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Suppression of the *Escherichia coli rpoH* Opal Mutation by Ribosomes Lacking S15 Protein

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Several suppressors (*suhD*) that can specifically suppress the temperature-sensitive opal *rpoH11* mutation of *Escherichia coli* K-12 have been isolated and characterized. Unlike the parental *rpoH11* mutant deficient in the heat shock response, the temperature-resistant pseudorevertants carrying *suhD* were capable of synthesizing σ^{32} and exhibiting partial induction of heat shock proteins. These strains were also cold sensitive and unable to grow at 25°C. Genetic mapping and complementation studies permitted us to localize *suhD* near *rpsO* (69 min), the structural gene for ribosomal protein S15. Ribosomes and polyribosomes prepared from *suhD* cells contained a reduced level (ca. 10%) of S15 relative to that of the wild type. Cloning and sequencing of *suhD* revealed that an IS*10*-like element had been inserted at the attenuator-terminator region immediately downstream of the *rpsO* coding region. The *rpsO* mRNA level in the *suhD* strain was also reduced to about 10% that of wild type. Apparently, ribosomes lacking S15 can actively participate in protein synthesis and suppress the *rpoH11* opal (UGA) mutation at high temperature but cannot sustain cell growth at low temperature.

Escherichia coli cells exhibit transient induction of a set of heat shock proteins in response to upshift of temperature (12, 33). A number of heat shock proteins and their genes have been identified and characterized, and the mechanisms regulating the heat shock response have been studied extensively (20). The heat shock induction occurs at the level of transcription (34), and the regulatory gene *rpoH* (*htpR*) and its product σ^{32} play a central role in the induction of heat shock genes (5, 19, 25, 35).

To further study the function and regulation of the heat shock response, we have isolated and characterized temperature-resistant pseudorevertants from various *rpoH* mutants. Extragenic suppressors that are involved in these reversions have been classified into several groups according to their map locations. We have previously reported on two of these suppressors obtained from *rpoH* amber mutants (30) or from the *rpoH* deletion mutant (11).

This paper deals with another class of suppressors, designated *suhD*, that can suppress the temperature-sensitive growth of an opal *rpoH* mutation and simultaneously render the cells cold sensitive. It will be shown that *suhD* drastically reduces the synthesis of a ribosomal protein S15 due to the insertion of an insertion element at the transcription attenuator-terminator region of the S15 gene (*rpsO*). Under these conditions, marked synthesis of the *rpoH* gene product σ^{32} was observed at the permissive temperature (40°C).

Mutations affecting ribosomal proteins, rRNA, or a polypeptide chain elongation factor as well as tRNAs have previously been shown to cause suppression or readthrough translation of opal codons (for an example, see reference 16). The present finding with *suhD* strains that produce ribosomes lacking S15 may provide a new clue to the mechanism of opal suppression, translational readthrough, and fidelity.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains used were derivatives of *E. coli* K-12. Wild-type strain MC4100 (1) and isogenic temperature-sensitive heat shock-defective *rpoH* mutants carrying a well-defined opal (*rpoH11*), amber (*rpoH6*, *rpoH16*, *rpoH18*, *rpoH165*), or missense (*rpoH15*) mutation have been described (29, 36, 37). The *suhD* revertants, KY1446 (*rpoH11 suhD2*) and KY1447 (*rpoH11 suhD3*), were isolated as temperature-resistant pseudorevertants of the *rpoH11* mutant (KY1430) in this study. F' plasmids used were originally supplied by B. Bachmann. pBR322-based plasmids carrying the *rpsO* region (pYN81, pYN81Δ2, and pYN115) have been described by Y. Nakamura (17). pKV41 and pKV42 were constructed by subcloning the *Hpa*I-*Hpa*I fragment (843 base pairs [bp]) or *Dra*I-*Eco*RI fragment (3.8 kilobases [kb]) from pYN81 (see Fig. 3 and 5A) into appropriate sites on pBR322. Opal (UGA) mutants of bacteriophage φ80 (Z. Chang, thesis, Kyoto University, Kyoto, Japan, 1988) and the *lacZ* (UGA) mutant (LS653) were obtained from H. Ozeki.

