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Isolation and Characterization of *Escherichia coli* Mutants That Lack the Heat Shock Sigma Factor σ^{32}

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The product of the *Escherichia coli rpoH* (*htpR*) gene, σ^{32}, is required for heat-inducible transcription of the heat shock genes. Previous studies on the role of σ^{32} in growth at low temperature and in gene expression involved the use of nonsense and missense *rpoH* mutations and have led to ambiguous or conflicting results. To clarify the role of σ^{32} in cell physiology, we have constructed loss-of-function insertion and deletion mutations in *rpoH*. Strains lacking σ^{32} are extremely temperature sensitive and grow only at temperatures less than or equal to 20°C. There is no transcription from the heat shock promoters preceding the *htpG* gene or the *groESL* and *dnaKJ* operons; however, several heat shock proteins are produced in the mutants. GroEL protein is present in the *rpoH* null mutants, but its synthesis is not inducible by a shift to high temperature. The low-level synthesis of GroEL results from transcription initiation at a minor σ^{70}-controlled promoter for the *groE* operon. DnaK protein synthesis cannot be detected at low temperature, but can be detected after a shift to 42°C. The mechanism of this heat-inducible synthesis is not known. We conclude that σ^{32} is required for cell growth at temperatures above 20°C and is required for transcription from the heat shock promoters. Several heat shock proteins are synthesized in the absence of σ^{32}, indicating that there are additional mechanisms controlling the synthesis of some heat shock proteins.

When cells or organisms are suddenly exposed to high temperature a set of heat shock proteins are transiently induced. The response is apparently universal, having been observed in members of all phylogenetic kingdoms (reviewed in reference 19). In addition, some heat shock proteins have been conserved during evolution. The eucaryotic heat shock proteins Hsp70 and Hsp83 have nearly 50% of their amino acid residues in common with their procaryotic homologs (3, 4). The functions of the heat shock proteins are not well understood. These proteins seem to be involved in a wide variety of cellular processes and are required for prolonged survival at high temperatures (19).

In *Escherichia coli* the heat shock response is rapidly induced. Within 1 min after a shift to high temperature, transcription initiation from the heat shock promoters increases, leading to the elevated synthesis of the heat shock proteins (reviewed in references 24 and 26). The heat shock promoters are recognized in vitro by RNA polymerase containing the 32-kilodalton σ subunit ($E\sigma^{32}$) but not by RNA polymerase containing the primary 70-kilodalton σ subunit ($E\sigma^{70}$) (8, 10, 12). Genetic studies from a number of laboratories support the idea that σ^{32}, the product of the *rpoH* gene, is required for the increased transcription of the heat shock genes after a shift to high temperature. Strains carrying *supC*(Ts), encoding a temperature-sensitive suppressor tRNA, and the *rpoH165* amber mutation fail to induce the synthesis of heat shock proteins and are temperature sensitive for growth (23, 26, 46). Several other *rpoH* nonsense and temperature-sensitive mutants show these phenotypes (14, 38). All of the studies indicate that σ^{32} is required for viability at high temperature and suggest that if σ^{32} is inactive or present in reduced amounts the induction of the heat shock proteins is prevented. Recent experiments have demonstrated a strong correlation between the concentration of σ^{32} in the cell and the expression of the heat shock genes, suggesting that transcription of the heat shock genes is directly controlled by the amount of σ^{32} present in the cell (11–13, 36).

The isolation and characterization of *rpoH* nonsense mutants from a parental strain without a suppressor tRNA (38) led to the proposal that the *rpoH* gene product is dispensable at low temperature (48). The unsuppressed *rpoH* nonsense mutants are viable, express heat shock proteins at near-normal levels at 30°C, and transcribe the heat shock genes from the $E\sigma^{32}$-controlled heat shock promoters (39; Y.-N. Zhou, N. Kusukawa, J. W. Erickson, and D. W. Cowing, unpublished data). These results indicate either that a form of RNA polymerase other than $E\sigma^{32}$ can recognize the heat shock promoters in vivo or that enough σ^{32} is produced in the *rpoH* nonsense mutants to allow cell growth and near-normal transcription of the heat shock genes at low temperature.

To determine whether σ^{32} is required for cell growth and for transcription of the heat shock genes, we constructed loss-of-function insertion and deletion mutations in *rpoH*. Our experiments provide direct proof that σ^{32} is required both for cell growth at temperatures above 20°C and for recognition of the heat shock promoters in vivo. The *rpoH* insertion and deletion mutants are extremely sensitive to heat; they are viable at ≤20°C, but do not survive at 25 or 30°C. In the *rpoH* insertion and deletion mutants there is no transcription from the $E\sigma^{32}$-controlled heat shock promoters; however, some of the heat shock proteins are made in the absence of σ^{32} because of transcription from other non-heat-shock promoters.

MATERIALS AND METHODS

Media. The Δ*rpoH30*::*kan* deletion mutant and its parental strains were grown in minimal medium E (42) supplemented with 0.25% glucose, 2 μg of thiamine per ml, and a mixture

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
JC7623	*recB21 recC22 sbc15 arg ara his leu pro thr*	16
CAG9275	JC7623 (λ25 *rpoH*$^+$)	This paper
CAG9280	JC7623 *rpoH120*::*kan* (λ25 *rpoH*$^+$)	This paper
CAG9288	JC7623 *rpoH120*::*kan zhg-21*::Tn*10* (λ25 *rpoH*$^+$)	This paper
KY1600	JC7623 *zhf-50*::Tn*10*	This paper
KY1620	JC7623 Δ*rpoH30*::*kan zhf-50*::Tn*10*	This paper
MG1655	Prototrophic *E. coli* K-12	CGSC[a]
CAG9291	MG1655 (λ25 *rpoH*$^+$)	This paper
CAG9301	MG1655 *rpoH120*::*kan zhg-21*::Tn*10*	This paper
MC4100	Δ(*argF-lac*)*205 araD139 rpsL150 thiA1 relA1 flb-5301 deoC1 ptsF25 rbsR*	M. Casadaban
KY1443	MC4100 [λpF13-(*groEp-lacZ*$^+$)]	39
KY1612	MC4100 Δ*rpoH30*::*kan zhf-50*::Tn*10* [λpF13-(*groE$_p$-lacZ*$^+$)]	This paper
KY1429	MC4100 *rpoH6*(Am) *zhf-50*::Tn*10*	This paper
CAG1951	rpoH165(Am) *supF*(Ts) *rpsL* Δ(*gpt-lac*)*5 trp*(Am) *thi ara galK2*	From CSH 26 (21)
EC514	MG1655 Δ(*dnaK-dnaJ*)*14 thr-34*::Tn*10*	E. A. Craig

[a] CGSC, *E. coli* Genetic Stock Center, Yale University.

of 18 L-amino acids (50 μg/ml each, excluding methionine and cysteine); peptone broth containing (per liter) 10 g of polypeptone (Wako Chemicals, Osaka, Japan), 5 g of NaCl, and 2.5 g of glucose (pH 7.4); or L broth (38). The *rpoH120*::*kan* insertion mutant and its progenitors were grown in M9-glucose (21) supplemented with all amino acids except those used to radioactively label proteins or in L broth. Antibiotics used were kanamycin (12 or 30 μg/ml), tetracycline (10 or 12 μg/ml), and ampicillin (100 μg/ml).

