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tuf Gene Dosage Effects on the Intracellular Concentration of EF-TuB

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In this paper we have studied the effect of raising the intracellular EF-Tu concentration on the expression of *tufB*. To this aim cells were transformed with multicopy plasmids carrying either *tufA* or *tufB*. The intracellular EF-Tu concentrations were determined by the specific immunoelectrophoresis assay described in the preceding paper in this journal.

We have cloned the *tufA* gene in a plasmid, containing the powerful major leftward promoter (P_L) of phage λ. Transcription from P_L can be repressed at low temperature by a temperature-sensitive repressor and activated by heat induction. Cloning occurred in two orientations in a single *Eco*RI site about 150 base pairs downstream of P_L. Cells carrying either plasmid were shown to contain an almost doubled amount of EF-Tu at temperatures from 28 °C to 37 °C. This indicates that transcription of *tufA* can proceed from a possible binding site for RNA polymerase on these cloned fragments. The EF-Tu level was further increased to about 30% of total cellular protein after a temperature shift from 37 °C to 43 °C.

The multicopy plasmid $pTuB_1$ described by Miyajima et al. [*FEBS Lett. 102*, 207–210 (1979)] and a derivative ($pTuB_0$, compare preceding paper in this journal) were used to study the expression of both chromosomal and plasmid-borne *tufB*. Transformation with either plasmid raised the intracellular EF-Tu concentration by 30–60% depending on the nutritional conditions.

Suppression of *tufB* expression was observed when the intracellular level of EF-Tu increased after transformation with all plasmids mentioned above. The results are in accord with the concept that EF-Tu acts as an autogenous feedback inhibitor involved in the regulation of *tufB*.

Regulation of the synthesis of EF-Tu is an intriguing process which so far is poorly understood. EF-Tu is present in the cell in large quantities and represents about 10% of the total cellular protein in rapidly growing cells [1]. Its synthesis is coordinately regulated with that of tRNA. The intracellular content of EF-Tu is maintained at a 1:1 molar ratio with tRNA under a wide variety of nutritional conditions [2]. Analysis of *Escherichia coli* tRNA resistance to periodate oxidation revealed that more than 80% of total tRNA is aminoacylated, suggesting that the main part of both EF-Tu and tRNA is present in ternary complexes EF-Tu · GTP · aminoacyl-tRNA [3,4]. EF-Tu is encoded by two unlinked genes, *tufA* and *tufB*, located at 72 and 88 min on the *E. coli* linkage map [5]. Interestingly *tufB* is cotranscribed with four upstream tRNA genes [6,7].

Studies from this laboratory (preceding paper [7a]) have shown that the relative amounts of EF-TuA and EF-TuB are constant and independent of the generation time. Reeh and Pedersen [8] studied the synthesis rates of EF-TuA and EF-TuB under different nutritional conditions and found that the relative expression of *tufA* and *tufB* is invariant with the growth rate. They also showed that the synthesis of EF-TuA and EF-TuB responds differently to a temperature shift and to amino acid starvation, indicating that both genes are subject to different regulatory mechanisms [8,9]. Recently, Gausing [10,11] reported on a strain of *E. coli* carrying an inactive *tufA* gene and showed that *tufB* expression was preferentially stimulated.

In previous papers we have presented evidence that EF-Tu itself is involved in the regulation of the expression of *tufB* but not in that of *tufA*. The evidence was based on two major observations. First it was found that a single-site mutation of *tufA* rendering EF-TuA resistant to the antibiotic kirromycin, affects the expression of *tufB* dramatically, but leaves the expression of *tufA* unaltered. Secondly, addition of EF-Tu to a coupled transcription/translation system, programmed with DNA from a plasmid ($pTuB_1$) harbouring the entire tRNA-*tufB* transcriptional unit, appeared to inhibit the synthesis of EF-Tu *in vitro* rather drastically [7a].

Another approach to study the expression of the *tuf* genes is the modulation of the intracellular concentration of EF-Tu. Previously [1] we demonstrated that insertion of bacteriophage Mu DNA into the coding part of *tufB* results in a complete elimination of EF-TuB from the cell and in a 40% drop in total EF-Tu concentration. Furthermore, transformation of the cell with a plasmid harbouring *tufB* caused an increase in EF-Tu level of about 30–60% depending on the nutritional conditions. Neither the reduction nor the increase in intracellular EF-Tu appeared to affect the expression of *tufA* [7a].

