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Duplication of the *tuf* Gene, Which Encodes Peptide Chain Elongation Factor Tu, Is Widespread in Gram-Negative Bacteria

DAVID FILER† AND ANTHONY V. FURANO*

Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20205

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The *tuf* gene which encodes peptide chain elongation factor Tu was found to be duplicated in nine enteric and four nonenteric gram-negative bacteria, but present only in one copy in two gram-positive genera. In two of the nonenteric gram-negative genera, *Pseudomonas* and *Caulobacter*, the duplicate *tuf* genes were found to be very close together on the chromosome, which contrasts with the situation in *Escherichia coli*, where they are more than 660 kilobases apart.

Naturally occurring, genetically stable duplications of protein-encoding genes are rare in procaryotes (18). The persistence of the *tuf* gene duplication in *Escherichia coli* and *Salmonella typhimurium* (8) even after the 10,000,000 or so years since their divergence (11) suggests that the *tuf* gene duplication serves an essential function for certain bacteria. We were therefore curious to learn how widespread the *tuf* gene duplication is among various procaryotes.

The following bacteria were examined: gram-negative enteric species—*E. coli*, *Shigella dysenteriae*, *Serratia marcescens*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella* ("*Proteus*") *morganii*, *Providencia stuartii*, *Edwardsiella tarda*, and *Erwinia carotovora*; gram-negative nonenteric species—*Pseudomonas putida*, *Caulobacter crescentus*, *Myxococcus xanthus*, and *Rhodospirillum rubrum*; gram-positive species—*Bacillus subtilis* and *Micrococcus luteus*. Bacterial DNAs were prepared from most of the bacteria as previously described (8). Samples of DNA from *M. morganii*, *P. stuartii*, *E. tarda*, and *E. carotovora* were generously supplied by Don J. Brenner, Centers for Disease Control, Atlanta, Ga.

To determine the number of *tuf* genes in the various bacteria, we took advantage of our recent findings (7; unpublished data) that these procaryotes contain DNA sequences of almost perfect homology to different portions of the *E. coli tuf* gene. Thus, hybridization of *E. coli tuf* gene sequences to replicas (blots) of agarose gel electropherograms of restriction endonuclease digests of these DNAs reveals the number of restriction endonuclease fragments that contain *tuf* homologous sequences. This number would be equivalent to the number of *tuf* genes in a given bacterial DNA if the endonuclease digestion went to completion and there were no sites for the endonuclease within the *tuf* gene. To control for the completion of endonuclease digestion, we included a small portion of bacteriophage λ DNA in the endonuclease reactions and used only those reactions that showed complete digestion of λ DNA. These digestion products were detected by hybridization with λ [^{32}P]DNA (Fig. 1, legend). To control for the second possibility, duplicate blots of a restriction endonuclease digest were hybridized with radioactive probes encoding either the N terminus or C terminus of EF-Tu. These portions of the *tuf* gene, referred to as N fragment or C fragment, are illustrated in Fig. 1 and were obtained by cloning into the appropriate plasmid vector, the indicated *tufA* gene fragment that was purified from *Sma*I or *Sma*I plus *Eco*RI endonuclease digests of the defective λ, λ d*fus-3* (12, 14). The detection of a particular radioactive band, using probes corresponding to either end of the *tuf* gene, would be most readily explained by the presence of an intact *tuf* gene in the particular band.

Two types of hybridization probes were prepared which gave identical results. For the first type, the entire C or N fragment was made radioactive in vitro with [α-^{32}P]TTP (250 to 400 mCi/μmol) by nick translation of the appropriate recombinant plasmid, using the method of Maniatis et al. (16). The plasmid DNA was prepared from chloramphenicol-treated cultures (4), using a modification of the method of Clewell and Helinski (5). The radioactive C or N fragments were excised from the plasmid vector by digestion with the appropriate restriction endonuclease(s), separated from unincorporated

† Present address: Institut Pasteur, 75724 Paris-Cedex 15, France.

