CONSTRUCTION AND CHARACTERIZATION OF THE TWO HYBRID ColEl PLASMIDS CARRYING *ESCHERICHIA COLI tufB* GENE

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1. Introduction

The polypeptide chain elongation factor Tu (EF-Tu) is coded by two distinct genes, one located at 72 min (*tufA*) and the other at 88 min (*tufB*) on the genetic map of *E. coli* [1]. In [2] we described the cloning of *tufA* gene into ColEl derivative plasmid RSF2124 (ColEl-Ap^r). The hybrid plasmid, designated as pTUAl, contained a 4.0 kilobase (8.5% λ-unit) *Eco*RI fragment derived from phage λ*fus*3 [3], and could direct the synthesis of EF-Tu in a cell-free transcription–translation coupled system after digestion with endonuclease *Eco*RI. Most of the nucleotide sequence of the *tufA* region coding for EF-Tu has been determined (Yokota et al., in preparation).

Although the products of the two genes, *tufA* and *tufB*, appear to be almost identical in terms of their physical, chemical, and catalytic properties [1,4–7] it would be of interest to compare, in a more direct manner, the identity of these two genes by analyzing their nucleotide sequences.

This paper describes the cloning of *tufB* on plasmid RSF2124. The DNA fragment carrying *tufB* was isolated from the *Eco*RI digests of DNA of the transducing phage λ*rif*^{d}18 [8] and ligated with the plasmid to produce two new hybrid plasmids designated as pTUB1 and pTUB2. The direction of the inserted fragment in pTUB1 and pTUB2 is found to be in an opposite orientation. Some properties of the hybrid plasmids are also described.

2. Materials and methods

λ*rif*^{d}18 DNA was prepared by the method in [9] from phage λ*cI857S7rif*^{d}18 obtained by heat induction of the culture of NO1736 (CA274(*trp, lac*) (λ*cI857S7xis6b515b519*) (λ*rif*^{d}18)) (kindly supplied by Dr M. Nomura). The DNA of the vector plasmid RSF2124 (ColEl-Ap^r) [10] was prepared as in [2] from the cleared lysate of TM201 (K12, *thy, trp, str*^{s} (RSF2124)).

For the isolation of the 8.6 kilobase (18.6% λ-unit) *Eco*RI fragment carrying *tufB*, 500 μg λ*rif*^{d}18 DNA was digested with *Eco*RI endonuclease for 16 h at 37°C, and the fragments were separated by electrophoresis on a 0.8% agarose gel at 4 V/cm for 10 h, using 40 mM Tris-acetate buffer (pH 7.5), containing 20 mM sodium acetate and 2 mM EDTA. After electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide, and the band corresponding to the 8.6 kilobase *Eco*RI fragment was cut out and homogenized in 20 ml 10 mM Tris–HCl buffer (pH 7.5) containing 2 mM EDTA. After standing overnight at 37°C, the gel was removed by centrifugation and the supernatant was concentrated to ~5 ml. The DNA was precipitated with ethanol, dissolved in a minimum volume of 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, and dialyzed against the same buffer solution.

For the construction of the recombinant molecule, 1 μg of the 8.6 kilobase *Eco*RI fragment and 1.6 μg *Eco*RI-cleaved RSF2124 DNA were incubated at

10°C for 5 h in 50 μl of a reaction mixture containing 66 mM Tris–HCl buffer (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, and 1.5 units of T4 ligase. After heating for 5 min at 65°C to inactivate the ligase, the reaction mixture was directly used to transform *E. coli* C600 (*thr, leu, thi, lac*) cells. Ampicillin-resistant, non-colicinogenic transformants were selected.

Other methods including the cell-free protein synthesis directed by plasmid DNA, and the DNA–RNA hybridization test were the same as in [2].

3. Results

Figure 1 shows the outline of the cloning experiments. Since plasmid RSF2124 possesses only a single *Eco*RI site in the structural gene for colicin E1, the insertion of the DNA fragment carrying *tufB* would render the transformants non-colicinogenic. At first, we tried to isolate *tufB*-carrying clones directly by ligating the mixture of *Eco*RI digests of λ*rif*^{d}18 DNA with vector DNA. However, none of the ampicilline-resistant, non-colicinogenic transformants isolated contained the 8.6 kilobase *Eco*RI fragment. Therefore, we decided to isolate first the fragment carrying *tufB* and then to construct the hybrid plasmid by ligation of the purified fragment with vector DNA.

