Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5798–5802, June 1994
Biochemistry

Identification of the *prfC* gene, which encodes peptide-chain-release factor 3 of *Escherichia coli*

(stop codon/peptide chain termination/release factors/nonsense suppression)

OSAMU MIKUNI*, KOICHI ITO*, JOHN MOFFAT†, KIYOYUKI MATSUMURA*, KIM MCCAUGHAN†, TAKAHIRO NOBUKUNI*, WARREN TATE†, AND YOSHIKAZU NAKAMURA*§

*Department of Tumor Biology, The Institute of Medical Science, The University of Tokyo, P.O. Takanawa, Tokyo 108, Japan; and †Department of Biochemistry, University of Otago, Dunedin, New Zealand

Communicated by Paul Schimmel, January 19, 1994 (received for review October 20, 1993)

ABSTRACT **The termination of protein synthesis in bacteria requires two codon-specific polypeptide release factors, RF-1 and RF-2. A third factor, RF-3, which stimulates the RF-1 and RF-2 activities, was originally identified in *Escherichia coli*, but it has received little attention since the 1970s. To search for the gene encoding RF-3, we selected nonsense-suppressor mutations by random insertion mutagenesis on the assumption that a loss of function of RF-3 would lead to misreading of stop signals. One of these mutations, named *tos-1* (for *t*ransposon-induced *o*pal *s*uppressor), mapped to the 99.2 min region on the *E. coli* chromosome and suppressed all three stop codons. Complementation studies and analyses of the DNA and protein sequences revealed that the *tos* gene encodes a 59,442-Da protein, with sequence homology to elongation factor EF-G, including G-domain motifs, and that the *tos-1* insertion eliminated the C-terminal one-fifth of the protein. Extracts containing the overproduced Tos protein markedly increased the formation of ribosomal termination complexes and stimulated the RF-1 or RF-2 activity in the codon-dependent *in vitro* termination assay. The stimulation was significantly reduced by GTP, GDP, and the β,γ-methylene analog of GTP, but not by GMP. These results fit perfectly with those described in the original publications on RF-3, and the *tos* gene has therefore been designated *prfC*. A completely null *prfC* mutation made by reverse genetics affected the cell growth under the limited set of physiological and strain conditions.**

The termination of translation in bacteria requires two codon-specific peptide-chain-release factors: release factor 1 (RF-1; UAG/UAA-specific) and release factor 2 (RF-2; UGA/UAA-specific) (1). Stop codon recognition by release factors holds considerable interest, since it entails protein–RNA recognition rather than a codon–anticodon (RNA–RNA) interaction, but the mechanism is still unknown (2, 3).

The genes encoding the *Escherichia coli* release factors RF-1 and RF-2 have been isolated (4, 5). The map position of RF-1 (designated *prfA*) is 27 min on the *E. coli* chromosome (6), and RF-2 (designated *prfB*) is at 62 min, within the same operon as the lysyl-tRNA synthetase gene (7). Several mutants of RF-1 and RF-2 have been isolated, and they often cause misreading of stop codons or frameshifting, as well as temperature-sensitive growth of the cells (8–11). Hence the reduced activity of release factors results in several translational errors *in vivo*, and these errors are probably caused by an abnormally long pausing of ribosomes at stop signals (12).

In *E. coli*, a third factor, RF-3, is known to stimulate the activities of RF-1 and RF-2; it binds guanine nucleotides but is not codon-specific (13, 14). This factor has received little attention since its initial characterization. RF-3 was shown to partially correct the very poor binding of RF-1 and RF-2 to L7/L12-depleted ribosomes (15). To investigate the biological significance and function of RF-3 in stop codon recognition, polypeptide release, and cell growth, we aimed to select a partial or complete gene-knockout mutant of RF-3 from nonsense suppressor mutants generated by a transposon randomly jumping into the chromosome. One of these mutations, designated *tos*, affected a gene whose protein product exhibits the reported properties of RF-3. In this article, we describe genetic, sequence,¶ and biochemical evidence that the *tos* protein is RF-3, facilitating a new phase of study on the RF-3 function after two decades of silence.‖

MATERIALS AND METHODS

Bacterial and Phage Strains. Bacterial strains (*E. coli* K-12) used were as follows: LS653 [*leu*(UGA) *lacZ659*(UGA)] (16), OM6 (LS653 *thyA*$^+$), OM240 (OM6 *tos-1*::ΔTn*10*), W3110 (F$^-$ prototroph), OM219 [W3110 *leu*(UGA) *lacZ659*(UGA) *rpsL*], OM298 (OM219 *tos-1*::ΔTn*10*), MC4100 (17), DEV1 [*lacZ105*(UAG)] (18), DEV14 [*lacZ659*(UAA)] (18), and DEV15 [*lacZ*(UGA)] (18). Strain MP347 [λ(*c*I857 ΔH1 *bio252*)] (19) cells were used as host cells to overproduce proteins by heat induction. λNK1098 ("λ hop") carrying a transposable ΔTn*10* element was used for insertion mutagenesis (20). Kohara's λ clone 673 of the ordered genomic library (21) was used as a source of DNA for the wild-type *tos* gene.

Selection of the *tos* Mutant. *E. coli* OM6 [*lacZ*(UGA) *leu*(UGA)] cells were infected with λNK1098 and tetracycline-resistant (Tet^r) colonies were selected on lactose-MacConkey plates containing Tet at 15 μg/ml at 32°C. Red or pink colonies were further scored for growth on minimal E agar plates (22) lacking leucine. One lactose-utilizing (Lac$^+$) leucine-synthesizing (Leu$^+$) colony (OM240) isolated from ≈8 × 10^5 Tet^r transposants was designated *tos* for *t*ransposon-induced *o*pal *s*uppressor and was studied further.

