Identification of the σ^E subunit of *Escherichia coli* RNA polymerase: a second alternate σ factor involved in high-temperature gene expression

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The *rpoH* gene of *Escherichia coli* encodes σ^{32}, the 32-kD σ-factor responsible for the heat-inducible transcription of the heat shock genes. *rpoH* is transcribed from at least three promoters. Two of these promoters are recognized by RNA polymerase containing σ^{70}, the predominant σ-factor. We purified the factor responsible for recognizing the third *rpoH* promoter (*rpoH* P3) and identified it as RNA polymerase containing a novel σ-factor with an apparent M_r of 24,000. This new σ, which we call σ^E, is distinct from the known σ factors in molecular weight and promoter specificity. σ^E holoenzyme will not recognize the σ^{70}- or σ^{32}-controlled promoters we tested, but it does transcribe the *htrA* gene, which is required for viability at temperatures >42°C. The in vivo role of σ^E is not known. The transcripts from the σ^E-controlled *rpoH* P3 and *htrA* promoters are most abundant at very high temperature, suggesting the σ^E holoenzyme may transcribe a second set of heat-inducible genes that are involved in growth at high temperature or in thermotolerance.

When *Escherichia coli* cells are transferred to temperatures >40°C, complex changes in cellular structure, composition, and in the patterns of gene expression occur. The most dramatic change in gene expression is the increased synthesis of the 17 heat shock proteins that is termed the heat shock response (for review, see Neidhardt and VanBogelen 1987). The temperature-regulated expression of the heat shock proteins requires RNA polymerase containing the σ^{32}-subunit ($E\sigma^{32}$) (Neidhardt and VanBogelen 1981; Yamamori and Yura 1982; Grossman et al. 1984; Cowing et al. 1985). Following transfer to a high growth temperature, the amount of σ^{32} increases, resulting in increased transcription initiation by $E\sigma^{32}$ at the heat shock promoters. This nearly 20-fold increase in σ^{32} concentration is short-lived, and by 20–30 min at the high growth temperature, the amount of σ^{32} and thus, the expression of the heat shock genes, declines to three to five times the value at low temperature (Straus et al. 1987). If the temperature to which the cells are shifted is not too high (≤44°C), the heat shock response is superimposed over an essentially normal pattern of gene expression, suggesting that the synthesis of most *E. coli* proteins is not affected greatly by such temperature shifts (Herendeen et al. 1979; Neidhardt et al. 1984; Neidhardt and VanBogelen 1987).

The heat shock response is more extreme when cells are shifted to a lethal temperature such as 50°C. Under these conditions, heat shock proteins account for the major fraction of protein synthesis (Neidhardt et al. 1984; Paek and Walker 1986; VanBogelen et al. 1987a). This extreme response occurs for two reasons: First, the synthesis of most non-heat shock proteins is turned off, probably because the activity of σ^{70}, the primary σ-factor, is reduced under these conditions (Erickson et al. 1987; Skelly et al. 1987). Second, the heat shock genes are transcribed at high constitutive rates, and their mRNAs are translated for as long as the cells are able to synthesize protein. The continued synthesis of the heat shock proteins is important for survival at 50°C because *E. coli* mutants that are unable to increase the synthesis of heat shock proteins die much more rapidly than wild-type cells after a shift to 50°C (Yamamori and Yura 1982; Neidhardt et al. 1984; J.W. Erickson and C.A. Gross, unpubl.).

Because σ^{32} is an unstable molecule ($t_{1/2}$ = 1 min), transcription of the heat shock genes requires continuous synthesis of σ^{32} (Straus et al. 1987). Under normal growth conditions, the *rpoH* gene, which encodes σ^{32}, is transcribed from three promoters, P1, P3, and P4; however, only P3 functions following a shift to 50°C (Erickson et al. 1987). The promoters P1 and P4, which are recognized by RNA polymerase containing σ^{70} ($E\sigma^{70}$), are shut off after shift to 50°C; therefore, at 50°C, σ^{32} synthesis depends solely on the transcript originating from *rpoH* P3.

We investigated the regulation of the *rpoH* P3 pro-

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moter because of its crucial role in high-temperature physiology. The simplest explanation for the strong transcription from P3 at 50°C was that it is recognized by a novel, heat-stable form of RNA polymerase, as our previous studies suggested that neither $E\sigma^{70}$ nor $E\sigma^{32}$ transcribed from this promoter. We could not, however, rule out the possibility that either $E\sigma^{70}$ or $E\sigma^{32}$, in conjunction with an auxiliary factor, transcribed from $rpoH$ P3.

To resolve this issue, we purified the factors required for transcription from $rpoH$ P3. The P3 promoter is recognized by a form of RNA polymerase containing a σ-subunit with an apparent M_r of 24,000. This 24-kD σ, which we call σ^E, is distinct from any known subunit of RNA polymerase and directs core RNA polymerase to initiate transcription from $rpoH$ P3 and from the promoter of the $htrA$ ($degP$) gene (Lipinska et al. 1988) that is required for growth and survival only at high temperature (Lipinska et al. 1989). We do not know what other genes are under the control of σ^E, but the properties of the $rpoH$ P3 and $htrA$ promoters suggest that σ^E regulates other genes that are involved in growth at, or in resistance to killing by, high temperatures.

Results

To identify the factor responsible for transcription from the $rpoH$ P3 promoter, we prepared cell-free extracts that transcribed from P3 and used those extracts to purify the factor and determine its biochemical function. The extracts were prepared from cells that had been shifted from 30 to 50°C for 45 min. These conditions were chosen because S1 mapping of $rpoH$ mRNA indicated that transcription from P3 was at a maximum between 30 and 60 min after a shift to 50°C, and that σ^{70} activity, as judged by transcription from $rpoH$ P1 and P4, was reduced greatly under these conditions (Erickson et al. 1987). This reduction in σ^{70} activity was important, because in vitro transcription from the σ^{70}-controlled P4 promoter could have obscured low-level transcription from the overlapping P3 promoter. To reduce the amount of σ^{70} further, most extracts were prepared from cells carrying the $rpoD800$ mutation, which results in an altered σ^{70} that is degraded rapidly at high temperature (Grossman et al. 1983). The experiments described in the following text demonstrate that $rpoH$ P3 is recognized by RNA polymerase, which contains a new σ-factor with an apparent M_r of 24,000.