Media and chemicals. Medium E (32) with 0.5% glucose and thiamine (2 μg/ml) was used as minimal medium. L broth has been described (29). L-[^{35}S]methionine (1,000 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were purchased from Radiochemical Centre, Amersham, England.

P1 transduction. Transduction was carried out by using P1 *vir* essentially as described (24).

Radioactive labeling and gel electrophoresis of proteins. Pulse labeling and gel electrophoresis of proteins were done as described previously (30). Cells growing exponentially in minimal medium with amino acids (except methionine and cysteine) were labeled with [^{35}S]methionine (2 μCi) for 1 min at 37°C (or 30°C) or after shift to 42°C. Labeled cells were treated and washed with 5% trichloroacetic acid and rinsed with acetone. Whole cell proteins were dissolved in sodium dodecyl sulfate (SDS) sample buffer and loaded onto an SDS-polyacrylamide gel (10%) for electrophoresis.

Immunoblotting of σ^{32} protein. Cells were lysed by boiling in 2% SDS, and proteins (50 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. σ^{32} protein was detected by using antiserum against σ^{32} (gift of D. Straus and C. Gross) and anti-rabbit immunoglobulin biotinylated antibody (Amersham International plc, Amersham, United Kingdom) (23). Staining was done with streptavidin-colloidal gold conjugate

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(Bethesda Research Laboratories, Gaithersburg, Md.) and a silver enhancement kit (E-Y Laboratories).

Determination of mRNA by S1 nuclease protection. S1 nuclease protection experiments were carried out essentially as described (15). RNA was extracted from cells grown in L broth at appropriate temperature, hybridized with probe DNA, digested with S1 nuclease, and analyzed by urea-polyacrylamide gel electrophoresis.

Preparation of ribosome and polyribosome fractions. Log-phase cells grown in L broth were collected and disrupted by sonication, and total ribosome fractions were prepared by the published procedure (8). Polyribosomes were prepared essentially as described (2). Cells were collected from 100 ml of L broth culture after mixing with ice and NaN_3 in the cold, and disrupted by repeated (4×) freezing and thawing in a dry ice-ethanol bath. Crude ribosomes obtained were then subjected to sucrose gradient (15 to 30%) centrifugation, and polyribosome fractions were pooled and collected by centrifugation.

Nucleotide sequence determination. The DNA fragment containing the *rpsO* region was inserted into an M13mp10 vector, and nucleotide sequence was determined by the dideoxy-chain termination method using 7-deaza-2-dGTP (14).

RESULTS

Isolation of *suhD* suppressor mutations. The *rpoH11* mutant (KY1430) is defective in the heat shock response and cannot grow at or above 37°C (29) due to the opal mutation at the residue 25 (tryptophan) of the σ^{32} protein (36, 37). Spontaneous temperature-resistant revertants of this mutant were obtained at a frequency of about 10^{-6} when selected at 42°C, and some (1 to 2%) exhibited very slow growth at low temperature (25 to 30°C). Such cold-sensitive revertants could be divided into several groups on the basis of complementation studies by using a set of F′ strains. A group of mutants whose cold-sensitive phenotype could be complemented by F′ plasmids (F120, F140, or F141) was designated *suhD* and examined in this study.

A series of P1 transduction experiments employing the revertant (*rpoH11 suhD*), the parental *rpoH11* mutant, and the wild type was carried out to see whether a *suhD* mutation is responsible for both the suppression of temperature-sensitive growth of *rpoH11* mutant and the cold-sensitive growth of revertants. That this is indeed the case was verified by the finding that the two phenotypes were always associated with each other among the transductants obtained. Table 1 summarizes growth characteristics of the representative transductants obtained in these experiments.

