Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8432–8436, November 1990
Genetics

Autogenous suppression of an opal mutation in the gene encoding peptide chain release factor 2

(peptide chain release factor/*prfB*/*supK*/translation termination/UGA suppression)

KOICHI KAWAKAMI AND YOSHIKAZU NAKAMURA

Department of Tumor Biology, The Institute of Medical Science, The University of Tokyo, P.O. Takanawa, Tokyo 108, Japan

Communicated by Allan B. Campbell, August 3, 1990 (received for review May 21, 1990)

ABSTRACT **The peptide chain release factor 2 (RF2) gene, *prfB*, was cloned from *Salmonella typhimurium* by DNA hybridization using the *Escherichia coli prfB* probe. The nucleotide and amino acid sequences of *prfB* are 87.0% and 95.6% homologous between *E. coli* and *S. typhimurium*, respectively, including an in-frame premature UGA stop codon at position 26, the site of +1 frameshift for mature RF2 synthesis. The *supK584* mutation, which had been isolated as a recessive UGA suppressor in *S. typhimurium*, caused an opal (UGA) substitution at amino acid position 144 in the *prfB* gene. Complementation, reversion, and gene fusion analyses led to the conclusion that *supK* is a *S. typhimurium* RF2 mutation and this opal RF2 mutation generates a UGA suppressor activity, presumably because of inefficient translation termination due to the reduced cellular level of RF2. In fact, suppression of the *supK* opal mutation results from a form of autogenous control of RF2 synthesis.**

Prokaryotic translation termination requires two different peptide chain release factors, which act at termination codons. Release factor 1 (RF1) catalyzes termination at UAG and UAA codons, and release factor 2 (RF2) catalyzes termination at UAA and UGA codons (1). In *Escherichia coli*, RF1 is encoded by the *prfA* gene, which maps at 27 min of the chromosome (2), whereas RF2 is encoded by the *prfB* gene, which maps at 62 min (3, 4). The *E. coli prfB* gene is in an operon with the *herC* gene (4), which affects maintenance of the ColE1 plasmid (5).

In *Salmonella typhimurium*, a recessive UGA suppressor mutation, *supK*, has been isolated and mapped at the 62-min region of the chromosome (6). Reeves and Roth (7) have observed reduced levels of tRNA methyltransferase activities in several *supK* strains and have suggested that an unmodified tRNA causes UGA suppression. However, we have assumed, on the basis of high homology between the *E. coli* and *S. typhimurium* genomes, that the *supK* mutation may affect the RF2 protein (4). Two lines of evidence are consistent with this assumption. First, the *supK* mutation of *S. typhimurium* is complemented by the *E. coli prfB* gene (4). Second, RF2 mutants of *E. coli* harbor a recessive UGA suppressor activity (8). In this article, we describe the DNA sequence and genetic studies of the *S. typhimurium* RF2 gene isolated from wild type and the *supK* mutant.* These results affirm the prediction that *supK* is a *S. typhimurium* RF2 mutation.

MATERIALS AND METHODS

Bacterial Strains. *S. typhimurium* LT2 strains used are ST3 (*metB-23 trpB-2*), GT68 (*supK584 hisG200*$_{UGA}$*pro688*::Tn*5 serA790*; a gift from G. R. Björk, Umeå University), KK651 (same as GT68 except rifampicin resistant and streptomycin resistant; mutations added by sequential selection of spontaneous resistant mutants of GT68), and KK665 (same as KK651 except *supK*$^+$ and *serA*$^+$; constructed from KK651 by P22 transduction). *E. coli* K-12 strains used are YN2970 (*prfB1 lacZ659*$_{UGA}$) and KK579 (*prfB286 lacZ659*$_{UGA}$) (8).

Media. L broth contains 1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. YT medium contains 1% Bacto-tryptone, 0.1% yeast extract, 0.25% NaCl, and 1% agar. Minimal E agar plates were described by Miller (9).

