1055, containing the *rpoE* gene under the exclusive T7 promoter expression system. When the T7 promoter was induced, the specific induction of an ~24 kDa protein was observed (Figure 4A). The same [^{35}S]methionine extract from the over-producing *rpoE* strain (DM1055) was analyzed by two-dimensional equilibrium PAGE (Figure 4B). The RpoE protein migrated with an apparent isoelectric point of ~5.2, just beneath the EF-Tu and OmpA proteins, consistent with the predicted amino acid sequence.

Purification of the σ^E transcription factor

The σ^E protein was purified from a total protein extract of *E.coli* strain DM1055 (or DM1056 for the mutant allele R178G), using the protocol described in Materials and methods (Figure 5). Fractions containing the RpoE protein, purified on the basis of its apparent molecular weight following separation on a 12.5% SDS gel or by monitoring its activity in run-off transcription assays using the *htrA* promoter, were pooled. It should be noted that only 10% of the RpoE protein expressed under the T7 promoter was recovered following disruption of the cells. Most of the RpoE protein was found along with the membrane fraction, the remaining soluble fraction being used for purification.

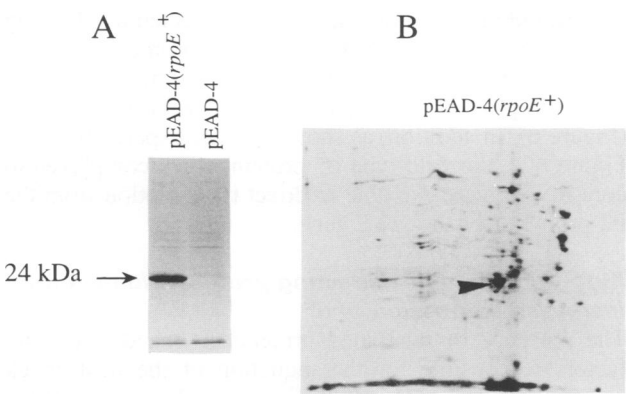


Fig. 4. Identification of the RpoE (σ^E) protein. (**A**) Cultures of strains carrying either vector alone or plasmid pDM1055 with the *rpoE* gene cloned under the T7 RNA polymerase promoter were grown at 37°C in M9 minimal medium. Expression of the T7 RNA polymerase was induced by addition of IPTG (5 mM). Following a 30 min incubation, cells were treated with rifampicin (200 μg/ml) for another 15 min and then labeled with [^{35}S]methionine (50 μCi/ml) for 5 min. The proteins were resolved by 12.5% SDS−PAGE and an autoradiogram of the dried gel is shown. (**B**) Two-dimensional equilibrium polyacrylamide gel electrophoresis of the [^{35}S]methionine cell extracts used in (A) mixed with a wild-type *E.coli* extract labeled after a 5 min temperature shift from 30 to 42°C. The proteins were resolved in the first dimension on 1.6% (pH 5.0–7.0) and 0.4% (pH 3.5–10.0) ampholines (Pharmacia) and by 12.5% SDS−PAGE in the second dimension. The arrow points to the position of the σ^E protein.

The purified rpoE gene product is transcriptionally active

To assay for the function of the protein encoded by the putative *rpoE* gene, we performed run-off transcription assays using the purified RpoE protein and a 550 bp DNA fragment containing the *htrA* promoter as the template. As can be seen in Figure 6, purified σ^E, in conjunction with the RNAP core enzyme, was able to specifically transcribe the *htrA* promoter *in vitro*. We also purified one of the mutant proteins RpoE178 (R178G), whose

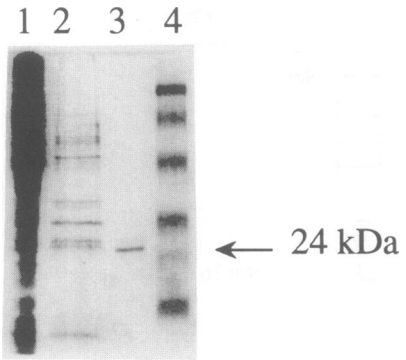


Fig. 5. Purification of the RpoE protein. A 1 l culture of strain DM1055 carrying the *rpoE* gene cloned under the T7 RNA polymerase promoter of the pEAD-4 vector was induced for 2 h with 5 mM IPTG. Cells were harvested and the extracted proteins subjected to the following purification steps: lane 1, whole protein extract after lysis of the cells by sonication and removal of the membrane and insoluble fractions by centrifugation (25 000 revs/min, 45 min); lane 2, protein pool containing σ^E after heparin–agarose chromatography; lane 3, σ^E protein after P11 phosphocellulose chromatography; lane 4, pre-stained molecular size standards from Bio-Rad (108, 87, 49, 32.5, 27 and 18.5 kDa). Proteins were separated by 12.5% SDS–PAGE; the gel was stained with Coomassie brilliant blue.

corresponding mutation was initially selected as blocking transcription from the *htrA* and *rpoH*P3 promoters *in vivo*. No transcriptional activity was associated with this mutant RpoE178 protein *in vitro*, as compared with wild-type σ^E (Figure 6). In addition, as shown in later experiments (see Figure 8C), the wild-type σ^E protein, when complexed to core RNAP, was also able to direct transcription from the P2 promoter of the *rpoE* gene.

Mutations in genes affecting protein folding show increased expression of σ^E

The presence of misfolded proteins is one of the main factors responsible for the induction of the heat shock response (Gross *et al.*, 1990; Georgopoulos *et al.*, 1994). Therefore, we used different mutant bacteria known to have severe defects in the regulation of the heat shock response to study their effect on σ^E activity. We have previously shown that *htrC* null mutants exhibit a protein profile different from that of isogenic wild-type bacteria; certain proteins are over-expressed, while many others are under-represented (Raina and Georgopoulos, 1990). We found that the *htrC* mutation resulted in constitutive induction of σ^E activity, leading to increased *lacZ* transcription (Table III). Judging from the apparent migration of one of the proteins (not previously described as a heat shock protein), we guessed that it could be the product of the *rpoE* gene. To test this prediction, we analyzed by two-dimensional equilibrium PAGE the purified σ^E protein mixed with ^{35}S-labeled extracts from *htrC* mutant bacteria. As can be seen in Figure 7, the protein over-produced in *htrC* mutant bacteria co-migrates with the purified σ^E protein.

