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Deletion and Insertion Mutations in the *rpoH* Gene of *Escherichia coli* That Produce Functional σ^{32}†

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***Escherichia coli* K-12 strain 285c contains a short deletion mutation in *rpoD*, the gene encoding the σ^{70} subunit of RNA polymerase. The σ^{70} protein encoded by this allele (*rpoD285*) is unstable, and this instability leads to temperature-sensitive growth. Pseudorevertants of 285c that can grow at high temperature contain mutations in the *rpoH* gene (encoding the heat shock σ factor σ^{32}), and their mutant σ^{70} proteins have increased stability. We characterized the alterations in three of these *rpoH* alleles. *rpoH111* was a point mutation resulting in a single amino acid substitution. *rpoH107* and *rpoH113*, which are known to be incompatible with *rpoD*$^+$, altered the restriction map of *rpoH*. *rpoH113* was deleted for 72 base pairs of the *rpoH* gene yet retained some σ^{32} activity. *rpoH107* had two IS*1* elements that flanked an unknown DNA segment of more than 6.4 kilobases inserted in the *rpoH* promoter region. The insertion decreased the amount of *rpoH* mRNA to less than 0.5% of the wild-type level at 30°C. However, the mRNA from several heat shock promoters was decreased only twofold, suggesting that the strain has a significant amount of σ^{32}.**

The *Escherichia coli* K-12 strain 285c is unable to grow at high temperatures. This strain contains a 42-base-pair (bp) internal deletion in *rpoD* (15, 16), the gene encoding σ^{70}, the major σ subunit of RNA polymerase. The temperature-sensitive phenotype of 285c results from the rapid proteolytic degradation of the mutant σ^{70} polypeptide at high temperatures (11, 13). Pseudorevertants of 285c that grow at 43.5°C are defective in the *rpoH* (*htpR*) gene, and these alleles (*rpoH107*, *-111*, *-112*, and *-113*) have been called Sde (for sigma defect expression) (13). The *rpoH* gene codes for σ^{32}, the σ factor responsible for transcription of genes that are transiently overexpressed after a temperature upshift (18, 21, 31, 32). The *rpoD285* gene product is stabilized by these Sde alleles, presumably because of altered expression of genes under the control of σ^{32} (1, 13). Two of the pseudoreversion mutations (*rpoH107* and *rpoH113*) are not compatible with the wild-type allele of *rpoD* (13; Yan-ning Zhou and Carol Gross, personal communication). In order to elucidate the nature of defects exhibited by Sde strains, we cloned and sequenced four Sde alleles of *rpoH*. The sequence change in *rpoH112* was presented previously (13). Here we report the sequences of the *rpoH107*, *rpoH111*, and *rpoH113* alleles. We found that those alleles that are incompatible with *rpoD*$^+$ have the greatest changes at the DNA level: *rpoH113* carries a deletion, and *rpoH107* carries an insertion.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used were P90A5c *thi lacZ4 argG75*, its *rpoD285* derivative 285c, and the *rpoH*-defective pseudorevertants of 285c PM107, PM111, PM112, and PM113, each carrying the Sde allele of *rpoH* whose allele number corresponds to the strain number (13). K165 *rpoH165*(Am) *supC*(Ts) comes from Cooper and Ruettinger (6). pAN4 contains the *rpoH*$^+$ allele of strain 285c cloned as a 1.5-kilobase (kb) *Hin*dIII-*Hpa*I fragment into pBR322 (5) cleaved with *Hin*dIII and *Pvu*II. pKH47 is a pBR322 derivative with IS*1* inserted at the *Eco*RI site, resulting in a duplication of the site so that the IS*1* element is flanked by *Eco*RI sites. pDBS9 contains a *Stu*I fragment of *E. coli* DNA that carries the *ftsX*, *ftsE*, and *rpoH* genes and was cloned into the *Eco*RV site of pBR322. The *E. coli* DNA fragment in pDBS9 was obtained from pLC31-16 (22).

S1 mapping. S1 mapping (3) was done as described previously (7, 8, 33) except that in vivo RNA was prepared by the method of Salser et al. (25). Experiments were quantified by densitometry.

