Journal of Bacteriology, Oct. 1995, p. 5547–5553 Vol. 177, No. 19
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Comparative Characterization of Release Factor RF-3 Genes of *Escherichia coli*, *Salmonella typhimurium*, and *Dichelobacter nodosus*

YOICHI KAWAZU, KOICHI ITO, KIYOYUKI MATSUMURA, AND YOSHIKAZU NAKAMURA*

Department of Tumor Biology, The Institute of Medical Science, The University of Tokyo, Tokyo 108, Japan

Received 22 May 1995/Accepted 27 July 1995

The termination of protein synthesis in bacteria requires two codon-specific release factors, RF-1 and RF-2. A gene for a third factor, RF-3, that stimulates the RF-1 and RF-2 activities has been isolated from the gram-negative bacteria *Escherichia coli* and *Dichelobacter nodosus*. In this work, we isolated the RF-3 gene from *Salmonella typhimurium* and compared the three encoded RF-3 proteins by immunoblotting and intergeneric complementation and suppression. A murine polyclonal antibody against *E. coli* RF-3 reacted with both *S. typhimurium* and *D. nodosus* RF-3 proteins. The heterologous RF-3 genes complemented a null RF-3 mutation of *E. coli* regardless of having different sequence identities at the protein level. Additionally, multicopy expression of either of these RF-3 genes suppressed temperature-sensitive RF-2 mutations of *E. coli* and *S. typhimurium* by restoring adequate peptide chain release. These findings strongly suggest that the RF-3 proteins of these gram-negative bacteria share common structural and functional domains necessary for RF-3 activity and support the notion that RF-3 interacts functionally and/or physically with RF-2 during translation termination.

The termination of translation in bacteria requires two codon-specific peptide chain release factors, RF-1 (UAG/UAA specific) and RF-2 (UGA/UAA specific) (32). A third factor, RF-3, is known to stimulate the activities of RF-1 and RF-2 and binds guanine nucleotides but is not codon specific (4, 6). The mechanism of stop codon recognition by release factors is still unknown (9, 36) but holds considerable interest since it entails protein-RNA recognition rather than the well-understood RNA-RNA recognition of codon-anticodon interaction.

The genes encoding the *Escherichia coli* RF-1 and RF-2 (ecRF-1 and ecRF-2, respectively) have been isolated. ecRF-1 was identified by a genetic screen for antisuppression against an amber suppressor tRNA (38), and ecRF-2 was identified by an antibody-probed screen for protein overexpression in the Clarke-Carbon *E. coli* library (5). The map position of RF-1 (designated *prfA*) is 27 min on the *E. coli* chromosome (28), and RF-2 (designated *prfB*) is at 62 min, within the same operon as the lysyl-tRNA synthetase gene (18). Several mutants of RF-1 and RF-2 have been isolated, and they often caused misreading of stop codons or frameshifting, as well as temperature-sensitive growth of the cells (11, 17, 19, 29). Hence, the reduced activity of release factors results in several translational errors in vivo, and these errors are likely caused by abnormally long pausing of ribosomes at stop signals (27).

Contrary to RF-1 and RF-2, RF-3 has received little attention since its initial characterization in the 1970s (4, 6, 13). RF-3 (initially called S or α) was isolated independently by two groups in 1969 as a stimulation factor for the in vitro termination reaction (4, 6). These investigators had shown that RF-3 stimulated the formation of ribosomal termination complexes and that stimulation was abolished by GTP or GDP. However, the biological significance of RF-3 in protein synthesis has been a long-standing puzzle. After two decades of silence, the gene for RF-3 was identified simultaneously by two groups and designated *prfC* (15, 21). The *prfC* gene maps at 99 min on the *E. coli* chromosome and encodes a 59,442-Da protein with sequence homology to elongation factor EF-G, including G-domain motifs. Mutations in *prfC* caused suppression of all three stop codons in vivo. The overproduced gene product markedly increased the formation of ribosomal termination complexes and stimulated the RF-1 or RF-2 activity in the codon-dependent in vitro termination reaction. These results firmly established the existence and biological importance of RF-3.

Upon revealing the *E. coli prfC* sequence, Billington et al. (2) noticed that a gene homologous to *E. coli prfC* is located downstream of the *lpsA* gene of *Dichelobacter nodosus*, which is responsible for a modification of the lipopolysaccharide. *D. nodosus* is a gram-negative strict anaerobe and is responsible for the invasive lesions at the skin-horn junction, which are associated with footrot in sheep. This disease has a significant economic impact on the Australian sheep meat and wool industries. However, the study of this organism or genes involved in virulence have been hampered by the lack of a genetic system in *D. nodosus*. No plasmids have been isolated from this organism, and no conjugation or transformation methods have been developed. The *D. nodosus prfC* homolog shares 62.2% protein sequence identity (78.5% similarity) to *E. coli prfC*; however, the activity of the gene product remains to be investigated.

We describe here the isolation and the complete sequence of the *prfC* gene of *Salmonella typhimurium* and the genetic characterization of three RF-3 proteins from *E. coli*, *S. typhimurium*, and *D. nodosus*. We conclude that the heterologous