To extend and generalize these findings further, we studied other mutant backgrounds which show defects in protein folding and increased *htrA* and *rpoH*P3 *lacZ* expression. These included mutations in the *dsbA*, *dsbC* and *trxB* genes, which we previously isolated because they resulted in a dithiothreitol (DTT)-sensitive phenotype,

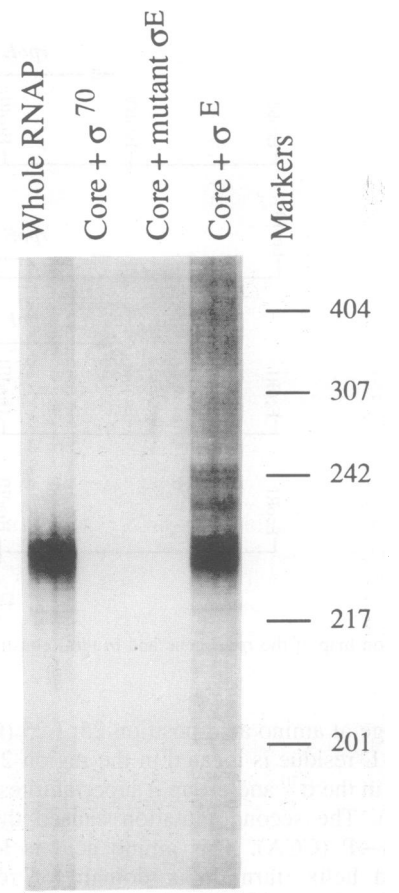


Fig. 6. *In vitro* transcription activity of the *htrA* promoter. The transcription reactions were performed using pSR1451 (containing the *htrA* promoter) as a template and the following different RNA polymerases, from left to right: whole RNA polymerase extracted from MC4100 at 37°C; RNA polymerase core (1 μg) mixed with the purified wild-type σ^{70} protein (2 μg); RNA polymerase core (1 μg) mixed with a purified mutant σ^E protein R178G (2 μg); RNA polymerase core (1 μg) mixed with a purified wild-type σ^E protein (2 μg). RNA samples (4 μl) labeled with [^{35}S]UTP were electrophoresed as described in Materials and methods.

Table III. β-Galactosidase activities of *lacZ* operon fusions to the *htrA* and *rpoH*P3 promoters tested in various mutant backgrounds

	β-Galactosidase activity (Miller units)	
	SR1710 = MC4100 ϕ (*rpoH*P3–*lacZ*)	SR1458 = MC4100 ϕ (*htrA*–*lacZ*)
Wild-type	149 ± 12	365 ± 14
htrC::Tn*10*	305 ± 21	883 ± 39
trxB::Tn*10*	292 ± 23	792 ± 31
dsbA::Tn*10*	314 ± 18	801 ± 34
dsbC::Tn*10*	230 ± 15	538 ± 33
htrA::Tn*10*	142 ± 14	357 ± 17
htrA::Tn*10 dsbA*::Tn*10*	715 ± 35	1502 ± 44

The respective mutations studied were transduced in the two strains SR1710 and SR1458 containing the two promoter fusions.

presumably due to the accumulation of partially folded proteins which either lack disulfide bonds or contain wrongly paired disulfides (Missiakas *et al.*, 1993b, 1994). As can be seen in Table III, strains carrying a *trxB*, *dsbA* or *dsbC* null mutation express increased σ^E activity and

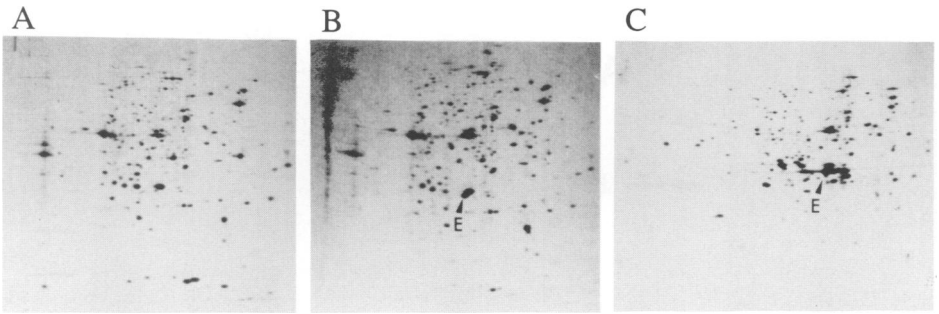


Fig. 7. The σ^E protein is over-expressed in certain mutant backgrounds. Two-dimensional equilibrium polyacrylamide gels were run under the same conditions as described in Figure 4B. Cell extract labeled with [^{35}S]methionine from (**A**) wild-type bacteria, (**B**) *htrC*::Tn*5* (SR381) and (**C**) *trxB*::Tn*10* (SR1748). In (B) and (C) the position of σ^E protein was verified by mixing samples with the purified protein and staining the gel with Coomassie brilliant blue before exposure. Autoradiograms of the dried gels are shown. The arrows point to the position of the σ^E protein.

the σ^E protein accumulates in such backgrounds, e.g. as shown for *trxB* mutant bacteria (Figure 7C). Interestingly, although an *htrA* null allele did not trigger a σ^E-dependent response, the double null mutant *dsbA htrA* exhibited a much higher β-galactosidase activity than the *dsbA* null mutant alone (Table III).

Transcriptional regulation of rpoE

In order to understand the nature and regulation of the *rpoE* promoter region, primer extension analyses were carried out with RNA isolated from wild-type bacteria grown at either 30 or 50°C. As shown in Figure 8A, there are two major putative transcriptional start sites located 174 nt (P1 = TTCTGT) and 75 nt (P2 = TTTGCT) upstream of the ATG start codon. There is also another minor start site located 121 nt upstream of the ATG start codon, but it is unclear whether it is a real one or the result of RNA processing. There are no obvious σ^{70} consensus promoter sequences upstream of the P1 transcriptional start site. The most interesting observation pertains to the P2 transcriptional start site. The sequences of the −10 and −35 regions of this promoter exhibit a significant homology with the two other known promoters recognized by the Eσ^E holoenzyme. As shown in Figure 8B, the alignment of the corresponding DNA sequences is perfect for the −35 region (6/6 nt) and nearly perfect for the −10 region (5/6 nt). There is also a significant homology among these promoters in the spacer region located between the −10 and −35 regions. As can be seen in Figure 8A, the number of transcripts from the P1 promoter exhibits a steady decline following a temperature shift to 50°C, in contrast to the P2 promoter.