Plasmids. Plasmid pNE30 was constructed by recloning the 10-kb *Bam*HI–*Eco*RI fragment containing the *tos* gene from Kohara's λ clone 673 into the same sites of pSU19 (23). Plasmids pNE36, pNE40, and pNE73 were subclone derivatives from pNE30 and contain relevant restriction fragments (Fig. 1). Plasmid pNE36 contains a single *Xma* I site in the coding sequence of *tos*; it was cleaved by *Xma* I and the ends were repaired with T4 DNA polymerase and ligated. The resulting plasmid, pKM1, does not produce the active Tos protein. Plasmid pTOSOP contains a λP_L promoter segment

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Abbreviations: RF, release factor; Tet, tetracycline; Cm, chloramphenicol; Kan, kanamycin; r, resistant; Lac$^+$, lactose-utilizing; Leu$^+$, leucine-synthesizing.

§To whom reprint requests should be addressed.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. D17724).

‖This work was presented at the EMBO Workshop on Alternate Readings of the Genetic Code, May, 1993, Parnasilla, Ireland.

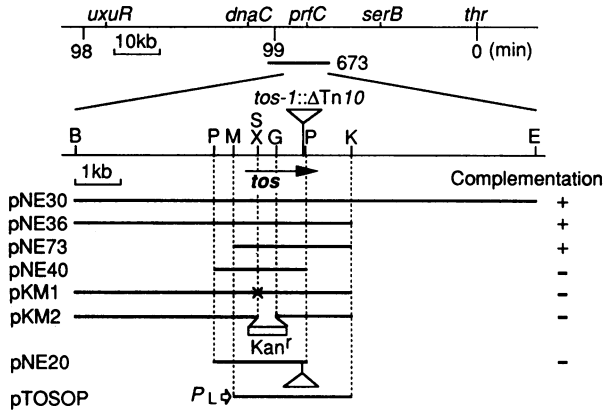

FIG. 1. Chromosomal structure of the *tos* region of *E. coli*. Genetic and physical maps are shown. Heavy lines indicate the bacterial DNA segments cloned in the Kohara phages and plasmids. The arrow represents the location and orientation of the *tos* gene, and the triangle indicates the site of ΔTn*10* insertion. The arrowhead indicates the λP_L promoter. The open box represents insertion of the Kan^r cassette. Only relevant restriction sites are included: B, *Bam*HI; P, *Pst* I; M, *Mlu* I; X, *Xma* I; S, *Sma* I; G, *Bgl* II; K, *Kpn* I; E, *Eco*RI. To test complementation activities of plasmids, OM298 cells [*lacZ659*(UGA) *leu*(UGA) *tos-1*::ΔTn*10*] were transformed with the indicated plasmids and their β-galactosidase levels and/or growth on a leucine-free minimal agar plate were examined. + denotes complementation (i.e., Lac$^-$ Leu$^-$ phenotype), and − denotes no complementation (i.e., Lac$^+$ Leu$^+$ phenotype).

immediately upstream of *tos* (see Fig. 1). The plasmid pKM2 substituted a kanamycin resistance (Kan^r) cassette for the 0.4-kb *Sma* I–*Bgl* II sequence of plasmid pNE36 (*tos*-Δ2::*kan*; see Fig. 1).

***In Vivo* Cloning of *tos-1*::ΔTn*10*.** *E. coli* strain OM240 (*tos-1*::ΔTn*10*) lysogenic for Mu *c*ts62 phage (24) was transformed with the pIT134 plasmid carrying a mini-Mu replicon (25) and used to prepare phage lysates by heat induction. Plasmid pNE16 was isolated from these lysates as a Tet^r plasmid as described previously (24, 25); it contained *tos-1*::ΔTn*10* and flanking sequences. The Tet^r 5-kb *Pst* I fragment was subcloned to pUC119 (26), giving rise to pNE20 (see Fig. 1).

Crude Extract of Overproduced Tos Protein. MP347 cells carrying pTOSOP (*tos*) were grown in 500 ml of LB medium (22) supplemented with chloramphenicol (Cm) at 10 μg/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·HCl (pH 7.8), 0.1 M KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, DNase I at 1 μg/ml, and 2 mM phenylmethylsulfonyl fluoride. After centrifugation for 15 min at 10,000 × *g* and for 4 hr at 45,000 × *g*, the supernatant was fractionated by adding solid ammonium sulfate. The proteins precipitated between 50% and 85% saturated ammonium sulfate were dissolved in 1 ml of solution containing 50 mM Tris·HCl (pH 7.8), 0.1 M KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol and were dialyzed against the same solution. A control extract for the *in vitro* activity assays was prepared in the same way from MP347 cells without the pTOSOP plasmid.

***In Vitro* Termination Assay.** Because of the extreme lability of overexpressed Tos protein, we employed the 50–85% saturated ammonium sulfate fraction of Tos for the *in vitro* termination assay as described previously (27). The substrate complex containing f[^{3}H]Met-tRNA in the ribosomal P site was prepared by incubating 50 pmol of 70S ribosomes, 2.5 nmol of AUG, and 25 pmol of f[^{3}H]Met-tRNA in 50 μl containing 20 mM Tris·HCl (pH 7.5), 0.15 M NH_4Cl, and 10 mM $MgCl_2$ at 30°C for 30 min. To detect termination, 5 μl of substrate was incubated with or without 200 ng of RF-1 and/or 5 μg of crude Tos protein in 50 μl containing 50 mM Tris acetate (pH 7.2), 50 mM KCl, and 30 mM magnesium acetate, for 30 min at 20°C. To terminate the reaction 200 μl of 0.1 M HCl was added, and free f[^{3}H]Met was extracted into 1 ml of ethyl acetate and the radioactivity was determined.