* Corresponding author. Mailing address: Department of Tumor Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan. Phone: 81-3-5449-5307. Fax: 81-3-5449-5415. Electronic mail address: nak@hgc.ims.u-tokyo.ac.jp.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant description[a]	Source or reference
E. coli K-12		
Strains		
DEV1	*lacZ105*(UAG)	14
DEV14	*lacZ659*(UAA)	14
DEV15	*lacZ*(UGA)	14
OM315	DEV1 *prfC*Δ*2*::*kan* (Km^r)	This work
OM316	DEV14 *prfC*Δ*2*::*kan* (Km^r)	This work
OM317	DEV15 *prfC*Δ*2*::*kan* (Km^r)	This work
W3110	Prototroph	Laboratory stock
KM3	W3110 *prfC*Δ*3*::*kan* (Km^r)	This work
OM220	W3110 *leu*(UGA) *prfB286*(Ts) *rpsL*	This work
MP347	λ(*c*I857 ΔH1 *bio252*) lysogen	12
MV1184	Δ(*lac-proAB*) Δ(*srl-recA*)*306*::Tn*10* (φ80 *lacZ*ΔM15) F′ (*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	37
JC7623	*recB21 recC22 sbcB15 rpsL*	8
Plasmids		
pNE73	*E. coli prfC* gene cloned in pSU19, Cm^r	21
pYK3	*E. coli prfC* gene cloned in pACYC184, Cm^r	This paper
pYK5	*E. coli prfC* gene cloned in pGEX, Ap^r	This work
pKM3	Same as pNE73 but with *prfC*Δ*3*::*kan* substitution	This work
pTOSOP	*E. coli prfC* gene fused to λ p_L promoter, Ap^r	21
S. typhimurium LT2		
Strains		
ST3	*metB-23 trpB-2*	Laboratory stock
GT66	*hisG200*(UGA) *supK599*(Ts)	G. R. Björk
Plasmids		
pYK1	*S. typhimurium prfC* gene cloned in pACYC184, Cm^r	This work
pSRF2	*S. typhimurium prfB* gene cloned in pACYC184, Cm^r	19
D. nodosus plasmid		
pJIR730	*D. nodosus prfC* gene cloned in pTZ19R, Ap^r	2

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Ts, temperature sensitive.

RF-3 proteins function in the intergeneric background of *E. coli*, since they are capable of complementing the ecRF-3 mutation. Moreover, multicopy expression of either of these *prfC* genes compensates for the defect of temperature-sensitive mutations in the *prfB* gene. The latter finding strongly suggests that RF-3 interacts functionally and/or physically in vivo with RF-2 in peptide chain termination.

MATERIALS AND METHODS

Bacterial strains, phage, and media. *E. coli* K-12, *S. typhimurium* LT2, and *D. nodosus* strains used are listed in Table 1. The *prfC*Δ*3* null mutant of *E. coli* was constructed by transformation of JC7623 (*recB recC sbcB*) cells with the 2.7-kb *Sac*I-*Sph*I fragment of pKM3 DNA (Fig. 1) by selecting for Km^r transformants. One of these Km^r alleles was transduced into W3110 cells and named KM3. Phage P1 *vir* was used for transduction of relevant *E. coli* alleles. Southern blot hybridization confirmed that the *prfC*Δ*3*::*kan* construct had substituted for the wild-type *prfC* sequence in KM3 (data not shown). λ ZAP phage (Stratagene) was used for construction of an *S. typhimurium* DNA library. Minimal medium was medium E (23) with appropriate supplements. LB medium was as described by Miller (23), and YT medium contained 1% Bacto Tryptone, 0.1% yeast extract, and 0.25% NaCl (25).

Plasmids. *E. coli*, *S. typhimurium*, and *D. nodosus* plasmids used are listed in Table 1. Plasmids pYK1 and pYK3 are derivatives of pACYC184 and encode *S. typhimurium* RF-3 (stRF-3) and ecRF-3, respectively. pYK1 was constructed by recloning the 3.0-kb *Sal*I DNA cloned in λSRF3 phage into the same site of pACYC184 (7). pYK3 was constructed by recloning the 2.4-kb *Eco*RI-*Hin*dIII DNA cloned in pNE73 (21) into the *Eco*RV site of pACYC184 after blunting *Eco*RI and *Hin*dIII ends with the Klenow fragment of DNA polymerase I. pJIR730 is a derivative of pTZ19R plasmid (20) and carries the 2.4-kb *Ssp*I fragment of the *D. nodosus prfC* gene (2). Plasmid pYK5 is a derivative of pGEX-2T (33) and encodes an RF-3 fusion protein with the C terminus of a 26-kDa glutathione *S*-transferase (GST). To construct pYK5, the 2.1-kb *Tth*111I-*Eco*RI fragment encoding ecRF-3 except for N-terminal two amino acids was cleaved from pNE73, and its *Tth*111I end was blunted and ligated with a *Bam*HI linker. The resulting *Bam*HI-*Eco*RI segment was ligated at the same sites of pGEX-2T, resulting pYK5. Plasmid pKM3 has a Km^r cassette in place of the 1,084-bp *Sma*I-*Bss*HII sequence of plasmid pNE73 (*prfC*Δ*3*::*kan*; Fig. 1).

Cloning of the *S. typhimurium prfC* gene. The size of a *Sal*I fragment encoding stRF-3 was estimated to be ca. 3.0 kb by Southern blot hybridization using the *E. coli prfC* probe. Hence, *Sal*I digests of *S. typhimurium* ST3 DNA were separated by 1.0% agarose gel electrophoresis, and DNA fragments ranging in size from 2.5 to 3.5 kb were eluted from gels and inserted into the *Xho*I site of λ ZAP vector. The resulting *S. typhimurium* library was screened by plaque hybridization using the 1,084-bp *Sma*I-*Bss*HII DNA probe encoding the internal part of the *E. coli*

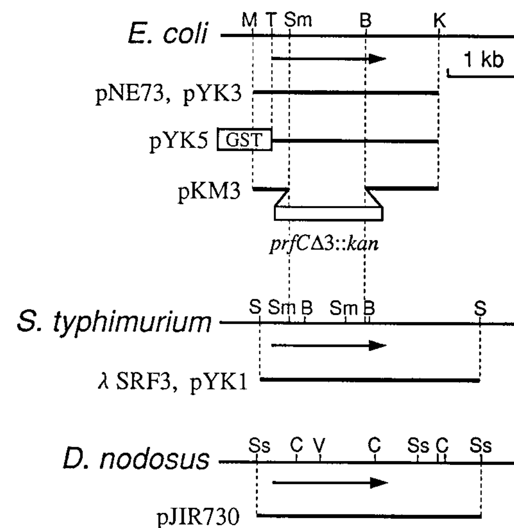

FIG. 1. Chromosomal structure of the *prfC* region. The physical maps of the *prfC* loci of *E. coli*, *S. typhimurium*, and *D. nodosus* are shown. Heavy lines indicate the bacterial DNA segments cloned on phages and plasmids. Arrows indicate locations and orientations of the RF-3 genes. Symbols: B, *Bss*HII; C, *Cla*I; K, *Kpn*I; M, *Mlu*I; S, *Sal*I; Sm, *Sma*I; Ss, *Ssp*I; T, *Tth*111I; V, *Eco*RV; GST, GST gene.

prfC gene (Fig. 1). After several rounds of screening procedures, one positive phage clone, λSRF3, was analyzed further.

