THE EXPRESSION OF THE CLONED *tufB* GENE IN VIVO

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1. Introduction

In [1], we have reported the construction and characterization of two hybrid ColE1 plasmids, pTUB1 and pTUB2, in which the 8.9 kilobases (kb) (18.6% λ-unit) *Eco*RI fragment derived from transducing phage λ*rif*^{d}18 was inserted in two different orientations into a ColE1 derivative plasmid RSF2124. The 8.9 kb fragment contained a part of *rrnB*, genes for 4 tRNAs (*glyT*, *tyrU*, *thrT*, and *thrU*), *tufB*, a gene for an unidentified protein 'U', and a part of *rplK* [2] (fig.1).

In a cell-free transcription–translation system, both pTUB1 and pTUB2 DNA could direct the synthesis of *tufB* mRNA and EF-Tu(B) (product of *tufB*) [1]. Furthermore, the analysis of the transcripts synthesized in the presence of purified RNA polymerase holoenzyme has revealed that the transcription was initiated at a point ~300 nucleotides upstream of the structural gene for *tufB* (Shibuya, A. M., Y. K., in preparation) and was specifically inhibited by low concentrations of ppGpp (A. M., Shibuya, Y. K., in preparation). These results suggests that the promotor gene for *tufB* which is under the stringent control may be located in close proximity to its structural gene.

Here, we have studied the expression of the cloned *tufB* gene within cells of the kirromycin-resistant mutant LBE2012 [3,4]. The antibiotic kirromycin inhibits protein synthesis by preventing the release of EF-Tu from ribosomes [5–7]. The mutant LBE2012 was originally thought to have the *tufA* mutant allele coding for a functional kirromycin-resistant EF-Tu(A) and the mutant *tufB* gene that produces a non-functional EF-Tu(B) [3,4]. However, the *tufB* product of LBE2012 is functional in the absence of kirromycin, but is inactivated in the presence of kirromycin (L.

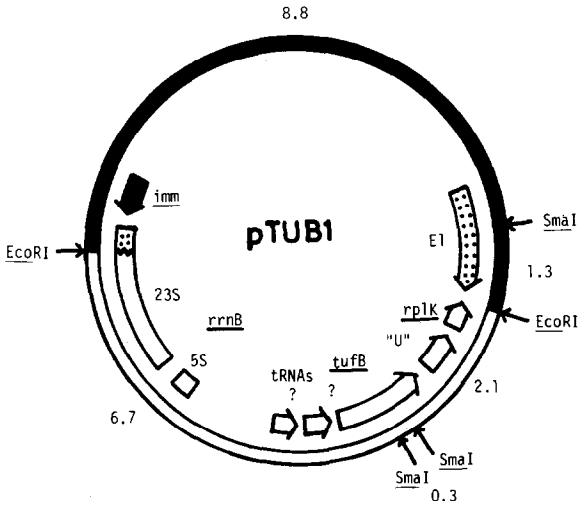

Fig.1. Physical and functional map of pTUB1. The black and white regions in the outside circle represent the vector and host genoms, respectively. Locations of *Eco*RI and *Sma*I restriction endonuclease cleavage sites and lengths of the restriction fragments (in kilobases) are shown. The major transcription units and their directions are indicated by the arcs with arrows inside the map of pTUB1. The gene *imm* (closed arc) codes for the ColE1 immunity. The ColE1 gene (dotted arc) is cleaved by *Eco*RI near the end, and the rest of the gene is fused to the portion of *rrnB* gene at one end of the 8.9 kb (18.6% λ-unit) *Eco*RI fragment. The size of the *Eco*RI fragment was calculated assuming 1% λ-unit as 480 basepairs [25]. The sum of the lengths of 4 restriction fragments in the *Eco*RI fragment determined by gel electrophoresis is 9.1 kb. The bacterial genes (open arcs) contain the part of *rrnB*, the genes for 4 tRNAs, *tufB*, the 'U' gene, and the part of *rplK*. The distance between the 4 tRNA genes and *tufB* are from J. D. Friesen (personal communication). It is not clear whether the tRNA genes (*thrU–tyrU*, and *glyT–thrT*) and *tufB* are transcribed as a single or multiple transcription unit.