Reagents. The Klenow fragment of DNA polymerase I, T4 DNA ligase, T4 polynucleotide kinase, and *Sac*I linkers were purchased from New England BioLabs, Beverly, Mass. Restriction endonucleases were from New England BioLabs or Promega Biotec, Madison, Wis. Calf intestine alkaline phosphatase and S1 nuclease were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Antisera against GroEL or DnaK were obtained from R. Hendrix (University of Pittsburgh) and C. Georgopoulos (University of Utah). L-[^{35}S]methionine (>1,400 Ci/mmol), L-[4,5-^{3}H]leucine (52 Ci/mmol), L-[4,5-^{3}H]lysine (46 Ci/mmol), [6-^{3}H]thymidine (21 Ci/mmol), and [α-^{32}P]dATP (3,000 Ci/mmol) were from Amersham Corp., Arlington Heights, Ill. [α-^{32}P]dCTP (752 Ci/mmol) was purchased from ICN Radiochemicals USA, Irvine, Calif. [γ-^{32}P]ATP was obtained from Amersham (3,000 Ci/mmol) or ICN Radiochemicals (5,000 Ci/mmol).

Strains, plasmids, and bacteriophages. The bacterial strains used are listed in Table 1. The *rpoH*$^+$ plasmids pKP11 (9, 31), pKV1, pKV3 (38), and pFN97 (25) have been described previously. Plasmid pKV7 was constructed by subcloning a 2.4-kilobase (kb) *Hpa*I fragment from pKV1 bearing *rpoH* and its flanking sequences (1.0 kb upstream and 0.5 kb downstream) into the *Pvu*II site of pBR322 (6). Plasmid pKV11 was derived from pKV7 by replacing the *Mlu*I-*Pvu*II segment (0.7 kb) with a 1.47-kb fragment containing the *kan* resistance element from pUC-4K (41). The *kan* gene was originally derived from Tn*903* (28). Plasmid pFN97 contains an intact *rpoH* gene and flanking sequences (133 base pairs [bp] upstream and 681 bp downstream). Plasmid pYN1 was made by cutting pFN97 with *Nco*I, filling in the ends with the Klenow fragment of DNA polymerase I, and ligating in a 1.25-kb *Hinc*II fragment that contains the *kan* resistance element from a modified pUC-4K. This version of pUC-4K (obtained from E. A. Craig) carries an uncharacterized deletion of about 250 bp from the region containing the *kan* resistance element. Plasmids pS2, pdnaK, and pC62.5 have been described previously (8) and were used for making probes for S1 mapping. pJB8 *groES*$^+$ and pKV101 *dnaK*$^+$ were also used for S1 mapping and were provided by C. Georgopoulos and R. Yano.

λpF13-(*groEp-lacZ*$^+$) phage carrying a heat shock promoter-*lacZ* operon fusion has been described previously (47). λ *i*^{21}*rpoH*$^+$ is an i^{21} derivative of λ5.1 of Oxender et al. (29) isolated for this study. λ25 *rpoH*$^+$ was made by inserting the bacterial DNA from pKP11 into Charon 25. pKP11 was cut with *Bam*HI and *Sal*I, and the ends were filled in with the Klenow fragment of DNA polymerase I. *Sac*I linkers were added, and the *rpoH* fragment was purified and ligated into Charon 25 cut with *Sac*I. Following in vitro packaging, λ25 *rpoH*$^+$ lysogens were isolated by their ability to complement the temperature-sensitive phenotype of CAG1951 [*rpoH165* (Am) *supF*(Ts)]. Culture supernatants from CAG1951(λ25 *rpoH*$^+$) were used to prepare high-titer stocks of λ25 *rpoH*$^+$.

Nucleic acid techniques. Isolation of plasmid and phage DNA, agarose gel electrophoresis, DNA ligations, DNA end labeling, nick translations, and restriction enzyme digestions were done as described by Maniatis et al. (20). In vitro transcriptions were done as described by Erickson et al. (9), except that linear pS2 DNA was the template. Total cellular RNA was isolated by the method of Salser et al. (34) or Aiba et al. (2). The methods of Berk and Sharp (5) were used for S1 mapping as described by Mori and Aiba (22) or Erickson et al. (9). *E. coli* chromosomal DNA was prepared as described by Silhavy et al. (35) or Saito and Miura (33). For Southern hybridization analysis of the *rpoH* deletion strains chromosomal DNA was electrophoresed in 1% agarose gels, blotted onto Zeta Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.), and hybridized with ^{32}P-labeled probe at 42°C in 4× SSPE (20) containing 0.5% skim milk (Snow Brand Co., Tokyo, Japan), 1% sodium dodecyl sulfate (SDS), 50% formamide, 10% dextran sulfate, and 0.5 mg of sheared salmon sperm DNA per ml. Blotted DNAs were washed successively (15 min each at room temperature) in 2× SSC (1× SSC is [pH 7.0] 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS, with a final high-stringency wash in 0.1× SSC–0.1% SDS at 50°C for 30 min (32). For Southern hybridization analysis of the *rpoH* insertion strain DNA was electrophoresed in 1% agarose gels, transferred to nitrocellulose (20), hybridized with ^{32}P-labeled probe for 20 h at 68°C, and washed twice with 1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.)–5 mM Tris (pH 8.0) at room temperature and seven times with 5 mM Tris (pH 8.0) at room temperature.

Construction and isolation of the *rpoH120*::*kan* mutation. Plasmid pYN1, containing the *kan* resistance cartridge at the *Nco*I site of *rpoH*, was cut with *Nde*I and transformed into strain CAG9275 (*recB recC sbcB*), which carries a λ25 *rpoH*$^+$ prophage. The Amp^s Kan^r colonies that grew at 30°C were verified by Southern blotting to contain the *rpoH120*::*kan* mutation in addition to the *rpoH*$^+$ gene carried on the λ25 *rpoH*$^+$ prophage. A Tn*10* transposon, *zhg-21*::Tn*10*, which is 80 to 90% linked to the chromosomal *rpoH* locus, was transduced into strain CAG9280 (*rpoH120*::*kan*[λ25 *rpoH*$^+$]), creating CAG9288 (*rpoH120*::*kan zhg21*::Tn*10*[λ25 *rpoH*$^+$]). Phage P1 was used to transduce the *rpoH120*::*kan*

TABLE 2. Transduction of *rpoH120*::*kan* into strains haploid and diploid for *rpoH*$^+$[a]

Recipient	Temp (°C)	No. of Tet^r colonies	No. of Kan^r colonies	% Linkage
MG1655	30	200	0	0
CAG9291 (λ25 *rpoH*$^+$)	30	200	178	89
MG1655	15	52	42 (all ts)	81
CAG9291 (λ25 *rpoH*$^+$)	15	50	44	88

[a] A P1 *vir* transducing lysate was prepared on CAG9298 [*rpoH120*::*kan zhg-21*::Tn*10* (λ25 *rpoH*$^+$)] and used to transduce the recipient strains to Tet^r at the indicated temperatures. Colonies were then scored for Kan^r and temperature sensitivity (ts).

mutation and the *zhg21*::Tn*10* insertion into strains MG1655 (*rpoH*$^+$) and CAG9291 [MG1655(λ25 *rpoH*$^+$)] at 15°C by selecting for Tet^r and screening for Kan^r and temperature sensitivity (Table 2). One of the Tet^r Kan^r temperature-sensitive transductants, CAG9301, was verified by Southern blotting to have the *rpoH120*::*kan* insertion and no other copies of *rpoH* (see Fig. 2).