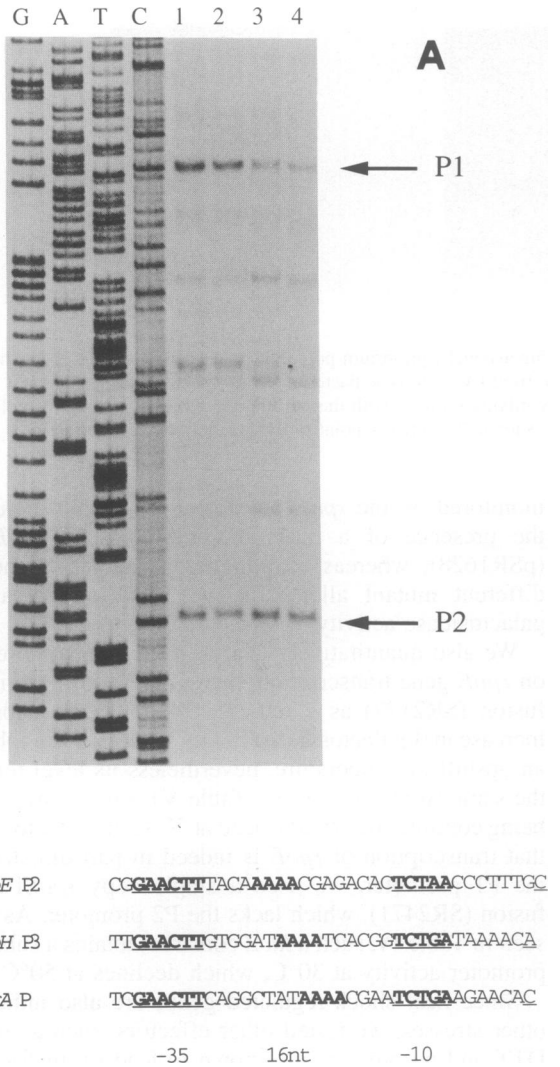
To confirm these results, we performed run-off transcription assays, using as a template plasmid pSR2189, which contains the P2 promoter region along with the flanking sequences of the *rpoE* gene (360 nt) cloned in Bluescript. As can be seen in Figure 8C, when RNAP core was mixed with purified σ^E protein, a unique message was synthesized, starting at the predicted position of the P2 promoter. We did not observe any synthesis of transcripts starting from the P1 promoter (174 nt) using either the Eσ^{70} or Eσ^E holoenzymes (Figure 8C). The positive σ^E autoregulatory circuit was further analyzed *in vivo* by constructing a single copy *lacZ* transcriptional fusion containing the promoter region of *rpoE* (SR2177). As shown in Table IV, the expression of β-galactosidase monitored by the *rpoE* promoter was greatly induced in the presence of a plasmid containing the *rpoE* gene (pSR1628), whereas introduction of either of the three different mutant alleles of *rpoE* led to decreased β-galactosidase activity.

We also quantitatively analyzed the temperature effect on *rpoE* gene transcription, using the *lacZ* transcriptional fusion (SR2177) as a reporter. Although no substantial increase in β-galactosidase activity was observed following an upshift in temperature, nevertheless its level remained the same from 30 to 50°C (Table V), suggesting that it is being continuously synthesized at 50°C. In order to confirm that transcription of *rpoE* is indeed in part directed from the P1 promoter, we used a single copy *rpoE*P1−*lacZ* fusion (SR2471), which lacks the P2 promoter. As can be seen in Table IV, such a *lacZ* construct retains a substantial promoter activity at 30°C, which declines at 50°C.

Since heat shock-regulated genes are also induced by other stresses, we tested other effectors, such as ethanol, DTT and puromycin, all known to lead to misfolding of proteins, in a strain carrying the *rpoE*−*lacZ* fusion. Again, β-galactosidase expression, although not significantly increased, nevertheless was maintained at a high level at concentrations of ethanol, DTT or puromycin which greatly reduced the rate of expression of housekeeping genes (Table V). It should be noted here that the values obtained for the *rpoE* promoter fusion are at least 2-fold higher than those seen with the σ^{32}-dependent *lon* heat shock promoter (one of the better expressed σ^{32}-inducible promoters), even at 50°C. All these results, taken together, provide convincing evidence that *rpoE* transcription is under both heat shock regulation (overall stress control) and positive autoregulation at the transcriptional level. Thus, the *rpoE* gene becomes the third member of the Eσ^E-dependent regulon.

The rpoE gene is essential for bacterial viability at temperatures above 40°C

To study the role of the *rpoE* gene in *E.coli* cell physiology in detail, a null mutation was constructed by substitution of an Ω Tet^R cassette for the *rpoE* coding region between the *Hin*dIII and *Hpa*I restriction sites. As expected from the phenotypic analysis of the *rpoE* point mutants, the *rpoE* Ω::Tet^R mutant bacteria exhibited a Ts$^-$ growth phenotype above 40°C in most of the genetic backgrounds tested, such as W3110, CA8000 or MG1655. This Ts$^-$



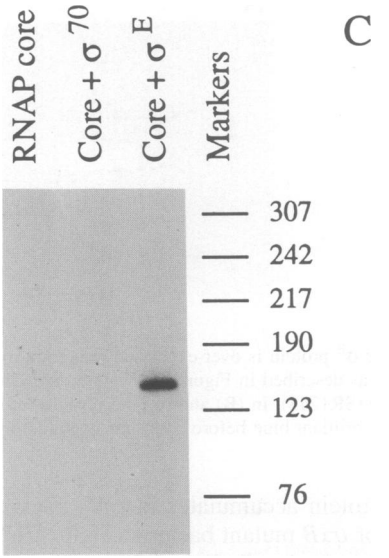


Fig. 8. Transcriptional regulation of the *rpoE* gene. (**A**) Mapping of the 5′ termini of the *rpoE* transcripts. Primer extension reactions of total cellular RNA hybridized to a ^{32}P-end-labeled DNA oligonucleotide probe complementary to nt 35–60 of the *rpoE* coding sequence. RNA was extracted from wild-type MC4100 bacteria grown at 30°C (lanes 1 and 2) or shifted to 50°C for 10 or 20 min (lanes 3 and 4 respectively). Lanes labeled G, A, T and C correspond to the dideoxy sequencing reactions carried out using the same oligonucleotide as the primer and pSR1628 as the DNA template. The two major promoters are indicated by bold arrows. (**B**) Sequence alignment of the *rpoE*P2, *htrA* and *rpoH*P3 promoters. (**C**) *In vitro* transcription assays using pSR2189 (containing the promoter region of *rpoE*) as a template and the following different RNA polymerases, from left to right: RNA polymearse core alone (1 μg); RNA polymerase core (1 μg) mixed with purified wild-type σ^{70} protein (2 μg); RNA polymerase core (1 μg) mixed with purified wild-type σ^{E} protein (2 μg). RNA samples (4 μl) labeled with [^{35}S]UTP were electrophoresed as described in Materials and methods.