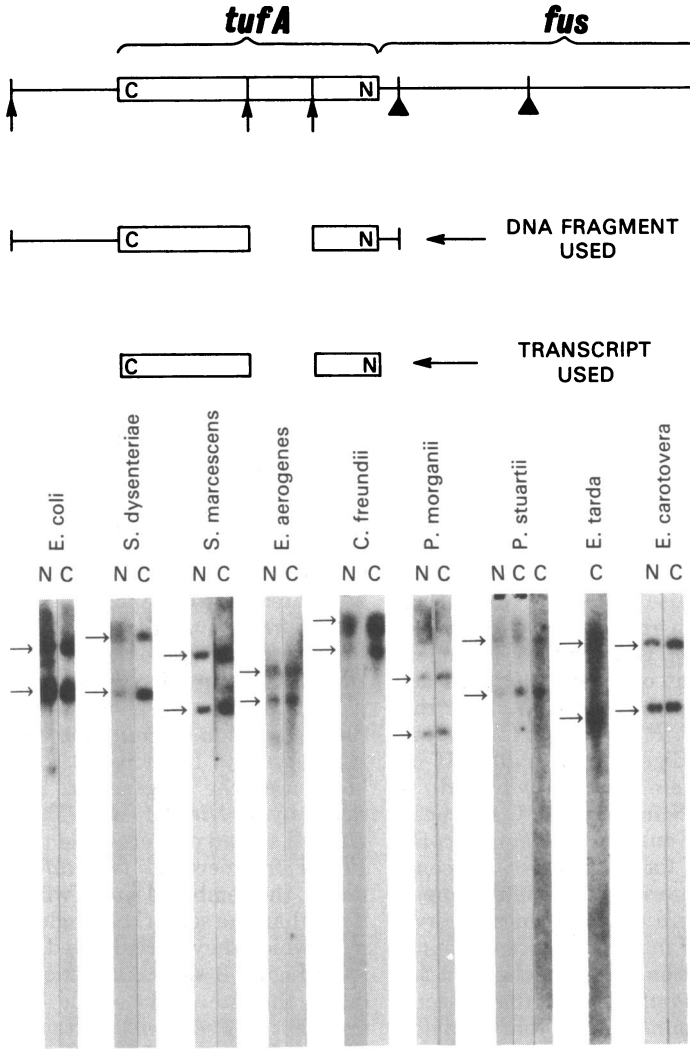

FIG. 1. *Hybridization between radioactive probes encoding the N terminus or C terminus of EF-Tu and the DNAs from various enteric bacteria. Schematic representations of the portion of the E. coli chromosome that contains the tufA and fus genes (top line) and of the cloned portions of the tufA gene (DNA fragment used) are shown. The arrows and triangles indicate the sites recognized by the restriction endonucleases SmaI and EcoRI, respectively. The gene fragment designated C contains 600 nucleotides of the tufA gene encoding the COOH-terminal half of EF-Tu as well as 500 nucleotides of flanking DNA (9). The fragment designated N contains 300 nucleotides that encode the NH_2-terminal portion of EF-Tu plus about 65 bases between the fus and tufA genes as well as the coding sequence of the last 25 or so amino acids of EF-G (23; Furano, unpublished data). Also shown is an autoradiogram of the hybrids formed between different tuf probes and DNA fragments from different bacteria. Various bacterial DNAs (10 to 20 μg) were mixed with about 0.4 μg of bacteriophage λ DNA (see text) and digested in 0.1-ml reactions (1 h at 37°C) containing 10 to 20 U of any one of several restriction enzymes that had been obtained from commercial sources (Bethesda Research Laboratories or New England Biolabs). The reactions were carried out according to the supplier's recommendations, and the mixtures were then adjusted to 0.25% sodium dodecyl sulfate, 30 mM sodium EDTA, 5% glycerol, and 0.05% bromophenol blue, heated at 65°C for 5 min, and divided into several portions which were subjected to electrophoresis (14 to 16 h at 20 V [constant voltage] in a horizontal direction in a 1.2% agarose gel [0.4 by 13.5 by 14.5 cm]) in 42 mM Tris-acetate buffer, pH 8.0, containing 20 mM sodium acetate and 1 mM sodium EDTA. After staining in an 0.5-μg/ml aqueous solution of ethidium bromide, the gel was treated (22) for the transfer of DNA to diazobenzyloxymethyl paper (1), using the method of transfer described by Mears et al. (17). After pretreatment of the paper (22), three separate strips, each containing any one of the bacterial DNAs, were hybridized separately with either [^{32}P]DNA or [^{32}P]RNA corresponding to either end of the tuf gene or with radioactive bacteriophage λ DNA that had been made radioactive by nick translation. The reactions*