Enzyme assay. Cells carrying heterologous *prfC* plasmids were grown in LB medium containing 15 μg of chloramphenicol or 50 μg of ampicillin per ml, and their β-galactosidase activities were determined as described previously (23).

Anti-RF-3 antibody. To prepare an immunogen, MV1184 cells were transformed with plasmid pYK5 and grown in 1 liter of LB medium containing 50 μg of ampicillin per ml at 37°C. One millimolar isopropyl-1-thio-β-D-galactoside was added to the exponentially growing culture to induce GST–RF-3 fusion protein synthesis. After 3 h of incubation, cells were harvested and lysed by sonication, and the soluble fraction was mixed with slurry of glutathione-agarose beads (Sigma) as described previously (33) according to the manufacturer's instructions. The GST–RF-3 fusion protein bound to the beads was collected by centrifugation and then incubated with the site-specific protease thrombin, which cleaves the site between the GST carrier and the RF-3 polypeptide. The final RF-3 polypeptide thus prepared has a substitution of the N-terminal sequence, Gly-Ser-Gly, for the authentic sequence, Met-Thr. For antibody generation, the RF-3 antigen was injected subcutaneously into mice (BALB/c; Japan SLC, Shizuoka, Japan) after emulsification with complete Freund's adjuvant. The mice were boosted 2, 4, and 6 weeks later, and blood samples were collected the following week.

Western blot (immunoblot) analysis. Protein determination was carried out by the Bio-Rad protein assay (Bio-Rad Laboratories). Bulk proteins prepared by sonication of cells were solubilized in 50 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–2% β-mercaptoethanol by boiling for 3 min, separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels, and electroblotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore). The blots were incubated with anti-ecRF-3 serum diluted 1:300 in Tris-buffered saline (24 mM Tris-HCl [pH 7.4], 2.7 mM KCl, 137 mM NaCl) supplemented with 5% skimmed milk (1 h, room temperature with rocking). After being washed three times, the blots were incubated with peroxidase-conjugated rabbit secondary antibody, anti-mouse immunoglobulin G (1:500 dilution in blocking buffer; Dako A/S) for 1 h. After being washed, the membranes were developed by immersion in Tris-buffered saline containing 0.4 mg of 3,3′-diaminobenzidine (Dojindo Laboratories, Tokyo, Japan) per ml and 0.03% H_2O_2.

RF-3 protein. Cells MP347 (λ *c*I857 lysogen) carrying the ecRF-3 overproduction plasmid pTOSOP (21) were grown in 500 ml of LB medium supplemented with 10 μg of chloramphenicol per ml at 32°C and exposed to 42°C for 90 min. The harvested cells (1 g of cell paste) were ground with alumina and suspended in 10 ml of solution containing 50 mM Tris-Cl (pH 7.8), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mg of DNase I per ml and 2 mM phenylmethylsulfonyl fluoride. After repeated centrifugation for 15 min at 10,000 × *g* and for 4 h at 45,000 × *g*, the supernatant was fractionated by adding solid ammonium sulfate. The crude RF-3 fraction, precipitated between 50 and 85% saturated ammonium sulfate concentrations, was subjected to DEAE-cellulose chromatography, using elution with a linear gradient of 0.1 to 0.45 M potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and 10% glycerol. The fractions containing RF-3 as judged by Coomassie blue staining of proteins after SDS-PAGE as well as by [α-^{32}P]GTP binding were combined and applied to a column of hydroxyapatite. Elution was achieved by using a linear gradient of 0.01 to 0.15 M potassium phosphate buffer containing 2 mM EDTA, 10% glycerol, and 0.5 mM dithiothreitol. The resulting RF-3 preparation was >85% pure, and its protein identity to RF-3 was confirmed by the N-terminal peptide sequence analysis.

Other DNA procedures. Double-stranded or single-stranded DNAs were sequenced by the dideoxynucleotide chain termination method with appropriate synthetic primers and [α-^{32}P]dCTP (31). DNA blot hybridization and plaque hybridization were conducted according to standard methods (30) except that plaque hybridization signals were detected by the nonradioisotope method using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham) as instructed by the manufacturer. The DNA and amino acid sequence comparison was carried out with the BESTFIT or PILEUP program from the Genetics Computer Group GCG program package (10).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with accession number D50496.

RESULTS

Isolation of the *S. typhimurium prfC* gene. *S. typhimurium* ST3 DNA was digested with several restriction enzymes and analyzed by Southern blot hybridization using the 1,084-bp *Sma*I-*Bss*HII DNA probe encoding the internal part of the *E. coli prfC* gene. A 3.0-kb *Sal*I DNA was detected (data not shown). Hence, a λ ZAP genomic library of ST3 DNA was constructed by using *Sal*I fragments ranging in size from 2.5 to 3.5 kb and fractionated by agarose gel electrophoresis. The library was screened with the same 1,084-bp probe, and phage λSRF3 carrying the 3.0-kb *Sal*I fragment was isolated (Fig. 1).

The DNA fragment cloned in λSRF3 was subcloned in plasmid pACYC184 and sequenced. The DNA sequence was very similar to that of the *E. coli prfC* region (Fig. 2). A putative *S. typhimurium prfC* gene encodes the same number of nucleotides and amino acids as the *E. coli* gene. The amino acid sequence is 96.2% (509/529) identical to *E. coli*, and the nucleotide sequence is 87.1% (1,382/1,587) identical in the coding region of *prfC* (Fig. 2). The calculated molecular mass is 59,590 Da.