Termination Complex Formation. The effect of Tos protein on incorporation of RF-2 into a termination complex was determined by adding the 50–85% saturated ammonium sulfate fraction of Tos or control extract and 75 pmol of [^{35}S]methionine-labeled RF-2 (20 cpm/pmol) to 200 μl containing 50 pmol of 70S ribosomes, 2 nmol of UAA, 20 mM Tris·HCl (pH 7.5), 0.1 M NH_4Cl, 20 mM $MgCl_2$, and 4% (vol/vol) ethanol. After 10 min at 4°C the ribosomes were pelleted by centrifuging at 95,000 rpm for 15 min in a Beckman Airfuge, and [^{35}S]RF-2 in the pellet and supernatant fractions was determined.

RESULTS

Transposon-Induced Nonsense Suppressor. Assuming that a loss-of-function mutation of RF-3 results in misreading of stop codons, we selected UGA suppressor mutants by random insertion mutagenesis using "λ hop" carrying ΔTn*10* transposon. OM6 [*lacZ*(UGA) *leu*(UGA)] cells were infected with λNK1098 and one Lac$^+$ Leu$^+$ Tet^r colony (OM240) was isolated. P1 phages grown on this mutant successfully cotransduced into strain OM6 the capacities for Lac$^+$ and Leu$^+$ along with resistance to Tet. These markers did not segregate during crosses (320/320), suggesting that the ΔTn*10* transposon insertion is solely responsible for UGA suppression. Hence, this mutation was named *tos-1*::ΔTn*10* (for *t*ransposon-induced *o*pal *s*uppressor).

The *tos-1*::ΔTn*10* allele was transduced into three isogenic strains carrying UAG, UAA, and UGA mutations in *lacZ*, and the β-galactosidase levels in these transductants were measured *in vitro*. [The *lacZ*(UGA) allele used was a leaky opal mutation.] The *tos-1* mutation stimulated the misreading of all three stop codons 2- to 3-fold, although the relative leakiness of the respective stop codons remained unchanged (Table 1). To test codon specificity in suppression, the *tos-1*::ΔTn*10* mutation was introduced into *trpA* strains containing several nonsense or missense (i.e., UGU, UGC, CGA, AGA, and UGG) mutations at codon position 211 or 234 (ref. 28). However, none of the missense *trpA* alleles were suppressed by *tos-1* (data not shown). These results suggested that the *tos-1* mutation affects the termination rather than the elongation of protein synthesis.

***In Vivo* Cloning and Detailed Mapping of the *tos* Mutation.** The *tos-1*::ΔTn*10* insertion mutation in OM240 was cloned *in vivo* by using the mini-Mu plasmid system. One of such plasmid derivatives, pNE20, which carries a 5-kb Tet^r *Pst* I segment, was radiolabeled with [α-^{32}P]dCTP and hybridized with the Kohara miniset genomic filter (supplied by Takara Shuzo, Tokyo) for physical mapping. The genomic DNA carried on pNE20 hybridized to clones 673 and 674 (data not shown). The *E. coli* DNAs in these clones overlap each other and are located at 99.2 min (4690-kb position) on the chro-

Table 1. Suppression of nonsense *lacZ* mutations by *tos-1*

Strain	β-Galactosidase activity, units		
	lacZ(UAG)	*lacZ*(UAA)	*lacZ*(UGA)
tos$^+$	14	0.7	93
tos-1::ΔTn*10*	32	3	277

Strains DEV1 [*lacZ*(UAG)], DEV14 [*lacZ*(UAA)], DEV15 [*lacZ*(UGA)], and their *tos-1*::ΔTn*10* transductants were grown at 37°C in LB medium supplemented with 0.5% glycerol and 1 mM isopropyl 1-thio-β-D-galactoside, and β-galactosidase activities were determined as described (22). The values are expressed as Miller units (22).

mosome (see Fig. 1). Consistent with the physical mapping data, the Tet[r] marker in OM240 was cotransducible by P1 phage with the *thr-3091*::Tn*10kan* transposon located at 0 min (CAG18425; ref. 29) at a frequency of 16% (31/200).

The genomic DNA in Kohara's clone 673 was digested with several restriction enzymes and examined by Southern blot hybridization analysis using the ^{32}P-labeled 5-kb fragment from pNE20 as a probe. The 10-kb *Bam*HI–*Eco*RI DNA, one of the positive fragments, was subcloned in plasmid pSU19. The resulting plasmid pNE30 and its derivative pNE36 abolished the UGA-suppressor activity of *tos-1* upon transformation of strain OM298 [*leu*(UGA) *lacZ*(UGA) *tos-1*::ΔTn*10*] (Fig. 1). On the other hand, the plasmid pNE40 carrying the 2-kb *Pst* I fragment or the plasmid pKM2 (*tos*-Δ2::*kan*), which substituted the Kan[r] segment for the 0.4-kb *Sma* I–*Bgl* II segment of pNE36, failed to complement the mutation (see Fig. 1). Finally, we found that plasmid pNE73 carrying the 2.6-kb *Mlu* I–*Kpn* I segment fully complements *tos-1* (Fig. 1). The restriction enzyme analysis of the pNE20 DNA revealed that the ΔTn*10* transposon inserted adjacent to the *Pst* I site (data not shown; see Fig. 1). These results indicated that the wild-type *tos* activity is encoded within the sequence between *Mlu* I and *Kpn* I, and the ΔTn*10* transposon truncates this activity.