Partial recovery of heat shock induction and σ^{32} synthesis. To determine if *suhD* can suppress the defective heat shock response of the *rpoH11* mutant, proteins pulse-labeled with [^{35}S]methionine at various times after temperature shift from 37 to 42°C were examined by SDS-polyacrylamide gel electrophoresis. Little or no induction of heat shock proteins occurred in the *rpoH11* mutant (29), whereas significant induction was observed in the revertants though it was delayed and less marked (for DnaK) than in the wild type (Fig. 1). Thus, *suhD* suppresses not only the defective cell growth at high temperature but also the defective heat shock response of the *rpoH11* mutant.

The above results suggested that some σ^{32} protein is produced in the revertants at high temperature. Immunoblotting of cell extracts by specific antiserum revealed that appreciable amounts of σ^{32} are synthesized at the permissive temperature in the *suhD* revertants but not in the parental *rpoH11* mutant (Fig. 2). In separate experiments, the *rpoH* mRNA level in *suhD* cells as determined by S1 nuclease protection was shown to be comparable to that in the wild type (data not shown). These results suggested that *suhD* acted at the level of translation as an informational suppressor, permitting the synthesis of partially active σ^{32} protein from the *rpoH11* (opal) mutant allele.

TABLE 1. Growth of cells on L agar plates[a]

Strain and genotype[b]	Growth at indicated temp (°C)[c]				
	30	34	37	40	42
MC4100 (wild type)	+	+	+	+	+
KY1430 (*rpoH11*)	+	+	−	−	−
KY1446 (*rpoH11 suhD2*)	−	−	+	+	+
KY1447 (*rpoH11 suhD3*)	−	−	+	+	+
Cs^+ td (*rpoH11 suhD2* × wild type)	+	+	−	−	−
(*rpoH11 suhD3* × wild type)	+	+	−	−	−
Ts^+ td (*rpoH11* × *rpoH11 suhD2*)	−	−	+	+	+
(*rpoH11* × *rpoH11 suhD3*)	−	−	+	+	+

[a] Cell suspensions were streaked on L agar plates and incubated for 18 h at the temperatures indicated.

[b] The upper four strains are described in Materials and Methods. Cold-resistant transductants (Cs^+ td) were obtained from crosses between a *suhD* strain (recipient) and wild type (donor) selecting for transductants at 25°C, whereas temperature-resistant transductants (Ts^+ td) were obtained from crosses between the *rpoH11* mutant (recipient) and a *suhD* strain (donor) selecting for transductants at 40°C on L agar. All the transductants tested, eight from each cross, exhibited similar growth characteristics as reported here.

[c] Growth is indicated as present (+) or absent (−).

***suhD* maps at the *rpsO* region.** The observation that the cold-sensitive phenotype of *suhD* revertants can be complemented by F120, F140, or F141 suggested that *suhD* may be located between 66 and 75 min on the *E. coli* chromosome. P1 transduction experiments permitted us to localize *suhD* between *argG* (69 min) and *zgi-132*::Tn*10* (68.5 min), the

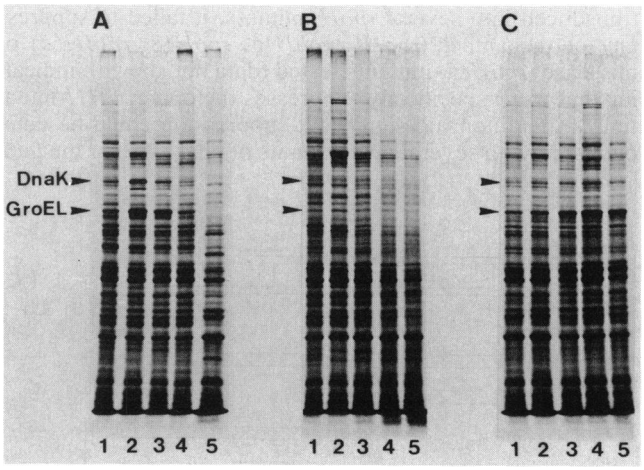

FIG. 1. Gel electrophoresis of proteins synthesized after temperature upshift. Cells of (A) wild type (MC4100), (B) the *rpoH11* mutant (KY1430), and (C) a *suhD* revertant (KY1447) were pulse-labeled with [^{35}S]methionine at 37°C (30°C for KY1430) or after shift to 42°C, and proteins (5.8×10^5 cpm) were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Lanes: 1, cells pulse-labeled at 37°C (30°C for KY1430); 2, 5 min at 42°C; 3, 20 min at 42°C; 4, 40 min at 42°C; 5, 60 min at 42°C.