Plasmid pYK1, which carries a 3.0-kb *Sal*I DNA segment (Fig. 1), was tested for ability to complement the ecRF-3 mutation, using *E. coli* OM315 through OM317, which carry a *prfC* null mutation and nonsense mutations in *lacZ*. Since the *prfC* mutation has the suppressor activities of all three nonsense alleles, strains carrying this mutation are capable of synthesizing severalfold more β-galactosidase than the isogenic *prfC*$^+$ strains. Plasmid pYK1 was introduced into these strains, and the β-galactosidase activities were measured. As shown in Table 2, plasmid pYK1 reduced the β-galactosidase expression and therefore complemented the *E. coli prfC* mutation. Even in the presence of wild-type *prfC* allele in the chromosome, plasmid pYK1 reduced leaky expression of *lacZ* nonsense alleles, but this may have been due to the increased gene dosage (Table 2). The protein synthesized from the recombinant DNA clone reacted with an antibody against ecRF-3 and is therefore structurally similar also (see below). These results led us to conclude that this protein-coding sequence comprises the functional stRF-3 gene as *prfC*.

Functional activity of dnRF-3 protein in *E. coli*. An RF-3 homolog (dnRF-3) encoded by the *D. nodosus* DNA in pJIR730 plasmid is a polypeptide of 531 amino acids with a calculated molecular mass of 59.9 kDa (2). Comparison of the amino acid sequence of this protein with sequences of RF-3 from *E. coli* and *S. typhimurium* showed 62.2 and 62.5% identity, respectively (Fig. 3). A murine polyclonal antibody against ecRF-3 was prepared to analyze structural relationships of these three RF-3 proteins (see Materials and Methods). The resulting antibody detected a single protein band among *E. coli* proteins by immunoblotting, which disappeared in the *prfC* null mutant (Fig. 4, lanes 2 and 6). As expected, both stRF-3 and dnRF-3 proteins reacted with the anti-ecRF-3 antibody. In agreement with their relative similarities to ecRF-3 at the amino acid sequence level, a greater amount of dnRF-3 (62.2% identity) protein than stRF-3 (96.2% identity) protein was needed for detection (Fig. 4, lanes 4 and 8). Under these conditions, heterologous RF-3 proteins exhibited slightly different migrations in SDS-PAGE: ecRF-3 migrates behind dnRF-3 but slightly faster than stRF-3.

When various amounts of bulk proteins in each sample were analyzed by SDS-PAGE and subsequent immunoblotting, the RF-3 level was seen to increase more than 10-fold by transformation with pYK1 (stRF-3), pYK3 (ecRF-3), or pJIR730 (dnRF-3) (Fig. 4). Therefore, the cellular RF-3 level reflects the copy number of *prfC*, suggesting that RF-3 synthesis is not negatively autoregulated.

To test the activity of the dnRF-3 homolog, *E. coli prfC* mutants OM315 through OM317 were transformed with pJIR730, and β-galactosidase activities were measured. As shown in Table 2, the dnRF-3 protein complemented the *E. coli prfC* null mutation and enhanced termination at the three stop codons in *lacZ* as well as its *E. coli* and *S. typhimurium* counterparts (Table 2). These immunological and intergeneric complementation data clearly indicate that the cloned *D. nodosus* DNA produces the active RF-3 protein.

Suppression of *prfB* mutations by multicopy expression of RF-3. It is known that RF-3 stimulates binding of RF-1 or