Phage and Plasmids. The structures of the phages and plasmids are shown in Fig. 1. Phage vectors λVIII (10) and λZAP (11) were used to construct wild-type ST3 DNA libraries. Phages λSRF2E and λSRF2H, which carry the COOH-terminal and NH_2-terminal regions of the *S. typhimurium prfB* gene, were isolated from size-fractionated *Eco*RI and *Hin*dIII fragment libraries by plaque hybridization using the *E. coli prfB* probe. The chromosomal DNA of the GT68 (*supK584*) strain was digested with *Eco*RI and *Pst* I and fractionated by agarose gel electrophoresis; 1.2-kilobase (kb) fragments were ligated into the pUC119 plasmid (12). The 1.2-kb *Eco*RI/*Pst* I fragment encoding the COOH-terminal part of the mutant *prfB* gene was cloned from this pUC119 library. The 1.8-kb *Hin*dIII fragment containing the NH_2-terminal part of the mutant *prfB* gene was cloned from a size-fractionated *Hin*dIII λZAP library of GT68 by screening with the wild-type *S. typhimurium prfB* probe. The mutant *prfB* gene was reconstructed by ligating these two fragments at the *Eco*RI site; ligation product yielded a 1.8-kb *Cla* I/*Pst* I fragment, which was subcloned between the *Cla* I and the *Sph* I sites of pACYC184 (13). The final product includes the entire *supK* (*prfB*) gene and was designated pSUPK. An intermediate copy number plasmid, pACYC184, was used as a vector of the *prfB* gene since structural aberrations can be induced when it is cloned in the high copy number plasmid. The *Cla* I/*Cla* I fragment of pSUPK, containing the *supK584* substitution, was replaced by the wild-type fragment, giving rise to pSRF2. Thus, pSUPK is structurally equivalent to pSRF2 except for mutation *supK584*. pSUPKΔH was constructed from pSUPK by deleting the COOH-terminal one-third of the *prfB* gene from the *Hin*cII site.

Plasmids pSRF2Z13, pSRF2Z229, and pSUPKZ229 carry a gene fusion between the *S. typhimurium prfB* and *E. coli lacZ* genes at the different junction points in the *prfB* sequence. The pSRF2 DNA was digested with *Eco*O109I, the 5′ protruding ends were filled in with the Klenow fragment of DNA polymerase I enzyme, and the resulting blunt ends were ligated to a *Bam*HI linker. Then, the DNA segment flanked by this linker-generated *Bam*HI site and the *Sal* I site, which leaves only the NH_2-terminal 13 amino acids of RF2, was replaced by the *Bam*HI/*Sal* I *lacZ* fragment of the pMC1403 plasmid (14), giving rise to pSRF2Z13. The *Eco*RV/*Sal* I

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: RF2, peptide chain release factor 2.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38590).

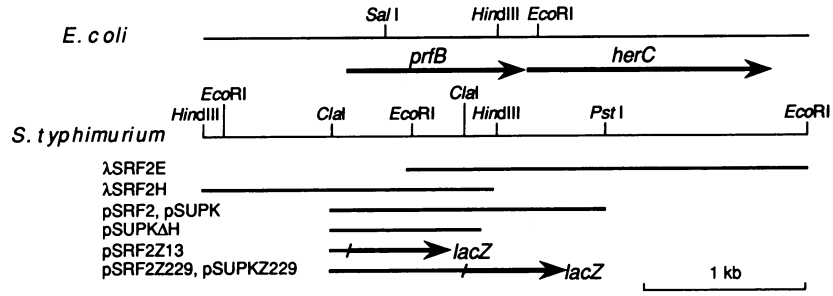

FIG. 1. Chromosomal structure of the *prfB* region of *S. typhimurium*. The physical maps of the *prfB* locus of *E. coli* and *S. typhimurium* are shown. Heavy lines indicate the bacterial DNA segments cloned on phages and plasmids. Arrows indicate location and orientation of the genes. The *lacZ* gene was fused to *prfB* at the points indicated in pSRF2Z13, pSRF2Z229, and pSUPKZ229.

segment of the pSRF2 and pSUPK plasmids, which leaves the NH_2-terminal 229 amino acids of RF2, was replaced by the *Sma* I/*Sal* I fragment of pMC1403, giving rise to pSRF2Z229 and pSUPKZ229, respectively.

Revertant Analysis of *supK584*. The mutation *supK* causes lethality at high temperature; 10 independent temperature-resistant revertant colonies were selected from strain GT68 at 43°C on YT plates. The revertant frequency was around 10^{-5}. The chromosomal DNAs of these revertant cells were used to amplify an internal *prfB* segment containing the *supK584* substitution site by the polymerase chain reaction method (15) using AAGTTGGCGCAGTTGGAATT and TCAATGACTTCTGTCTTGAA as primers, AmpliTaq DNA polymerase (Cetus), and Zymoreactor apparatus (ATTO, Tokyo, Japan). The amplified 176-base-pair (bp) double-stranded DNA was directly sequenced by the dideoxynucleotide chain-termination method.