Construction and isolation of the Δ*rpoH30*::*kan* deletion. Plasmid pKV11, which has a large deletion of the *rpoH* gene and a *kan* resistance cartridge inserted in the position of the deletion, was cut with *Pst*I and *Eco*RI and transformed into strain KY1600 (*recB recC sbcB zhf-50*::Tn*10*) at 20°C. Many of the Amp^s Kan^r cells were temperature sensitive, indicating that they carried the Δ*rpoH30*::*kan* deletion. The temperature sensitivity could be complemented by either λ i^{21} *rpoH*$^+$ or pKV3 which carries *rpoH*$^+$. The presence of the Δ*rpoH30*::*kan* mutation was confirmed by Southern blotting. The Δ*rpoH30*::*kan* deletion was transduced into strain KY1443 (MC4100 λpF13(*groEp-lacZ*$^+$) by using phage P1 grown on KY1620, carrying plasmid pKV3 to improve the phage yield, and selecting for Tet^r at 20°C and scoring for Kan^r and temperature sensitivity. One of the Tet^r Kan^r temperature-sensitive mutants was isolated and named KY1612. The presence of the Δ*rpoH30*::*kan* deletion was verified by Southern blotting (see Fig. 2).

Measurements of protein synthesis. For the experiment shown in Fig. 5, log-phase cells in minimal medium supplemented with 18 amino acids were pulse-labeled with [^{35}S]methionine, precipitated with trichloroacetic acid, and prepared for gel electrophoresis as described previously (38). To measure GroEL and DnaK synthesis, we grew cells to an optical density at 450 nm of 0.3 to 0.4 in M9 glucose medium plus 19 amino acids at 16.5°C. Portions (1 ml) of culture were pulse-labeled with 60 μCi of [^{35}S]methionine for 4 min at 16.5°C or for 2 min following a shift to 30 or 42°C and chased with unlabeled methionine. The cells were harvested and lysed in 50 mM Tris hydrochloride–2% SDS at 100°C for 3 min. GroEL and DnaK proteins were immunoprecipitated with antisera provided by R. Hendrix and C. Georgopoulos by using *Staphylococcus aureus* cells as described previously (15). To correct for losses, an extract of strain MG1655 labeled with [^{3}H]leucine and [^{3}H]lysine after the shift from 30 to 42°C was used as a source of [^{3}H]GroEL and [^{3}H]DnaK. Equal counts per minute of the ^{3}H-labeled extract were added to each sample. Samples were analyzed on SDS–7.5% polyacrylamide gels (17), and the relative syntheses of GroEL and DnaK were determined by double-label quantitation as described previously (13).

RESULTS

Isolation of mutants with an inactive *rpoH* gene. The first step in the isolation of *rpoH* null mutants was to construct

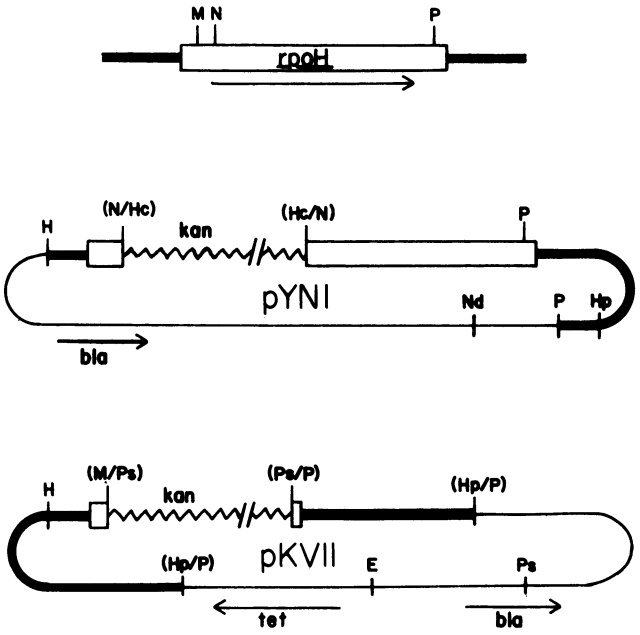

FIG. 1. Plasmids used to inactivate the *rpoH* gene. Plasmid pYN1 was created by inserting the *kan* gene from pUC4K at the *Nco*I site in the *rpoH* gene of pFN97. Plasmid pKV11 was created by deleting the *Mlu*I-*Pvu*II fragment of pKV7 and replacing it with the *kan* gene from pUC4K. Symbols: ▬, chromosomal DNA inserts; ——, pBR322-derived DNA; ∿∿∿, DNA from pUC4K. Only the chromosomal DNA is drawn to scale. Restriction sites: E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nco*I; P, *Pvu*II; Hc, *Hin*cII; Hp, *Hpa*I; Nd, *Nde*I; Ps, *Pst*I. The sites in parentheses were destroyed during construction.

plasmids that carried insertion or deletion mutations in the *rpoH* gene (Fig. 1). The *rpoH* insertion mutation was made by inserting a *kan* resistance gene at the *Nco*I site in the *rpoH* gene, creating plasmid pYN1. This insertion mutation, called *rpoH120*::*kan*, interrupts the translation of σ^{32} at amino acid 44 (18, 48) and prevents transcription of the portion of *rpoH* downstream of the insertion (J. W. Erickson, unpublished data). The *rpoH* deletion mutation, Δ*rpoH30*::*kan*, was made by removing a 737-base *Mlu*I-*Pvu*II fragment that corresponds to the amino acid residues 23 through 268 of σ^{32} (a total of 283 amino acids) (18, 48) and replacing it with a *kan* resistance gene, creating plasmid pKV11. The *rpoH* insertion and deletion mutations were transferred from the plasmids to the *E. coli* chromosome by the procedure of Winans et al. (45) as described in Materials and Methods.

To determine whether *rpoH* null mutants were viable, we transduced the *rpoH120*::*kan* mutation into strains haploid (MG1655) or diploid (CAG9291) for *rpoH*$^+$ by selecting for transfer of the linked transposon *zhg-21*::Tn*10*. When the transductants were plated at 30°C Kan^r colonies appeared only in the progeny of the strain that carried two copies of *rpoH*$^+$; however, at 15°C Kan^r colonies were recovered with nearly equal frequencies in both strains (Table 2). None of the Kan^r transductants from the strain haploid for *rpoH* were able to grow at 25 or 30°C. These results suggest that *rpoH*$^+$ is not required for growth at 15°C, but is essential at 25 or 30°C.