Of the 28 ampicilline-resistant, non-colicinogenic clones isolated by this procedure, 6 were found to contain the 8.6 kilobase *Eco*RI fragment. These clones are classified into two groups depending on the orientation of the inserted fragment (see fig.1). Both pTUB1 and pTUB2 yielded 10.7 and 8.6 kilobase fragments upon digestion with *Eco*RI (fig.2A), whereas after digestion with *Sma* I, pTUB1 and pTUB2 formed 15.3 and 3.3 kilobase and 11.2 and 7.4 kilobase fragments, respectively (fig.2B). The presence of a 0.3 kilobase *Sma* I fragment was not detected on this gel electrophoresis, but was demonstrated using the 4% polyacrylamide gel (data not shown). From these results, the orientation of the inserted DNA fragment in pTUB1 and pTUB2 was deduced as depicted in fig.1.

The ability of pTUB1 and pTUB2 DNA to direct the synthesis of EF-Tu was examined in a cell-free transcription-translation coupled system developed as in [2]. As shown in fig.3, a considerable amount of EF-Tu was synthesized when DNA from pTUB1 (slot 2), pTUB2 (slot 3), or λ*rif*^{d}18 (slot 4) was used as a template. On the other hand, no protein migrating

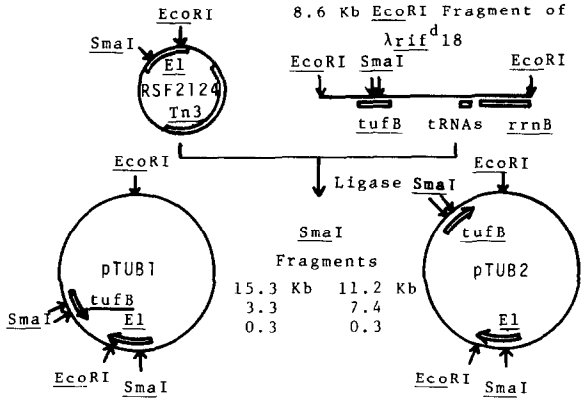

Fig.1. Outline of the cloning experiments. The organization of genes in λ*rif*^{d}18, and RSF2124 is based on [3] and [11], respectively. The predicted size of the *Sma*I fragments of pTUB1 and pTUB2 is shown in kilobases.

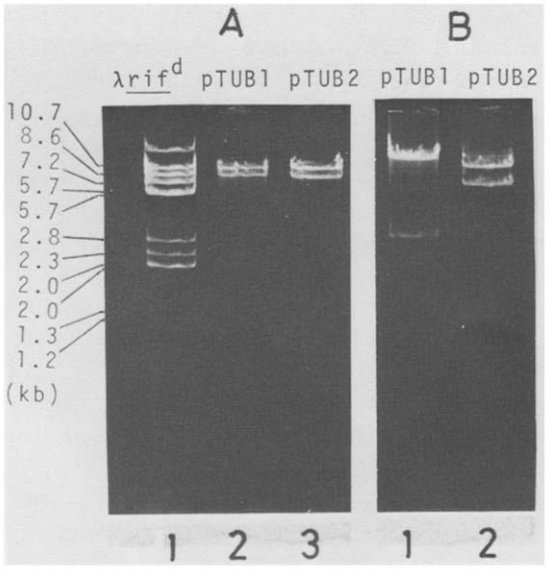

Fig.2. Agarose gel electrophoresis of *Eco*RI- and *Sma*I-digested DNA fragments. Electrophoresis was carried out as in section 2. (A) *Eco*RI-digested DNA fragments of λ*rif*^{d}18 (slot 1), pTUB1 (slot 2) and pTUB2 (slot 3). (B) *Sma*I-digested DNA fragments of pTUB1 (slot 1) and pTUB2 (slot 2).

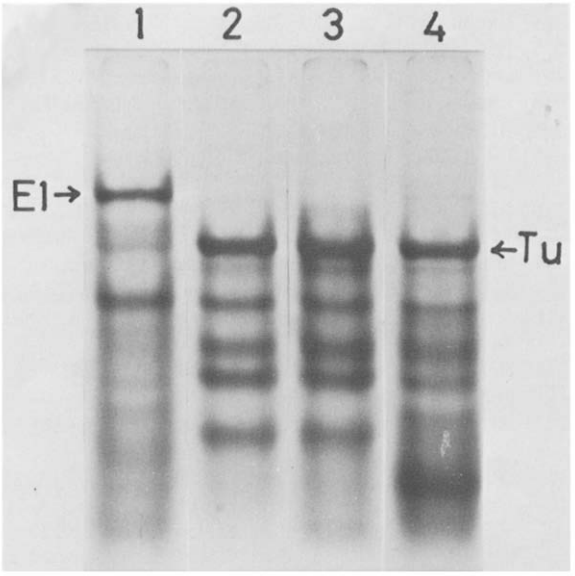

Fig.3. SDS–polyacrylamide gel electrophoresis of proteins synthesized in a cell-free system. The procedures for the DNA-directed cell-free protein synthesis were the same as in [2]. The templates used were DNAs from RSF2124 (slot 1), pTUB1 (slot 2), pTUB2 (slot 3), and λ*rif*^{d}18 (slot 4).