Other Procedures. Southern blot hybridization, plaque hybridization, and colony hybridization were conducted by standard methods (16). The dideoxynucleotide chain-termination method has been described (17, 18). The β-galactosidase activity was determined as described (8) except L broth was used for cultivating *S. typhimurium* transformants.

RESULTS

Cloning and Sequencing the *S. typhimurium* RF2 Gene. *S. typhimurium* ST3 DNA was digested with *Eco*RI or *Hin*dIII and analyzed by Southern blot hybridization using the 1062-bp *Sal* I/*Eco*RI DNA probe encoding the COOH-terminal part of the *prfB* gene and the NH_2-terminal part of the *herC* gene of *E. coli* (Fig. 1). A 2.5-kb *Eco*RI DNA and a 1.8-kb *Hin*dIII DNA were detected (data not shown). Hence, independent digestion products of ST3 DNA by *Eco*RI and *Hin*dIII were size-fractionated by agarose gel electrophoresis and genomic DNA libraries using λ phage vectors were constructed. The *Eco*RI library was screened with the same 1062-bp probe, and the λSRF2E phage carrying the 2.5-kb *Eco*RI fragment was isolated (Fig. 1). Then, the *Hin*dIII library was screened by using the 1.0-kb *Eco*RI/*Hin*dIII *S. typhimurium* DNA, and the λSRF2H phage carrying the 1.8-kb *Hin*dIII fragment was isolated (Fig. 1).

The DNA fragments cloned in λSRF2E and λSRF2H were subcloned in the plasmid pUC119 and sequenced. The DNA sequence was highly homologous to the *E. coli prfB* region (Fig. 2). A putative *S. typhimurium prfB* gene encodes the same number of nucleotides and amino acids as the *E. coli* gene and also contains an in-frame UGA codon at position 26. Therefore, it appears that the mature *S. typhimurium* RF2 protein is synthesized by +1 frameshift at the opal codon, as has been shown for the *E. coli prfB* gene. The amino acid sequence is 95.6% (349/365) identical to *E. coli* and the nucleotide sequence is 87.0% (956/1099) identical in the coding region of *prfB* (Fig. 2).

The pSRF2 plasmid, which carries a 1.8-kb *Cla* I/*Pst* I DNA segment (Fig. 1) was used in a test to complement the *E. coli* RF2 mutation. The *E. coli* strains YN2970 and KK579 carry different missense UGA suppressor mutations in the *prfB* gene and a UGA mutation in *lacZ*. Plasmid pSRF2 was introduced into these strains and the β-galactosidase activities were measured. As shown in Table 1, the plasmid pSRF2 complemented the *prfB* mutations. These results led us to conclude that this protein-coding sequence, including a +1 frameshift site, encodes a functional *S. typhimurium* RF2 protein. We refer to the gene as *prfB*.

Identification of the *supK584* Mutation. The *supK* mutation of *S. typhimurium* confers a recessive UGA suppressor activity at 30°C and causes lethality at 41°C (6). The *prfB* gene was cloned from the *supK584* strain by colony and plaque hybridization. The nucleotide sequence analysis of the mutant DNA disclosed a single base substitution of A for G at position 433 (Fig. 3). No other alteration was found within the coding sequence of *prfB*. This substitution generates a UGA nonsense codon for a UGG tryptophan codon at amino acid position 144 (Fig. 2).

The pSRF2 plasmid encoding the wild-type RF2 protein eliminated the UGA suppression activity of both the *E. coli prfB* mutants as discussed above and the *S. typhimurium supK584* mutant (Fig. 4). In contrast, the pSUPK plasmid carrying the *supK584* mutant RF2 gene failed to complement these mutations (Table 1 and Fig. 4). However, the suppression efficiency in strains with the pSUPK plasmid was slightly reduced compared to those with vector plasmid pACYC184. This small but significant reduction of the UGA suppressor activity caused by pSUPK is due to the leakiness of the opal UGA mutation rather than to the partial activity of the opal RF2 fragment. This is suggested because the pSUPKΔH plasmid, which produces a truncated RF2 protein larger than the opal fragment (Fig. 1), did not reduce the UGA suppressor activity (Table 1 and Fig. 4). In addition to the UGA suppressor activity, the *supK584* strain is heat sensitive for growth; ability of the strain to grow at 42°C was restored fully by plasmid pSRF2, poorly by pSUPK, but not at all by pSUPKΔH (Fig. 4); this is consistent with the fact that these plasmids complement UGA suppression activity.