phenotype is more severe than those exhibited by bacteria carrying an *htrA*::Tn*10* null mutation, suggesting that the *rpoE* gene regulates some additional genes whose products are essential for bacterial growth at high temperatures. Consistent with these results, induction of *rpoE* leads to the increased synthesis of at least 10 proteins (Figure 9). It should be noted that, as seen with the *rpoE* point mutants L25P or R178G, the *rpoE*::Ω Tet^{R} null mutant does not show a severe growth defect in some wild-type strains, such as MC4100. In such backgrounds, the Ts^{-} phenotype was only observed at temperatures above 42°C.

Homologies with other sigma factors

Sequence comparison showed that at the amino acid level the *rpoE* gene product bears a very high degree of identity (66%) with the putative transcription factor AlgU/AlgT from *P.aeruginosa* (Martin *et al.*, 1993a; DeVries and Ohman, 1994). RpoE is also highly homologous to a subset of the group 3 family of sigma factors, which includes CarQ from *Myxococcus xanthus* (McGowan *et al.*, 1993), CnrH from *Alacaligenes entrophus* (Liesegang *et al.*, 1993), FliA from *Salmonella typhimurium* (Helmann and Chamberlin, 1987; Ohnishi *et al.*, 1990) and SpoOH (σ^{H}) from *Bacillus subtilis* (Dubnau *et al.*, 1988). We, and others (Deretic *et al.*, 1994; Lonetto *et al.*, 1995), have noted that in this sub-group the AlgU/AlgT, CarQ and CnrH proteins are involved in the regulation of primarily extracytoplasmic functions. It has been shown that AlgU in *P.aeruginosa* regulates alginate biosynthesis via AlgD and, likewise, CarQ regulates the synthesis of extracytoplasmically localized carotenoids. These homologies within this sub-family have been recently discussed by Lonetto *et al.* (1994).

Discussion

In the present study, the cloning of the σ^{E}-encoding *rpoE* gene was facilitated by the isolation of *trans*-acting mutations which specifically blocked transcription from the two known Eσ^{E}-dependent promoters, *htrA* and *rpoH*P3. Such mutations were identified because they conferred a Lac^{-} phenotype in strains carrying the *htrA*−*lacZ* or *rpoH*P3−*lacZ* transcription fusions. These findings were further complemented by the cloning of genes whose over-production, when expressed from a multicopy plasmid, led to a simultaneous increase in the level of *rpoE* activity. Following the mutagenesis approach, we identified two more loci in addition to the *rpoE* gene, mapping either to 40 min or to the *cpxR*/*cpxA* regulon (Weber and Silverman, 1988; Dong *et al.*, 1993).

Table IV. The *rpoE* gene product positively regulates its own gene transcription from the P2 promoter

Temperature (°C)	β-Galactosidase activity (Miller units)	
	30	50
Wild-type		
SR2195 = MC4100 ϕ (*rpoE–lacZ*)	972 ± 24	1224 ± 41
Wild-type with *rpoE*$^+$ plasmid (pSR1628)		
SR2195/pSR1628 (*rpoE*$^+$)	2370 ± 70	2410 ± 65
Mutant background		
SR2195 *rpoE* R178G	235 ± 17	187 ± 15
SR2195 *rpoE* S172P	227 ± 21	182 ± 14
SR2195 *rpoE* L25P	374 ± 29	256 ± 21
The distal P1 promoter of *rpoE* gene is not autoregulated		
SR2471 = MC4100 ϕ (*rpoE*P1–*lacZ*)	336 ± 19	254 ± 17
SR2471 *rpoE* R178G	323 ± 21	241 ± 18

Assays were performed after a 15 min incubation following the temperature shift.

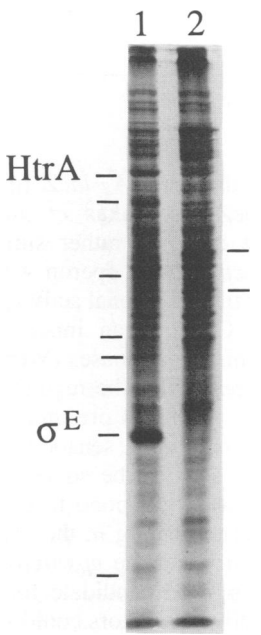


Fig. 9. RpoE induction leads to altered protein synthesis. Exponentially growing cultures of BL21 carrying pDM1055 (*rpoE*$^+$) were labeled with [^{35}S]methionine for 2 min following a 15 min induction with 1 mM IPTG (lane 1) or without any induction (lane 2). The autoradiogram of a dried gel is shown. The positions of the major proteins induced along with σ^E and HtrA are indicated on the left side of lane 1. The positions of some proteins which show a marked reduction in synthesis following σ^E induction are shown on the right side.

Interestingly, the gene mapping to the 40 min region was also obtained with the 'multicopy' approach. Our preliminary results suggest that the 40 min gene product is part of a phosphorylation/dephosphorylation pathway leading to activation of the σ^E-dependent response (D.Missiakas and S.Raina, unpublished results).