Southern hybridizations. DNA was digested with restriction enzymes, electrophoresed in agarose, transferred to nitrocellulose membrane filters (Schleicher & Schuell), and hybridized with ^{32}P-labeled, nick-translated DNA (19). The three probes used in the experiments were pAN4, which carries the 1.5-kb *Hin*dIII-*Hpa*I fragment that contains the *rpoH* gene, the 1.8-kb *Pst*I-*Hin*dIII fragment from pDBS9 that contains the *ftsE* and *ftsX* genes, and the 0.6-kb *Pst*I-*Eco*RI fragment from pKH47 that contains most of IS*1*.

RESULTS AND DISCUSSION

Cloning of the mutant alleles. The *rpoH* gene is carried on a 1.5-kb *Hin*dIII-*Hpa*I fragment (Fig. 1A). We cleaved total *E. coli* DNA from each of the Sde mutants with *Hin*dIII and *Hpa*I, purified the 1.5-kb fragments, and ligated them to the purified 2.3-kb *Hin*dIII-*Pvu*II fragment of pBR322 (5). Plasmids containing *rpoH* were selected by complementation of the growth defect of *E. coli* K165, a strain unable to grow at 37°C because it contains a defective *rpoH* allele (6, 22). The desired clones were ampicillin-resistant K165 cells that were able to grow at 37°C. Using this procedure, we obtained clones of *rpoH111*, *-112*, and *-113*.

The restriction maps of *rpoH111* and *rpoH112* proved identical to that of the wild type. In contrast, the *rpoH113* allele was missing the *Pst*I site (Fig. 1A). This change could be recognized in a Southern transfer hybridization (27) by comparing DNAs from PM113 (*rpoH113*) and 285c (*rpoH*$^+$) after digestion with *Eco*RV and *Pst*I and probing with labeled pAN4, which contains a 1.5-kb *Hin*dIII-*Hpa*I *rpoH*$^+$

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† Dedicated to Arthur Kornberg on the occasion of his 70th birthday.

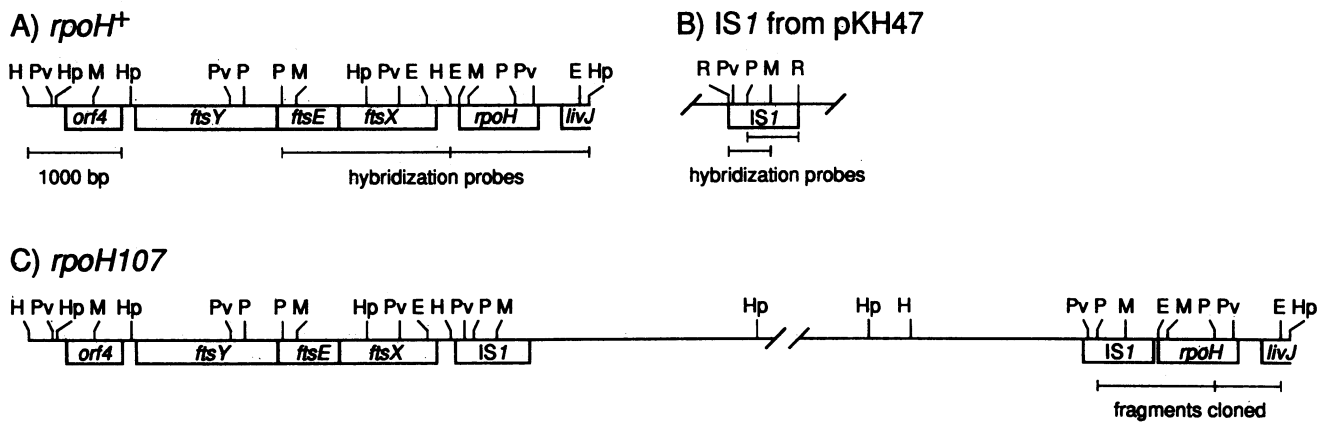

FIG. 1. (A) Restriction map of the normal *rpoH* gene and flanking DNA, as derived from published sequence and restriction maps (10, 17, 18, 32). The probes from this region that were used in Southern hybridization experiments are indicated below the map. (B) Restriction map of the IS*1* region of pKH47. The 0.6-kb *Pst*I-*Eco*RI and 0.4-kb *Eco*RI-*Mlu*I fragments used in Southern hybridization experiments are indicated below the map. (C) Restriction map of *rpoH107* allele, as derived from our DNA sequence analysis and from restriction analysis by Southern hybridizations. The restriction fragments that were cloned from PM107 for sequencing are indicated under the map. Restriction sites: E, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; M, *Mlu*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI.