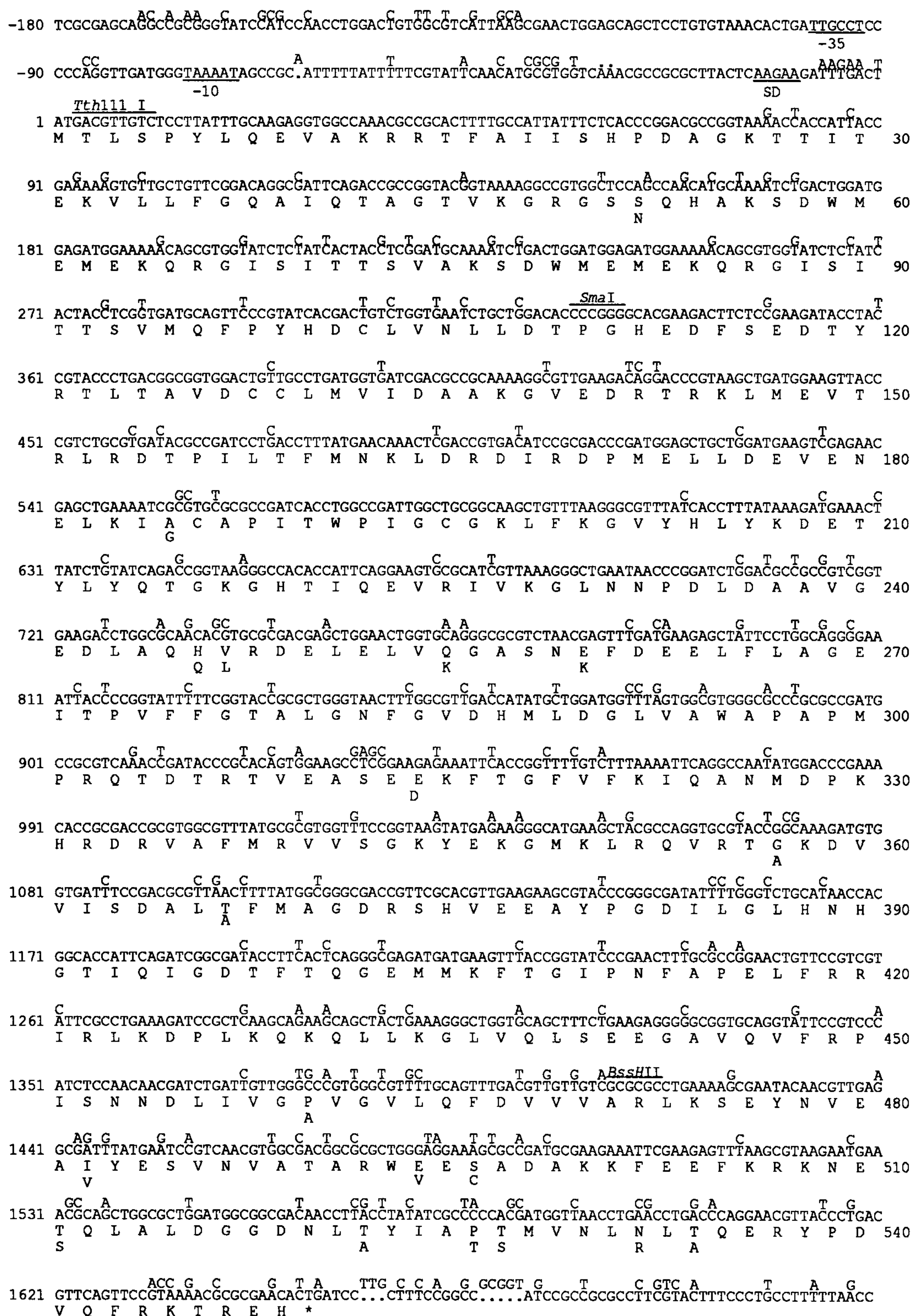

FIG. 2. Nucleotide and deduced amino acid sequences of the *S. typhimurium prfC* gene. Nucleotides and amino acids of the *E. coli prfC* gene that differ from those of *S. typhimurium prfC* are indicated immediately above and below the sequence, respectively. Dots indicate deletion of a base for optimal sequence alignment. Nucleotide and amino acid coordinates are counted from the initiation site of the *prfC* coding sequence. Consensus −35 and −10 sequences of a σ^{70} promoter and a putative ribosomal binding site (SD) are underlined. The restriction enzyme sites used for DNA manipulation are shown.

TABLE 2. Intergeneric complementation of *E. coli prfC*

Plasmid	β-Galactosidase activity[a]					
	prfC^{+} strain			*prfC*Δ2 strain		
	UAG	UAA	UGA	UAG	UAA	UGA
pACYC184	3.3	0.05	118	9.6	0.06	510
pYK1 (stRF-3)	1.2	0.03	52	0.9	0.03	98
pYK3 (ecRF-3)	1.2	0.03	57	0.9	0.03	100
pTZ19R	1.0	0.01	185	5.9	0.01	246
pJIR730 (dnRF-3)	0.2	0.01	42	0.5	0.01	37

[a] Strains DEV1 [*lacZ*(UAG) *prfC*$^{+}$], DEV14 [*lacZ*(UAA) *prfC*$^{+}$], and DEV15 [*lacZ*(UGA) *prfC*$^{+}$] and their *prfC*Δ*2*::*kan* (Km^{r}) transductants, OM315 through OM317, were transformed with derivatives of pACYC184 (Cm^{r}) or pTZ19R (Ap^{r}). Transformants were grown at 37°C in LB medium containing 15 μg of chloramphenicol or 50 μg of ampicillin per ml, 0.5% glycerol, and 1 mM isopropyl-1-thio-β-D-galactoside, and β-galactosidase activities were measured as described by Miller (23). Values are expressed as Miller units (23).

RF-2 to ribosomes and stimulates the rate of peptide chain release in the RF-1- or RF-2-dependent in vitro termination reaction. This finding suggests cooperative binding of RF-3 with RF-1 or RF-2 to ribosomes or a functional interaction between these release factors. Hence, we investigated the effect of multicopy expression of heterologous RF-3 genes on two RF-2 mutations, *prfB286* of *E. coli* (14, 22) and *supK599* of *S. typhimurium* (26). These two alleles are temperature-sensitive lethal at 42°C and suppress UGA mutations. As shown in Table 3, both *prfB286* and *supK599* cells became viable at 42°C

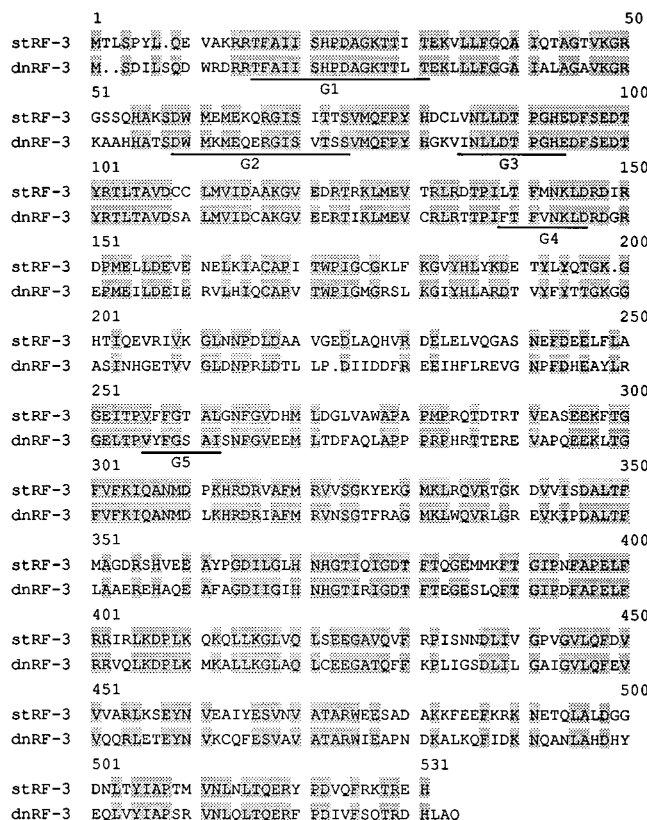

FIG. 3. Amino acid comparison between stRF-3 and dnRF-3 (2). The comparison was done with the BESTFIT program. G-domain motifs, G1 through G5, are shown by bars, and the identical amino acids are shown by shading. Dots indicate deletion of a base for optimal sequence alignment.