Transcription from rpoH P3 using cell-free extracts

We prepared microscale extracts (Gross et al. 1976) from $rpoD800$ cells and assayed their ability to transcribe from $rpoH$ P3 by S1 mapping the in vitro transcripts. With the $rpoH^+$ plasmid pKP11 as template, the extracts produced a single RNA species that had the same 5' end as the in vivo P3 transcript (Fig. 1). There was no transcription from the σ^{70}-controlled $rpoH$ P1 and P4 promoters, showing that the in vitro extracts accurately reflected transcription in vivo (Fig. 1). We also tested the
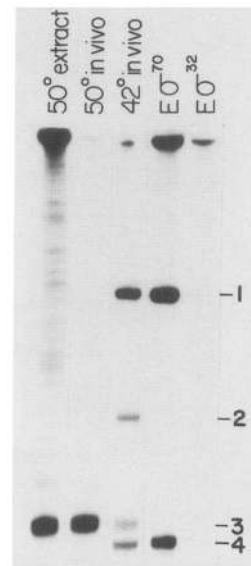

Figure 1. S1 mapping of in vitro and in vivo $rpoH$ transcripts. 5'-End-labeled $rpoH$ probe was hybridized to RNA synthesized in vitro or in vivo and S1 mapped. In vitro RNA was isolated from reactions that contained pKP11 as template, and as sources of RNA polymerase, 5 μl of an extract prepared from $rpoD800$ cells that had been shifted from 30 to 50°C for 45 min, 0.17 μg $E\sigma^{70}$, or 0.25 μg $E\sigma^{32}$. In vivo RNA was isolated from strain SC122 either 20 min after shift from 30 to 42°C (40 μg RNA used in the hybridization) or 60 min after shift to 50°C (5 μg RNA used in the hybridization). The protected fragments corresponding to the transcripts initiated from $rpoH$ P1, P3, and P4 are marked, as is the fragment corresponding to the SC122-specific transcript mRNA-2 (Erickson et al. 1987).

50°C extracts for the presence of $E\sigma^{32}$ by looking for transcripts from the $E\sigma^{32}$-dependent promoters $dnaK$ P1 and $groE$ pHS (Cowing et al. 1985). As expected, $E\sigma^{32}$ was present in the 50°C extracts (data not shown), but pure $E\sigma^{32}$ does not recognize P3 (Erickson et al. 1987; Fujita and Ishihama 1987).

Although S1 mapping was appropriate to identify an extract that contained the factor that recognizes P3, it was a laborious way to assay a large number of fractions during a purification. We simplified the transcription assay by cloning the $rpoH$ promoters in front of an efficient ρ-independent transcription terminator, so that transcripts initiated from each promoter could be distinguished directly, based on their sizes. With plasmid pJET41 (containing the $rpoH$ promoters and the terminator) as a template, the transcripts initiated from $rpoH$ P1, P3, and P4 are 428, 295, and 290 nucleotides, respectively. The data in Figure 2 demonstrate the validity of the transcription assay. When 50°C extracts (prepared from both $rpoD^+$ and $rpoD800$ cells) were used to transcribe pJET41, a single RNA species of ~295 bases was produced (Fig. 2). This RNA was shown to originate from $rpoH$ P3 by S1 mapping (data not shown) and by comparison with the 428- and 290-base transcripts made in vitro by $E\sigma^{70}$ (Fig. 2). This more sensitive assay confirmed that the extracts contained no σ^{70} activity be-
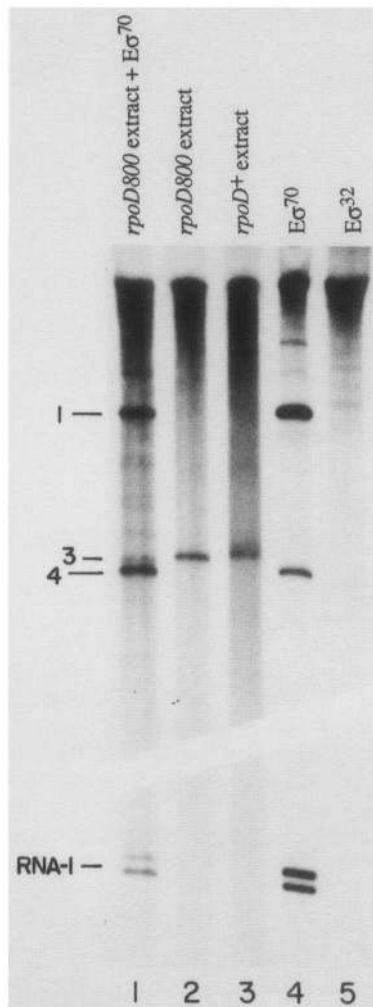
Figure 2. Transcription from the *rpoH* promoters in vitro. In vitro transcription reactions contained supercoiled pJET41 as template and the following sources of RNA polymerase: extracts (5 μl each) prepared from *rpoD*+ or *rpoD800* cells that had been shifted from 30 to 50 °C for 45 min (lanes *1–3*), 0.1 μg pure Eσ^{70} (lanes *1* and *4*), or 0.2 μg Eσ^{32} (lane *5*). Pure Eσ^{70} was added to the 50°C extract in the reaction loaded in lane 1. RNA samples labeled with ^{32}P were concentrated and electrophoresed as described in Materials and methods. The transcripts from the *rpoH* P1, P3, and P4 promoters are marked, as are the plasmid *ori* region RNA-1 transcripts. The central portion of the micrograph has been removed to allow a larger image to be shown.

cause there was no transcription from *rpoH* P1 or P4, or from the plasmid RNA-1 promoter.