DNA fragment (Fig. 1A). The two hybridizing fragments from the *rpoH*$^+$ strain (0.63 and 0.67 kb) became fused to create a slightly undersized single fragment of 1.2 kb in the *rpoH113* strain (Fig. 2, lanes A and B). This result suggested that *rpoH113* contains a deletion of about 100 bp in the region of the *Pst*I site. When the experiment was repeated with DNAs digested with *Hin*dIII and *Pvu*II and hybridized with labeled pAN4 (Fig. 1A), the *Hin*dIII-*Pvu*II fragment that included the first 940 bp of the *rpoH* gene became about 70 bp shorter as a result of the *rpoH113* mutation (Fig. 2, lanes C and D).

We were unable to clone *rpoH107* by using the strategy that was successful for the other three mutant alleles. We used Southern hybridization with the same pAN4 *rpoH*$^+$ probe to determine whether the 1.5-kb *Hin*dIII-*Hpa*I fragment was present in PM107. This analysis indicated that *rpoH107* was not carried on the normal 1.5-kb *Hin*dIII-*Hpa*I fragment but on a 4.0-kb fragment (Fig. 1C; Fig. 2, lanes E and F). Further experiments revealed that the *Hin*dIII site immediately upstream of *rpoH* was missing in PM107, and the upstream end of the 4.0-kb fragment resulted from a new *Hin*dIII site 2.5 kb upstream of the *rpoH* gene in PM107 (Fig. 1C).

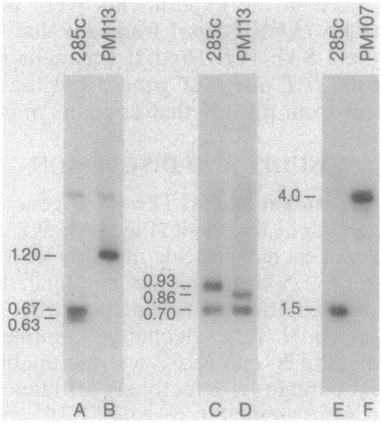

FIG. 2. Southern hybridizations with the *rpoH107* and *rpoH113* alleles. In lanes A (strain 285c, *rpoH*$^+$) and B (strain PM113, *rpoH113*), the DNA samples were treated with *Eco*RV and *Pst*I to give 0.67-kb and 0.63-kb fragments and a 1.2-kb fragment. In lanes C (*rpoH*$^+$) and D (*rpoH113*), DNA was treated with *Hin*dIII and *Pvu*II to give 0.93-kb and 0.86-kb fragments, in addition to their common 0.7-kb fragment. In lanes E (*rpoH*$^+$) and F (strain PM107, *rpoH107*), DNA was treated with *Hin*dIII and *Hpa*I to give 1.5-kb and 4.0-kb fragments. The last pair of lanes are from a separate experiment.

We were unable to clone this 4.0-kb fragment by complementation of the growth defect of K165. Instead, we cloned the *rpoH107* allele in two parts. Restriction mapping with Southern blots revealed a new *Pst*I site 0.5 kb upstream from the *rpoH* gene in PM107 (Fig. 1C). We used this site and the *Pst*I site in the *rpoH* gene to clone the upstream portion of the gene as a 1.2-kb *Pst*I fragment, and we cloned the downstream portion of the gene as a 0.7-kb *Pst*I-*Eco*RV fragment (Fig. 1C). The correct fragments were identified by colony hybridization (14) with labeled pAN4 *rpoH*$^+$ DNA as a probe.

Sequence analysis. The *rpoH* gene has been sequenced independently by two groups, but the two sequences disagree at eight positions (18, 32). Therefore, we determined the sequence of the *rpoH* gene from strain 285c in the regions where the discrepancies occurred in order to ascertain the correct sequence, and then we sequenced the mutant alleles (Fig. 3). The correct nucleotide sequence and the deduced amino acid sequence of *rpoH*$^+$ and the positions of the mutations are presented in Fig. 4.