We confirmed by Southern blotting that several of the temperature-sensitive Tet^r Kan^r colonies carried the

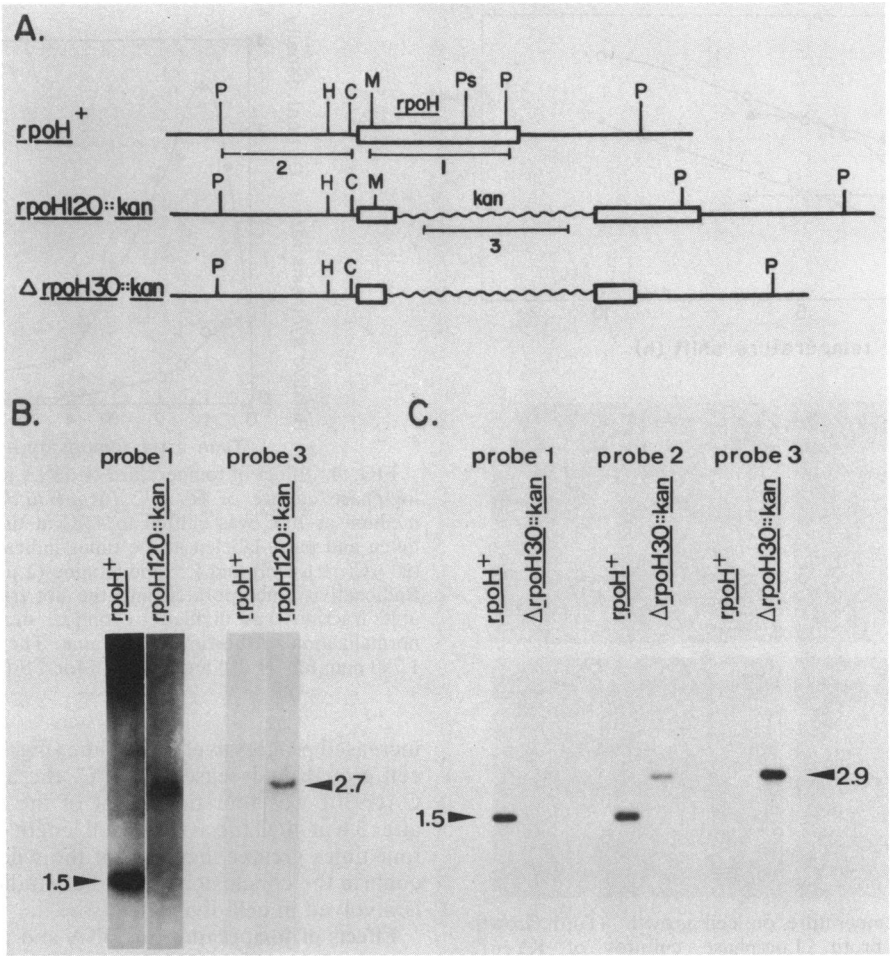

FIG. 2. Confirmation of the structures of the *rpoH* insertion and deletion mutations. (A) Expected structures of the *rpoH* region of the chromosome from the *rpoH*$^+$ and the putative *rpoH*::*kan* strains. Symbols: □, *rpoH* coding sequences, ∿, DNA from pUC4K. The three probes used for hybridization are indicated under the restriction maps. Restriction sites: P, *Pvu*II; H, *Hin*dIII; C, *Cla*I; M, *Mlu*I; Ps, *Pst*I. (B and C) Southern hybridizations to confirm the structures of the *rpoH* insertion and deletion mutations. Chromosomal DNAs were cut with *Pvu*II, separated on agarose gels, transferred, and hybridized with probe 1, probe 2, or probe 3 as described in Materials and Methods. The numbers at the sides indicate the apparent sizes of DNA in kilobases. Panel B shows the results obtained with MG1655 (*rpoH*$^+$) and CAG9301 (*rpoH120*::*kan*). Panel C shows the results with MC4100 (*rpoH*$^+$) and KY1612 (Δ*rpoH30*::*kan*).

rpoH120::*kan* mutation. Figure 2B shows the results obtained with the *rpoH120*::*kan* mutation in strain MG1655. Hybridization of *rpoH* DNA (probe 1) with chromosomal DNA from MG1655 (*rpoH*$^+$) cut with *Pvu*II revealed a single 1.5-kb *Pvu*II fragment. The 1.5-kb band was missing in the temperature-sensitive Tet^r Kan^r transductant CAG9301 and was replaced with a band of 2.7 kb, as expected from insertion of the 1.25-kb *kan* resistance cassette in the *rpoH* gene. The same 2.7-kb band was detected when the DNA was hybridized with the *kan* resistance gene-specific probe (probe 3), confirming that the 2.7-kb band contained the *rpoH120*::*kan* insertion. To aid in the characterization of the *rpoH* null mutants, we transduced the Δ*rpoH30*::*kan* allele into a derivative of strain MC4100 by using the linked *zhf-50*::Tn*10* marker. The Southern blots shown in Fig. 2C confirm that the Δ*rpoH30*::*kan* deletion was present in the MC4100 derivative KY1612. *rpoH* DNA (probe 1) hybridized to the 1.5-kb *Pvu*II fragment in strain MC4100 (*rpoH*$^+$), but did not hybridize with DNA from KY1612, consistent with the presence of an extensive deletion in *rpoH*. When KY1612 DNA was hybridized with a probe containing DNA from either the region upstream of *rpoH* (probe 2) or the *kan* resistance gene (probe 3), a single ca. 2.9-kb band was observed, as was expected from the size of the *rpoH* deletion and the length of the *kan* insertion. Experiments with chromosomal DNAs cut with both *Bam*HI and *Pst*I confirmed the presence of Δ*rpoH30*::*kan* in KY1612 (data not shown).

Effects of temperature on growth of the *rpoH* null mutants. Mutants without a functional *rpoH* gene are viable only at or below 20°C and have growth defects even at low temperature. At 20°C the doubling times of KY1612 (Δ*rpoH30*::*kan*) are about 2.5 times longer than those of the parental strain MC4100 in minimal medium E, peptone broth, or L broth. Providing a wild-type *rpoH* gene in *trans* restores the ability of the *rpoH* insertion and deletion mutants to grow at high temperatures, indicating that the growth defect is caused by a lack of σ^{32}. The permissive growth temperature of the *rpoH* mutants is affected by antibiotics. CAG9301 (*rpoH120*::*kan zhg-21*::Tn*10*) grew on LB plates at temperatures up to 20°C. When the LB plates contained either kanamycin (30 μg/ml) or tetracycline (10 μg/ml) the maximum growth temperature of CAG9301 was about 15°C, and when both antibiotics were included no colonies were found at any

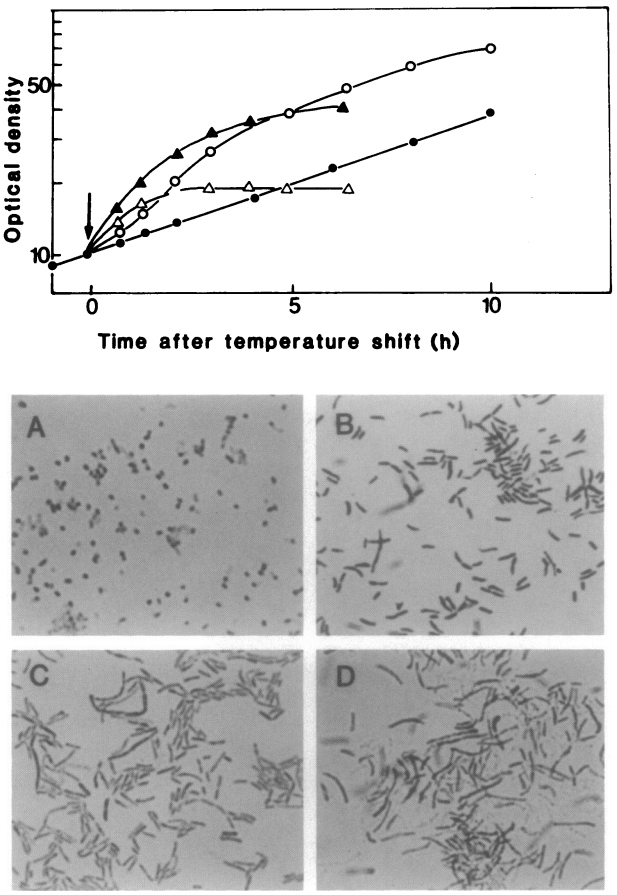

FIG. 3. Effect of temperature on cell growth. (Top) Growth kinetics in peptone broth. Log-phase cultures of KY1612 (Δ*rpoH30*::*kan*) grown in peptone broth at 20°C were transferred at time zero to higher temperatures as indicated. The optical density was monitored in a Klett-Summerson colorimeter with a no. 54 filter. Symbols: ●, 20°C; ○, 25°C; ▲, 30°C; △, 40°C. (Bottom) Cell morphology in L broth. Strains were grown in L broth at 20°C and transferred to higher temperatures as in the above experiment. After 3 h at each temperature, cells were fixed in 8% Formalin for 15 min, centrifuged, and washed and suspended in water. Cells were spread onto microslides and stained with an eosin-Giemsa solution. Similar results were obtained with peptone broth. (A) *rpoH*$^+$ (MC4100) at 20°C; (B) Δ*rpoH30*::*kan* (KY1612) at 20°C; (C) Δ*rpoH30*::*kan* at 30°C; (D) Δ*rpoH30*::*kan* at 40°C.