deoxynucleoside triphosphate by chromatography on Sephadex G-100, and then separated from the DNA of the vector by electrophoresis in 1% (wt/vol) gels of low-melting agarose (Bethesda Research Laboratories). The second type was RNA transcripts of the *tuf*-containing portion of the cloned *tufA* fragments. The transcripts were synthesized from the appropriate recombinant plasmid in vitro by using [α-^{32}P]UTP (50 to 100 mCi/μmol) and purified by using methods described in detail earlier (7, 8) by hybridization to DNA of the defective λ bacteriophage, λ d*rif*^{d}18. This DNA contains the *tufB* gene (13, 15), and because none of the DNA that flanks the *tufA* gene is homologous to λ d*rif*^{d}18 (8), this hybridization yields *tuf* transcripts that are essentially free of non-*tuf* sequences.

Figure 1 shows that all of the enteric bacterial DNAs digested with *Eco*RI contained two radioactive bands after hybridization with either the N fragment or C fragment probes. Two radioactive bands were also found in *Bam*HI or *Hin*dIII digests. Such results can only be explained by duplicate *tuf* genes in these genomes.

Figure 2 shows that only one band was detected in *Eco*RI digests of the DNA from the nonenteric organisms, *P. putida* or *C. crescentus*. However, after digestion with *Bam*HI or *Hin*dIII, two bands were detected by hybridization with either the N fragment or C fragment probe. Although only one *tuf*-containing *Eco*RI fragment is indicated for *M. xanthus*, a second must also be present unresolved from the upper region of the gel where we sometimes observe nonspecific binding of the probe, since we found that secondary digestion of *Eco*RI-digested *M. xanthus* DNA with *Bam*HI reveals this *tuf*-containing fragment (data not shown) but does not alter the mobility of the *Eco*RI fragment indicated in Fig. 2. Our preparation of *R. rubrum* DNA inhibited *Eco*RI, but both *Bam*HI and *Hin*dIII digests of this DNA contained two *tuf*-containing fragments.

In many cases, the hybridization signal with the C fragment was more intense than with the N fragment. With *M. xanthus*, the N fragment bands were too weak to be reproduced photographically. These results are consistent with our previous findings (7), which showed that sequences homologous to the C terminus of the *E. coli tuf* gene are the most highly conserved *tuf* sequences.

We could not detect more than one restriction fragment that contained *tuf* sequences in the two gram-positive genera that we examined, *B. subtilis* and *M. luteus*. We used six different restriction endonucleases: *Eco*RI, *Hin*dIII, *Bam*HI, *Pst*I, *Sal*I, and *Bgl*II. Some of the data for *B. subtilis* are shown in Fig. 2. This result with *B. subtilis* was consistent with the genetic evidence of Smith and Paress (20), showing that *B. subtilis* contains only one *tuf* gene.

The presence of the *tuf* homologous sequences of *C. crescentus* and *P. putida* on a single *Eco*RI endonuclease fragment suggested that the duplicate *tuf* genes in these organisms could either be located very close together or, less likely, on *Eco*RI fragments of identical length in each of these bacterial genomes. Each *tuf*-containing *Eco*RI fragment is long enough to accommodate two 1.2-kilobase *tuf* genes, which is the size of the *tuf* genes in *E. coli* (2, 9, 23), 3 kilobases in *P. putida*, and 18 kilobases in *C. crescentus*. In both cases, the *tuf* genes are presumably separated by a *Hin*dIII site. Thus, digestion by *Hin*dIII should convert the single *tuf*-containing *Eco*RI fragment to two *tuf*-containing fragments, the combined size of which should not be more than the size of the single *Eco*RI fragment from which they were derived.

As Figure 3 shows, digestion by *Hin*dIII reduced the size of the single 3-kilobase *Eco*RI fragment of *Pseudomonas* to two smaller fragments of about 1.5 kilobases and 1.8 kilobases each. In the case of *C. crescentus*, the size of the 18-kilobase *tuf*-containing fragment was reduced to two fragments of about 2.1 and 9 kilobases each.