To firmly establish that the UGA suppressor activity of the *supK584* mutant is caused by the opal mutation in the *prfB* gene but not by some other mutation(s) in some other gene(s), revertants of the *supK584* mutant of strain GT68 were isolated and analyzed. Of 10 temperature-resistant colonies, 4 were His^- and had lost the ability to suppress the UGA mutation *hisG200*; 6 revertants retained the ability to suppress this mutation. A 176-bp chromosomal DNA region containing the opal substitution (nucleotide positions 326–501 in Fig. 1) was amplified directly by the polymerase chain reaction method from these revertant chromosomal DNAs and sequenced. The 4 His^- revertants had changed the UGA codon to UGG (i.e., true reversion) and the latter 6 His^+ still

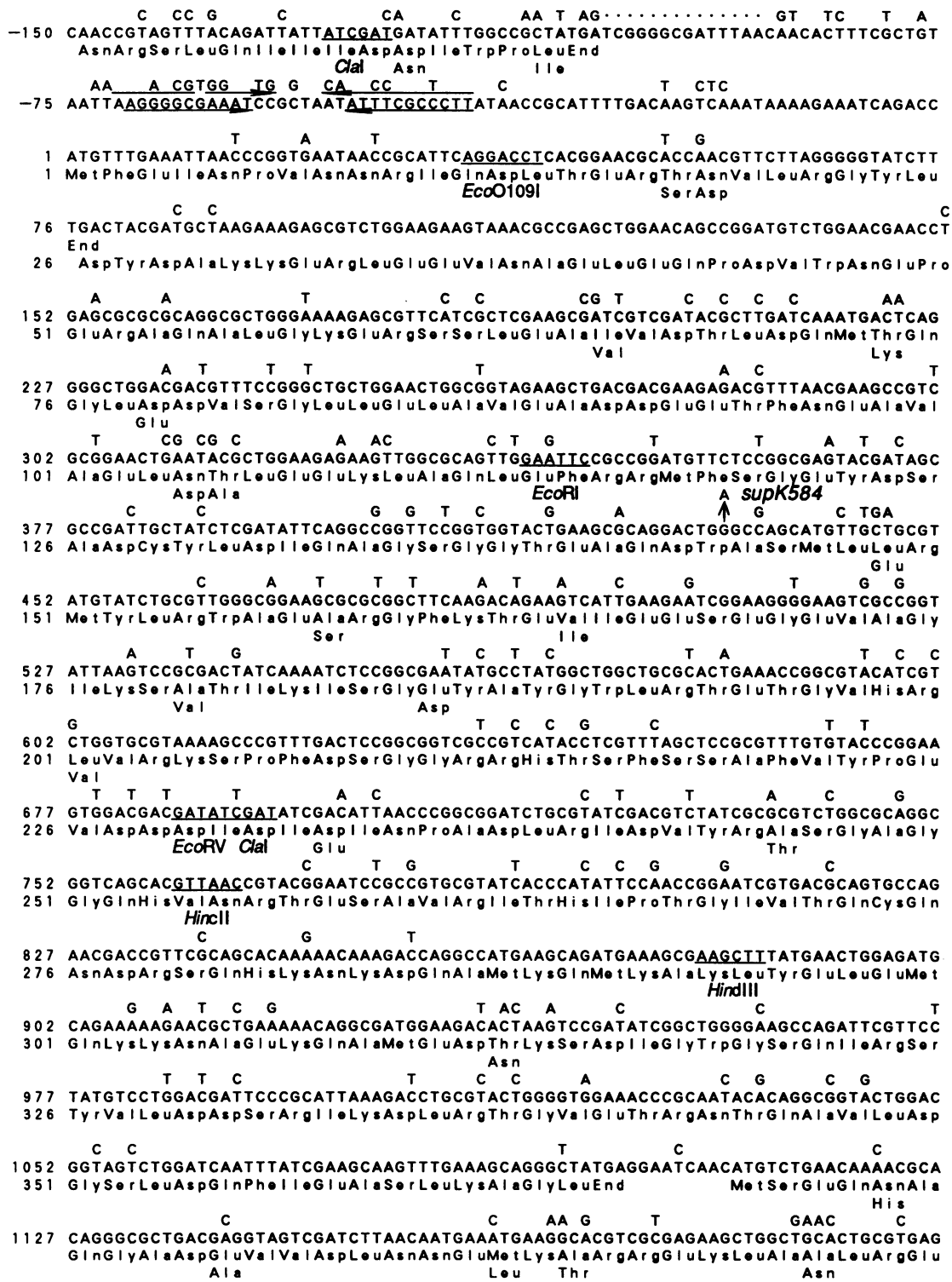

FIG. 2. Nucleotide and deduced amino acid sequences of the *S. typhimurium prfB* gene. Nucleotides and amino acids of the *E. coli prfB* gene that differ from those of *S. typhimurium prfB* are indicated. ●, Deletion of a base in the *E. coli prfB* sequence. The numbers of the nucleotide and amino acid positions are counted from the initiation site of the *prfB* coding sequence. The restriction enzyme sites used for DNA manipulation are shown. Arrows upstream of the *prfB* gene indicate a dyad symmetry. The *supK584* mutation substitutes an A residue for a G residue at the position indicated.

carried the UGA codon at amino acid position 144 (data not shown). These results led us to conclude that the opal substitution in the RF2 gene of *supK584* is solely responsible for both the UGA suppression and the temperature sensitivity of growth.

Autogenous Suppression. Since the *prfB*-encoded protein is essential, it seems extraordinary that a nonsense mutation in the *prfB* gene generates an opal suppressor activity while causing only temperature-sensitive lethality. This can be explained by assuming that the reduction in RF2 level caused by the opal mutation causes inefficient termination of translation and leads to suppression of its own nonsense UGA codon. To test this assumption, expression of the RF2 gene in the wild-type and mutant cells was analyzed by using gene fusions (Fig. 1). Three *prfB–lacZ* fusion plasmids were constructed. pSRF2Z13 and pSRF2Z229 carry NH_2-terminal 13-