Nucleotide Sequence of the *tos* Gene. Using several subclone derivatives from pNE36, we determined 2.5 kb of DNA sequence at the *Pst* I–*Kpn* I region. An open reading frame (ORF) encoding a 59,574-Da protein was identified at this region (Fig. 2). Eight base pairs upstream of this ORF, there is a canonical Shine–Dalgarno sequence (AAGAAG). The transcription start site was determined by primer extension analysis at the adenine nucleotide 56 bp upstream of the translation start site, which is preceded immediately by canonical σ^{70} promoter elements (−10 and −35 motifs; see Fig. 2). Ten base pairs downstream of the stop codon there is a potential ρ-independent termination signal (see Fig. 2). The mutant DNA sequence of pNE20 contained the ΔTn*10* insertion at nucleotide position 1278 from the predicted start codon (see Fig. 2). Therefore, taking the genetic complementation data (Fig. 1) into consideration, we concluded that the deduced sequence corresponds to the *tos* gene. The protein encoded by the ORF is predicted to contain 529 amino acids, and the *tos-1*::ΔTn*10* insertion eliminated 104 amino acids at the C terminus of the protein.

The proteins encoded by the plasmids were analyzed by the *in vitro* coupled transcription/translation system. Consistent with the sequence data, plasmids pNE30 and pNE36 synthesized a protein of ≈60 kDa, whereas the plasmid pNE20 carrying *tos-1*::ΔTn*10* synthesized a shortened product with a molecular mass of ≈48 kDa (data not shown). The *tos*-encoded protein was overproduced from plasmid pTOSOP, partially purified by DEAE-cellulose and hydroxyapatite column chromatography (procedures will be published elsewhere), and subjected to N-terminal protein sequence analysis. The deduced peptide sequence was TLSPYLQEV, which agreed with the predicted translation start site, provided that the N-terminal methionine was removed post-translationally. Therefore, the mature Tos protein consists of 528 amino acids and has a molecular mass of 59,442 Da.

Tos protein has significant sequence homology with *E. coli* elongation factor EF-G and other translation factors (such as IF2 or EF-Tu) that contain G domains. Tos and EF-G have 95 identical amino acids (28% identity) and 72 conservative amino acids in the N-terminal 336 amino acid overlap including the G-domain motifs (see Fig. 2; data not shown), suggesting how the protein might be involved in translation.

Complete Gene Knockout Experiment. The fact that the *tos-1*::ΔTn*10* mutation eliminates the C-terminal one-fifth of the polypeptide without affecting cell viability suggested that the *tos* gene itself is dispensable. This assumption was tested by introducing a *tos* deletion, *tos*-Δ2::*kan* (pKM2; see Fig. 1), into the chromosome from plasmids by targeted recombination. The *tos*-Δ2::*kan* construct substituted a Kan[r] cassette