The *rpoH112* and *-111* mutations were single-base-pair changes. The *rpoH112* mutation had been mapped to the downstream end of the gene by marker rescue with a set of deletion plasmids (13), and the DNA sequence of this region indicated that *rpoH112* had a leucine-to-tryptophan change at amino acid 278 (13) (Fig. 4). The *rpoH111* mutation had not been mapped previously. We therefore sequenced the entire gene (Fig. 3) and found a single-base-pair substitution, causing an amino acid change from aspartic acid to glycine at amino acid 179 (Fig. 4).

The *rpoH113* mutation had been mapped between the *Pst*I and *Pvu*II sites in the downstream portion of the gene (13), and the *rpoH113* allele was missing the *Pst*I site. We determined the sequence of the *rpoH113* allele around the

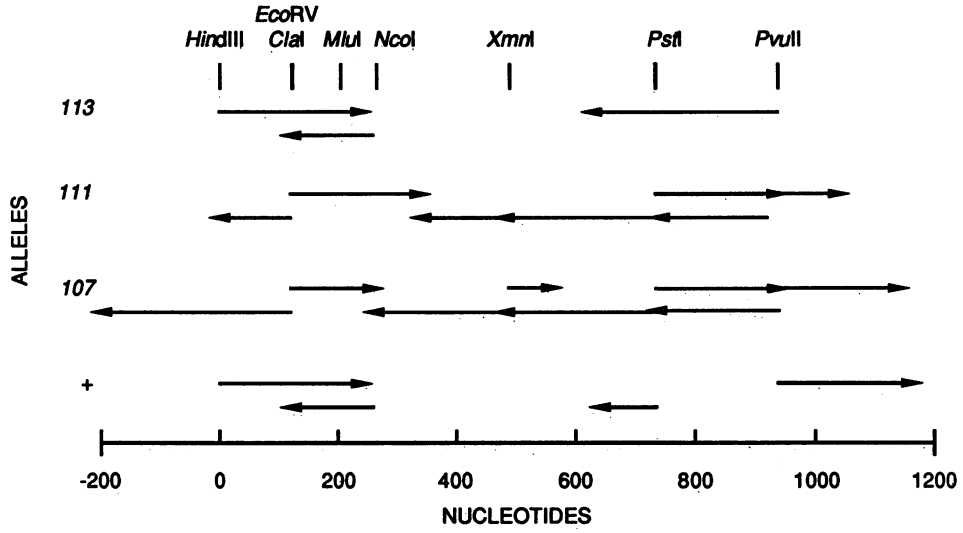

FIG. 3. Sequencing strategy. The Maxam-Gilbert sequencing method (20) was used for *rpoH113* and for nucleotides 1 to 200 of the wild-type allele. Arrows indicate the sequence obtained in the 5'-to-3' direction on each strand of DNA. The Sanger et al. method (26) with ^{35}S label (4) was used for *rpoH107* and the remainder of the wild-type allele. The *rpoH111* allele was sequenced by using modified T7 RNA polymerase (29).

missing *Pst*I site (Fig. 3) and found that it lacked 72 nucleotides coding for amino acids 178 to 201 (Fig. 4). The deletion event that created the *rpoH113* allele was probably a single crossover between the two CAGGA sequences beginning at nucleotides 663 and 735 (Fig. 4). This hypothesis is suggested by the results of Farabaugh et al., who observed that repeated sequences of five or more nucleotides occurred at the ends of the majority of spontaneous deletions in the *lacI* gene (9).

The *rpoH107* mutation had been mapped to the upstream end of the gene (13). Furthermore, as described above, restriction mapping revealed the presence of new DNA upstream of *rpoH*, as evidenced by the absence of the *Hin*dIII site adjacent to the upstream end of *rpoH*$^+$ and by the presence of new *Hin*dIII and *Pst*I sites (Fig. 1). We sequenced the entire *rpoH* coding region in the *rpoH107* plasmid as well as 400 nucleotides of DNA upstream from the start of translation (Fig. 3). We found that the sequence of *rpoH107* was identical to that of the wild-type gene throughout the coding sequence and for 39 bp upstream. Beyond this, however, the sequence corresponded to the right end of the insertion sequence IS*1* (24), as shown in Fig. 1C and 4.