Fractionation of the transcription extracts

Preliminary experiments using the microscale 50°C extracts indicated that the factor that recognized P3 copurified with RNA polymerase on single-stranded DNA agarose and DEAE-cellulose columns but that it was separated into two components on a Bio-Rex 70 column (data not shown). To obtain sufficient material to iden-

tify the factor responsible for transcription from P3, we scaled up the purification, using standard techniques for the large-scale isolation of RNA polymerase (Burgess and Jendrisak 1975). We found that the activity that transcribes from *rpoH* P3 copurified with core RNA polymerase through chromatography on single-stranded DNA agarose, Sephacryl S-400, and DEAE-cellulose resins (data not shown). When the pooled fractions from the DEAE column were passed through a Bio-Rex 70 column, none of the resulting fractions retained the ability to recognize P3. The flowthrough fraction was inactive (as expected, because it should lack core RNA polymerase), as was the 0.6 M NaCl eluate, which contained core RNA polymerase (Fig. 3).

Because σ^{70} can be separated from core RNA polymerase on Bio-Rex 70 (Lowe et al. 1979), we wanted to determine whether the P3 activity had been separated into a σ and a core component. When the flowthrough fraction and the 0.06 M NaCl eluate were mixed, the ability to transcribe from *rpoH* P3 was restored (Fig. 3), suggesting that the activity had been separated into a σ-factor and core RNA polymerase. This was confirmed by mixing the flowthrough fraction with pure core RNA polymerase and demonstrating that the mixture transcribed from the *rpoH* P3 promoter (Fig. 3).

To assess the purity of the P3-specific factor, we separated the proteins from the Bio-Rex 70 column fractions on an sodium dodecyl sulfate (SDS)–polyacrylamide gel, and silver stained it (Wray et al. 1981) to visualize the proteins. The fraction that was loaded onto the Bio-Rex 70 column consisted of core RNA polymerase, with a few minor contaminants. The 0.6 M NaCl eluate was almost indistinguishable from the fraction that was loaded onto the column, whereas the highly purified flowthrough fraction contained <1% of the total protein (Fig. 4). To identify the proteins in the flowthrough fraction, we loaded 150 times more sample on an SDS–polyacrylamide gel, silver stained it, and observed a number of proteins, including the α-, β-, and β′-subunits of RNA polymerase (Fig. 4). This result was surprising, as the flowthrough fraction appeared to be dependent on added core RNA polymerase. We do not have a good explanation for the presence of the core subunits. It is not due to overloading the Bio-Rex 70 column because none of the flowthrough fraction was retained on a second Bio-Rex 70 column (data not shown). It may be that inactive core or proteolyzed subassemblies of RNA polymerase do not bind to Bio-Rex 70 and appear in the flowthrough fraction.

Identification of a 24-kD protein as the rpoH P3-specific σ-factor

To identify which of the proteins present in the Bio-Rex 70 flowthrough fraction was the σ-factor, we purified the proteins by eluting them from SDS-polyacrylamide gels and renatured them using the procedure of Hager and Burgess (1980). Figure 5 (left) shows a silver-stained SDS–polyacrylamide gel identical to the Coomassie-blue-stained gel that was used as the source of protein.
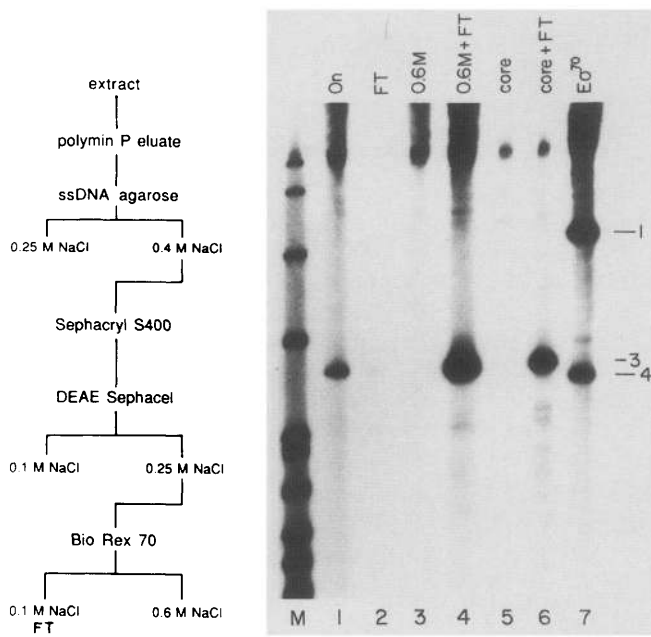
Figure 3. Purification of the *rpoH* P3-specific transcription factor. (*Left*) Schematic diagram of the purification strategy; (*right*) transcription from *rpoH* P3 with Bio-Rex 70 column fractions. In vitro reactions contained pJET41 as template, and as sources of protein, 2 μl of the Bio-Rex 70 onput (lane *1*), 4 μl of the Bio-Rex 70 flowthrough (lane *2*), 0.6 μl of the 0.6 M NaCl eluate from the Bio-Rex 70 column (lane *3*), 4 μl of flowthrough and 0.6 μl of the 0.6 M eluate (lane *4*), 0.6 μg pure core RNA polymerase (lane *5*), 0.6 μg core RNA polymerase mixed with 4 μl of the Bio-Rex 70 flowthrough fraction (lane *6*), or 0.3 μg pure Eσ⁷⁰ holoenzyme (lane *7*). The positions of the transcripts from the *rpoH* P1, P3, and P4 promoters are marked. (Lane *M*) DNA size markers of 622, 527, 404, 309, 242, 238, 217, 201, and 190 nucleotides (5'-end-labeled *Hpa*II digest of pBR322).