temperature. We do not understand why the presence of the antibiotics decreases the temperature at which the *rpoH120*::*kan* mutant can grow.

After a shift to high temperature the growth rate of KY1612 increased immediately, but after further incubation growth ceased and partial cell lysis, as judged by a decrease in optical density, ensued (Fig. 3A). A comparison of the viable-cell count with the increase in optical density suggested that cell division was inhibited after a shift to high temperature. After a shift from 16.5 to 30°C the optical density at 450 nm of the CAG9301 culture increased about 16-fold, but the viable-cell count did not even double (data not shown).

To determine whether cell division was inhibited in the *rpoH* mutants, KY1612 and CAG9301 were examined to see whether they formed filaments. At 20°C the *rpoH* mutant cells were slightly longer than the wild type, and after shifts to higher temperatures the average cell length of the mutants

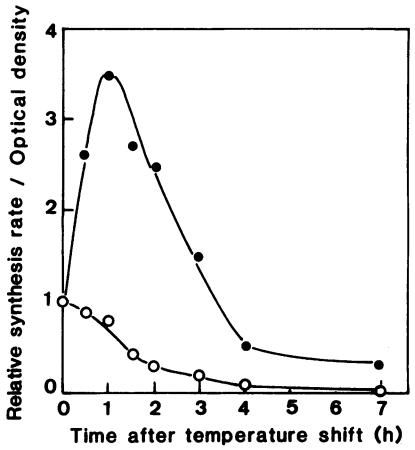

FIG. 4. Effect of temperature on DNA and protein synthesis. A log-phase culture of KY1612 (Δ*rpoH30*::*kan*) grown in minimal medium at 20°C was shifted to 42°C at time zero. Samples were taken and pulse-labeled at the times indicated with [^{3}H]thymidine (20 μCi; 1 μg/ml) and [^{35}S]methionine (2 μCi; 1 μg/ml) for 5 min. Radioactivity incorporated into the hot trichloroacetic acid-insoluble fraction was divided by optical density and plotted after normalization to the time zero value. The time zero values were 1,790 cpm for ^{3}H (○) and 360 cpm for ^{35}S (●).

increased progressively with time (Fig. 3B to D). By the time cell growth had ceased at 30°C, the average cell length of CAG9301 was four times that of the wild type. Similarly, after 3 h at 40°C the average cell length of KY1612 was about four times greater than that of the wild type. These results confirm the conclusions of earlier studies (40, 48) that *rpoH* is involved in cell division.

Effects of temperature on DNA and protein synthesis. We examined the effect of high temperature on DNA and protein synthesis in KY1612 (Δ*rpoH30*::*kan*) by pulse-labeling cells with a mixture of [^{3}H]thymidine and [^{35}S]methionine before and after a shift from 20 to 42°C. The rate of protein synthesis increased for about 1 h (ca threefold) and then decreased, whereas the rate of DNA synthesis steadily decreased at the high temperature (Fig. 4). Evidently DNA synthesis is inhibited much earlier than protein synthesis. In the wild type, incorporation of both [^{3}H]thymidine and [^{35}S]methionine was two to three times higher than in the Δ*rpoH30*::*kan* mutant at 20°C, reflecting the difference in growth rates, and incorporation of each was further increased after the temperature shift (data not shown). The inhibition of DNA synthesis in the mutant may explain the formation of the elongated cells at high temperature.

Expression of heat shock proteins in the absence of σ^{32}. The *rpoH* nonsense and missense mutants that have been studied previously (23, 38, 46, 48) synthesize heat shock proteins at nearly wild-type levels at 30°C, but do not increase heat shock protein synthesis in response to high temperature. We wanted to see whether any heat shock proteins were synthesized in the absence of σ^{32} and, if they were, whether this synthesis was heat inducible. KY1612 (Δ*rpoH30*::*kan*) and MC4100 were pulse-labeled with [^{35}S]methionine at 20°C and at various times after the shift to 42°C, and the proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. In contrast to the wild-type strain, which had a marked induction of heat shock protein synthesis, in the Δ*rpoH30*::*kan* mutant the synthesis of the heat shock proteins was not induced (Fig. 5). Several proteins, including one of 26 kilodaltons (Fig. 5), were overpro-

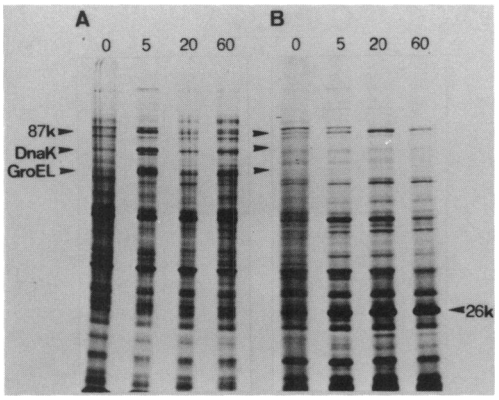

FIG. 5. SDS-polyacrylamide gel patterns of proteins synthesized. Log-phase cultures of the wild type and the isogenic Δ*rpoH30*::*kan* mutant in minimal medium were pulse-labeled with [^{35}S]methionine (20 μCi/ml) for 3 min at 20°C or after transfer to 42°C, and whole-cell proteins were analyzed by SDS-gel electrophoresis. Numbers at the top designate the times after temperature shift in minutes. (A) *rpoH*$^+$ (MC4100). (B) Δ*rpoH30*::*kan* (KY1612).

duced in the mutant after the shift to high temperature. This has not been investigated further.

Because of the background of non-heat-shock proteins in Fig. 5, we were unable to determine whether any heat shock proteins were synthesized at low levels in the Δ*rpoH30*::*kan* mutant at low or at high temperatures. We first attempted to resolve the heat shock proteins by two-dimensional gel electrophoresis (27), but we were unable to reliably identify the heat shock proteins in the wild-type strain at 16°C (data not shown). To increase our ability to detect small amounts of heat shock proteins, we used antibodies raised against two heat shock proteins, GroEL and DnaK, to immunoprecipitate proteins from the *rpoH120*::*kan* mutant. CAG9301 (*rpoH120*::*kan*) was pulse-labeled with [^{35}S]methionine at 16.5°C and after a shift to 30 or 42°C. The proteins were immunoprecipitated and visualized by autoradiography following separation on SDS-polyacrylamide gels. The radiolabeled proteins were excised from the gels and counted to quantify the experiments.