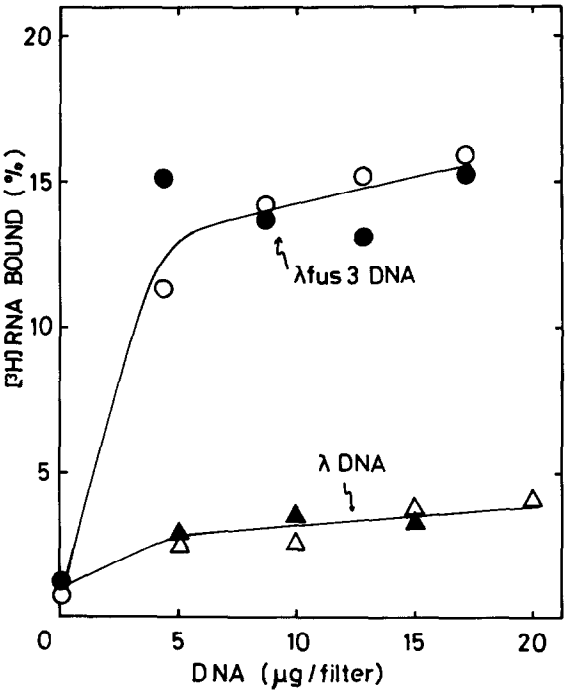

Fig.4. DNA–RNA hybridization. About 33 000 cpm of ^{3}H-labeled RNA transcribed from pTUB1 DNA (●,▲) or 25 000 cpm of ^{3}H-labeled RNA from pTUB2 DNA (○,△) were hybridized with various amounts of either λ*fus*3 (●,○) or λ (▲,△) DNA. In the ordinate, the extent of hybridization is shown as % of the DNA–RNA hybrid formed with an excess (20 μg) of pTUB1 DNA. The experimental details were as in [2].

to the position of EF-Tu was formed in the presence of RSF2124 DNA (slot 1). The identity of this protein with EF-Tu was confirmed further by specific immune precipitation with anti-EF-Tu antibody followed by SDS–gel electrophoresis [12] and also by two-dimensional gel electrophoresis according to [13] (data not shown).

In [2], we showed that the ^{3}H-labeled RNA synthesized with *E. coli* RNA polymerase holoenzyme under the direction of λ*rif*^{d}18 DNA, could form a DNA–RNA hybrid with pTUA1 DNA. We therefore investigated the possibility that pTUB DNA could direct the synthesis of mRNA for EF-Tu which, in turn, is capable of forming a hybrid with λ*fus*3 DNA containing *tufA*. As shown in fig.4, ~10% of the total RNA transcripts from pTUB1 or pTUB2 DNA could form a hybrid with λ*fus*3 DNA, whereas little hybrid formation was observed with λDNA.

4. Discussion

These experiments, two types of hybrid plasmids carrying *tufB* have been isolated. Since the bacteria harboring either pTUB1 or pTUB2 grow at a normal growth rate, we cannot explain why we failed to obtain the same clones by random cloning of the *Eco*RI digests into the vector plasmid.

Measurements of the intracellular level of EF-Tu indicated that there is not much overproduction of EF-Tu in transformants carrying pTUB1 or pTUB2 (data not shown). However, the *tufB* gene cloned on these hybrid plasmids is definitely expressed, since the introduction of pTUB1 or pTUB2 DNA into a kirromycin-resistant mutant (LBE2012 [14], kindly supplied by Dr L. Bosch) altered the phenotype of the mutant to kirromycin-sensitive (A. M., unpublished). The fact that both pTUB1 and pTUB2, having the inserted bacterial chromosome in an opposite orientation, could not only be transcribed, but also direct

the synthesis of EF-Tu both in vivo and in vitro, suggests that the promoter for EF-Tu is present in the inserted *Eco*RI fragment.

The synthesis of EF-Tu is known to be under the influence of stringent control [12,15–17]. To study the in vitro regulation of EF-Tu biosynthesis, it is desirable to have a DNA fragment which carries both the promoter and the structural gene for EF-Tu in close proximity. Since the promoter for *tufA* is located upstream of the whole *str* operon and not on the 4.0 kilobase *Eco*RI fragment cloned on pTUA1, the new plasmids pTUB1 and pTUB2 having the promoter for EF-Tu would be much more advantageous for such a purpose.

References

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