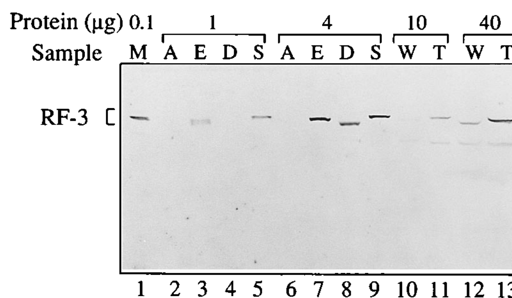

FIG. 4. Immunoblots of heterologous RF-3 proteins. *E. coli* cells transformed with plasmids carrying the heterologous *prfC* genes were grown in LB medium supplemented with 15 μg of chloramphenicol or 50 μg of ampicillin per ml at 32°C. Bulk proteins were separated by SDS-PAGE, and RF-3 proteins synthesized in the transformants were analyzed by immunostaining with an anti-ecRF-3 mouse antibody as described in Materials and Methods. Samples: M, purified ecRF-3 (0.1 μg); A, *E. coli* KM3 (*prfC*Δ*3*) carrying pACYC184; E, *E. coli* KM3 (*prfC*Δ*3*) carrying pYK3 (ecRF-3); D, *E. coli* KM3 (*prfC*Δ*3*) carrying JIR730 (dnRF-3); S, *E. coli* KM3 (*prfC*Δ*3*) carrying pYK1 (stRF-3); W, *E. coli* W3110 (no plasmid); T, *S. typhimurium* ST3 (no plasmid). Lanes: 2 through 5, 1 μg of bulk protein; 6 through 9, 4 μg of bulk protein; 10 and 11, 10 μg of bulk protein; 12 and 13, 40 μg of bulk protein.

upon transformation with either plasmid pYK1 (stRF-3) or plasmid pYK3 (ecRF-3). Consistently, test strains failed to grow or grew only poorly on minimal agar media, revealing that suppression of UGA mutations in *leu* or *his* was diminished or reduced by these plasmids even in the intergeneric background. Moreover, multicopy dnRF-3 (pJIR730) also suppressed intergenerically these RF-2 mutations (Table 3). These results are interpreted as indicating that the reduced activity of mutant RF-2 proteins was compensated for by the increased expression of RF-3. This inference suggests a cooperative interaction between RF-3 and RF-2, which might be prerequisite to the formation of ribosomal termination complexes.

DISCUSSION

In this work, we have isolated and sequenced a gene for RF-3 from *S. typhimurium* and characterized its biological activity together with those of other two relevant RF-3 genes, one from *E. coli* and the other from a pathogenic bacterium, *D.*

TABLE 3. Intergeneric suppression of *prfB* mutations[a]

Plasmid	Growth at 42°C[b]		Growth on minimal plates[c]	
	prfB286	*supK599*	*prfB286*	*supK599*
pACYC184	−	−	+	+
pYK1 (stRF-3)	++	+	−	+/−
pYK3 (ecRF-3)	++	+	−	+/−
pJIR730 (dnRF-3)	+	+/−	−	−
pSRF2 (stRF-2)	++	++	−	−

[a] *E. coli* OM220 [leu(UGA) *prfB286*(Ts)] and *S. typhimurium* GT66 [*his*(UGA) *supK599*(Ts)] cells were transformed with plasmids carrying RF-3 homologs, and the effect of heterogeneous RF-3 expression on RF-2 mutations was examined.

[b] Temperature-sensitive lethality was examined by streaking transformant cells on YT (strain OM220) or LB (strain GT66) agar medium containing 15 μg of chloramphenicol or 50 μg of ampicillin per ml and incubating them at 42°C for 12 h. Symbols: ++, normal growth; +, slow growth; +/−, poor growth; −, no growth.

[c] Suppression of *E. coli leu*(UGA) and *S. typhimurium his*(UGA) alleles was examined by growth of transformants on leucine- or histidine-free minimal agar medium E supplemented with 0.5% glucose and 15 μg of chloramphenicol or 50 μg of ampicillin per ml at 32°C. Symbols: +, growth; +/−, poor growth; −, no growth.

TABLE 4. Comparison of amino acid sequences of regions G1 to G5 in GTP-binding proteins

Protein[a]	Sequence[b]				
	G1 [XOOOOGXXGXGKS]	G2 [D− (X) $_n$ −T]	G3 [OJOODXAGJX]	G4 [OOOONKXD]	G5 [OOXXSAX]
ecRF-3	14 tfaiiSHpDaGKTTit	57 DwmemEkqRGISItts	83 vnllDtPGHe	137 ltfmNKlD	253 vffgTAl
stRF-3	14 tfaiiSHpDaGKTTit	57 DwmemEkqRGISItts	83 vnllDtPGHe	137 ltfmNKlD	253 vffgTAl
dnRF-3	14 tfaiiSHpDaGKTTlt	57 DwmkmEqeRGISVtss	83 vnllDtPGHe	137 ftfnNKlD	252 vyfgSAi
ecEF-G	12 NigisAHiDaGKTTtt	51 DwmeqEqeRGITItsa	84 iniiDtPGHv	138 iafvNKmD	263 vtcgSAf
ecEF-Tu	13 NvgtiGHvDhGKTTlt	50 DnapeEkaRGITInts	76 yahvDcPGHa	131 ivflNKcD	169 ivrgSAl
ecIF2	393 vvtimGHvDhGKTsll	414 tkvasgeagGITqhig	440 itflDtPGHa	494 vvavNKiD	530 fvhvSAk
ecERA	10 fiaivGrpnvGKSTll		58 aiyvDtPGlh	120 ilavNKvD	150 ivpiSAe
scRAS1	12 KiVVVGgGGVGKSALT	39 YDPTIEDSY	60 LDILDTAGQE	119 vvVGNKlD	149 flETSAK
scRAS2	12 KlVVVGgGGVGKSALT	39 YDPTIEDSY	60 LDILDTAGQE	119 vvVGNKsD	149 flETSAK

[a] sc, *Saccharomyces cerevisiae.*

[b] Amino acid sequences of putative regions G1 through G5 in RF-3 or more representatives, plus a consensus sequence for the G domains (3). Numbers represent amino acid residue numbers. Underlining indicates residues conserved in nearly all GTP-binding proteins; capital letters indicate amino acid identities used to align proteins to a particular family. Amino acids are designated according to the single-letter code. In consensus sequences, X indicates any amino acid, whereas O and J represent hydrophobic and hydrophilic residues, respectively.

nodosus. The deduced protein sequence of stRF-3 is highly homologous to that of ecRF-3, while dnRF-3 exhibits less identity (61.4%). Nevertheless, these heterologous RF-3 proteins reacted with a polyclonal antibody against ecRF-3 and showed equally good intergeneric complementation activities in the *prfC* mutant of *E. coli*. Therefore, regardless of differing among one-third of its total amino acid sequence from ecRF-3, the dnRF-3 protein retains subdomains required for peptide chain termination in *E. coli.*