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   1 CTGCAGCGGATGTTTCTTGATTACAGCGTCACCTTTACCGCTGAAGATTTTCAGGACTATCAGGCCGTTAACAAGCCACTGTGGGTGGATTATCAAAACGGCGCGATCACTTCATTACAGCTTCA
 126 GCACGGGCGGTTTGAGAGCTGGGCCGAACGGCTGAACGTCGAGCCAGGTAAACTCAACGAAGCCTTTATTAATGCGATGGCGGAAATCTGCACGCCGCTGCCGGGCGCGGTTTCTCTGCTTAACG
 251 CCATTCGTGGCAACGCCAAAATCGGCATCATCACCAACGGCTTTAGTGCCTTGCAACAGGTGCGTCTGGAACGCACGGGCCTGCGTGATTACTTCGATTTGCTGGTGATTTCCGAAGAAGTTGGC
 376 GTTGCCAAACCGAATAAGAAAATTTTCGATTATGCGCTGGAACAGGCGGGCAATCCTGACCGTTCACGCGTGCTGATGGTTGGCGACACTGCCGAGTCCGATATTCTCGGTGGCATCAACGCCGG
 501 GCTTGCGACCTGCTGGCTGAATGCACACCATCGCGAGCAACCAGAAGGCATCGCGCCCACCTGGACCGTTTCTTCGTTGCACGAACTGGAGCAGCTCCTGTGTAAACACTGATTGCCTCCCCCCC
                                                                                                                       -35
 626 GTTGATGGGTAAAATAGCCGCAATTTTTCGTTTTCAACAAGCGCGGCGCGATGCCGCTTACTCAAGAAGAAAGAATTATGACGTTGTCTCCTTATTTGCAAGAGGTGGCGAAGCGCCGCACTTTT
                -10      *                                     SD          M  T  L  S  P  Y  L  Q  E  V  A  K  R  R  T  F    16
 751 GCCATTATTTCTCACCCGGACGCCGGTAAGACTACCATCACCGAGAAGGTGCTGCTGTTCGGACAGGCCATTCAGACCGCCGGTACAGTAAAAGGCCGTGGTTCCAACCAGCACGCTAAGTCGGA
     A  I  I  S  H  P  D  A  G  K  T  T  I  T  E  K  V  L  L  F  G  Q  A  I  Q  T  A  G  T  V  K  G  R  G  S  N  Q  H  A  K  S  D    58
 876 CTGGATGGAGATGGAAAAGCAGCGTGGGATCTCCATTACTACGTCTGTGATGCAGTTTCCGTATCACGATTGCCTGGTTAACCTGCTCGACACCCCGGGGCACGAAGACTTCTCGGAAGATACCT
     W  M  E  M  E  K  Q  R  G  I  S  I  T  T  S  V  M  Q  F  P  Y  H  D  C  L  V  N  L  L  D  T  P  G  H  E  D  F  S  E  D  T  Y 100
1001 ATCGTACCCTGACGGCGGTGGACTGCTGCCTGATGGTTATCGACGCCGCAAAAGGTGTTGAAGATCGTACCCGTAAGCTGATGGAAGTTACCCGTCTGCGCGACACGCCGATCCTCACCTTTATG
      R  T  L  T  A  V  D  C  C  L  M  V  I  D  A  A  K  G  V  E  D  R  T  R  K  L  M  E  V  T  R  L  R  D  T  P  I  L  T  F  M  141
1126 AACAAACTTGACCGTGATATCCGCGACCCGATGGAGCTGCTCGATGAAGTTGAGAACGAGCTGAAAATCGGCTGTGCGCCGATCACCTGGCCGATTGGCTGCGGCAAGCTGTTTAAAGGCGTTTA
     N  K  L  D  R  D  I  R  D  P  M  E  L  L  D  E  V  E  N  E  L  K  I  G  C  A  P  I  T  W  P  I  G  C  G  K  L  F  K  G  V  Y 183
1251 CCACCTTTATAAAGACGAAACCTATCTCTATCAGAGCGGTAAAGGCCACACCATTCAGGAAGTCCGCATTGTTAAAGGGCTGAATAACCCGGATCTCGATGCTGCGGTTGGTGAAGATCTGGCAC
      H  L  Y  K  D  E  T  Y  L  Y  Q  S  G  K  G  H  T  I  Q  E  V  R  I  V  K  G  L  N  N  P  D  L  D  A  A  V  G  E  D  L  A  Q 225
1376 AGCAGCTGCGTGACGAACTGGAACTGGTGAAAGGCGCGTCTAACGAGTTCGACAAAGAGCTGTTCCTTGCGGGCGAAATCACTCCGGTATTCTTCGGTACTGCGCTGGGTAACTTCGGCGTCGAT
      Q  L  R  D  E  L  E  L  V  K  G  A  S  N  E  F  D  K  E  L  F  L  A  G  E  I  T  P  V  F  F  G  T  A  L  G  N  F  G  V  D   266
1501 CATATGTTGGATGGCCTGGTGGAGTGGGCACCTGCGCCGATGCCGCGTCAGACTGATACCCGTACCGTAGAAGCGAGCGAAGATAAATTTACCGGCTTCGTATTTAAAATTCAGGCCAACATGGA
      H  M  L  D  G  L  V  E  W  A  P  A  P  M  P  R  Q  T  D  T  R  T  V  E  A  S  E  D  K  F  T  G  F  V  F  K  I  Q  A  N  M  D 308
1626 CCCGAAACACCGCGACCGCGTGGCGTTTATGCGTGTGGTGTCCGGTAAATATGAAAAAGGCATGAAACTGCGCCAGGTGCGCACTGCGAAAGATGTGGTGATCTCCGACGCGCTGACCTTTATGG
      P  K  H  R  D  R  V  A  F  M  R  V  V  S  G  K  Y  E  K  G  M  K  L  R  Q  V  R  T  A  K  D  V  V  I  S  D  A  L  T  F  M  A 350
1751 CGGGTGACCGTTCGCACGTTGAAGAAGCGTATCCGGGCGATATCCTCGGCCTGCACAACCACGGCACCATTCAGATCGGCGACACCTTTACCCAGGGTGAGATGATGAAGTTCACCGGTATTCCG
      G  D  R  S  H  V  E  E  A  Y  P  G  D  I  L  G  L  H  N  H  G  T  I  Q  I  G  D  T  F  T  Q  G  E  M  M  K  F  T  G  I  P   391
1876 AACTTCGCACCAGAACTGTTCCGTCGTATCCGCCTGAAAGATCCGCTGAAGCAAAAACAGCTGCTCAAAGGGCTGGTACAGCTTTCCGAAGAGGGCGCGGTGCAGGTGTTCCGTCCAATCTCCAA
      N  F  A  P  E  L  F  R  R  I  R  L  K  D  P  L  K  Q  K  Q  L  L  K  G  L  V  Q  L  S  E  E  G  A  V  Q ↑V  F  R  P  I  S  N   433
2001 CAACGATCTGATCGTTGGTGCAGTTGGTGTGCTGCAGTTTGATGTGGTGGTAGCGCGCCTGAAGAGCGAATACAACGTTGAAGCAGTGTATGAGTCAGTCAACGTTGCCACTGCCCGCTGGGTAG
      N  D  L  I  V  G  A  V  G  V  L  Q  F  D  V  V  V  A  R  L  K  S  E  Y  N  V  E  A  V  Y  E  S  V  N  V  A  T  A  R  W  V  E 475
2126 AATGTGCAGACGCGAAGAAATTCGAAGAGTTCAAGCGTAAGAACGAAAGCCAACTGGCGCTTGATGGCGGCGATAACCTCGCTTACATCGCTACCAGCATGGTCAACCTGCGCCTGGCACAGGAA
      C  A  D  A  K  K  F  E  E  F  K  R  K  N  E  S  Q  L  A  L  D  G  G  D  N  L  A  Y  I  A  T  S  M  V  N  L  R  L  A  Q  E   516
2251 CGTTATCCGGACGTTCAGTTCCACCAGACCCGCGAGCATTAATTCTTGCCTCCAGGGCGCGGTAGCCGCTGCGCCCTGTCAATTTCCCTTCCTTATTAGCCGCTTACGGAATGTTCTTAAAACAT
      R  Y  P  D  V  Q  F  H  Q  T  R  E  H  END                 ------->     <-------
2376 TCACTTTTGCTTATGTTTTCGCTGATATCCCGAGCGGTTTCAAAATTGTGATCTATATTTAACAAAGTGATGACATTTCTGACGGCGTTAAATACCGTCAATACGT
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FIG. 2. Nucleotide and deduced amino acid sequences of the *E. coli tos* gene. The nucleotide position is counted from the *Pst* I site and the amino acid position is counted from the initiation site of the *tos* coding sequence. Consensus −35 and −10 sequences of a σ^{70} promoter and a putative ribosomal binding site (SD) are underlined. The underlined amino acids denote the N-terminal protein sequence determined in this study. G-domain motifs are shown by boxes. The putative ρ-independent terminator signal is indicated by arrows of a dyad symmetry. The transcription start site determined by primer extension analysis is shown by the asterisk. The vertical arrow represents the site of the *tos-1*::ΔTn*10* insertion at nucleotide position 1980.