The gel was cut into seven slices covering the molecular weight range from 36,000 to 16,500, and the proteins in each gel slice were eluted, renatured, mixed with pure core RNA polymerase, and assayed for activity. The only protein capable of directing transcription from the *rpoH*

P3 promoter was contained in slice 5, implicating the strongly stained 24-kD protein as the P3-specific σ-factor (Fig. 5). In similar experiments, proteins ranging from 14 to 200 kD were examined with the same result —only the protein in the gel slice containing the 24-kD protein was active in stimulating transcription from *rpoH* P3.
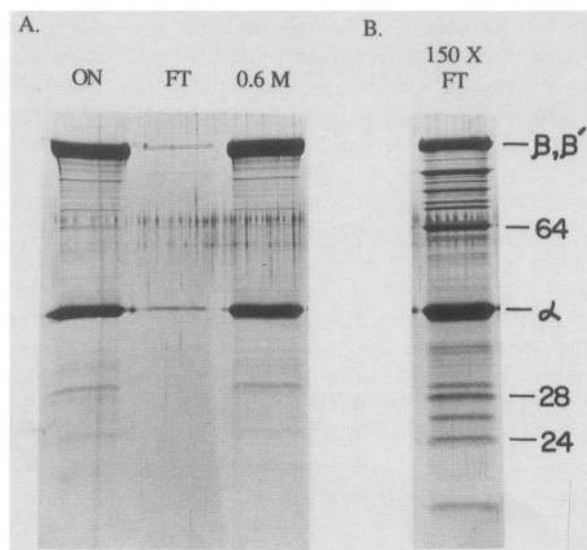
To confirm our identification of the 24-kD protein as the P3-specific σ-factor, we repeated the SDS–polyacrylamide gel renaturation experiment using proteins eluted from a silver-stained gel. Silver staining allowed the precise excision of the 24-kD protein, as well as several gel slices above and below it. Once again, σ activity was recovered only from the gel slice that contained the 24-kD protein (data no shown). The efficiency of elution and renaturation from the silver-stained gel was ~100-fold lower than from unstained or Coomassie-blue-stained gels, but the activity was easily distinguished from the background.

Our experiments demonstrate that the 24-kD protein is a novel RNA polymerase σ-factor. The purified protein confers a unique promoter specificity upon core RNA polymerase, and it has a different mobility on SDS–polyacrylamide gels (24 kD) than the previously identified *E. coli* σ factors, σ⁷⁰ (90 kD), σ⁵⁴ (75 kD), σ³² (32 kD), and σᶠ (28 kD) (Lowe et al. 1979; Grossman et al. 1984; Hunt and Magasanik 1985; Arnosti and Chamberlin 1989).

Originally, σ-factors were named with a superscript designating the molecular weight of the protein as estimated from its mobility on SDS–polyacrylamide gels. This has become confusing because most σ-factors were renamed when their true molecular weights were established. To avoid similar problems, we will call the 24-kD σ-factor, σᴱ, with the expectation that the gene encoding it will be named *rpoE* in accordance with the accepted nomenclature (Hayward and Scaife 1976).

Figure 4. Proteins present in the Bio-Rex 70 column fractions. (*A*) Proportional volumes of the Bio-Rex 70 onput (ON), flowthrough (FT), and 0.6 M NaCl eluate (0.6 M) were electrophoresed in 13% polyacrylamide–SDS gels, and the proteins were visualized by silver staining. (*B*) A volume of the flowthrough fraction 150 times greater than that loaded in *A* was concentrated by precipitation with trichloroacetic acid and treated as in *A*. The β-, β'-, and α-subunits of RNA polymerase are indicated. The molecular weights indicated were determined by comparison with the mobility of bovine serum albumin (BSA) (66 kD), ovalbumin (43 kD), α-chymotrypsin (25 kD), β-lactoglobulin (18 kD), and lysozyme (14 kD) (not shown).

Figure 5. Renaturation of the P3-specific σ-factor after SDS–polyacrylamide gel electrophoresis. (*Left*) A silver-stained 13% polyacrylamide–SDS gel containing a 100 μl sample of the flowthrough fraction from the Bio-Rex 70 column. A protein sample identical to the one shown was stained with Coomassie blue, and the Coomassie-stained lane was cut into sections as indicated. The proteins from gel sections 1–7 were eluted, renatured (765 μl total volume), and assayed for σ-factor function. (*Right*) In vitro transcription assays with the proteins eluted from the SDS–polyacrylamide gel. pJET41 was transcribed with 0.3 μg of core RNA polymerase plus 3 μl of the Bio-Rex 70 flowthrough (lane *on*), core RNA polymerase alone (lane *core*), or core RNA polymerase supplemented with 25-μl samples (3.3% of the total) of the proteins eluted from gel sections 1–7 (lanes *core + 1–7*). In this experiment the transcription reactions contained 33 mM Tris (pH 7.9), 8 mM $MgCl_2$, 140 μg/ml BSA, 160 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 5% glycerol, and 27 mM guanidine hydrochloride.

Structure of the rpoH P3 promoter

The σ^E-controlled P3 and the σ^{70}-controlled P4 promoters overlap extensively. To distinguish the two promoters and to isolate and identify P3, we constructed deletions that eliminated P4 function but that retained a functional P3. To eliminate P4, we synthesized two double-stranded oligonucleotides that lack a portion of the predicted −10 region of P4 but should contain all of P3. These oligonucleotides were cloned in front of a promoterless *galK* gene, creating pJEK60 and pJEK61 (Fig. 6). Galactokinase was expressed from both plasmids in vivo, suggesting that the P3 promoter was functional. This was confirmed in three ways. First, galactokinase synthesis was measured in vivo by pulse-labeling and was found to be induced by shift to 43 or 50°C (data not shown). Second, RNA was isolated from cells carrying pJEK60 or pJEK61, both at 30°C and after shift to 50°C, and then S1 mapped. In both strains a major plasmid-encoded transcript of the correct size was found, and these transcripts were induced strongly after shift to 50°C (Fig. 6). Third, $E\sigma^E$ recognized the P3 promoters of pJEK60 and pJEK61 and synthesized transcripts in vitro that were identical to those made in vivo. No specific transcripts

were detected from either plasmid using $E\sigma^{70}$, confirming that the P4 promoter was destroyed in the cloning (Fig. 6).