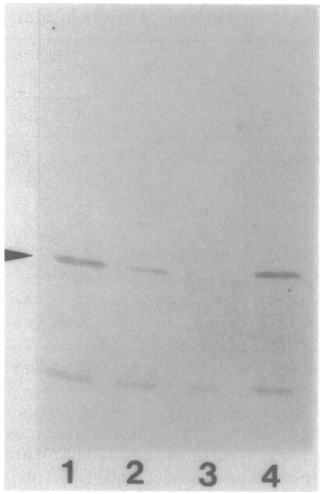

FIG. 2. Immunoblotting of σ^{32} protein. Proteins from log-phase cells grown at 30 or 40°C were separated by gel electrophoresis and blotted onto membrane, and σ^{32} protein was detected by specific antiserum as described in Materials and Methods. Arrowhead indicates σ^{32} protein. Lanes: 1, MC4100 at 30°C; 2, MC4100 at 40°C; 3, KY1430 (*rpoH11*) at 30°C; 4, KY1447 (*rpoH11 suhD3*) at 40°C.

cotransduction frequencies with *suhD* being 64 and 50%, respectively (data not shown). Further experiments with plasmids that carry part of this chromosomal region revealed that the cold sensitivity of the revertants can be complemented by pYN115 carrying the entire *rpsO-pnp* operon or by pKV41 carrying only *rpsO* but not by plasmids lacking the intact *rpsO* (Fig. 3). These results indicated that *suhD* is located at or around *rpsO*, the gene for ribosomal protein S15. It should be noted that the *suhD* strains harboring pYN115 or pKV41 are unable to grow at 40°C (Fig. 3). All these results indicate that *suhD* is recessive to *suhD*$^+$, namely *suhD* somehow inactivates the *rpsO* function, and that the inactivation of *rpsO* function in turn suppresses the *rpoH11* (opal) mutation.

suhD may represent an opal suppressor. When *suhD* was transduced into several *rpoH* mutants, it failed to suppress any of the amber (*rpoH6*, *rpoH16*, *rpoH18*, *rpoH165*) or missense (*rpoH15*) mutants tested (data not shown), indicating that *suhD* specifically suppresses the opal *rpoH11* mutation. In addition, no significant suppression could be demonstrated with several opal mutants of phase φ80 or the *lacZ*

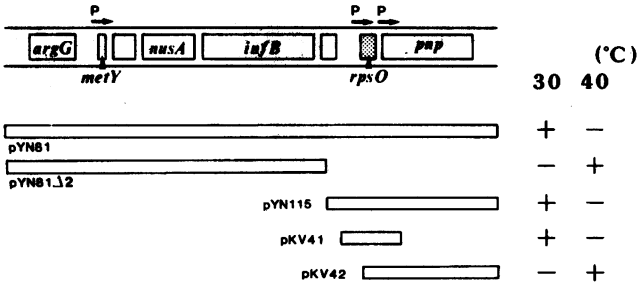

FIG. 3. Complementation of cold-sensitive growth phenotype of *suhD* strains by recombinant plasmids. Symbols: ▩, genes of the *rpsO* region of the *E. coli* chromosome, drawn approximately to scale; □, chromosomal regions carried by the plasmids. P, Promoters. Growth of derivatives of *suhD* revertants (KY1446 and KY1447) harboring each plasmid at 30 or 40°C is indicated to the right.

(opal) mutant (LS653). While available evidence suggests that *suhD* acts as an opal (UGA) suppressor by affecting certain ribosomal function, the question of suppressor specificity remains to be determined by further study.

Reduced level of ribosomal protein S15. To examine whether *suhD* affects the level or property of S15, ribosomes were prepared from *suhD* cells and analyzed by two-dimensional polyacrylamide gel electrophoresis (4) (Fig. 4A through C). In contrast to the wild-type ribosome that contains unit amounts of S15 relative to other proteins, the ribosomal fractions from *suhD* cells contain markedly reduced amounts (ca. 10%) of S15. The position of the S15 spot on the gel seemed unaffected, and no new spot was detected. This suggested that the majority of ribosome produced in *suhD* cells do not contain S15.