for the 0.4-kb *Sma* I–*Bgl* II fragment of plasmid pNE36, eliminating the internal one-fourth of the Tos protein including the G domains (see Fig. 2), thereby inactivating the gene completely. When OM6 cells [*lacZ*(UGA) *leu*(UGA)] were transformed with the wild-type and mutant plasmids, Lac^+ Leu^+ suppressor colonies appeared at high frequencies (i.e., 10^{-4}) from transformants with pKM2 compared with those from pNE36, which generated Lac^+ Leu^+ colonies at the spontaneous reversion rate ($\approx 10^{-6}$). P1 phages grown on these Lac^+ Leu^+ colonies cotransduced the UGA suppressor activity to OM6 with Kan^r. The chromosomal DNAs of these Kan^r UGA-suppressor transductants were examined by polymerase chain reaction using synthetic primers, and it was confirmed that the *tos*-Δ2::*kan* construct had substituted for the wild-type *tos* sequence (data not shown).

The W3110 strain having the *tos*-Δ2 mutation was viable under any growth conditions tested; however, the generation time was slightly but significantly increased (Table 2). On the other hand, this null mutation caused severe defects in cell growth of MC4100 in Bacto tryptone YT medium at high temperatures, and the mutant cells failed to form colonies on glucose-minimal solid medium at 42°C (Table 2). Although the reason underlying the different growth phenotypes remains to be clarified, these results suggested that the Tos protein participates in the cell growth at least under the limited set of conditions.

***In Vitro* Stimulation of Peptide Chain Release.** We increased the expression of the *tos* gene by introducing the strong, inducible P_L promoter of λ phage to make pTOSOP. When P_L was induced, Tos constituted a quarter of the total protein 1.5 hr after induction at 42°C (Fig. 3*A*), and the majority of the protein was present in the soluble form upon cell lysis. Tos-overexpressed extracts were further fractionated by stepwise precipitation with ammonium sulfate, and the 50–85% saturated ammonium sulfate fraction was used in the *in vitro* termination assay to determine whether it would stimulate RF-1 and RF-2 activities. This partially purified extract markedly stimulated the rate of f[^{3}H]Met release in the UAG-dependent RF-1 reaction (Fig. 3*B*), while the control extract from the plasmid vector without the *tos* gene did not enhance the reaction. Peptide release by both RF-1 and RF-2 was stimulated in a codon-specific manner; no release was observed with RF-1 in the presence of UGA or with RF-2 and UAG (not shown). These stimulatory effects by Tos were further enhanced by limiting the dose of the termination codon, UAG, *in vitro* (Fig. 3*C*), suggesting that Tos increases the formation of termination complexes. Indeed, the effect of Tos on *in vitro* termination was more dramatic when the formation of UAA-dependent termination complexes was examined directly by monitoring the interaction of [^{35}S]RF-2 with ribosomes (Fig. 3*D*). The proportion of ribosomes occupied by RF-2 increased in a linear fashion from 25% to 50% as the partially purified Tos was titrated into the stoichiometric assay. The activity of the Tos protein in these assays disappeared very rapidly at 50°C, suggesting that Tos is quite heat labile (data not shown). These properties of Tos

Table 2. Generation time of null *tos* mutants

Medium	Temp., °C	Generation time, min			
		W3110	W3110*tos*-Δ2	MC4100	MC4100*tos*-Δ2
YT	30	58	70	59	71
	42	41	59	50	218
ME	30	106	110	118	259
	42	90	105	230	NG

YT medium (9) contained 1% Bacto tryptone, 0.1% yeast extract, and 0.25% NaCl, and ME was minimal medium E (22) supplemented with 0.5% glucose and arginine at 50 μg/ml. NG, no growth.