A second σ^E-controlled promoter

Transcription of the *E. coli htrA* (high temperature requirement) gene is induced independently of *rpoH* and σ^{32}, by exposure to high temperature (Lipinska et al. 1988). When Lipinska et al. (1988) sequenced the *htrA* promoter, they noted a striking resemblance to the sequence of *rpoH* P3 and proposed that *htrA* would be transcribed by $E\sigma^E$. The S1 mapping experiment shown in Figure 7 confirms that the *htrA* promoter is recognized by $E\sigma^E$. In vivo, the *htrA* transcript is induced after shift from 30 to 50°C. In vitro, the *htrA* transcript is synthesized by $E\sigma^E$ but not by $E\sigma^{70}$ (Fig. 7). These experiments and those of Lipinska et al. (1988) establish *htrA* as the second *E. coli* gene known to be transcribed by $E\sigma^E$.

The sequences of the *htrA* and *rpoH* P3 promoters are shown in Figure 8A. There are four segments of common sequence in the two promoters. The sequence GAACTT probably corresponds to the upstream (−35) portion of each promoter, and the sequence TCTGA most likely represents the downstream (−10) region. The probable −10 and −35 regions are separated by a spacer of 16 bp. The spacer regions of both promoters contain the sequence ATAAA, but its position is offset by 2 bp in one promoter relative to the other. The sequences near the transcription start sites are also similar; however, these sequences are not required for promoter function (Fig. 6)

The sequences of the *rpoH* P3 and *htrA* promoters do not resemble any of the known *E. coli* or *Bacillus* promoter consensus sequences (Helmann and Chamberlin 1988). They do, however, resemble the *dagA* (agarase) P1 and P2 promoters from *Streptomyces coelicolor* (Buttner et al. 1988) , and the *phl* (phospholipase A1) promoter of *Serratia liquefaciens* (Givskov et al. 1988) (Fig. 8B). The *dagA* P2 promoter is recognized by the minor $E\sigma^{28}$ form of *S. coelicolor* RNA polymerase, but it is not known which holoenzyme transcribed from the P1 promoter (Buttner et al. 1988).

The *S. liquefaciens phl* promoter is known to be expressed in *E. coli* (Givskov et al. 1988), but we know of no published information regarding its activity at high temperature in either organism.

Discussion

Purification of σ^E

We identified and purified a novel *E. coli* σ-factor, which we call σ^E. RNA polymerase containing σ^E recognizes the P3 promoter of *rpoH*, the gene that encodes σ^{32}, the heat shock σ-factor. $E\sigma^E$ was purified by chromatography on single-stranded DNA agarose, Sephacryl S-400, and DEAE-Sephacryl. The σ^E subunit was separated from core RNA polymerase by chromatography on Bio-Rex 70. To prove that σ^E functions as a σ-factor and that it is distinct from any of the known σ-factors, we isolated the

Figure 6. Isolation of the *rpoH* P3 promoter. (*Top*) DNA sequence of the region containing the *rpoH* P3 and P4 promoters. The nontemplate strand is shown. The −35 and −10 regions of the σ70-controlled *rpoH* P4 promoter are underlined, and the transcription start sites are marked with the numbered arrows. Plasmid pJEK5 contains an additional 62 bp from the *rpoH* locus to the right of the sequence shown. Plasmids pJEK60 and pJEK61 contain the 40- and 37-bp sequences shown and no additional *rpoH* DNA. The plasmid-derived sequence that abuts the right-hand end of the *rpoH* DNA in both plasmids is CTAGAG-GATCCCC. (*Bottom*) S1 mapping of in vitro and in vivo transcripts from pJEK60 and pJEK61. DNA probes were prepared from pJEK60 and pJEK61 and were 5'-end-labeled at the *Bss*HII site in the vector located 250 nucleotides downstream from the 3' end of the *rpoH* P3 promoter insert. In vitro RNA was prepared from transcription reactions that contained 0.85 μg of Eσ70, 0.5 μg of Eσ32, or 0.7 of μg core RNA polymerase plus 8 μl of the Bio-Rex 70 flowthrough (Eσ^E). In vivo RNA was extracted from cells grown at 30°C or 20 min after shift to 50°C. Hybridizations contained 10 μg of 30°C RNA or 5 μg of 50°C RNA. *rpoH* P3 transcripts (indicated by arrows) initiate within the vector sequences immediately 3' of the insert and should protect fragments of ~247 nucleotides (pJEK60) or ~244 nucleotides (pJEK61). The 3-base difference in lengths of protected fragments derive from the different sizes of the inserts in the two plasmids. The origins of the smaller protected fragments in the in vivo RNA lanes are not known but may reflect processing or degradation of these plasmid-derived transcripts.

plasmid	promoter		
pJEK5	P3+ P4+	AAGCTTGCATTGAAC <u>TTGTGG</u> ATAAAATCACGGTCTGA<u>TAAAAC</u>AGTGAAT
pJEK60	P3+ P4−	AAGCTTGCATTGAAC <u>TTGTGG</u> ATAAAATCACGGTCTGA<u>TAA</u>
pJEK61	P3+ P4−	AAGCTTGCATTGAAC <u>TTGTGG</u> ATAAAATCACGGTCTGA
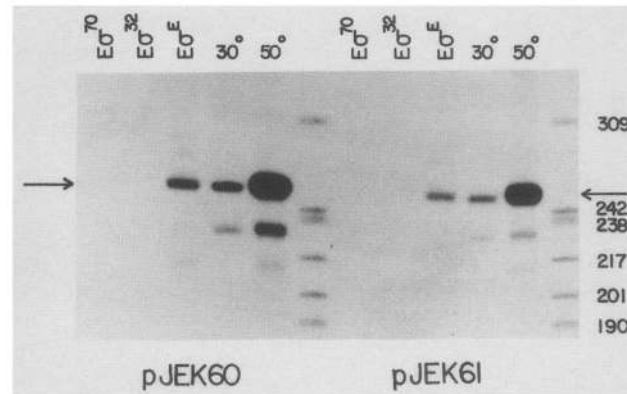
protein from SDS–polyacrylamide gels, renatured it, and showed that renatured σ^E directed core RNA polymerase to initiate transcription from *rpoH* P3 in vitro.