Further analysis of Southern blots revealed that the restriction map upstream of *rpoH* was completely altered in PM107, and the pattern could not be explained by the simple insertion of a single IS*1* between *rpoH* and the adjacent *ftsX* gene (Fig. 1). To determine whether PM107 contained additional IS*1* elements not present in 285c, we probed *Pst*I-digested DNA from 285c and PM107 with a labeled 0.6-kb *Pst*I-*Eco*RI fragment from pKH47 that contains a portion of IS*1* (Fig. 1B). We identified two IS*1*-containing *Pst*I fragments in PM107 that did not appear in 285c (data not shown), and one of these fragments was 1.2 kb long, as predicted by the locations of the *Pst*I sites in IS*1* and *rpoH* in PM107 (Fig. 1C). This is the fragment that we cloned.

Because the cell division genes *ftsE* and *ftsX* are thought to be essential (10), we checked to see whether they were still present in PM107. Southern hybridizations with a labeled 1.8-kb *Pst*I-*Hin*dIII *ftsE*- and *ftsX*-containing fragment from pDBS9 as a probe (Fig. 1A) confirmed that both genes were present (data not shown). To determine whether a second IS*1* element was inserted adjacent to the *ftsX* gene in PM107, we carried out another Southern hybridization experiment. We blotted chromosomal DNA digested with *Mlu*I and probed it with the 1.8-kb *Pst*I-*Hin*dIII fragment containing *ftsE* and *ftsX* (Fig. 1A) and separately with the 0.4-kb *Eco*RI-*Mlu*I IS*1*-containing fragment from pKH47 (Fig. 1B). A 2.2-kb fragment hybridized to both probes (data not shown), indicating that an IS*1* element was adjacent to *ftsX* in the orientation shown in Fig. 1C. In summary, a DNA segment of unknown length flanked by two IS*1* elements was inserted between the *ftsX* and *rpoH* genes in PM107. Restriction mapping by Southern hybridization (data not shown) indicated that the insertion must be greater than 8 kb in length.

Phenotypes of the *rpoH* deletion and insertion mutants. Strains entirely lacking σ^{32} because of null mutations in *rpoH* are unable to grow at temperatures above 20°C whether they carry the *rpoD*$^+$ or *rpoD285* allele (33; Y.-N. Zhou and C. A. Gross, personal communication). Since the *rpoH113 rpoD285* double mutant grows at 30°C, the internally deleted *rpoH* gene must encode a partially functional σ^{32} protein. Further evidence for this comes from the fact that the *rpoH113* allele was cloned because of its ability to complement the *rpoH165*(Am) mutation. Thus, amino acids 178 to 201 of σ^{32}, which are deleted in *rpoH113* (Fig. 4), are not essential for minimal activity. The region deleted contained no basic amino acids, four serines, and six aspartic acids. One of the aspartic acids, at position 179, is important for σ^{32} function, because its conversion to glycine in *rpoH111* allowed the normally temperature-sensitive *rpoD285* mutant to grow at 43.5°C. However, since the *rpoH111* allele is compatible with *rpoD*$^+$, its defect is not as great as that of the 72-bp deletion of *rpoH113*. The 24 amino acids that were deleted in *rpoH113* lay in a region of the protein that is not particularly well conserved among σ factors (15a, 18, 32); however, most of the sigma factors have a short stretch of acidic residues in the same relative position. The *rpoH113* mutation, in single copy, was not compatible with a wild-type *rpoD* gene, but multiple copies of the *rpoH113* allele allowed an *rpoD*$^+$ *rpoH165*(Am) strain