A variety of approaches were used to demonstrate that the cloned *rpoE* gene indeed codes for σ^E, namely: (i) the purified RpoE protein when added to the RNA polymerase core enabled it to specifically transcribe these two promoters; (ii) out of the 12 Ts$^-$ point mutations isolated which mapped in the *rpoE* gene, we over-expressed and purified one of the corresponding mutant proteins and showed that, unlike the wild-type RpoE protein, it was unable to direct transcription from the *htrA* promoter; (iii) the predicted amino acid sequence of RpoE bears a high degree of homology with the group 3 class of sigma factors (Dubnau *et al.*, 1988; Martin *et al.*, 1993a; Lonetto *et al.*, 1994); (iv) among the 12 point mutations obtained in the *rpoE* gene we sequenced three of them and found them to be located in regions that code for highly conserved amino acid sequences, such as region 2.1 (L25P), implicated in core binding, or region 4.2 (S172P and R178G), containing the conserved helix–turn–helix motif, both of which are highly conserved among sigma factors (Lonetto *et al.*, 1992); (v) RpoE exhibited properties similar to other *E.coli* sigma factors during the various purification steps; and (vi) part of the sequence of the cloned *rpoE* gene was found to be identical to that of one of the five tryptic digest fragments of the previously gel-purified σ^E protein (these peptide sequences were kindly provided by J.Mecsas and C.Gross).

To understand the nature of the σ^E regulon in general and regulation of the *rpoE* gene in particular, we undertook a number of studies. We first looked at the migration profile of the purified RpoE protein on two-dimensional gels to see if it had been previously identified, particularly in those genetic backgrounds known to have defects in the correct folding of proteins. We found that RpoE (σ^E) corresponded to a protein previously shown to be induced in bacteria carrying a null mutation in the *htrC* gene (Raina and Georgopoulos, 1990). We have previously shown that in such a genetic background, the Eσ^{32}-dependent heat shock response is constitutively expressed at elevated levels. Interestingly, null mutations in either *trxB*, *dsbA* or *dsbC* similarly lead to increased σ^E activity. *trxB*, *dsbA* and *dsbC* mutations were isolated because they affect the proper folding of disulfide bond-containing proteins usually found in the periplasmic space or in the outer membrane (Missiakas *et al.*, 1993b, 1994). Thus, it is not surprising that a combination of *dsbA* and *htrA* null mutations exhibited a 6-fold increase in σ^E activity, which is much higher than that observed with a *dsbA* mutant alone. Obviously, with the lack of both the DsbA and HtrA proteins, many unfolded proteins may accumulate which might otherwise have been substrates for HtrA proteolytic activity. Increased σ^E activity was also found in bacteria over-expressing the HtrL/RfaL protein. We have observed that in such bacteria aberrantly charged lipopolysaccharides (LPS) accumulate, leading to an altered ratio of outer membrane proteins (OMPs) associated with the membrane (D.Missiakas and S.Raina, unpublished results). A similar phenotype is exhibited by bacteria carrying mutations in genes affecting LPS biogenesis in general, such as *rfaD* (*htrM*) (Raina and Georgopoulos, 1991; Schnaitman and Klena, 1993). Therefore, it seems reasonable to assume that events affecting protein folding, both in the periplasm and the outer membrane, or altering the composition of LPS, thereby leading to 'misfolded' OMPs, specifically trigger the σ^E-dependent stress response. This suggestion is an extension of the finding

Table V. β-galactosidase activity of *lacZ* operon fusions to the promoter region of the *rpoE* gene carrying both P1 and P2

	β-Galactosidase activity (Miller units)		
	Temperature (°C)		
	30	42	50
MC4100 (λRS74-*plac*)	28 ± 2	24 ± 2	27 ± 2
SR1421 = MC4100 *lon*::λ*plac*Mu53	310 ± 12	627 ± 19	640 ± 21
SR2195 = MC4100 ϕ (*prpoE–lacZ*)	972 ± 24	1022 ± 30	1224 ± 41
SR1588 = MC4100 ϕ (*phtrL–lacZ*)	227 ± 14	243 ± 15	183 ± 12
	Ethanol (%)		
	0	4	10
SR2195 = MC4100 ϕ (*prpoE–lacZ*)	861 ± 37	979 ± 37	1043 ± 42
SR1588 = MC4100 ϕ (*phtrL–lacZ*)	232 ± 14	208 ± 12	134 ± 10
	Puromycin (μM)		
	0	70	
SR2195 = MC4100 ϕ (*prpoE–lacZ*)	861 ± 37	1255 ± 32	
SR1588 = MC4100 ϕ (*phtrL–acZ*)	225 ± 14	144 ± 9	
	DTT (mM)		
	0	10	
SR2195 = MC4100 ϕ (*prpoE–lacZ*)	861 ± 37	1047 ± 47	
SR1588 = MC4100 ϕ (*phtrL–lacZ*)	225 ± 14	164 ± 12	

All assays were performed after a 15 min incubation following either a temperature shift or addition of chemicals.

by Mecsas *et al.* (1993), namely that the $E\sigma^E$ regulon responds to changes in the outer membrane.

We did not find any specific growth conditions which led to a striking overall increase or decrease in expression of the *rpoE* gene. Rather, it seemed that *rpoE* gene transcription is sustained at high temperatures (50°C) or under conditions which induce misfolding of proteins. Mapping analysis of the *rpoE* promoter region revealed two transcriptional start sites, P1 and P2. The most interesting finding is that the constitutive transcription observed at 50°C emanates from the P2 promoter, whereas under the same conditions the transcriptional activity of the P1 promoter is declining. The P2 promoter, as shown in this study, is positively autoregulated, being transcribed by $E\sigma^E$ itself. However, other mechanisms of regulation are also likely to be involved, as judged by the very high accumulation of the RpoE protein in *htrC*, *dsb* and *trxB* mutant bacteria. The amount of σ^E protein accumulating in these cases is higher than what would be expected from the overall enhanced transcription of the *rpoE* gene. It is possible that σ^E behaves like other minor sigma factors, such as σ^{32} or σ^{38}, which are extremely unstable under normal growth conditions, yet accumulate under specific stress conditions (Tilly *et al.*, 1989; Gross *et al.*, 1990; Takayanagi *et al.*, 1994). Also, post-translational modification processes, such as phosphorylation/dephosphorylation of RpoE or interaction with a putative heat labile protein, could account for its accumulation and activity without additional synthesis.