Polyribosomes that are actively engaged in protein synthesis were prepared from one of the *suhD* strains (KY1447) and similarly examined for S15 contents. The polyribosome fraction of *suhD* cells also contained reduced amounts (ca. 10%) of S15, compared with that of the wild type (Fig. 4, D through E). Apparently, ribosomes lacking S15 can actively participate in protein synthesis. Consistent with this finding is the observation that the rate of bulk protein synthesis in *suhD* cells is not appreciably different from that of the wild type at the permissive temperature. As will be shown below, the reduced level of S15 in the *suhD* ribosome is primarily due to reduced synthesis of S15.

Cloning and sequencing of *suhD*. To determine the site of *suhD* mutation more precisely, the *argG-rpsO-pnp* region of DNA from the *suhD3* strain (KY1447) was cut out by restriction endonucleases *Eco*RI and *Hin*dIII and ligated with pBR322 digested with the same enzymes, and recombinant plasmids that can complement *argG* were selected. Restriction analysis of the cloned fragment revealed that a 1.4-kb fragment had been inserted into the *Hpa*I fragment (843 bp) containing *rpsO*. The nucleotide sequence analysis of this region (from the upstream *Hpa*I site to a part of the insert, including the entire *rpsO* gene) revealed that the insertion occurred immediately downstream of *rpsO*, within the inverted repeat sequence presumably involved in transcription termination or attenuation (Fig. 5). No sequence alteration was detected in the promoter or the *rpsO* coding region when compared with the published results for wild-type *rpsO* (21, 28). Comparison of the proximal part (206 bp) of the inserted sequence with the known insertion element sequences indicated a very high homology (97%) with IS*10* (6). We conclude that the *suhD3* suppressor represents insertion of an IS*10*-like element at the *rpsO* downstream region.

Reduced level of *rpsO* mRNA. Since the level of S15 in the ribosome of *suhD3* strain was specifically reduced and the insertion was found at the putative transcription attenuator-terminator region for *rpsO*, it seemed likely that *suhD* affected the synthesis or the stability of *rpsO* mRNA. Quantitative S1 nuclease protection experiments with two kinds of DNA probe revealed that *rpsO* mRNA in the *suhD* strain was reduced to about 10% that of wild type or of the parental *rpoH11* mutant at 40°C (Fig. 6). The reduced level of *rpsO* mRNA may be explained either by instability of mRNA due to the insertion at the 3′ end of RNA or by interference with the *rpsO* transcription caused by transcription in the opposite direction initiated from within the insertion. In any event, the insertion appears to reduce the amount of functional *rpsO* mRNA which, in turn, reduces the synthesis of S15 protein.