TABLE 3. Relative synthesis of heat shock proteins

Temp (°C)	Time (min)	Rate of synthesis of[a]: GroEL		DnaK	
		rpoH$^+$ cells	*rpoH120*::*kan* cells	*rpoH*$^+$ cells	*rpoH120*::*kan* cells
16.5	0	1.0	0.34	1.0	<0.02
30	10	1.0	0.31	0.5	<0.02
42	5	10.3	0.36	10.5	0.06
42	10	9.1	0.18	6.2	0.06

[a] Data are expressed as rates of synthesis in M61655 (*rpoH*$^+$) or CA69301 (*rpoH120*::*kan*) relative to either GroEL or DnaK in strain MG1655 at 16.5°C.

In the *rpoH120*::*kan* mutant GroEL was synthesized at about 30% of the wild-type rate at 16.5°C, but GroEL synthesis did not increase after the shift to high temperature (Fig. 6A; Table 3). DnaK protein could not be detected in the *rpoH120*::*kan* mutant at 16.5°C or after the shift to 30°C, but its synthesis increased following a shift to 42°C (Fig. 6B). Quantification of the data showed that the rate of DnaK synthesis in the *rpoH120*::*kan* mutant at 42°C was less than

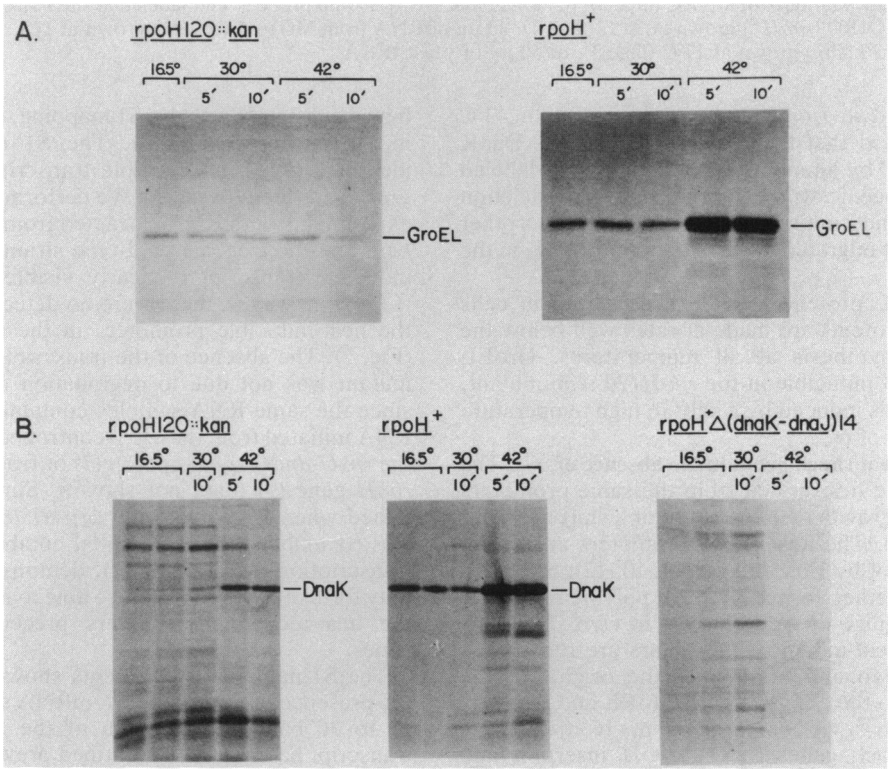

FIG. 6. Heat shock protein synthesis before and after a shift to high temperature. Stains CAG9301 (*rpoH120*::*kan*), MG1655 (*rpoH*$^+$), and EC514 (*rpoH*$^+$ Δ*dnaKJ*) were pulse-labeled with [^{35}S]methionine at 16.5°C and at the indicated times after a shift to 30 or 42°C. Samples were collected and immunoprecipitated as described in Materials and Methods by using either anti-GroEL (A) or anti-DnaK (B) antibody.