In our earlier work related to the regulation of *htrA* and *rpoH*P3 promoters (Missiakas *et al.*, 1993a) we identified a gene, designated *htpY*, whose product positively modulates the heat shock response. One intriguing observation was that HtpY over-expression induces a higher β-galactosidase activity with the *htrA–lacZ* fusion as compared with *rpoH*P3–*lacZ* (Missiakas *et al.*, 1993a). In the present study we obtained rather similar results, since mutations in the *cpxR/cpxA* operon were found to more specifically affect transcriptional activity of the *htrA–lacZ* promoter fusion. CpxA is an inner membrane protein which resembles histidine kinases (Weber and Silverman, 1988) and CpxR resembles the regulatory OmpR protein (Dong *et al.*, 1993). CpxR is presumed to be the cognate regulator for the membrane sensor CpxA (Dong *et al.*, 1993). Thus, there seem to be some additional elements in addition to σ^E which respond to extracytoplasmically generated signals (misfolding in the periplasmic space or the outer membrane) and the *cpxA/cpxR* two-component system could be a good candidate for transducing such signals. Such putative activators could serve as discriminators of promoter usage within the σ^E regulon, explaining the differential transcriptional activities we observed between the two *lacZ* fusions (similar results have been obtained by P.Danese and T.Silhavy, personal communication). It is also tempting to hypothesize that part of the reason why the σ^E protein remained associated with the membranes during the early purification steps is a possible direct interaction with the membrane-bound CpxA protein or with some additional membrane-associated response regulators, rather than to an aggregation problem.

Another interesting point emerging from our results is the influence of gene(s) located downstream of *rpoE*. Clones including both this region and the *rpoE* gene led to a down-regulation (~4-fold) of σ^E activity, as judged by β-galactosidase activity driven from either the *htrA* or *rpoE*P2 promoters. Similar results were found when the region responsible for this down-regulation effect was subcloned and provided *in trans* to a plasmid carrying the

minimal *rpoE* coding region (D.Missiakas and S.Raina, unpublished results). There are already known cases of specific negative elements that may act like anti-sigma factors. For example, MucA/B or CarR are the 'anti-factors' of the σ^E-related sigma factors AlgU and CarQ respectively (Martin *et al.*, 1993b; McGowan *et al.*, 1993).

The induction of σ^E leads to a simultaneous increase in the rate of synthesis of at least 10 different proteins. Further studies are needed to define the corresponding genes. In our previous screens, aimed at identifying genes whose expression is required at high temperature (*htr* genes; Raina and Georgopoulos, 1990), we identified an operon containing a gene called *htrG*, whose transcription is heat shock regulated, but in a σ^{32}-independent manner (S.Raina, unpublished results). Interestingly, the promoter of this gene resembles that of the *htrA*, *rpoH*P3 and *rpoE*P2 promoters. Thus, one of the σ^E-induced polypeptides could be the HtrG protein necessary for *E.coli* growth at 43°C. Another likely expectation would be that σ^E transcribes some of the genes whose products are directly required for preventing protein misfolding in extracytoplasmic cell compartments.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table I.

Media and chemicals

Luria–Bertani (LB) broth, MacConkey medium and M9 minimal medium were prepared as described by Miller (1992). M9 was supplemented with glucose (0.2%), thiamine (2 μg/ml), $MgSO_4$ (1 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.1 mM) and $FeCl_3$ (0.3 mM). Whenever necessary, glucose was replaced by succinate (0.2%) as the carbon source. For [^{35}S]methionine labeling experiments, the M9 high-sulfur medium, supplemented with a mixture of defined amino acids, was used as previously described (Missiakas *et al.*, 1993a). When necessary, the media were supplemented with ampicillin (100 μg/ml), tetracycline (15 μg/ml) or kanamycin (50 μg/ml). The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was dissolved in *N,N*-dimethylformamide and was added to a final concentration of 40 μg/ml in the agar medium. Ortho-nitrophenyl β-D-thiogalactoside (tONPG) (Berman and Beckwith, 1979) was added to a final concentration of 1 mM.

Construction of lacZ fusions

The promoter-containing region of the *htrA* gene was first amplified by the polymerase chain reaction (PCR) method (Innis *et al.*, 1990) using the primers 5′-TAGCCATCCAGATCTCGAGCGGCTT-3′ and 5′-CTCAGAGCCAGTCGACTCAGTGCTAA-3′, based on the published sequences of the *htrA* and *dgt* genes (Lipinska *et al.*, 1988; Wurgler *et al.*, 1990). This amplified DNA fragment contains a terminator located between the *dgt* and *htrA* genes. The *htrA* promoter was first cloned into the *Sal*I and *Bam*HI sites of the pSK vector (Bluescript; Stratagene) and then directionally cloned into the promoter-probing vector pRS415 (Simons *et al.*, 1987), resulting in plasmid pSR1451. Single copy lysogens were made using λRS45 (Simons *et al.*, 1987) to monitor the transcriptional strength of the *htrA*–*lacZ* fusion (SR1458). Similarly, *rpoH*–*lacZ* fusions were constructed by first amplifying the P3 promoter region, using the primers 5′-ATACTCTTTCCCGGGAATGGGTTCCGTAGC-3′ and 5′-AGGTTATCATTCACTGAATTCTCAGACCGT-3′. This fusion contains only the P3 promoter plus the two putative DnaA box sequences. As a control, another *rpoH*–*lacZ* fusion was constructed by PCR, by amplifying the DNA region which contains the P3, P4 and P5 promoters but lacks the P1 promoter, using the primers 5′-CTGTGCCAAAGCTTAAGAGCA-3′ and 5′-TCCTCAATCGATATCTTCTGGCGC-3′.

To study transcriptional regulation of the *rpoE* gene, we also constructed a fusion between the *rpoE* promoter region and *lacZ*. This region was amplified by PCR using the primers 5′-CACATAGCGGGAATTCAGACTCGCCA-3′ and 5′-TGGTGGAGGATCCGGTGGTGA-3′ and cloned into the *Eco*RI and *Bam*HI sites of the promoter-probing vectors pRS528 and pRS550 (Simons *et al.*, 1987), resulting in plasmids pSR2177 and pSR2180 respectively. These promoter fusions were subsequently transferred to the chromosome in a single copy form, using the λRS45 vector as described above. The same DNA fragment from pSR2177 was cloned in pSK, resulting in pSR2189, which was used, in turn, as a template for the *in vitro* run-off transcription assays using purified σ^E protein. Since it turned out that *rpoE* has at least two promoters, an additional single copy *lacZ* fusion was constructed which contained only the P1 promoter. To achieve this, the entire region containing the P2 promoter and downstream sequences was removed by digesting pSR2189 with the restriction enzymes *Bsa*BI (a unique site located between the P1 and P2 promoters) and *Eco*RI. The remaining region was then subcloned as a *Bam*HI–*Bsa*BI fragment into promoter probe vector pRS528 using *Bam*HI and *Sma*I sites and subsequently transferred to the chromosome in a single copy form.