Table 1. Complementation of the *prfB* mutations

Strain (relevant genotype)	β-Galactosidase activity			
	pACYC184	pSRF2	pSUPK	pSUPKΔH
YN2970 (*prfB1 lacZ659*$_{UGA}$)	122	4.6	106	146
KK579 (*prfB286 lacZ659*$_{UGA}$)	118	5.1	87	134

YN2970 and KK579 cells were transformed with the indicated plasmids by selecting for chloramphenicol resistance. Transformant cells were grown at 32°C in the presence of 0.5% glycerol, 10 μg of chloramphenicol per ml, and 1 mM isopropyl β-D-thiogalactoside as described (8). The β-galactosidase activity of the exponentially growing cultures is expressed as units per min per mg of protein.

and 229-amino acid portions of the wild-type *prfB* gene, respectively; thereby the fusion points are before and after the +1 frameshift site at position 26. Plasmid pSUPKZ229 is structurally equivalent to plasmid pSRF2Z229 except for the *supK584* opal substitution at position 144. Therefore, the UGA suppression efficiency can be deduced by comparing the β-galactosidase levels of strains carrying plasmids pSRF2Z229 and pSUPKZ229. As shown in Table 2, expression of the *prfB–lacZ* fusion protein with the *supK584* opal mutation (pSUPKZ229) was greatly reduced in the wild-type strain. However, synthesis of this mutant fusion protein was increased in the *supK584* strain, indicating that the opal RF2 mutation is autogenously suppressed under these conditions. The +1 frameshift efficiency at the early UGA codon (codon 26) was also increased by suppressor mutation *supK584*. Thus, a reduced cellular level of the mature RF2 protein in the opal mutant is the most probable reason for the increased UGA suppression activity. The efficiency of translation termination at the *supK584* opal codon was higher at 42°C than at 37°C in both the *supK584* and wild-type strains. This might explain, at least in part, the temperature-sensitive growth of the *supK584* mutant.