Promoter specificity of Eσ^E

RNA polymerase containing σ^E has a unique promoter specificity. Eσ^E did not initiate transcription from the three σ70-controlled promoters we tested (Figs. 1–3, and 5) or from the σ32-controlled promoters; *dnaK* P1, *groE* pHS, and *rpoD* pHS (data not shown). So far we have found two promoters that are recognized by Eσ^E: the *E. coli rpoH* P3 and the *htrA* promoters. The sequences of the σ^E-controlled promoters are shown in Figure 8A. The sequence comparison suggests that, as with most types of bacterial promoters, σ^E-controlled promoters have conserved sequences that are analogous to the −10 and −35 regions of σ70-controlled promoters (Reznikoff et al. 1985; Helmann and Chamberlin 1988). In addition, the sequence ATAAA, located between the putative −10 and −35 regions is present in both promoters. The sequences of these conserved regions are different from the known σ70-, σ32-, σ^F-, or σ54-controlled promoters (Hawley and McClure 1983; Cowing et al. 1985; Arnosti and Chamberlin 1989; Ninfa et al. 1989). Consistent with the differences in promoter sequences, neither Eσ70 nor Eσ32 transcribes from the *htrA* or *rpoH* P3 promoters in vitro (Erickson et al. 1987; Figs. 1, 2, and 7). Our results conflict with those of Fujita and Ishihama (1987), who reported that P3 (P2* in their paper) was recognized by Eσ70. Their claim was based on low-level in vitro

transcription from P3 by Eσ70. We believe their results were due to contamination with Eσ^E because none of the steps in their RNA polymerase purification would have separated Eσ70 and Eσ^E.

The existence of σ^E explains the synthesis of the heat shock proteins at lethal temperatures

At 50°C, a temperature too high for growth and lethal under prolonged exposure, the heat shock proteins are virtually the only proteins synthesized (Neidhardt et al. 1984; Paek and Walker 1986). Neidhardt et al. (1984) suggested originally that the exclusive synthesis of the heat shock proteins could occur if σ32 was active and σ70 was inactive at 50°C. This proposal now has experimental support. First, there is no transcription from the σ70-controlled *rpoH* P1 and P4 promoters at times after shift to 50°C when the σ32-controlled *groE* pHS promoter remains active (Erickson et al. 1987; unpubl.). Second, transcription extracts prepared from cells shifted to 50°C contain active σ32 but do not contain detectable σ70 activity (Fig. 2; data not shown). The decrease in σ70 activity may seem paradoxical, given that σ70 is a heat shock protein; however, there are other suggestions that σ70 is less active at high temperature. Skelly et al. (1987) quantified the amount of Eσ70 in extracts made from cells shifted from 33 to 40 or 45°C and found that it decreased with increasing temperature. Although the amount of Eσ70 decreased, the amount of σ70 protein in the extracts increased, suggesting that at high temperatures a modification of σ70, or of core, prevents or de-

Figure 7. Eσ^E transcribes from the *htrA* promoter. Comparison of in vivo and in vitro *htrA* transcripts by S1 mapping. The schematic diagrams the S1 mapping strategy. A 778-bp *PstI* (Ps)–*BstEII* (Bs) DNA fragment from pBL133, 5'-end-labeled at the *BstEII* site, was used as probe. Transcripts initiated from the *htrA* promoters should protect a 619-nucleotide fragment (arrow) from digestion by S1 nuclease. In vitro RNA was synthesized with 0.43 μg of Eσ^{70}, 0.5 μg of Eσ^{32}, or 0.35 μg of core RNA polymerase plus 2 μl of the Bio-Rex 70 flowthrough (Eσ^E), using pBL133 as template. In vivo RNA was isolated from strain SC122 grown at 30°C or 20 min after shift to 50°C. Hybridizations were done with 40 μg of 30°C RNA and 10 μg of 50°C RNA. DNA size markers of 622 and 527 nucleotides are present in the flanking lanes. The origin of the smaller protected fragment in the 50°C lane is not known, but it may reflect degradation of the RNA at the lethal temperature.

creases the association of σ^{70} with core RNA polymerase.

σ^{32} is degraded rapidly at 50°C (A. Grossman, W. Walter, and C. Gross, unpubl.) and must therefore be synthesized continuously if the cell is to maintain the expression of the heat shock genes. Because σ^{32} is not autoregulatory (Bloom et al. 1986; Tilly et al. 1986; Erickson et al. 1987; Fujita and Ishihama 1987), it was not clear how its synthesis could be maintained at 50°C if the *rpoH* promoters were recognized solely by the Eσ^{70}. Our demonstration that *rpoH* P3 is recognized by Eσ^E, which is active at 50°C, explains this aspect of the heat shock response. It is the transcription from the σ^E-dependent *rpoH* P3 promoter that allows σ^{32} to be made and the heat shock genes to be transcribed at 50°C.

σ^E and the regulation of rpoH transcription

At normal growth temperatures, the transcripts from P3 represent a small fraction of the *rpoH* mRNA. This fraction increases with increased temperature, but even at 43.5°C, the transcript from P3 represents only ~15% of

the total *rpoH* mRNA (Erickson et al. 1987; Fujita and Ishihama 1987; J. Erickson and C. Gross, unpubl.). We argued previously (Erickson et al. 1987; Straus et al. 1987) that the transcription of *rpoH* is of little regulatory significance for the heat shock response. This is because the increase in *rpoH* mRNA synthesis after temperature upshift (Erickson et al. 1987) is too slow and too small to account for the transient 20-fold increase in σ^{32} concentration that leads to the heat shock response (Straus et al. 1987). Our argument did not, however, imply that the increased transcription of *rpoH* after temperature upshift is unimportant. In fact, it is likely that the level of *rpoH* mRNA determines the steady-state level of σ^{32}, because there is a good correlation between the steady-state amount of σ^{32} and the *rpoH* mRNA concentration (Erickson et al. 1987; Straus et al. 1987; D. Straus, J. Erickson, and C. Gross, unpubl.). Viewed in this way, the role of σ^E in *rpoH* transcription is as part of the mechanism that determines the amount of *rpoH* transcription appropriate for the growth temperature. At low temperatures the contribution of σ^E is small, but as the temperature increases, it becomes quantitatively more important as transcription from P3 is induced and the amount of active σ^{70} declines.