To compare our data with a known σ^{32} heat shock-dependent promoter, we used strain SR1421, a derivative of MC4100 carrying the *lon*::λ-*plac*Mu53 transcriptional fusion. This promoter fusion was originally isolated from a library of insertions, using the λ*plac*Mu53 transposon (Bremer *et al.*, 1985). This particular single copy fusion was selected as a strong heat-inducible promoter fused to the *lacZ* gene. Using the *Sst*I and *Eco*RI restriction enzymes, part of the *lacZ* gene from λ*plac*Mu53 and the adjacent regions were cloned into the multicopy vector pRS415. Sequence analysis showed that this DNA corresponds to the *lon* promoter, which is exclusively transcribed by Eσ^{32} (Gross *et al.* 1990).

Isolation of trans-acting mutations

Trans-acting mutations which abolish or reduce β-galactosidase expression from both *htrA*–*lacZ* and *rpoH*P3–*lacZ* transcriptional fusions were isolated through two independent genetic approaches. First, saturated mini-Tn*10* (Tet^R) and mini-Tn*10* (Kan^R) pools were prepared in the wild-type strain MC4100 (Lac^-) at 22°C. P1 bacteriophage lysates prepared on these pools were subjected to *in vitro* mutagenesis with hydroxylamine (Miller, 1992). Such mutagenized bacteriophage P1 lysates were used to transduce strains SR1458 and SR1710, which carry the single copy fusions *htrA*–*lacZ* or *rpoH*P3–*lacZ* respectively, at 22°C. Lac^- transductants were retained and further tested. Alternatively, a *mutD* mutation was transduced into MC4100 and such a strain was used as a host to construct mutagenized mini-Tn*10* libraries. These strains, carrying either Kan^R or Tet^R, were initially grown on minimal medium (M9) and then shifted for several generations to rich medium supplemented with casamino acids and thymidine, thus resulting in the accumulation of point mutations. The putative point mutations were transduced into SR1458 and SR1710 using bacteriophage P1, by screening for Lac^- or reduced β-galactosidase activity. To directly select for Lac^- events, transductants were also plated on minimal medium with succinate (0.2%) as the sole carbon source, supplemented with tONPG and X-gal (Berman and Beckwith, 1979). Bacteria that can hydrolyze tONPG are killed, thus directly selecting for Lac^- derivatives. Lac^- isolates obtained with either of these two approaches were subsequently checked for their Ts^- phenotype, as well as inability to accumulate heat shock proteins at 50°C (see text).

Mapping and complementation analysis of the Lac^- mutations

Since we obtained a relatively large number of Lac^- Ts^- mutant candidates, we chose to further characterize only those which did not exhibit a heat shock response at 50°C, a temperature at which the *rpoH* gene is exclusively transcribed from its σ^E-dependent P3 promoter. To assign such mutations to different complementation groups and perform the exact linkage analysis, we took advantage of the mini-Tn*10* markers (Tet^R and Kan^R) closely linked to the mutations causing the Lac^- Ts^- phenotypes. In addition, a cosmid library (in the cosmid cloning vector pREG153; O'Connor *et al.*, 1989) was used to isolate clones that could both recombine the mini-Tn*10* markers and complement the Ts^- phenotype. DNA prepared from such recombinant cosmids were subsequently ^{32}P-labeled by random priming (Sambrook *et al.*, 1989) and used to map the mutations by hybridization to the ordered λ *E.coli* chromosomal DNA library (Kohara *et al.*, 1987). The cosmids carrying the corresponding wild-type gene were further used to subclone the minimal complementing DNA fragments.

Cloning of the rpoE gene

Chromosomal DNA isolated from the *E.coli* wild-type strain MC4100 was used to construct a library in the p15A-based vector pOK12 (Viera and Messing, 1991), as previously described (Missiakas *et al.*, 1993b). This library was used to transform three different strains containing

trans-acting mutations (SR1576, SR1502 and SR1723) and the single copy fusions *htrA*−*lacZ* or *rpoH*P3−*lacZ*. The wild-type *rpoE* gene was cloned on the basis of simultaneous complementation of both the Ts$^-$ and Lac$^-$ phenotypes. A 3.3 kbp *Sau*3A fragment was first cloned (pSR1546). Further subcloning resulted in the construction of pDM619, which contains a minimal 1.6 kbp *Ssp*I−*Eco*RI DNA fragment and is able to rescue all the phenotypic defects of the group of mutations mapping at 56 min on the *E.coli* chromosome. The over-expression of RpoE (σ^E) was achieved by first amplifying the minimal coding region of the *rpoE* gene by PCR, using the primers 5′-GACTTTACCTCATATG-AGCGAGCA-3′ and 5′-TGCATGCCTGGATCCCTTATCCAG-3′. The resulting amplified DNA product was cloned into the T7 promoter expression vector pEAD-4 (pDM1055) and its wild-type DNA sequence verified. The pEAD-4 vector was a kind gift from Dr S.Doering.

In a complementary approach, we directly used the p15A-based multicopy pOK12 library to select for genes whose presence in high copy leads to increased expression of the two known σ^E-dependent promoters. Fourteen plasmid clones were thus selected on MacConkey plates at 30°C, based on the fact that their presence led to higher β-galactosidase activity when transformed in strains SR1458 (*htrA*−*lacZ*) and SR1710 (*rpoH*P3−*lacZ*). The restriction enzyme digestion pattern of these 14 plasmids showed that they carried four different chromosomal inserts. Four plasmid clones (represented by plasmids pSR1546 and pSR1628 in Table I) were retained and shown to contain the wild-type *rpoE* gene. These plasmids carry a common 1.4 kb *Sau*3A fragment, in either orientation with respect to the T7 promoter present on the pOK12 vector.

The various genes cloned using the two approaches described above were mapped by hybridizing ^{32}P-labeled, nick-translated complementing plasmid DNAs to the ordered *E.coli* genomic library (Kohara *et al.*, 1987). The location of these mutations was further confirmed by linkage to nearby known genetic markers using bacteriophage P1 and strains CAG18481 and CAG18480 (Singer *et al.*, 1989), which carry a Tn*10* insertion at 55 and 55.7 min respectively on the *E.coli* chromosome, for transduction experiments.