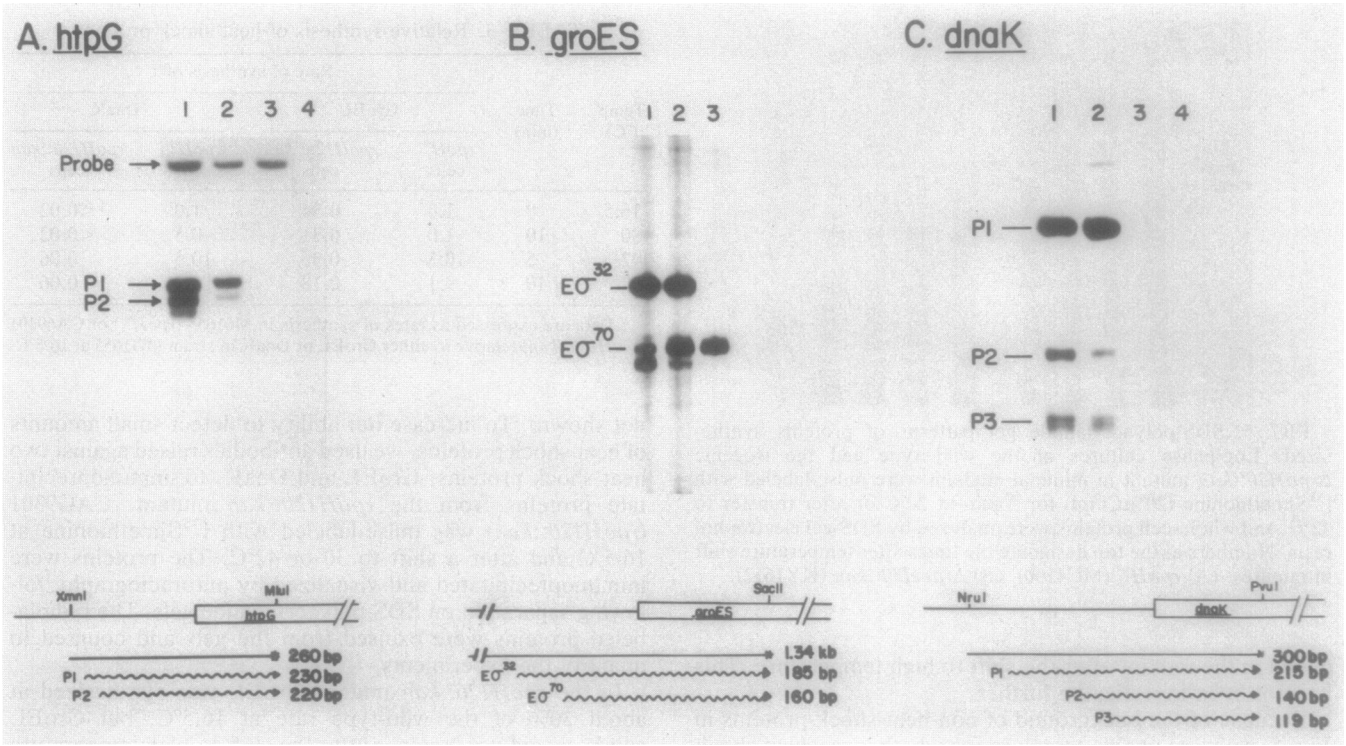

FIG. 7. S1 mapping of the 5′ ends of heat shock gene transcripts. The schematics illustrate the relevant genes and restriction sites. The straight lines below the restriction maps indicate the probes used for S1 mapping. The wavy lines indicate the transcripts. The RNAs are labeled as originating from *htpG* promoters P1 and P2; the *groE* $E\sigma^{32}$-controlled or the *groE* $E\sigma^{70}$-controlled promoter (see Fig. 8A); or the *dnaK* promoters P1, P2, and P3. (A) *htpG* mRNA. A 260-bp probe 5′ end labeled at the *Mlu*I site was hybridized with 40 μg of RNA from MC4100 (*rpoH*$^+$) grown at 30°C (lane 1), 40 μg of RNA from MG1655 (*rpoH*$^+$) grown at 17°C (lane 2), 80 μg of RNA from CAG9301 (*rpoH120*::*kan*) grown at 17°C (lane 3), or 50 μg of yeast tRNA. (B) *groES* mRNA. A 1.34-kb probe 5′ end labeled at the *Sac*II site was hybridized with 40 μg of RNA from MC4100 (*rpoH*$^+$) grown at 30°C (lane 1), 40 μg of RNA from MG1655 (*rpoH*$^+$) grown at 17°C (lane 2), or 80 μg of RNA from CAG9301 (*rpoH120*::*kan*) grown at 17°C (lane 3). (C) *dnaK* mRNA. A 300-bp probe 5′ end labeled at the *Pvu*I site was hybridized with 40 μg of RNA from MC4100 (*rpoH*$^+$) grown at 30°C (lane 1), 40 μg of RNA from MG1655 (*rpoH*$^+$) grown at 17°C (lane 2), 160 μg of RNA from CAG9301 (*rpoH120*::*kan*) grown at 17°C (lane 3), or 50 μg of yeast tRNA.

1% of that observed in *rpoH*$^+$ cells after a shift to 42°C (Table 3). We verified that the protein identified as DnaK was actually DnaK by immunoprecipitating pulse-labeled proteins from an isogenic strain (EC514) that has a deletion of the *dnaK* gene and confirming that there were no other cellular proteins that migrated to the position of DnaK in the gel (Fig. 6B).

DnaK and GroEL proteins can be synthesized in cells lacking σ^{32}. Both proteins are made at rates well below the wild-type rate of synthesis at all temperatures. GroEL synthesis is not heat inducible in the *rpoH120*::*kan* mutant, but DnaK synthesis is induced by a shift to high temperature even in the absence of σ^{32}.

Transcription of heat shock genes in the absence of σ^{32}. The heat shock genes are transcribed from the same promoters during steady-state growth at 30°C and after a shift to higher temperatures (8, 37). The heat shock promoters are recognized by $E\sigma^{32}$ but not by $E\sigma^{70}$ in vitro (8, 10, 12); however, it is not known whether forms of RNA polymerase other than $E\sigma^{32}$ can recognize these promoters in vivo. To determine whether the heat-inducible promoters are recognized only by $E\sigma^{32}$ in vivo and to identify the origins of the transcripts leading to the expression of GroEL and DnaK in the mutants lacking σ^{32}, we compared the in vivo transcripts of several heat shock genes in the *rpoH* insertion and deletion mutants with those in the wild type.

The locations of the heat-inducible promoters for *groE*, *dnaK*, and *htpG* (encoding the heat shock protein C62.5) have been established by S1 mapping, promoter cloning, and in vitro transcription (8). The S1 mapping experiments identified two heat-inducible transcripts for *htpG*, one for *groE*, and three for *dnaK*. We performed similar S1 mapping experiments with RNA extracted from CAG9301 (*rpoH120*::*kan*) and the isogenic wild-type strain MG1655. Each heat-inducible transcript is clearly visible in the *rpoH*$^+$ strain (Fig. 7); however, there were no detectable transcripts from the heat-inducible promoters in the *rpoH120*::*kan* mutant (Fig. 7). The absence of the transcripts in the *rpoH120*::*kan* mutant was not due to degradation of the RNA samples, since the same RNA samples contained normal amounts of RNA initiated from the $E\sigma^{70}$-controlled promoters preceding the *rpsU-dnaG-rpoD* operon (7) or from those preceding the *rpoH* gene (9) (data not shown). Similar results were obtained when RNA from the Δ*rpoH30*::*kan* mutant KY1612 was S1 mapped. These results, combined with the in vitro transcription data (8, 10, 12), demonstrate that $E\sigma^{32}$ is the only form of RNA polymerase able to initiate transcription at the heat-inducible promoters preceding the heat shock genes.

The S1 mapping experiments shown in Fig. 7B revealed the presence of a second *groE* mRNA species that is initiated 25 to 30 bases downstream of the $E\sigma^{32}$ transcript. This transcript had not been identified previously (8, 10) because it represents a small fraction of the total *groE* transcripts at 25 to 42°C. At 17°C the shorter transcript accounts for about 25% of the total *groE* mRNA in *rpoH*$^+$ cells and was the only

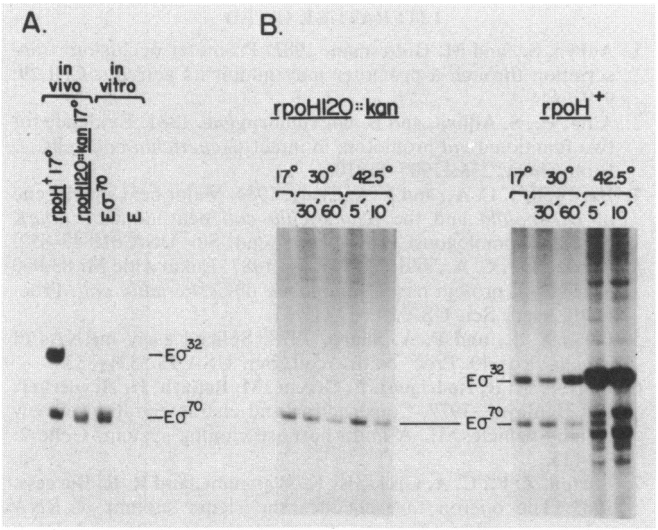

FIG. 8. S1 mapping of the 5′ ends of in vitro and in vivo *groE* transcripts. (A) In vitro and in vivo transcription of *groE*. The 5′-end-labeled probe (Fig. 7B) was hybridized with in vivo RNA from MG1655 (*rpoH*$^+$) or CAG9301 (*rpoH120*::*kan*) grown at 17°C, with RNA synthesized in vitro from plasmid pS2 by using RNA polymerase containing σ^{70} ($E\sigma^{70}$), or core RNA polymerase (E). (B) S1 mapping of *groE* mRNA following the temperature shift. The 5′-end-labeled probe was hybridized to 30 μg of RNA isolated from MG1655 (*rpoH*$^+$) or CAG9301 (*rpoH120*::*kan*) at 17°C or at the indicated times after the shift to 30 or 42.5°C.

groE transcript present in either the *rpoH* insertion or deletion mutants. An examination of the DNA sequence (8) of the *groE* promoter region revealed a sequence resembling the $E\sigma^{70}$ promoter consensus having the sequence TGGTCA in the −35 region, a 17-bp spacer, and a −10 region sequence of TAAGCT. In vitro transcription experiments were done to identify which form of RNA polymerase is responsible for the smaller *groE* transcript. The results (Fig. 8A) indicate that the promoter for the second *groE* transcript is recognized by $E\sigma^{70}$ and not by $E\sigma^{32}$.