Bosch, personal communication). Since kirromycin-sensitivity is dominant over kirromycin-resistance [3,4], the introduction of plasmids bearing the wild-type allele of *tufB* into LBE2012 would yield kirromycin-sensitive transformants if the cloned *tufB* gene is expressed in vivo. These results indicate that *tufB* in both pTUB1 and pTUB2 is indeed expressed in transformed cells. Although the copy number of pTUB1 was ~20 in the transformants, the rate of the synthesis of EF-Tu did not increase appreciably. This indicates the presence of an additional regulatory mechanism that maintains the normal cellular level of EF-Tu.

2. Materials and methods

2.1. *Chemicals*

[^{3}H]Leucine, [^{3}H]serine and [^{3}H]thymidine were obtained from the Radiochemical Centre, Amersham. Kirromycin was a kind gift from Professor H. Wolf, Tübingen University. In some experiments it was replaced by Aurodox (X5108) [8] kindly supplied by Roche.

2.2. *Bacterial strains and plasmids*

The kirromycin-resistant mutant LBE2012 (*tufA*, *tufB*, *xyl*) as well as its parental strain LBE1001 (F^-, su^-) [3,4] were kindly donated by Dr L. Bosch, Leiden University. The ColE1-derivative plasmids RSF2124 (ampicillin-resistant) [9] and pCR1 (kanamycin-resistant) [10] were obtained from Dr K. Sakaguchi, Mitsubishi Kasei Life Science Institute and Dr M. Yoshikawa of our institute, respectively. The isolation of plasmid pTUB1 was as in [1].

2.3. *Transformation of* E. coli *cells*

Transformation experiments done as in [11]. When pTUB1 or RSF2124 DNA was used for transformation, ampicillin-resistant transformants were selected on the L-broth plates containing 25 μg ampicillin/ml. For curing of the cells harboring pTUB1 with pCR1 DNA, the selection was carried out on L-broth plates containing 25 μg kanamycin/ml. Kanamycin-resistant colonies were isolated and purified by two successive transfers on kanamycin-containing plates. After the second transfer, most of the transformants with pCR1 (kanamycin-resistant) had lost ampicillin resistance, indicating that plasmid pTUB1 which is incompatible with pCR1 [12,13] had been eliminated.

2.4. *Measurements of kirromycin-resistance*

The kirromycin-resistance of the transformant cells was assayed essentially as in [3]. The cells were grown at 37°C in an M9 medium supplemented with 0.4% glucose. At the mid-log phase, the culture was divided into 0.1 ml aliquots to which 2 mM EDTA and varying amounts of kirromycin (0–200 μg/ml) were added. After incubation for 5 min at 37°C, 5 nmol [^{3}H]leucine (50 Ci/mol) were added to each tube and the mixtures were incubated further for 20 min at 37°C. The radioactivity incorporated into the hot 4% perchloric acid-insoluble fraction was determined.

2.5. *Differential rate of EF-Tu synthesis*

Escherichia coli cells were grown in 2 ml 0.4% glucose-M9 medium at 37°C. When A_{660} reached 0.1, the culture was supplemented with 5 μCi [^{3}H]serine (20 Ci/mmol) and incubated for 2 min at 37°C. The [^{3}H]serine was then chased with an excess of unlabeled serine (1 mM) for 3 min at 37°C. The synthesis of total cellular protein was determined by incorporation of the radioactivity into the hot perchloric acid-insoluble fraction, and that of EF-Tu by immunoprecipitation–electrophoresis as in [14]. The differential rate of the synthesis of EF-Tu was calculated as:

$$\frac{\text{Rate of the synthesis of EF-Tu}}{\text{Rate of the synthesis of total cellular protein}} \times 100$$