Disruption of the rpoE gene

To construct a null allele of the *rpoE* gene, we first removed the *rpoE* coding sequence between the unique *Hpa*I and *Hin*dIII sites, creating a deletion of 48 amino acids in the N-terminal region. Next, this truncated *rpoE* construct was treated with T4 DNA polymerase to make the ends blunt. An Ω Tet^R cassette was excised as a 2.1 kb *Sma*I fragment from pHP45 (Fellay *et al.*, 1987) and inserted in the truncated *rpoE* DNA coding region, resulting in plasmid pSR2364. The *Kpn*I- and *Xba*I-digested fragment, containing the disrupted *rpoE* gene with the Ω Tet^R cassette and flanking sequences from the *nadB* gene, was cloned into phagemid vector pBIP (Slater and Maurer, 1993) at the *Sca*I site. The resulting plasmid, pSR2370, was transformed into JM109, which carries an F′. The null mutation was then transferred to the chromosome in the wild-type strain CA8000 (F$^+$) using M13 amber phage f1R189, using the phagemid-based allele replacement system as outlined by Slater and Maurer (1993). The Tet^R, sucrose-resistant colonies were selected and tested for the loss of plasmid sequences.

RNA isolation and mapping of 5′ termini

Total cellular RNA was isolated by using the hot SDS−phenol extraction procedure (Sambrook *et al.*, 1989). To define the transcriptional start site(s) of the *rpoE* gene, ~10 ng of an oligonucleotide probe 5′-TTTCTGATCTCCCTTCTGGACCCGTT-3′, which is complementary to nucleotide positions 60–35 of the *rpoE* sequence, was annealed to 10 μg of total cellular RNA. The annealed primer was extended with AMV reverse transcriptase (Promega), essentially as previously described (Raina and Georgopoulos, 1990). The primer extension products were electrophoresed on the same gel as the dideoxy sequencing reactions, using the same primer.

Protein purifications

Escherichia coli bacteria carrying plasmid pDM1055 (*rpoE*$^+$) were induced with 5 mM IPTG at an optical density of 0.2 at 600 nm for 2 h. Cells were resuspended in buffer A (50 mM Tris−HCl, pH 7.2, 0.5 mM EDTA, 10% v/v glycerol) and lysed by sonication. The lysate was centrifuged at 25 000 r.p.m. for 45 min at 4°C. Proteins recovered in the soluble fraction were precipitated with ammonium sulfate (0.6 g/ml), dialyzed for 6 h in buffer B (50 mM Tris−HCl, pH 7.2, 0.5 mM EDTA, 0.1 M NaCl, 5 mM 2-mercaptoethanol, 10% v/v glycerol) and loaded onto a Q-Sepharose column (2×12 cm; Pharmacia) equilibrated in buffer B. The adsorbed proteins were eluted with a linear NaCl gradient (0.1–0.6 M). The σ^E protein was eluted at a concentration of ~0.4 M NaCl. Fractions containing σ^E, as judged by its migration in 12.5% SDS−PAGE, were concentrated by ammonium sulfate precipitation (0.6 g/ml). The pellet was dialyzed for 6 h in buffer C (10 mM imidazole, pH 7.0, 0.5 mM EDTA, 0.1 M NaCl, 5 mM 2-mercaptoethanol, 10% v/v glycerol) and loaded onto a heparin−agarose column (1×6 cm; Sigma). The σ^E protein was eluted at a concentration of ~0.35 M NaCl of a linear gradient 0.1–0.7 M NaCl. Fractions containing the σ^E protein were pooled, dialyzed directly against buffer C and loaded onto a P11 column (1×6 cm; Whatman). The protein was eluted with ~0.35 M NaCl, using a linear NaCl gradient (0.1–0.7 M). Fractions containing purified σ^E protein, as judged by Coomassie brilliant blue stained SDS−PAGE, were pooled, dialyzed against buffer D (40 mM HEPES, pH 7.6, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.2 M NaCl, 10% v/v glycerol) and directly used in the run-off transcription assays. The mutant σ^E protein (R178G) was purified under the same conditions as used for the wild-type protein. For over-expression purposes, the mutant allele was cloned in the same manner as the wild-type *rpoE*$^+$ gene into the T7 promoter expression vector pEAD-4 (pDM1056). Protein concentrations were estimated using the Bradford assay (Bio-Rad) and the purified proteins were stored at −80°C.

The RNAP core and the σ^{70} protein were purified as described (Burgess and Jendrisak, 1975).

Biochemical assays

The β-galactosidase activities were determined by the method of Miller (1992). The bacterial cultures were grown overnight in either M9 minimal or LB rich medium at 30°C, diluted 1:100 and allowed to reach an optical density of 0.2 at 600 nm. When necessary, aliquots of the cultures were maintained at either 30°C or shifted to 42 or 50°C, or under the various physiological conditions specified in Tables II−V. Samples were assayed in duplicate and the data presented here are the average of three independent experiments.

Run-off transcription experiments were performed as described by Grossman *et al.* (1984) and Wang and Kaguni (1989). The transcriptional activity of the purified σ^E protein was measured by using as a template a 550 bp DNA fragment from pSR1451 containing the promoter region of the *htrA* gene. The 20 μl reaction mixtures contained 20 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.2 M NaCl, 0.2 mM of each rNTP (1 μCi = 37 kBq [α-^{35}S]UTP), 1 μg pSR1451, 2 μg RNAP core and 1 μg σ^E protein. To allow the formation of the open complex, DNA templates were first incubated with RNAP (E) or, as a control, with core RNAP alone in a transcription buffer lacking the rNTPs. Next, rNTPs were added and the reaction mixtures were further incubated for 10 min in the presence of [α-^{35}S]UTP. After completion, the reactions were chased with an excess of unlabeled UTP (0.2 mM). The reaction products were analyzed on a 5% acrylamide gel containing 7 M urea. To estimate the size of the products of the reaction, a *Msp*I digest of pBR322 DNA, end-labeled with [γ-^{32}P]ATP, was electrophoresed on the same gel. Run-off transcription experiments, to verify the transcriptional start site(s) of the *rpoE* gene, were carried out under the above-described conditions. In this case, the template DNA was a 360 bp DNA fragment from plasmid pSR2189, which contains the promoter region as well as adjacent flanking areas of the *rpoE* gene.

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