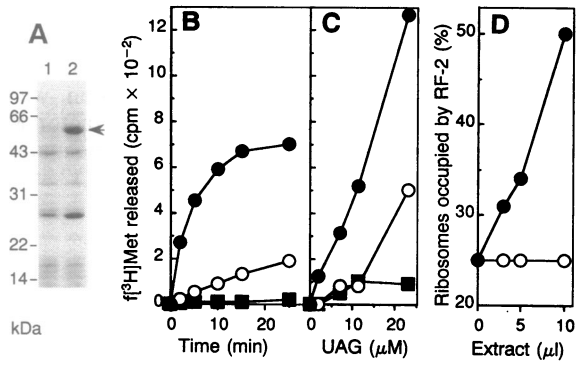

FIG. 3. Protein expression and effect of Tos protein on *in vitro* activity of RF-1 and RF-2. MP347 cells with or without pTOSOP (*tos*) plasmid were grown, proteins were overexpressed by P_L induction at 42°C, and the 50–85% saturated ammonium sulfate fraction was prepared. (*A*) Overexpression of the Tos protein. Lysates of pTOSOP-bearing cells before or after heat induction were prepared and subjected to $NaDodSO_4$/PAGE followed by Coomassie blue staining. Lane 1, 32°C; lane 2, 42°C. Sizes of marker proteins run in parallel are represented at left and the Tos protein is marked by the arrow. (*B*) Effect on rate of UAG-dependent fMet release by RF-1. f[^{3}H]Met release from the [f[^{3}H]Met-tRNA^{fMet}-AUG-ribosome] was determined at the indicated times as described in the text. Reaction mixtures contained 20 μM UAG, RF-1 (○), Tos (■), or RF-1 plus Tos (●). Background counts of 400 cpm were subtracted. (*C*) Stimulation of f[^{3}H]Met release by Tos at limiting codon concentrations. Reaction mixtures contained the indicated amounts of UAG trinucleotide, RF-1 (○), Tos (■), or RF-1 plus Tos (●). Background counts of 400 cpm were subtracted. (*D*) Incorporation of ^{35}S-labeled RF-2 protein into termination complexes with 70S ribosomes and UAA in the presence of the indicated amounts of Tos (●) or control (○) extract.

are consistent, not only qualitatively but also quantitatively, with the published characteristics of RF-3.

Guanine Nucleotide Inhibition. Another unique feature of RF-3 is that GTP causes it to lose its stimulatory effect on termination (13). This was confirmed for Tos in the *in vitro* termination reaction when it was assayed in the presence or absence of GTP (Fig. 4*A*) and for binding of [^{35}S]RF-2 to the ribosome (data not shown). Furthermore, previous studies of RF-3 have shown that this effect is not limited to GTP; GDP and the nonhydrolyzable analog guanosine 5′-[β,γ-methylene]triphosphate also inhibit the stimulation, whereas GMP and nonguanine nucleotides have no effect. Fig. 4*B* shows that this rather unusual profile of activity is reproduced with the Tos protein.

The Tos protein has consensus G-domain motifs in the N-terminal region, similar to other G proteins (see Fig. 2). It

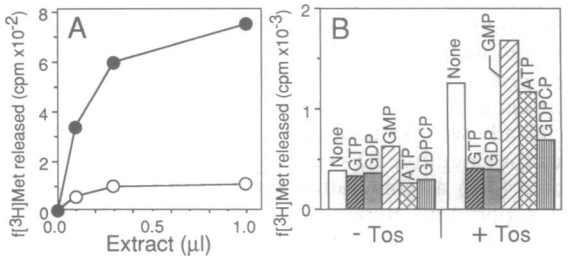

FIG. 4. Effect of GTP and nucleotide analogs on the activity of Tos protein *in vitro*. (*A*) Effect of GTP on release-stimulating activity. The indicated amounts of Tos extract were added to the RF-1 *in vitro* termination assay as described in the text, in the presence (○) or absence (●) of GTP (1 mM). (*B*) Comparison of effects of nucleotides on release-stimulating activity. Reaction mixtures contained 20 μM UAG, RF-1 with or without Tos protein, and 100 μM indicated nucleotide or analog. GDPCP, guanosine 5′-[β,γ-methylene]triphosphate. Background counts (400 cpm) obtained in the absence of RF-1 were subtracted.

retains all conserved amino acids. In fact, overproduced Tos protein bound [α-^{32}P]GTP by UV crosslinking and by a filter assay (data not shown). Taking this information and the other genetic and biochemical results into consideration, we conclude that the *tos* gene encodes the release factor RF-3. Hence, the gene has been designated *prfC* and the mutations *tos-1* and *tos*-Δ2 are designated *prfC1* and *prfC*Δ2, respectively.

DISCUSSION

The biological significance of RF-3 in protein synthesis has been a long-standing puzzle. 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 (13, 14). These investigators showed that RF-3 stimulated the formation of ribosomal termination complexes and that stimulation was abolished by GTP or GDP. However, the genetic or biological significance of this factor was not resolved by the initial studies or subsequent studies of the 1970s (13, 14, 30). In the present study, we have successfully identified a gene, originally named *tos*, for RF-3 and designated it *prfC*, consistent with the previous designations of *prfA* and *prfB* for RF-1 and RF-2, respectively.

The Tos protein (i.e., cloned RF-3) markedly stimulated the rate of fMet release in the RF-1- or RF-2-dependent *in vitro* termination reaction. The activity exhibited the same profile of inhibition by guanine nucleotides as reported for RF-3. Goldstein and Caskey (30) proposed that RF-3 alone stimulates the formation of ribosomal termination complexes, while in the presence of GTP or GDP, RF-3 facilitates dissociation of this intermediate, thus catalyzing the cycling of RF. The stimulation of release was greater at low codon concentration, and the enhancement of termination complex formation was more directly demonstrated than in previous studies by measuring the incorporation of ^{35}S-labeled RF-2 into ribosomal termination complexes. These data are completely consistent with those reported previously, and they provide solid biochemical evidence for the identity of the gene product as RF-3. The activity of Tos as monitored by GTP binding or stimulation of fMet release was drastically reduced upon purification or storage, indicating that overproduced RF-3 protein is unstable.