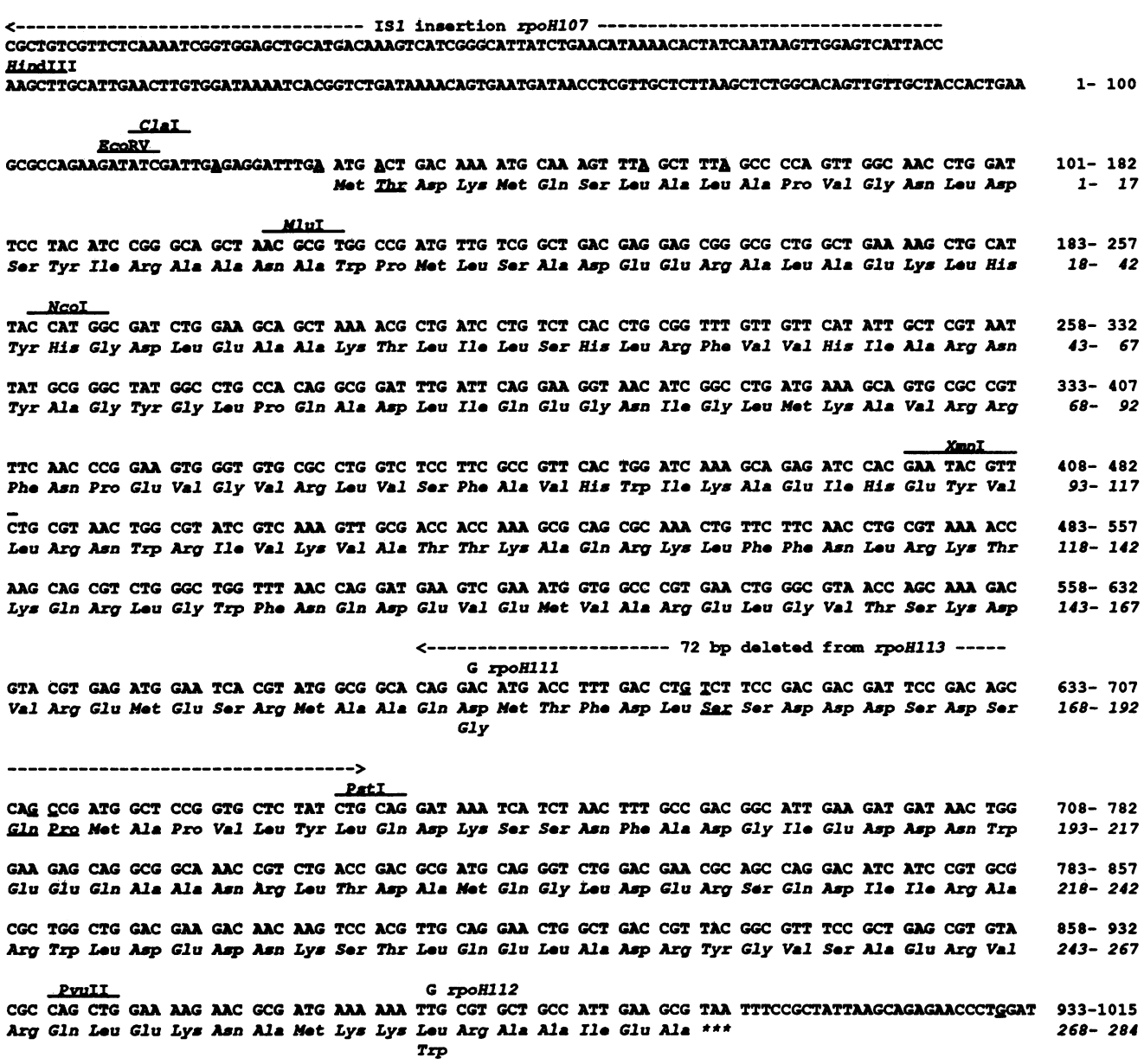

FIG. 4. DNA sequence of *rpoH* and the locations of the *rpoH107*, *-111*, *-112*, and *-113* mutations. The underlined nucleotides and amino acids are those about which two previous reports disagree (18, 32). The restriction sites used for sequencing are indicated. Nucleotide and corresponding amino acid numbers are shown to the right. Specific nucleotide changes and corresponding amino acid changes are shown above and below the sequences, respectively. Dashed arrows indicate the bases replaced by the IS*1* insertion in the *rpoH107* mutation and the bases deleted in the *rpoH113* mutation.

to grow at 37°C. This defect might be due to poor binding of the mutant σ^{32} to core RNA polymerase. Such poor binding could result from a loss of some of the amino acids directly involved in core binding or from an alteration in the tertiary structure of σ^{32}. An alternative possibility is that *rpoH113* mutant σ^{32} is altered in promoter recognition and that this leads to an imbalance of gene expression that can be suppressed by the *rpoD285* mutation. It is reasonable to believe that *rpoH113* mutant σ^{32} may have altered promoter recognition, since the deleted 24 amino acids lay between the domains of σ^{32} that are thought to recognize the −35 and −10 regions of heat shock promoters (C. Gross, R. Losick, and M. Susskind, personal communication).