3. Results

The kirromycin-sensitive or -resistant phenotype of LBE1001, and LBE2012 and its transformants with pTUB1 or RSF2124 were assessed by their ability to incorporate labeled leucine into protein in the presence of various concentrations of kirromycin. As shown in fig.2, incorporation of the labeled amino acid was not inhibited by kirromycin in the kirromycin-resistant mutant LBE2012 or in its transformant with the vector plasmid RSF2124. On the other hand, the transformant of LBE2012 with pTUB1, LBE2012 (pTUB1) was as sensitive as the kirromycin-sensitive parental strain LBE1001. These results indicate that the kirromycin-resistant phenotype of LBE2012 was altered to give the kirromycin-sensitive phenotype upon introduction of the plasmid pTUB1, and suggest

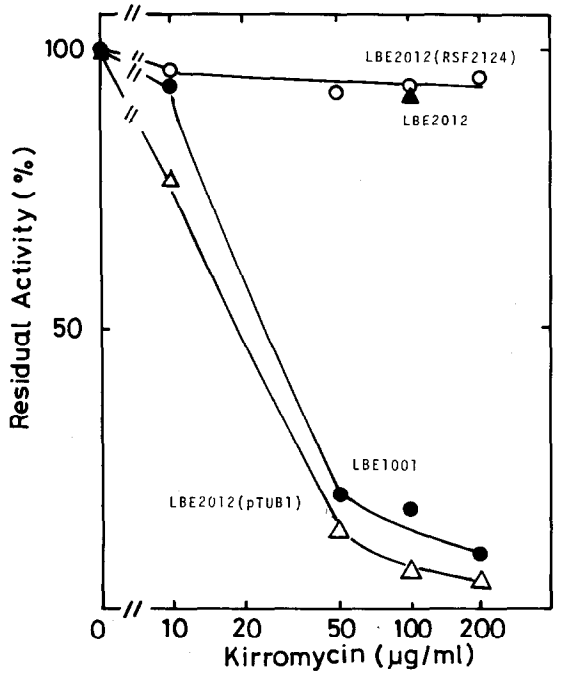

Fig.2. Suppression of kirromycin-resistance in LBE2012 by transformation with pTUB1. The activity to incorporate [^{3}H]leucine into protein of LBE1001 (•), LBE2012 (▲), LBE2012 (RSF2124) (○) and LBE2012 (pTUB1) (△) cells was plotted as a function of kirromycin concentrations. Assay conditions were as in section 2.

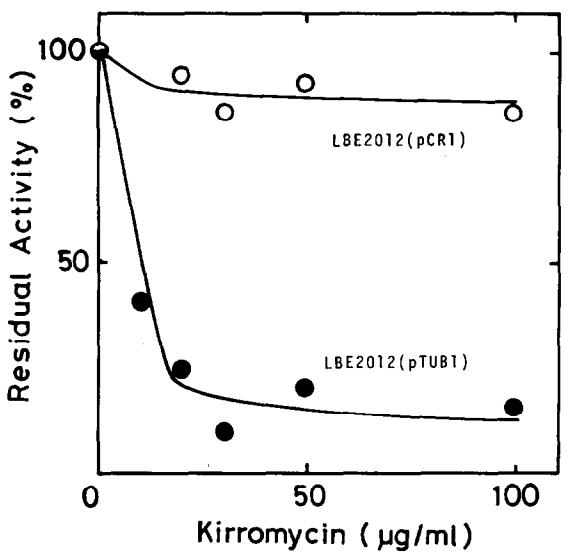

Fig.3. Displacement of pTUB1 in LBE2012 cells by pCR1. Incorporation of [^{3}H]leucine into protein at various concentrations of kirromycin was measured with LBE2012 (pTUB1) (•) and LBE2012 (pCR1) (○) cells. The latter strain was constructed by transformation of the former with pCR1 followed by selection with kanamycin. For details, see the text.

that the cloned wild-type *tufB* is expressed in the transformant cells.