A second heat shock regulon

The *htrA* (*degP*) gene is required for growth and survival of *E. coli* at temperatures >42°C (Lipinska et al. 1989). *htrA* encodes a 51-kD precursor protein that is processed to the 48-kD periplasmic protease DegP (Strauch and Beckwith 1988; Lipinska et al. 1989). The exact function of DegP is not known, but strains with an *htrA* :: Tn*10* insertion cease macromolecular synthesis and lyse soon after shift to high temperature (Lipinska et al. 1989).

Figure 8. Comparison of the DNA sequences of the *htrA* and *rpoH* P3 promoters with other promoters that may be recognized by Eσ^E. (*A*) The *E. coli htrA* and *rpoH* P3 promoter sequences. The nontemplate strands are shown. The transcription start sites are outlined, and the common sequences are shown in boldface type. The putative −10 and −35 regions are underlined. (*B*) DNA sequences of three heterologous promoters that are similar to the σ^E-controlled *htrA* and *rpoH* P3 promoters. The start points of transcription are outlined and common sequences in the putative −10 and −35 regions are shown in boldface type. The *dagA* P1 and P2 promoters are from *S. coelicolor* (Buttner et al. 1988), and the *phl* promoter is from *S. liquefaciens* (Givskov et al. 1988).

Transcription of *htrA* is induced strongly in vivo after shift to high temperature, but the increased transcription is independent of σ^{32} (Lipinska et al. 1988). Lipinska et al. (1988) mapped the 5' end of the *htrA* transcript and found that the sequence of the putative promoter was almost identical to *rpoH* P3. Using the criteria of in vitro transcription by pure $E\sigma^E$ and induction of transcription after shift to 50°C in vivo, we confirmed that the *htrA* promoter is recognized by $E\sigma^E$ (Fig. 7), making it the second *E. coli* gene known to be under the control of σ^E.

Given that σ-factors tend to regulate global changes in gene expression, we think it unlikely that the *rpoH* P3 and the *htrA* promoters are the only ones recognized by $E\sigma^E$. Until now, most studies of gene expression at high temperature have focused on the heat shock genes, which are under the control of *rpoH* and transcribed by $E\sigma^{32}$. The emphasis on the heat shock genes has obscured the fact that there are a number of σ^{32}-independent genes that are expressed at higher levels after shift to high temperature (Herendeen et al. 1979; Neidhardt et al. 1982, 1983; Yamamori and Yura 1982; Neidhardt and VanBogelen 1987). We believe that some of these genes are transcribed by $E\sigma^E$ and constitute a second group of coordinately regulated heat-inducible genes.

The existence of this second high-temperature regulon raises a problem with existing terminology. Previously, the term "heat shock genes," has been used to describe only those genes that are regulated by σ^{32} and are heat inducible. To avoid confusion, we suggest that the different heat-inducible genes should be referred to as σ^{32} or σ^E dependent in cases of ambiguity.

What is the role of σ^E?

What are the likely functions of σ^E? It is clear that the σ^E-controlled *htrA* gene is required for growth and survival at temperatures >42°C (Lipinska et al. 1989) and that the σ^E-dependent transcription of *rpoH* is necessary for the synthesis of σ^{32} at lethal temperatures; therefore, we expect that other σ^E-controlled genes will have functions related to growth or survival at high temperature. One possible function for σ^E is in thermotolerance, i.e., the inducible resistance to killing at high temperature. Until recently, it was thought that the heat shock proteins were the primary components of thermotolerance; however, it has now been demonstrated that the induction of the heat shock genes is not sufficient to induce thermotolerance (VanBogelen et al. 1987a). One implication of these results is that treatments inducing thermotolerance must induce or activate a set of proteins distinct from the heat shock proteins. Transcription by $E\sigma^E$ is increased by some of the conditions that induce thermotolerance, such as a shift to 42°C (Erickson et al. 1987; Fujita and Ishihama 1987) or addition of ethanol to 10% (J. Erickson and C. Gross, unpubl.). $E\sigma^E$ is also active at lethal temperatures, making it a likely candidate to be a regulator of the σ^{32}-independent genes involved in thermotolerance.

We emphasized possible roles of σ^E at high temperatures because of the properties of two *E. coli* genes that are transcribed by $E\sigma^E$. It is also possible that σ^E is im-

portant at low temperatures. In this regard, the similarity between the *ropH* P3 and *htrA* promoters and the *S. coelicolor* and *S. liquefaciens* promoters is interesting. If these promoters are recognized by $E\sigma^E$, the σ-factors that direct RNA polymerase to recognize these promoters in *S. coelicolor* and *S. liquefaciens* are likely to be homologous to *E. coli* σ^E. If there is conservation of function among the σ-factors from these diverse organisms, we may be able to learn something about the function of *E. coli* σ^E by analogy. The only connection we know of between the *S. coelicolor dagA*, *S. liquefaciens phl*, and *E. coli htrA* genes is that they encode secreted (extracellular or periplasmic) hydrolytic enzymes. It may be that $E\sigma^E$ transcribes a set of genes encoding secreted proteins; however, most of the known genes that encode periplasmic or extracellular proteins appear to be transcribed by $E\sigma^{70}$ (Oliver 1985; Randall et al. 1987).

The available data suggest that σ^E can control the synthesis of σ^{32} under extreme environmental conditions and that σ^E activity is required for survival at high temperature. Transcription by $E\sigma^E$ appears to be induced by high temperature and by exposure to high concentrations of ethanol. When the analysis of the *rpoE* gene is begun, these observations and the possible connection between σ^E and secreted proteins will provide starting places in the search for the functions of σ^E.