There exist two main regions whose amino acids are highly conserved among the three RF-3 proteins, amino acid positions 14 to 148 and 250 to 470 (Fig. 3). The former represents the guanine nucleotide binding domains G1 through G4. These G domains are well conserved among all GTPase proteins (3), but we find that Ser-68 in the G2 domain of all three RF-3 proteins substitutes for the threonine residue highly conserved in other GTP-binding proteins (Table 4). RF-3 may be the first noted exception to the G2 consensus sequence. In every case, the basic function of G domain is to switch the protein conformation between two alternative states, depending on whether GTP or GDP is bound to the active site. We assume that the G-domain function of RF-3 is also to switch the state of ribosomal termination complexes. Ongoing experiments suggest that a GTP analog stimulates binding of RF-3 to ribosomes (on state), whereas GDP dissociates the complexes (off state) (24). This might reflect the process of termination cycle mediated by GTP hydrolysis.

The latter conserved region of RF-3 proteins, positions 250 to 470, comprises a G5 subdomain and protein part similar to elongation factor EF-G. Ævarsson et al. (1) have solved the three-dimensional structure of *Thermus thermophilus* EF-G and proposed that three subdomains of RF-3 are well conserved among six subdomains of EF-G: subdomains G, G′, and II (Fig. 5). It is noteworthy that subdomain G′ is less conserved than subdomain G among dnRF-3 and ec/stRF-3. Subdomain G′ has been proposed to function as an intrinsic exchange factor modulating the binding of the guanine nucleotides and facilitating their exchange. It remains to be investigated whether RF-3 per se hydrolyzes GTP as efficiently as EF-G even in the absence of other modulating factors. Although the G5 domain is involved in guanine nucleotide binding in the p21^{ras} protein (3), it cannot be unambiguously identified by amino acid sequence in all of the subfamilies of GTPases. Nevertheless, RF-3 conserves the G5 motif 110 amino acids downstream of G4, with the one exception that a threonine residue at position 257 of ecRF-3 and stRF-3 proteins substitutes for the serine residue highly conserved in other GTP-binding proteins. Though the original report by Ævarsson et al. (1) did not demonstrate conservation of domain III in RF-3,

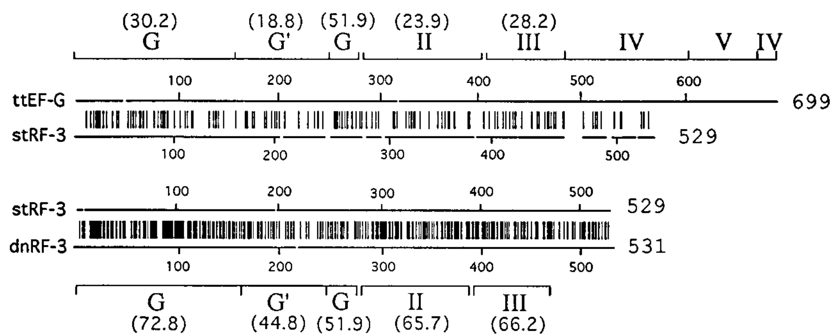

FIG. 5. Schematic structural alignment of elongation factor EF-G and heterologous RF-3 proteins. Structural alignment between stRF-3 and *T. thermophilus* EF-G (ttEF-G) or ecRF-3 was done with the BESTFIT program. Vertical bars represent conserved amino acids. Lines represent polypeptides with some gaps in the alignment. The subdomain regions are indicated at the top and bottom, and the percent polypeptide identities are shown in parentheses. The G domain is strictly conserved. Subdomain G′ has been proposed to function as an intrinsic exchange factor modulating the binding of the guanine nucleotides and facilitating their exchange. Domain II is conserved among translation factors and together with domain G makes a common structural unit possibly responsible for similar interaction with the ribosome. Domain III has been proposed to be involved in the interaction with RNA and seems to be conserved among EF-G and three RF-3 proteins regardless of the argument by Ævarsson et al. (1).

three RF-3 proteins seem to conserve this domain as well (Fig. 5).

The role of RF-3 in peptide chain termination is, at least in part, to stimulate binding of RF-1 and RF-2 to ribosomal termination complexes. The present finding that expression of RF-3 from multicopy plasmids gives rise to suppression of temperature-sensitive mutations in RF-2 supports this idea and is explained by assuming that the mutant RF-2 protein binds ribosomes less efficiently under normal conditions and binds better in the presence of oversynthesized RF-3. In support of this view, we have seen that the *prfB286*-encoded protein shows a reduced ability to bind ribosomes in vitro (35). Consistent with multicopy suppression of *prfB* by RF-3, one class of temperature-resistant revertants isolated from the *prfB286*(Ts) mutant acquired up-promoter mutations in *prfC* (unpublished data). Contrary to *prfB286*, a temperature-sensitive RF-1 mutation, *prfA1*, was not suppressed by multicopy RF-3 plasmids used in this study, but specific amino acid alterations in RF-3 restored the growth of *prfA1* cells (unpublished data). It remains to be investigated whether these different effects of RF-3 overexpression reflect differences in altered protein's activity of RF-1 and RF-2 or a potential preference of UGA stop codon by RF-3 as proposed recently (16). Nevertheless, these observations provide genetic evidence for cooperative and/or physical interaction between RF-3 and RF-2 (as well as RF-1) in the formation of ribosomal termination complexes. Further comparisons of RF-3 homologs from different organisms might facilitate the study of structure-function relationships of RF-3.

ACKNOWLEDGMENTS

We thank Stephen J. Billington and Glenn R. Björk for the bacterial strain and plasmid used in this study, Naohiro Seo for expertise in preparation of murine polyclonal antibodies, and Bradford S. Powell for critical review of the manuscript.

This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan, and the Human Frontier Science Program (awarded in 1993).