To exclude an alternate possibility that the above observation is due to the recombination of *tufB* in plasmid DNA with chromosomal genes for *tufA* or *tufB*, the following experiments were done. Since two different ColE1 plasmids are mutually incompatible in the same host [12,13], the introduction of the kanamycin-resistant ColE1 plasmid pCR1 into LBE2012 (pTUB1) and selection for kanamycin-resistance would result in the elimination of pTUB1. The results in fig.3 indicate that the kirromycin-resistant phenotype was completely restored on displacement of pTUB1 by pCR1, thus proving unequivocally that the kirromycin-sensitivity in LBE2012 (pTUB1) is due to the direct expression of *tufB* in the cloned plasmid.

Since the replication of ColE1 plasmids is under 'relaxed' control [15], the copy number of pTUB1 in the transformant cells is increased. As shown in table 1, there are ~20 copies of plasmids in LBE2012 cells infected with either pTUB1 or its vector RSF2124. We then measured the rate of synthesis of EF-Tu in cells transformed with pTUB1 or RSF2124, to see whether there is any overproduction of EF-Tu due to the increase in the number of the functional *tufB* gene within the cells. As shown in

Table 1
Lack of gene dosage effect on the synthesis of EF-Tu

Strain	Copy number of plasmids[a]	Differential rate of EF-Tu synthesis[b] (%)
LBE2012 (RSF2124)	23, 14, 18	4.9
LBE2012 (pTUB1)	24, 17	5.1

[a] Plasmid copy numbers in LBE2012 were determined by CsCl–ethidium bromide centrifugation of the [^{3}H]thymidine-labeled cells lysates according to [16]. The radioactivity found at the position of the covalently closed circular plasmid DNA was used to calculate the plasmid copy number. The values are expressed as plasmid copies per chromosome equivalent and were calculated assuming M_r 2.5 × 10^9 for the *E. coli* chromosome, 7.4 × 10^6 for RSF2124, and 1.3 × 10^7 for pTUB1. The individual values were obtained from separate experiments

[b] Determined as in section 2

table 1, there was practically no increase in the differential rate of the synthesis of EF-Tu in LBE2012 cells containing multicopies of *tufB*.

4. Discussion

These experiments prove that *tufB* gene cloned on a ColE1 plasmid pTUB1 [1] is expressed in the kirromycin-resistant mutant LBE2012. With the results of the cell-free system [1] and those to be reported elsewhere (see section 1), it appears that *tufB* possesses a promotor adjacent to its structural gene. The plasmid pTUB2 which contains the inserted 8.9 kb *Eco*RI fragment in an opposite orientation was expressed equally well in the cell-free system [1] but somewhat weakly in LBE2012 (not shown). This might be due to the presence of a small deletion in pTUB2 upstream of its coding region (A. M., Y. Takebe, unpublished). Another plasmid pTUA1 which contains a 4 kb (8.5% λ-unit) *Eco*RI fragment derived from phage λ*fus*3 was only weakly expressed in the cell-free system due to the lack of the natural promoter in the cloned fragment [17]. The expression of pTUA1 in whole cells of LBE2012 assessed as in the present experiment was also weak (A. M., unpublished).

The synthesis of EF-Tu was not appreciably increased in the transformant LBE2012 (pTUB1) cells, in spite of the presence of ~20 copies of the plasmid. It is of interest to note that the lack of gene dosage effect has been reported for the synthesis of ribosomal proteins [18–24] and of the β and β′ subunits of RNA polymerase [17]. In some cases [20,21, 23], it has been shown that the rate of transcription of mRNA increases as the number of the corresponding gene copies increases, yet no dosage effect was observed in the synthesis of the respective proteins. This suggests that expression of these genes may be regulated by post-transcriptional control, including the inactivation or degradation of mRNA. This regulation was interpreted by a model in which free ribosomal proteins, when overproduced, selectively inactivate their own mRNA by a feedback mechanism [19,20,24]. It remains to be seen whether the similar regulatory mechanism may be present in the case of EF-Tu biosynthesis.

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