The *rpoH107* mutation did not disrupt the *rpoH* structural gene; instead, it contained two IS*1* elements, which flanked a DNA segment of unknown length, inserted at a position which would separate the *rpoH* gene from its promoters (8). Since the *rpoH107 rpoD285* double mutant grew at temperatures up to 43.5°C, we were interested in determining whether there was any transcription of *rpoH* in this strain. S1 mapping experiments identified a low-abundance *rpoH* transcript that probably originated from within the proximal IS*1* element (Fig. 5). From the experiment shown in Fig. 5 and S1 mapping data for the 3′ end of the *rpoH* transcript (data not shown), we estimate that at 30°C the *rpoH107* mutation reduces the amount of *rpoH* mRNA to less than 0.5% of that seen in the *rpoD285* mutant.

Strains that lack σ^{32} because of a deletion of *rpoH* only

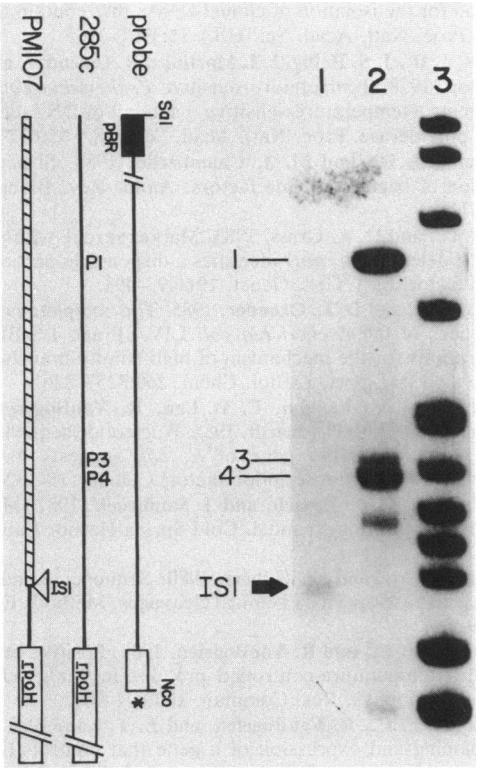

FIG. 5. S1 mapping of the *rpoH* transcripts in the *rpoH107 rpoD285* mutant. The schematic illustrates the strategy used to map both the wild-type and mutant *rpoH* transcripts. The probe was 5'-end labeled (*) at the *Nco*I site and contained 624 bp of *E. coli* DNA upstream of *rpoH* (open box) as well as 276 bp derived from pBR322 (solid box). Strain 285c contains a wild-type *rpoH* gene and the three *rpoH* promoters P1, P3, and P4. PM107 contains two IS*1* elements that flank DNA of unknown length (hatched bar) inserted 40 bp upstream of *rpoH*. Probe was hybridized to 100 μg of RNA from PM107 grown at 30°C (lane 1) or to 10 μg of RNA from 285c grown at 30°C (lane 2). A 5'-end labeled *Hpa*II digest of pBR322 served as size standards (lane 3). Protected fragments arising from the three *rpoH* promoters are marked 1, 3, and 4, and the arrow indicates a fragment that corresponds to an RNA whose 5' end is at or upstream of the IS*1* insertion point. The band below 4 may be a degradation product or a transcript derived from a recently identified promoter, P5 (H. Nagai, R. Yano, T. Yura, J. Erickson, and C. Gross, unpublished data).