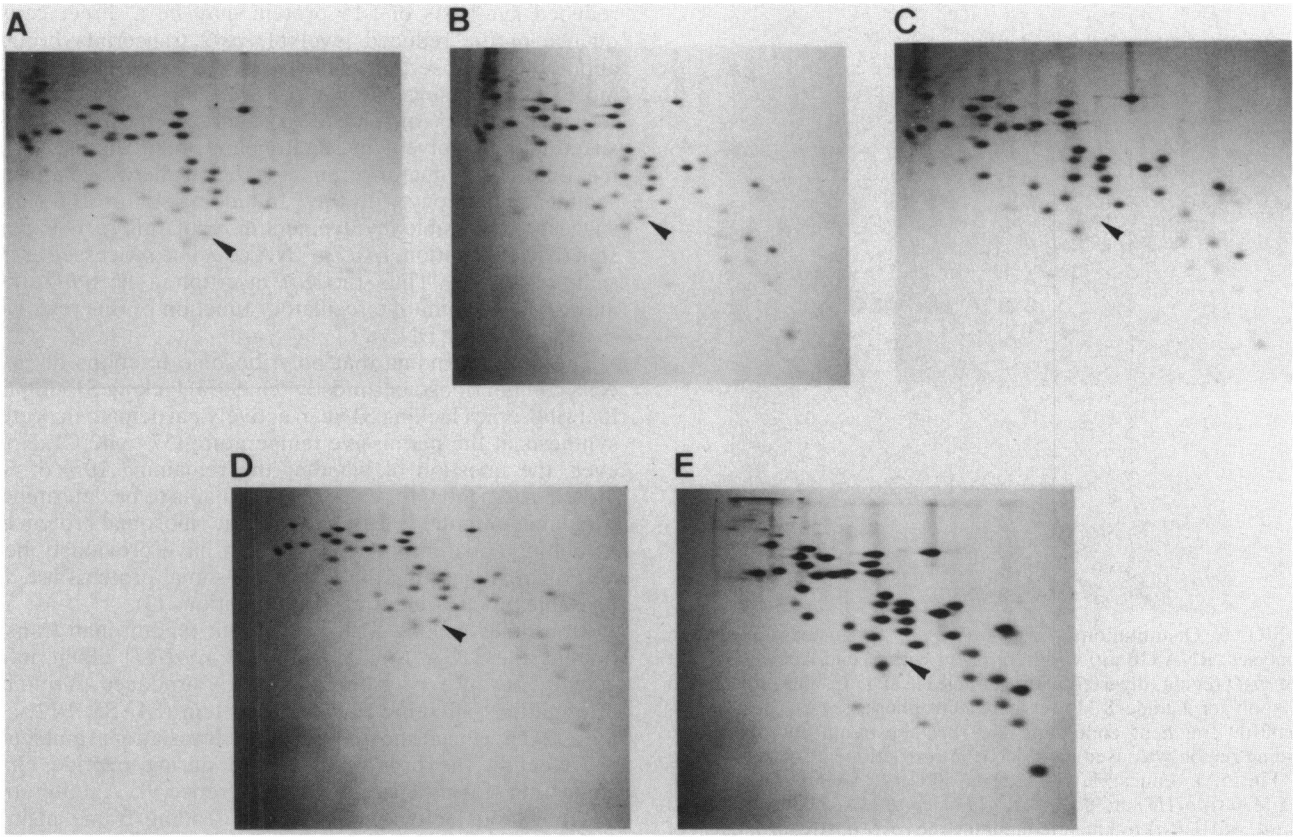

FIG. 4. Two-dimensional gel electrophoresis of ribosomal proteins. Total ribosome fractions (A, B, and C) were prepared from log-phase cells grown in L broth at 30 or 40°C. (A) MC4100 at 40°C; (B) KY1430 (*rpoH11*) at 30°C; (C) KY1447 (*rpoH11 suhD3*) at 40°C. Polyribosome fractions (D and E) were prepared by sedimenting total ribosome fractions through sucrose gradients (15 to 30%). The fractions were pooled, collected by centrifugation, and analyzed as described above. (D) MC4100 at 40°C; (E) KY1447 at 40°C. Arrowheads, S15 protein.

DISCUSSION

The *suhD* suppressors examined in this study can suppress both temperature-sensitive growth of the *rpoH11* mutant and the induction of heat shock proteins due to the resumed synthesis of σ^{32} protein. The suppressors do not seem to suppress any of the amber or missense *rpoH* or other opal mutations tested, suggesting codon or context specificity in suppression. However, the *rpoH* mutant could be a very sensitive test for suppression because the mutant σ^{32} produced by suppression could be greatly stabilized. A rigorous test of suppressor specificity would require