REFERENCES

1. **Ævarsson, A., E. Brazhnikov, M. Garber, J. Zheltonosova, Y. Chirgadze, S. Al-Karadaghi, L. A. Svensson, and A. Liljas.** 1994. Three-dimensional structure of the ribosomal translocase: elongation factor G from *Thermus thermophilus*. EMBO J. **13:**3669–3677.
2. **Billington, S. J., B. H. Jost, and J. I. Rood.** 1995. A gene region in *Dichelobacter nodosus* encoding a lipopolysaccharide epitope. Microbiology **141:** 945–957.
3. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
4. **Capecchi, M. R., and H. A. Klein.** 1969. Characterization of three proteins involved in polypeptide chain termination. Cold Spring Harbor Symp. Quant. Biol. **34:**469–477.
5. **Caskey, C. T., W. C. Forrester, W. Tate, and C. D. Ward.** 1984. Cloning of the *Escherichia coli* release factor 2 gene. J. Bacteriol. **158:**365–368.
6. **Caskey, T., E. Scolnick, R. Tompkins, J. Goldstein, and G. Milman.** 1969. Peptide chain termination, codon, protein factor, and ribosomal requirements. Cold Spring Harbor Symp. Quant. Biol. **34:**479–488.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
8. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167:**327–335.
9. **Craigen, W. J., C. C. Lee, and C. T. Caskey.** 1990. Recent advances in peptide chain termination. Mol. Microbiol. **4:**861–865.
10. **Devereux, J., P. Haeberili, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
11. **Elliott, T., and X. Wang.** 1991. *Salmonella typhimurium prfA* mutants defective in release factor 1. J. Bacteriol. **173:**4144–4154.
12. **Epp, C., and M. L. Pearson.** 1976. Association of bacteriophage lambda *N* gene protein with *E. coli* RNA polymerase, p. 667–691. *In* R. Losick and M. Chamberlin (ed.), RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Goldstein, J. L., and C. T. Caskey.** 1970. Peptide chain termination: effect of protein S on ribosomal binding of release factors. Proc. Natl. Acad. Sci. USA **67:**537–543.
14. **Göringer, H. U., K. A. Hijazi, E. J. Murgola, and A. E. Dahlberg.** 1991. Mutations in 16S rRNA that affect UGA (stop codon)-directed translation termination. Proc. Natl. Acad. Sci. USA **88:**6603–6607.
15. **Grentzmann, G., D. Brechemier-Baey, V. Heurgue, L. Mora, and R. H. Buckingham.** 1994. Localization and characterization of the gene encoding release factor RF3 in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **91:**5848–5852.
16. **Grentzmann, G., D. Brechemier-Baey, V. Heurgué-Hamard, and R. H. Buckingham.** 1995. Function of polypeptide chain release factor RF-3 in *Escherichia coli*. RF-3 action in termination is predominantly at UGA-containing stop signals. J. Biol. Chem. **270:**10595–10600.
17. **Kawakami, K., T. Inada, and Y. Nakamura.** 1988. Conditionally lethal and recessive UGA-suppressor mutations in the *prfB* gene encoding peptide chain release factor 2 of *Escherichia coli*. J. Bacteriol. **170:**5378–5381.
18. **Kawakami, K., Y. H. Jönsson, G. R. Björk, H. Ikeda, and Y. Nakamura.** 1988. Chromosomal location and structure of the operon encoding peptide-chain-release factor 2 of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:** 5620–5624.
19. **Kawakami, K., and Y. Nakamura.** 1990. Autogenous suppression of an opal mutation in the gene encoding peptide chain release factor 2. Proc. Natl. Acad. Sci. USA **87:**8432–8436.
20. **Mead, D. A., E. Szczesna-Skorupa, and B. Kemper.** 1986. Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1:**67–74.
21. **Mikuni, O., K. Ito, J. Moffat, K. Matsumura, K. McCaughan, T. Nobukuni, W. Tate, and Y. Nakamura.** 1994. Identification of the *prfC* gene, which encodes peptide-chain-release factor 3 of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **91:**5798–5802.
22. **Mikuni, O., K. Kawakami, and Y. Nakamura.** 1991. Sequence and functional analysis of mutations in the gene encoding peptide-chain-release factor 2 of *Escherichia coli*. Biochimie **73:**1509–1516.
23. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Moffat, J., W. Tate, and Y. Nakamura.** Unpublished data.
25. **Nakamura, Y., S. Mizusawa, D. L. Court, and A. Tsugawa.** 1986. Regulatory defects of a conditionally lethal *nusA*ts mutant of *Escherichia coli*; positive and negative modulator roles of NusA protein in vivo. J. Mol. Biol. **189:**103–111.
26. **Reeves, R. H., and J. R. Roth.** 1971. A recessive UGA suppressor. J. Mol. Biol. **56:**523–533.
27. **Roesser, J. R., Y. Nakamura, and C. Yanofsky.** 1989. Regulation of basal level expression of the tryptophane operon of *E. coli*. J. Biol. Chem. **264:** 12284–12288.
28. **Ryden, M., J. Murphy, R. Martin, L. Isaksson, and J. Gallant.** 1986. Mapping and complementation studies of the gene for release factor 1. J. Bacteriol. **168:**1066–1069.
29. **Ryden, S. M., and L. A. Isaksson.** 1984. A temperature-sensitive mutant of *Escherichia coli* that shows enhanced misreading of UAG/A and increased efficiency for some tRNA nonsense suppressors. Mol. Gen. Genet. **193:**38–45.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Scolnick, E., R. Tompkins, T. Caskey, and M. Nirenberg.** 1968. Release factors differing in specificity for terminator codons. Proc. Natl. Acad. Sci. USA **61:**768–774.
33. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67:**31–40.
34. **Tanabe, K., S. Takasaki, J. Watanabe, A. Kobata, K. Egawa, and Y. Nakamura.** 1989. Glycoproteins composed of major surface immunodeterminants of *Pneumocystis carinii*. Infect. Immun. **57:**1363–1368.
35. **Tate, W., and Y. Nakamura.** Unpublished data.
36. **Tate, W. P., and C. M. Brown.** 1992. Translational termination: "stop" for protein synthesis or "pause" for regulation of gene expression. Biochemistry **31:**2443–2450.
37. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
38. **Weiss, R. B., J. P. Murphy, and J. A. Gallant.** 1984. Genetic screen for cloned release factor genes. J. Bacteriol. **158:**362–364.