Although there is no easy way to prove that

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with radioactive RNA were incubated at 65°C in 1% glycine (1), 2× SSC, 0.1% (wt/vol) sodium dodecyl sulfate, and 1% (vol/vol) phenol. When DNA was used, the reactions were incubated at 60°C in 2× SSC, 0.25% (wt/vol) sodium dodecyl sulfate, 1% glycine, 5× Denhardt solution without bovine serum albumin (6), and 100 μg of sonicated salmon sperm DNA per ml. After 18 h, the strips were washed three times with 200 ml of 2× SSC-0.1% sodium dodecyl sulfate at the hybridization temperature, dried, and exposed to X-ray film at −70°C with an intensifying screen. The autoradiograms shown here are of the bacterial DNAs that had been digested with EcoRI. The hybridizations with E. coli, S. marcescens, E. aerogenes, C. freundii, P. morganii (first two strips), and E. carotovora were carried out by using RNA transcripts, whereas those with S. dysenteriae, the third P. stuartii strip, and E. tarda were carried out with a nick-translated probe. Some of the dark marks at the top of the strips shown here and in Fig. 2 are registration or reference marks applied to the X-rays that were not completely cropped from the photograph. The lower (tufA) and upper (tufB) tuf bands in the E. coli tracks are about 4.3 and 9.3 kilobases, respectively.

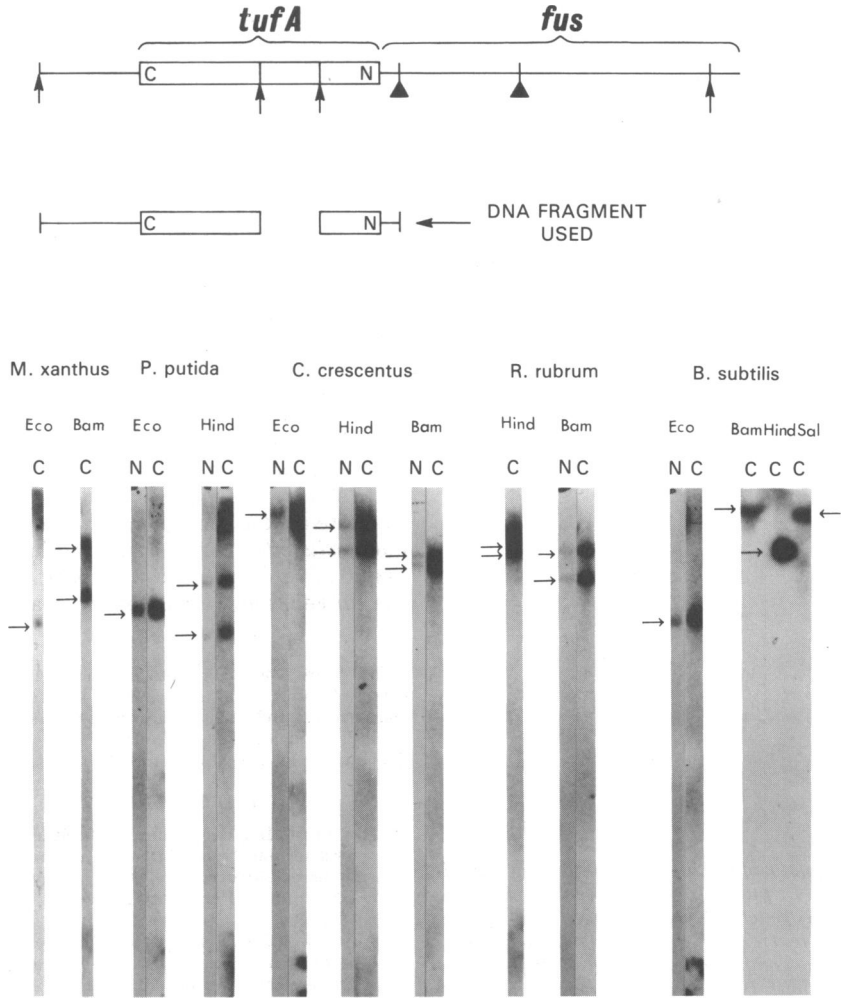

FIG. 2. *Hybridization between radioactive tuf sequences and the DNAs from various nonenteric bacteria. These experiments were carried out as described in the legend to Fig. 1, using a nick-translated probe. As indicated in Fig. 3, the single EcoRI fragment in the P. putida lanes is about 3 kilobases, whereas the single EcoRI fragment in the C. crescentus lanes is about 18 kilobases.*