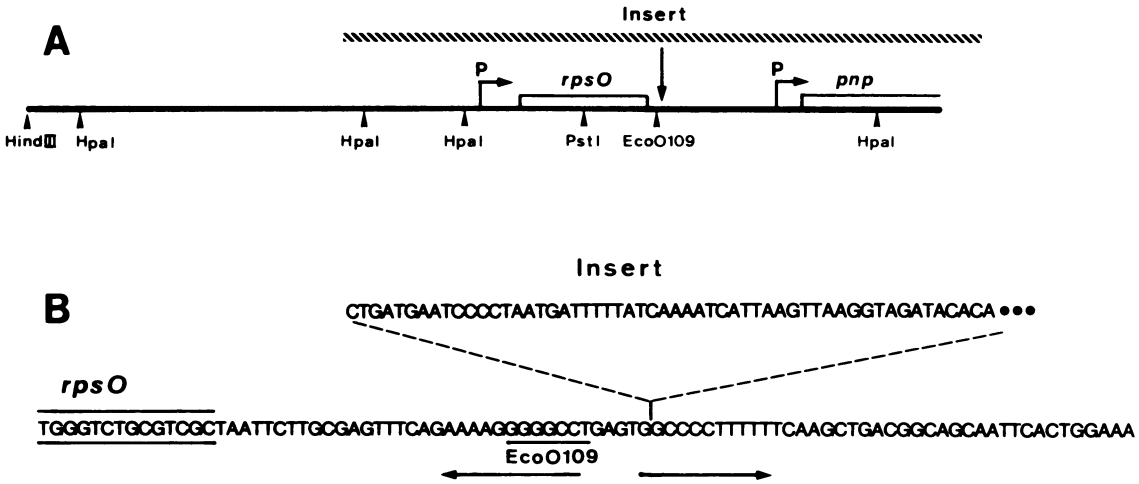

FIG. 5. (A) Structure of the *rpsO* region of the chromosome in the *suhD* strains. Insert represents an IS*10*-like element, and open boxes (□) indicate structural parts of genes. Arrowheads, Restriction enzyme cleavage sites. P, Promoters. (B) Nucleotide sequence around the insertion site. The insert was found within the inverted repeat sequences.

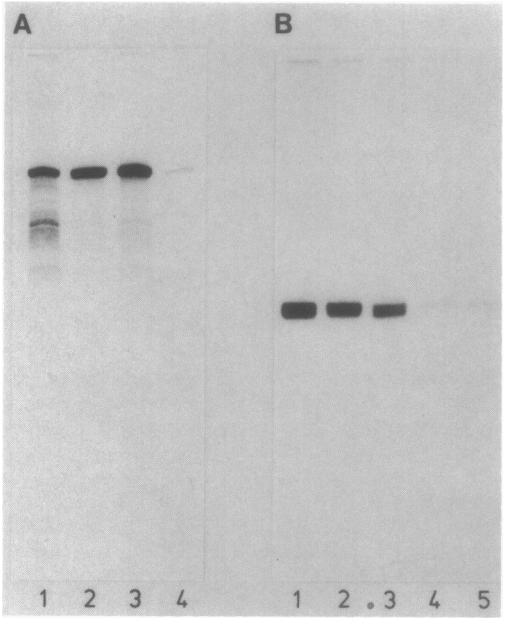

FIG. 6. Quantitation of *rpsO* mRNA by S1 nuclease protection analyses. RNA (10 μg) was hybridized with 5′ end-labeled DNA of the *rpsO* region, digested with S1 nuclease at 45°C, and subjected to 5% polyacrylamide–8 M urea gel electrophoresis. (A) The *Hin*dIII-*Eco*0109 fragment containing the *rpsO* upstream and the whole coding region was used as probe (the restriction sites are indicated in Fig. 5A). Lanes: 1, MC4100 at 30°C; 2, MC4100 at 40°C; 3, KY1430 (*rpoH11*) at 30°C; 4, KY1447 (*rpoH11 suhD3*) at 40°C. (B) The *Hin*dIII-*Pst*I fragment containing the *rpsO* upstream and half of the coding region was used as probe. Lanes: 1, MC4100 at 30°C; 2, MC4100 at 40°C; 3, KY1430 (*rpoH11*) at 30°C; 4, KY1447 (*rpoH11 suhD3*) at 37°C; 5, KY1447 at 40°C.

more systematic analysis of additional opal and other mutations.