DISCUSSION

The *prfB* gene of *S. typhimurium* is highly homologous to that of *E. coli*: 87.0% in the nucleotide sequence and 95.6% in the amino acid sequence. Both genes contain an in-frame UGA codon at amino acid position 26 and require a +1 frameshift at this codon to permit complete translation of the *prfB* mRNA. This in-frame UGA stop codon is preceded by leucine tRNA codon CUU and a Shine–Dalgarno-like sequence AGGGGG 3 bases upstream of CUU. Weiss *et al.* (19) have demonstrated that these structures are required for

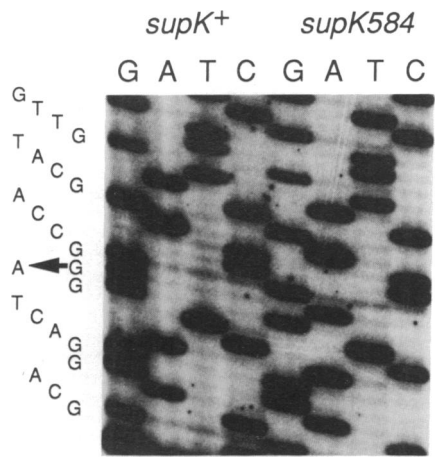

FIG. 3. DNA sequence of the wild-type and *supK584 prfB* genes. Arrow indicates a substitution by *supK584*.

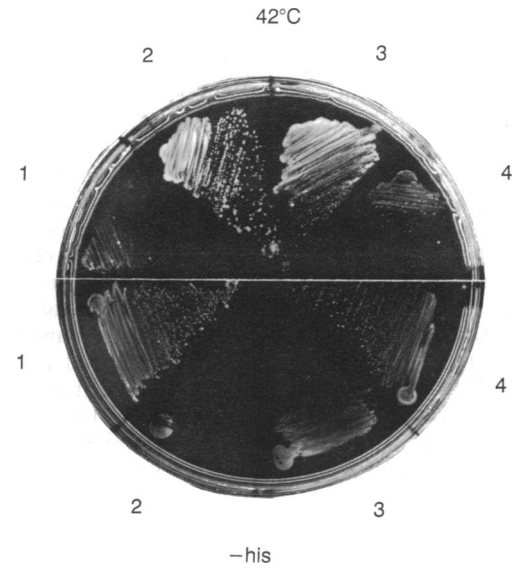

FIG. 4. Complementation of the *supK* mutation. KK651 (*supK584 hisG200*$_{UGA}$) cells were transformed with the respective plasmids and examined for the mutant phenotype. (*Upper*) Transformant cells were streaked on a YT plate and incubated at 42°C for 24 hr. (*Lower*) Transformant cells were streaked on a minimal agar plate containing 0.5% glucose, 0.002% proline, and 0.002% serine and incubated at 37°C for 48 hr. The following plasmids were used: 1, pACYC184; 2, pSRF2; 3, pSUPK; 4, pSUPKΔH.

frameshift in the *prfB* gene of *E. coli*. This "frameshift window" sequence of the *prfB* gene is precisely conserved in *S. typhimurium*. Downstream of *E. coli prfB*, the *herC* gene, which affects maintenance of the ColE1 plasmid, is located in the same operon (4, 5). The *herC* sequence also followed *prfB* in the *S. typhimurium* genome (Fig. 2). Another homologous open reading frame ends at a TGA 107 bp upstream of *prfB* in *S. typhimurium* and at a TAG 93 bp upstream of *prfB* in *E. coli*. Thus, the *prfB* region is highly homologous between *E. coli* and *S. typhimurium*, including its flanking sequences.

The present work demonstrates that the *supK* mutation, a recessive UGA suppressor mutation, affects the *S. typhimurium prfB* gene. First, the RF2 gene isolated from the *supK584* strain carried an opal mutation at amino acid position 144. Second, the *prfB* gene from *supK584* causes a reduction in translation termination at UGA codons. Finally, *supK584* revertants, which lost the UGA suppressor activity and temperature sensitivity, carried a UGA-to-UGG change.

Table 2. Efficiency of +1 frameshift and UGA suppression in the *supK* mutant

Strain (relevant genotype)	Relative activity of β-galactosidase			
	pSRF2Z13	pSRF2Z229	pSUPKZ229	
	37°C	37°C	37°C	42°C
KK665 (*supK*$^+$)	100	60	4	2
KK651 (*supK584*)	100	95	11	7

S. typhimurium KK665 and KK651 cells were transformed with the indicated plasmids by selecting for chloramphenicol resistance. Transformant cells were grown in L broth in the presence of 10 μg of chloramphenicol per ml. Cultures grown at 37°C were inoculated into the same broth and grown at 37°C or at 42°C for 3 hr until the optical density at 600 nm increased ≈6-fold. The β-galactosidase activity of the exponentially growing cultures is expressed as units per min per mg of protein. The values represent β-galactosidase activities relative to those of pSRF2Z13.