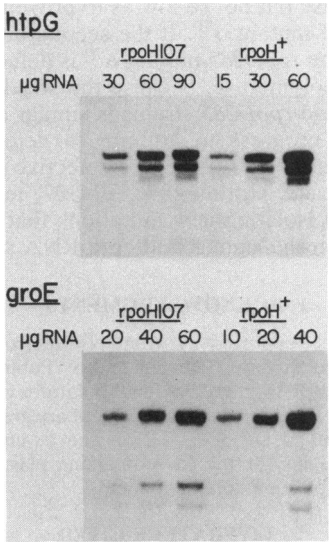

FIG. 6. S1 mapping of heat shock gene transcripts. The indicated amounts of RNA from strains PM107 (*rpoH107*) and 285c (*rpoH*$^+$) grown at 30°C were hybridized to 5'-end-labeled *htpG* (top) or *groE* (bottom) probes (7, 33) and mapped with S1.

grow at temperatures ≤20°C (33); therefore, the ability of the *rpoH107 rpoD285* double mutant to grow at high temperatures was surprising, given that there was so little *rpoH* transcription. This ability to grow at high temperatures could be the result of a suppressor mutation that bypasses the need for σ^{32} or by a mechanism that results in increased expression (such as the *suhA* mutation [30]) or increased activity of σ^{32}. Since there is no transcription from heat shock promoters in strains that lack σ^{32} (33), we examined the transcription of the heat shock genes *htpG* (2, 7) and *groES* (7) by S1 mapping to distinguish these possibilities. In the *rpoH107 rpoD285* mutant, the levels of the *groES* and *htpG* heat shock promoter transcripts were depressed only twofold relative to the *rpoD285* strain (Fig. 6). These results suggest that the *rpoH107 rpoD285* mutant has a greater amount of σ^{32} than would be predicted from the abundance of *rpoH* mRNA and therefore suggest that PM107 has some means of elevating the level or the activity of σ^{32}. Some clues as to how this may occur were provided by the work of Straus et al. (28), who showed that σ^{32} is unstable, with a half-life of 1 min, and that the synthesis of σ^{32} is translationally regulated. The ability of PM107 to transcribe from the heat shock promoters at nearly wild-type levels could be explained if the rate of translation of *rpoH* mRNA was maximized, if σ^{32} was stabilized, or if the activity of σ^{32} was increased after translation. Given these possibilities, we envision three mechanisms which could increase the amount or activity of σ^{32}. First, the *rpoH* mRNA may be translated more efficiently due to its altered structure in PM107. Second, there may be some kind of "physiological adaptation" to increase σ^{32} synthesis or stability to counter the lowered transcription of *rpoH*. Third, there may be a "suppressor" mutation that is distinct from *rpoH107*. In addition, there may be some combination of these three mechanisms. The fact that we were unable to clone the *rpoH107* allele by complementation (using the same strategy that was successful for cloning the *rpoH*$^+$, -111, -112, and -113 alleles) is consistent with the existence of an unlinked suppressor.

We cannot rule out the possibility that an as yet unidentified σ factor with a promoter specificity similar to that of σ^{32} could be activated in *E. coli rpoD285 rpoH107*. However, Noriko Kusukawa and Takashi Yura (personal communication) have selected a number of mutants that grow at high temperatures in the absence of σ^{32}. These strains grow at high temperatures because they have increased amounts of *groE* mRNA and protein, but they do not transcribe from the heat shock promoters. This indicates that they bypass the need for σ^{32} without inducing a σ factor with an overlapping promoter specificity.

It is not clear why the *rpoH107* mutation cannot coexist with a wild-type *rpoD* gene. Presumably this lethal interaction reflects some imbalance in gene expression. As is the case with the *rpoH113* allele, two general explanations for the suppression by *rpoD285* exist. First, *rpoD285* mutant σ^{70} may be altered so that it no longer properly recognizes certain promoters, and this results in a more appropriate ratio between some σ^{70}- and σ^{32}-controlled proteins. The second possibility is that the level of σ^{32} in PM107 is too low to compete effectively with wild-type σ^{70} for binding to core

RNA polymerase but not so low as to prevent σ^{32} binding in the presence of mutant σ^{70}. If the second idea is correct, it suggests that the *rpoD285* mutant σ^{70} is defective in binding to core RNA polymerase, because the steady-state level of σ^{70} in *rpoD*$^+$ and *rpoD285* strains is similar at 30°C. Experiments are in progress to attempt to determine whether *rpoD285* σ^{70} (and *rpoH113* σ^{32}) are defective in core binding. If this is the case, strains 285c, PM107, and PM113 may prove useful in isolating new mutations that alter the interactions between the sigmas and core RNA polymerase.

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