The results of transduction mapping and complementation tests indicated that *suhD* affected the ribosomal protein gene *rpsO*. Cloning and sequencing of one of the *suhD* suppressors revealed that an IS*10*-like element had been inserted at the attenuator-terminator site located downstream of *rpsO*. No other alteration was detected in the promoter or coding region, strongly suggesting that the IS*10*-like insert is solely responsible for the observed reduction in *rpsO* mRNA and protein, as well as for cold-sensitive growth.

Either or both of the following mechanisms may explain the reduced level of *rpsO* mRNA observed. First, the insertion at the transcriptional attenuator-terminator region may cause mRNA instability by affecting its 3′-terminal structure. Deletion of the 3′ end of a ribosomal protein (S20) operon has previously been shown to cause faster mRNA degradation, suggesting the importance of the mRNA 3′ end for stability (13). Second, *rpsO* transcription of opposite direction per se or the anti-mRNA initiated from a promoter(s) located within the insert may interfere with the normal *rpsO* transcription. Indeed, one of the promoters on IS*10* is known to be located near its terminal and promotes outward transcription (toward *rpsO*). Such an IS*10*-mediated transcription of adjacent genes has been reported for a number of genes in *E. coli* (10).

Since both the *rpsO* mRNA level and the S15 protein level of isolated ribosomes of *suhD* suppressor strains were reduced to similar extents (ca. 10% of wild type), and since no sequence alteration was found in the *rpsO* coding region, the reduced synthesis of S15 protein may be a direct consequence of the reduced level of *rpsO* transcripts brought about by the insertion. However, the situation might be more complex, since *rpsO* expression may be actively controlled. The lack of gene dosage effect on S15 synthesis suggests the involvement of autogenous regulation (27). The fact that the attenuator-terminator region shows a significant sequence homology with the S15 binding site on 16S rRNA suggests its possible involvement in regulating *rpsO* expression (28). In addition, *rpsO* mRNA could be processed at this same region (22). Thus, the IS*10* insertion in the *suhD* strain may affect the putative regulatory function of this region on *rpsO* mRNA (or DNA).

The present finding that polyribosome fractions of *suhD* cells contain ribosomes mostly (ca. 90%) lacking S15 implies that ribosomes lacking S15 can actively participate in protein synthesis at the permissive temperature (37 to 42°C). However, the question of whether the remaining 10% of S15 protein is essential for cell growth remains to be determined. A number of mutants lacking a specific ribosomal protein but exhibiting apparently normal growth have previously been described, suggesting that some ribosomal proteins are dispensable at least under certain conditions (3).

Ribosomes lacking S15 must permit readthrough translation of the UGA (opal) codon (of *rpoH11*) albeit infrequently, because σ^{32} protein is actually produced. A number of mutations affecting ribosomal protein (S4, S5, S12, and L7/L12) have been shown to increase translational ambiguity by affecting the proofreading step during peptide chain elongation (for an example, see reference 9). An alteration in elongation factor EF-Tu affects reading frame maintenance or tRNA selection, causing frameshift translation, readthrough of nonsense codons, and missense errors (31). A mutated 16S rRNA (16) and polypeptide releasing factor RF-II (Chang, thesis) were recently found to suppress UGA codons specifically. Although the precise role of S15 is not known, the ribosome lacking S15 would cause UGA-specific suppression if it were involved in releasing polypeptides in concert with RF-II.

In reconstitution experiments of 30S ribosome in vitro, binding of S15 to 16S rRNA appears to be essential for binding of S6 and S18, suggesting that S15 elicits certain conformational change of 16S rRNA by binding to it (7, 26). However, the results here indicate that ribosomes lacking S15 but containing S6 and S18 can be formed in vivo at high temperature and are active in protein synthesis. Many of the cold-sensitive mutants have been shown to be defective in ribosome assembly and accumulate intermediate particles (18). The cold sensitivity of the *suhD* strains suggests the possibility that the requirement for S15 in ribosome assembly is more stringent at low temperature than at high temperature. Although ribosomes lacking S15 remain active in polypeptide chain growth, their function must be sufficiently altered so as to exhibit reduced fidelity, possibly by affecting tRNA selection. Further analysis is needed to understand the detailed mechanism of suppression in this system.

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