A gene, *miaD*, whose disruption mutant, *miaD*::ΔTn*10kan*, restores a leaky *miaA*(UAA) mutation has been mapped at the 99-min region (31). The *miaA* gene product mediates the first step of 2-methylthio-N^6-(Δ^2-isopentenyl) adenosine-37 (ms^2i^6A-37) synthesis that is required for the tRNA modification. Connolly and Winkler (31) have speculated that *miaD* causes a decrease in ms^2i^6A-37 tRNA demodification or an increase in *miaA* gene expression posttranscriptionally. We assume that the *miaD* mutation occurs in the *prfC* gene and the reduced level of RF-3 results in suppression of a leaky *miaA*(UAA) mutation.

Originally we also undertook another genetic approach to screen for a putative RF-3 gene among suppressor mutations selected from a temperature-sensitive RF-2 strain. One of these suppressor mutations coincided with *tos-1* (unpublished). This evidence encouraged us to study further the *tos-1* mutation and its gene product to determine whether it was indeed RF-3. Several observations in our laboratory now provide evidence that the activities of mutant RF-1 or RF-2 proteins can be compensated for by altering or overexpressing the RF-3 protein in *E. coli* (K.M., O.M., and Y.N., unpublished data). Further experiments along these lines are necessary to investigate the functional and physical interactions between RF-3 and RF-1 or RF-2.

We thank M. Yamasaki for N-terminal protein sequencing, R. Buckingham for unpublished information, M. Murgola for *trpA* strains, and C. Prescott for DEV strains. This work was supported in part by grants from the Ministry of Education, Science and Culture, Japan (Y.N.), the Human Frontier Science Program (awarded in 1993; Y.N. and W.T.), the Health Research Council, New Zealand (W.T.), and the Howard Hughes Medical Institute (W.T. is an International Research Scholar).

1. Scolnick, E., Tompkins, R., Caskey, T. & Nirenberg, M. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 768–774.
2. Tate, W. P. & Brown, C. M. (1992) *Biochemistry* **31**, 2443–2450.
3. Craigen, W. J., Lee, C. C. & Caskey, C. T. (1990) *Mol. Microbiol.* **4**, 861–865.
4. Weiss, R. B., Murphy, J. P. & Gallant, J. A. (1984) *J. Bacteriol.* **158**, 362–364.
5. Caskey, C. T., Forrester, W. C., Tate, W. & Ward, C. D. (1984) *J. Bacteriol.* **158**, 365–368.
6. Ryden, M., Murphy, J., Martin, R., Isaksson, L. & Gallant, J. (1986) *J. Bacteriol.* **168**, 1066–1069.
7. Kawakami, K., Jönsson, Y. H., Björk, G. R., Ikeda, H. & Nakamura, Y. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5620–5624.
8. Rydén, S. M. & Isaksson, L. A. (1984) *Mol. Gen. Genet.* **193**, 38–45.
9. Kawakami, K., Inada, T. & Nakamura, Y. (1988) *J. Bacteriol.* **170**, 5378–5381.
10. Kawakami, K. & Nakamura, Y. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8432–8436.
11. Elliott, T. & Wang, X. (1991) *J. Bacteriol.* **173**, 4144–4154.
12. Roesser, J. R., Nakamura, Y. & Yanofsky, C. (1989) *J. Biol. Chem.* **264**, 12284–12288.
13. Caskey, T., Scolnick, E., Tompkins, R., Goldstein, J. & Milman, G. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 479–488.
14. Capecchi, M. R. & Klein, H. A. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 469–477.
15. McCaughan, K. K., Ward, C. D., Trotman, C. N. A. & Tate, W. P. (1984) *FEBS Lett.* **175**, 90–94.
16. Soll, L. (1974) *J. Mol. Biol.* **86**, 233–243.
17. Casadaban, M. (1976) *J. Mol. Biol.* **104**, 541–555.
18. Göringer, H. U., Hijazi, K. A., Murgola, E. J. & Dahlberg, A. E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6603–6607.
19. Epp, C. & Pearson, M. L. (1976) in *RNA Polymerase*, eds. Losick, R. & Chamberlin, M. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 667–691.
20. Way, J. C., Davis, M. A., Morisato, D., Roberts, D. E. & Kleckner, N. (1984) *Gene* **32**, 369–379.
21. Kohara, Y., Akiyama, K. & Isono, K. (1987) *Cell* **50**, 495–508.
22. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
23. Martinez, E., Bartolomé, B. & de la Cruz, F. (1988) *Gene* **68**, 159–162.
24. Groisman, E. A. & Casadaban, M. J. (1986) *J. Bacteriol.* **168**, 357–364.
25. Ito, K., Kawakami, K. & Nakamura, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 302–306.
26. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
27. Tate, W. P. & Caskey, C. T. (1990) in *Ribosomes and Protein Synthesis, A Practical Approach*, ed. Spedding, G. (IRL, Oxford), pp. 81–100.
28. Murgola, E. J. & Hijazi, K. A. (1983) *Mol. Gen. Genet.* **191**, 132–137.
29. Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. W. & Gross, C. A. (1989) *Microbiol. Rev.* **53**, 1–24.
30. Goldstein, J. L. & Caskey, C. T. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 537–543.
31. Connolly, D. M. & Winkler, M. E. (1991) *J. Bacteriol.* **173**, 1711–1721.