These data clearly indicate that the phenotypes of mutation *supK584* are due to a *prfB* opal mutation.

The *prfB–lacZ* gene fusion experiments revealed that the *supK584* opal mutation reduces the cellular amount of RF2 but causes suppression of UGA codons in the *prfB* gene. This is consistent with the previous observation that the increased gene dosage of the *prfB* gene reduces the leakiness of the UGA codon (8). The UGA suppression activity of mutation *supK584* leads to autogenous suppression of its own opal *prfB* mutation, thereby generating another feedback regulatory loop for the synthesis of RF2 in the mutant. The *supK584* mutation also enhanced the normal feedback regulation at the +1 frameshift at the 26th UGA codon. Craigen and Caskey (20) showed that synthesis of RF2 is reduced by adding excess RF2 protein to the *in vitro* coupled transcription/translation system. Our result is consistent with this *in vitro* observation and may be the first *in vivo* evidence for autogenous control of RF2 synthesis by a +1 translational frameshifting.

Why does the *supK584* strain exhibit temperature sensitivity? The suppression efficiency of the *supK584* UGA codon in the *supK584* mutant was lower at 42°C than at 37°C (Table 2). It is possible that the lower amount of the RF2 protein produced in mutant *supK584* at high temperature (≈10% of the wild type) is insufficient for cell growth. However, another possibility cannot be excluded at present: a nontryptophan amino acid may be incorporated in the 144th opal position in *supK584* and this mutant RF2 protein may lose activity at high temperature.

Reeves and Roth (7) observed low levels of tRNA methyltransferase in the *supK584* strain. The present work demonstrated that the *supK584* mutation is not a mutation in the structural gene for tRNA methyltransferase but a mutation in the *prfB* gene. We assume that RF2 may play a crucial role in expression of a tRNA methyltransferase gene. Further studies will be needed to disclose the relationship between RF2 and tRNA methyltransferase.

We thank G. R. Björk for the *S. typhimurium* strain GT68, K. Itoh for help in DNA cloning and sequencing, O. Mikuni for discussion, D. L. Court for critical reading of the manuscript, and K. Egawa for continuous support of this project. This work was supported in part by grants from The Ministry of Education, Science and Culture, Japan.

1. Scolnick, E., Tompkins, R., Caskey, T. & Nirenberg, M. (1968) *Proc. Natl. Acad. Sci. USA* **61,** 768–774.
2. Ryden, M., Murphy, J., Martin, R., Isaksson, L. & Gallant, J. (1986) *J. Bacteriol.* **168,** 1066–1069.
3. Craigen, W. J., Cook, R. G., Tate, W. P. & Caskey, C. T. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3616–3620.
4. Kawakami, K., Jönsson, Y. H., Björk, G. R., Ikeda, H. & Nakamura, Y. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5620–5624.
5. Kawakami, K., Naito, S., Inoue, N., Nakamura, Y., Ikeda, H. & Uchida, H. (1989) *Mol. Gen. Genet.* **219,** 333–340.
6. Reeves, R. H. & Roth, J. R. (1971) *J. Mol. Biol.* **56,** 523–533.
7. Reeves, R. H. & Roth, J. R. (1975) *J. Bacteriol.* **124,** 332–340.
8. Kawakami, K., Inada, T. & Nakamura, Y. (1988) *J. Bacteriol.* **170,** 5378–5381.
9. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Murray, N. E. & Murray, K. (1974) *Nature (London)* **251,** 476–481.
11. Short, J. M., Fernandez, J. M., Sorge, J. M. & Huse, W. D. (1988) *Nucleic Acids Res.* **16,** 7583–7600.
12. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153,** 3–11.
13. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
14. Casadaban, M. J., Chou, J. & Cohen, S. N. (1980) *J. Bacteriol.* **143,** 971–980.
15. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
16. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Basic Methods in Molecular Biology* (Elsevier, New York).
17. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
18. Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4767–4771.
19. Weiss, R. B., Dunn, D. M., Dahlberg, A. E., Atkins, J. F. & Gesteland, R. F. (1988) *EMBO J.* **7,** 1503–1507.
20. Craigen, W. J. & Caskey, C. T. (1986) *Nature (London)* **322,** 273–275.