Materials and methods

Strains and plasmids

Strains SC122 ($rpoD^+$) (Cooper and Ruettinger 1975) and CAG510 (SC122 *rpoD800*) were used as sources of transcription extracts and in vivo *rpoH* and *htrA* mRNA. MC1061 Δ(*ara-COIBA*, *leu*)7697, *araD139*, Δ(*lac*)X74, *galK*, *galU*, *rpsL*) (Casadaban and Cohen 1980), carrying plasmids pJEK60 or pJEK61, was used to analyze expression from the isolated P3 promoter (Fig. 6). Plasmid pUC19-*spf'* was made by cloning a 132-bp fragment containing most of the *spf* gene and its transcription terminator (Joyce and Grindley 1982) into pUC19 (Yanisch-Perron et al. 1985). The HindIII–BamHI portion of the polylinker is located upstream of the terminator so that transcripts terminate either 103 or 104 bases from the last position of the BamHI site. pUC19-*spf'* carries the σ^{70}-dependent ColE1 RNA-1 promoter, which produces transcripts of 102 and 108 nucleotides. pJET41 was made by inserting a blunt-ended 895-bp SphI–MluI fragment from pKP11 (Paek and Walker 1986; Erickson et al. 1987) that contains the *rpoH* promoters and the first 22 codons of *rpoH* into pUC19-*spf'* that had been cleaved with SphI and SalI and made blunt-ended. The *rpoH* P3$^+$–*galK*$^+$ fusion plasmids pJEK60 and pJEK61 were made by ligating double-stranded oligonucleotides (synthesized by the University of Wisconsin Biotechnology Center) containing the P3 promoter (Fig. 6) into pKO500 [a derivative of pKO1 (McKenney et al. 1981), with the multiple cloning site from M13mp11] that had been cut with HindIII and XbaI. The sequences of the inserts were confirmed by dideoxy sequencing (Tabor and Richardson 1987). pBL133, which contains the *htrA* gene, has been described (Lipinska et al. 1988).

Purification of σ^E

For the large-scale purification of σ^E, CAG510 was grown in a 10-liter fermentor at 30°C, shifted to 50°C for 45 min, chilled

on ice, and harvested by filtration. The procedure of Burgess and Jendrisak (1975) was used through the $(NH_4)_2SO_4$ precipitation step. The $(NH_4)_2SO_4$ precipitates (starting from 80 grams of cells) were resuspended in TGED [10 mM Tris-HCl (pH 7.9), 10% glycerol, 0.1 mM EDTA, 1 mM DTT], diluted to a conductivity equal to TGED + 0.25 M NaCl, and loaded onto a 25-ml single-stranded DNA agarose column (Arndt-Jovin et al. 1975). The column was washed with TGED + 0.25 M NaCl, and $E\sigma^E$ was eluted with TGED + 0.4 M NaCl. The fractions were precipitated with $(NH_4)_2SO_4$ (3.5 g/ml), resuspended in TGED + 0.4 M NaCl, and loaded onto a 400-ml Sephacryl S-400 column (Lowe et al. 1979). The active fractions were pooled, diluted to TGED + 0.1 M NaCl, and loaded onto a 4-ml DEAE-Sephacel column. $E\sigma^E$ was eluted with TGED + 0.25 M NaCl. The fractions were diluted to TGED + 0.1 M NaCl and passed through a 4-ml Bio-Rex 70 column. σ^E activity was found in the flowthrough fraction and core RNA polymerase was eluted with TGED + 0.6 M NaCl. To purify σ^E further, portions of the flowthrough were acetone precipitated, resuspended in SDS sample buffer, and electrophoresed through SDS–polyacrylamide gels (Laemmli 1970; Hager and Burgess 1980). σ^E was identified by staining with Coomassie blue or silver (Wray et al. 1981), cut from the gel, eluted, and renatured as described (Hager and Burgess 1980).

In vitro transcription

Transcription reactions (100 μl) contained 0.3–0.5 μg plasmid DNA in 30 mM Tris-HCl (pH 7.9), 180 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 100 μg/ml of acetylated BSA, 200 μM each ATP, GTP, and CTP, and 15 μM UTP with 5–10 μCi [α-^{32}P]UTP. Core RNA polymerase, $E\sigma^{70}$, and $E\sigma^{32}$ were prepared as described (Lowe et al. 1979; Erickson et al. 1987). Cell-free transcription extracts (Gross et al. 1976) were made from SC122 or CAG510 that had been shifted from 30 to 50°C for 45 min. The step 4 extracts (Gross et al. 1976) were used in the transcriptions. The forms and amounts of RNA polymerase used are noted in the figure legends. Reactions were incubated at 37°C for 10 min and stopped by the addition of 100 μl of [4 M NH_4^+ acetate (pH 7), 40 mM EDTA, 0.3% (wt/vol) SDS, 400 μg/ml tRNA], and precipitated with 220 μl of isopropanol. Samples were washed with 70% ethanol, resuspended in 80% formamide-containing marker dyes, and electrophoresed in 5 or 6% (wt/vol) polyacrylamide gels containing 50% (wt/vol) urea (Maxam and Gilbert 1980). For preparation of RNA for S1 mapping, ^{32}P was omitted and UTP was present at 200 μM. In vitro RNA was isolated as described (Erickson et al. 1987).

S1 nuclease mapping

S1 nuclease mapping (Berk and Sharp 1977) was performed as described for rpoH mRNA (Erickson et al. 1987) or htrA mRNA (Lipinska et al. 1988). Samples were electrophoresed on DNA sequencing gels containing either 6% (rpoH) or 4.5% (htrA) polyacrylamide and 50% (wt/vol) urea (Maxam and Gilbert 1980). The method of Salser et al. (1967) was used to isolate in vivo RNA.

Acknowledgments

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Identification of the sigma E subunit of Escherichia coli RNA polymerase: a second alternate sigma factor involved in high-temperature gene expression.

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