the *tuf* homologous sequences that we have detected are part of genes that encode a protein which is the functional counterpart of EF-Tu, we referred to such sequences as *tuf* genes. This assumption seems warranted in the case of the *Enterobacteriaceae*, where in the degree of sequence homology to the *E. coli tuf* gene is very high (7, 8), including the relative location of restriction endonuclease restriction sites within the gene (8; unpublished data). Furthermore, even genera such as *Bacillus* and *Caulobacter*, which are evolutionarily very distant from each other and from *E. coli*, contain an EF-Tu molecule which, when complexed with their respective elongation factor Ts, can substitute for the *E. coli* EF-Tu:EF-Ts to form an active replicase for the *E. coli* phage Qβ (21; L. Stringfellow and T. Blumenthal, personal communication). Although it is possible that the *tuf* homologous sequences encode non-EF-Tu proteins in these procaryotes, it seems reasonable at present to refer to the *tuf* homologous sequences as *tuf* genes.

The finding that the duplication of the *tuf* gene is widespread among the gram-negative genera suggests that for these bacteria, the *tuf* gene duplication serves an important function. This suggestion is supported by the fact that in

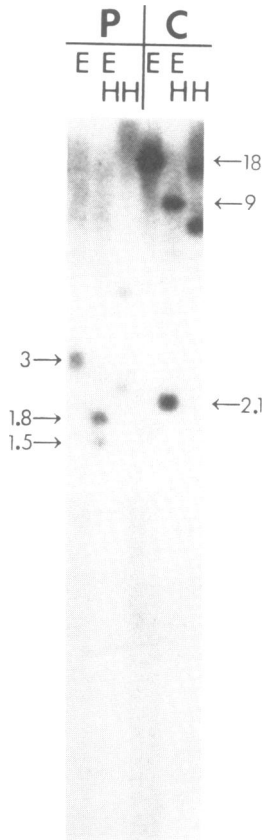

FIG. 3. *Cleavage of the tuf gene-containing EcoRI fragment from P. putida or C. crescentus DNA by HindIII. These reactions were carried out as described in the text except that after the incubation with EcoRI was completed, a portion of the mixture was incubated with HindIII for an additional 1 h at 37°C. That portion of each reaction that did not receive HindIII was also incubated for an additional 1 h. At the end of the reaction, the DNA was concentrated by precipitation with 2 volumes of ethanol. The DNA pellets were washed with 95% ethanol, dried, dissolved in the agarose gel sample buffer (see legend to Fig. 1), heated at 65°C, and the subjected to electrophoresis. The electrophoretic position and size (in kilobases) of some of the tuf-containing fragments are indicated by the arrows. The sizes were determined by comparison to the mobility of the restriction fragments of λ DNA that were detected by hybridization with radioactive λ DNA in a separate hybridization reaction (see legend to Fig. 1). P and C denote P. putida and C. crescentus, respectively, and E and H stand for EcoRI and HindIII, respectively. Sequences corresponding to the C fragment shown in Fig. 1 were used as the hybridization probe.*

E. coli, the *tufA* and *tufB* genes are almost identical (2, 23). However, only one *tuf* gene was present in the two gram-positive genera that we examined. Although this is too small a sample to make any generalizations, it would be interesting to know whether a single *tuf* gene is typical of gram-positive genera which may have diverged from the gram-negative genera early in the phylogenetic history of procaryotes (11).

While this work was in progress, our attention was called to the following correlation: in *B. subtilis*, the *strA* locus, which is genetically linked to the single *tuf* gene, is also linked to the *rif* locus. In *E. coli*, one of the *tuf* genes (*tufA*) is linked to *rpsL* (formerly *strA*), and the other (*tufB*) is linked to *rif*, but the *rpsL* and *rif* loci are genetically unlinked, being more than 660 kilobases apart (3, 19; I. Smith, personal communication). In *P. putida*, where the two *tuf* genes are very close together, the *str* and *rif* loci are also linked (10). If, as has been proposed (11), *Pseudomonas* spp. represent an intermediate state between the evolutionary events that separate genera such as *Bacillus* and the *Enterobacteriaceae*, then a single translocation event might have caused the transposition of one of the *tuf* genes and a part of the nearby chromosome.

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