The $E\sigma^{70}$-controlled *groE* transcript is present in sufficient quantity to account for the synthesis of GroEL protein in the *rpoH120*::*kan* mutant at 17°C (Fig. 7B and 8B). The amount of the $E\sigma^{70}$-controlled transcript remained constant after the shift to 30 or to 42°C in the *rpoH120*::*kan* mutant (Fig. 8B), consistent with the observation that the rate of GroEL protein synthesis does not vary with temperature (Fig. 6A). In the *rpoH*$^+$ strains the amount of the $E\sigma^{70}$ transcript decreased with increasing temperature concomitant with the increase in the amount of the $E\sigma^{32}$ transcript. This probably occurs because of promoter occlusion (1); i.e., the high-level transcription from the upstream $E\sigma^{32}$ promoter prevents transcription initiation from the downstream $E\sigma^{70}$ promoter.

We have not yet succeeded in mapping the 5′ ends of the *dnaK* transcripts at either 16 or 42°C in the *rpoH120*::*kan* or Δ*rpoH30*::*kan* mutant. We presume that this is due to their low abundance, since we expect any transcripts to be present at less than 1% of wild-type levels after a shift to 42°C. We are attempting to map the *dnaK* mRNA by using more-sensitive techniques and probes that extend further upstream of *dnaK*.

DISCUSSION

The transient overproduction of heat shock proteins that occurs after a shift to high temperatures is caused by a transient increase in transcription initiation from the promoters for the heat shock genes. $E\sigma^{32}$ is required for the increased transcription from the heat shock promoters and for viability at high temperature. Because no loss-of-function mutations affecting σ^{32} had been obtained previously, it was not known whether the basal rate of transcription from heat shock promoters at low temperature required $E\sigma^{32}$ or whether σ^{32} was essential for viability at low temperature. To determine the role of σ^{32} at low temperatures, we constructed insertion and deletion mutations in *rpoH*, the gene encoding σ^{32}, and analyzed their effects on the cell.

The *rpoH* insertion or deletion mutations confer the most temperature-sensitive growth phenotype yet described for *E. coli*. Mutants lacking σ^{32} are viable; however, they grow only at temperatures of ≤20°C. Even at 16°C the *rpoH* insertion or deletion mutants had growth defects. The mutants grew more slowly than the wild type at all temperatures, and some filamentous cells were observed even at 16.5°C. Cells lacking σ^{32} are also hypersensitive to the presence of antibiotics in the growth medium.

Some heat shock proteins are expressed in strains lacking σ^{32}, but they are present at greatly reduced levels. We examined the expression of two heat shock proteins, GroEL and DnaK, in detail and observed different results for each. In the *rpoH120*::*kan* mutant at 16.5°C GroEL was expressed at about 30% of the rate in wild-type cells, whereas DnaK could not be detected at this temperature. After a shift to 42°C DnaK could be detected, but was made at less than 1% of the wild-type rate. The cell division defect of the *rpoH* insertion and deletion mutants may result from the lack of DnaK, since *dnaK* mutants form filaments (30). The reduced growth rates, the hypersensitivity to antibiotics, and the selective inhibition of DNA synthesis in the *rpoH* insertion or deletion mutants may also result from decreased expression of heat shock proteins. However, σ^{32} may be more directly involved in the process of DNA replication, as seems to be the case with mini-F plasmid replication (43, 44).

Transcription of the heat shock genes is reduced or eliminated in the absence of σ^{32}. The heat-inducible transcripts of *htpG*, *groE*, and *dnaK* are absent in the *rpoH* insertion or deletion mutants. This indicates that $E\sigma^{32}$ transcribes from the heat shock promoters at all temperatures and that it is the only form of RNA polymerase to do so. This agrees with the results obtained in in vitro transcription experiments (8, 10, 12), which indicated that $E\sigma^{70}$ could not recognize the heat shock promoters. At least some of the heat shock genes have alternate non-heat-shock promoters. In addition to the heat shock promoter, *groE* is preceded by an $E\sigma^{70}$ promoter. In the absence of competing $E\sigma^{32}$, expression from the *groE* $E\sigma^{70}$ promoter is invariant with temperature. These results suggest that the heat-inducible regulation of *groE* is superimposed on an alternate mode of expression independent of $E\sigma^{32}$. This is also true of *rpoD*, which is transcribed both from a heat shock promoter and from two strong $E\sigma^{70}$ promoters (7, 37). It would appear that *dnaK* has an alternate non-$E\sigma^{32}$ promoter; however, we have not yet succeeded in mapping the 5′ end of the *dnaK* transcript in the *rpoH* deletion or insertion mutants. Since the *dnaK* mRNA species has not been identified in the mutants, we do not know whether the mechanism of increased DnaK protein synthesis at 42°C is due to a transcriptional or a translational mechanism.

Previous work on *rpoH* amber mutations in strains without a suppressor tRNA led to the conclusion that σ^{32} was essential for growth only at high temperatures (48). The *rpoH* deletion and insertion mutants described here are,

however, significantly more sensitive to high temperature than the isogenic *rpoH* amber mutants are. Two lines of evidence have been used to demonstrate that small amounts of functional σ^{32} are present in the *rpoH* amber mutants. First, the *rpoH* amber mutants contain significant quantities of transcripts initiated from the heat shock promoters preceding *dnaK* and *groE* (39; Zhou et al., unpublished data). In contrast, there is no transcription from these heat shock promoters in the *rpoH* deletion or insertion strains. Second, RNA polymerase prepared from the *rpoH6* amber mutant after temperature upshift catalyzes weak but significant transcription from the heat shock promoters in vitro (39). We do not know why there is so much σ^{32} in the *rpoH* amber mutants in the apparent absence of a suppressor tRNA; however, the results of P1 transduction experiments (Zhou, unpublished data) suggest the possible involvement of a suppressor closely linked to the *zhf-50*::Tn*10* that was originally used to cotransduce the *rpoH* nonsense mutations. The work of Straus et al. (36) also provides a possible explanation. In wild-type cells σ^{32} is unstable, being degraded with a half-life of 1 min during steady-state growth. If σ^{32} were stabilized in the *rpoH* amber mutants, σ^{32} could accumulate to significant levels because of the small amount of σ^{32} synthesized owing to natural suppression of the amber mutation. Other possible explanations would include increased synthesis of σ^{32} owing to increased levels of *rpoH* mRNA or increased translation initiation from the *rpoH* mRNAs. The final answer to this question, however, must await further investigation.

To further study the physiological role of σ^{32} and the heat shock proteins, we have recently isolated numerous temperature-resistant derivatives of the *rpoH* deletion mutants that vary in their maximum growth temperatures. All suppressors analyzed carry mutations located upstream of the *groE* operon that increase expression of *groE*. There is a strong correlation between the amounts of the GroE proteins in the suppressors and the maximum permissive growth temperature (N. Kusukawa and T. Yura, Gene Dev., in press). These results suggest that the amounts of the GroE proteins may determine the maximum growth temperature. At 16°C the level of *groE* mRNA (and GroE protein) in mutants lacking σ^{32} is 30% of that in the wild type, because of expression of the *groE* operon from the σ^{70}-controlled promoter. This amount of *groE* expression may be sufficient to allow the growth of *rpoH* null mutants below 20°C but not at higher temperatures. If this idea is correct, the *groE* genes should be essential for growth at all temperatures in both wild-type and *rpoH* deletion strains. Further studies of the *rpoH* deletion and insertion mutants and their suppressors should provide valuable information on the roles of σ^{32} and other factors related to the heat shock response and on the general problem of global control mechanisms in bacteria.

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