In the present paper we have investigated the effect of such modulations on the expression of *tufB*. We show that raising the intracellular level of EF-Tu by transformation with plasmids harbouring either *tufA* or *tufB* suppresses the expression of chromosomal *tufB*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains used for transformation are listed in Table 1. *Escherichia coli* strains C 600 carrying $pTuA_1$ and LBE

Table 1. *Strains of* E. coli *K12 used in this study*
The designations A_S, A_R, B_S and B_O refer respectively to a wild-type *tufA* product, a kirromycin-resistant *tufA* product, a wild-type *tufB* product, and an altered *tufB* product, which properties have been described previously [13–15]. In the phenotype description, Kir^r is kirromycin resistance; Rif^r is rifampicin resistance, and UV^s is ultraviolet sensitivity. For the isolation of these mutant strains see Van der Meide et al. [1]. For the introduction of the *recA*$^-$ allele, bacteria were treated with trimethoprim to select *Thy*$^-$ cells as described by Miller [28]. *Thy*$^-$ cells were subsequently crossed with a Hfr strain KA 273 (Hfr, $recA_{56}$, *thr*, *ile*). Selection was for *Thy*$^+$ cells and screening for ultraviolet sensitivity

Strain	EF-Tu symbols	Genotype	Phenotype
LBE 11001	A_SB_S	$recA_{56}$	UV^s
LBE 12020	A_SB_O	*tufB*, *rpoB*, $recA_{56}$	UV^s, Rif^r
LBE 12021	A_RB_O	*tufA*, *tufB*, *rpoB*, $recA_{56}$	UV^s, Rif^r, Kir^r
PM 1505	A_S	*tufB*::*(Mu)*, *rpoB*, $recA_{56}$	UV^s, Rif^r
PM 1455	A_R	*tufA*, *tufB*::*(Mu)*, *rpoB*, $recA_{56}$	UV^s, Rif^r, Kir^r

2012,A_RB_O carrying $pTuB_1$ were a generous gift from Dr Y. Kaziro.

Plasmids pcI(857) and pPLa2311 were kindly donated to us by Dr W. Fiers [12]. Plasmid pcI(857) carries a mutated cI gene of phage λ, coding for a temperature-sensitive repressor, which inhibits specifically the initiation of transcription at the P_L promoter of this phage at the permissive temperature. At high temperature this repressor becomes inactive. Plasmid pPLa2311 carries the O_LP_L region of phage λ. The construction of plasmids pGp81 and pGp82 is described below. Plasmid $pTuB_O$ is identical to $pTuB_1$, except that the former plasmid codes for a *tufB* product (EF-TuB_O) differing from wild-type EF-Tu in isoelectric point [13–15].

$pTuA_1$ [16] and pPLa2311 [12] DNA (4 µg of each) were digested to completion with *Eco*RI in 100 mM Tris/HCl, pH 7.6, 10 mM $MgCl_2$ and 50 mM NaCl at 37 °C separately. Subsequently the restriction enzymes were inactivated by heating for 10 min at 65 °C. The digestion products were mixed and ligated for 16 h at 14 °C using T4 DNA ligase in a total volume of 50 µl. The ligase buffer was 50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 20 mM dithiothreitol and 1 mM ATP. The ligation mixture was used to transform *E. coli* strain M 5219 [12]. This strain harbours a defective, non-excisable λ prophage carrying a mutated cI gene that codes for a temperature-sensitive repressor. The transformed cells were incubated for 2 h in rich medium at 28 °C to permit phenotypic expression. Cells carrying plasmid DNA of pPLa-2311 were selected on plates containing kanamycin at a final concentration of 50 µg/ml. The plasmid DNA of 10 transformants was digested to completion with *Eco*RI and analyzed by electrophoresis on 1% agarose gels. Four recombinant plasmids appeared to contain the 4000-base *Eco*RI fragment from $pTuA_1$. These fragments were formally identified as identical to the 4000-base *Eco*RI fragment on the basis of their electrophoretic mobility on agarose gels. The orientation of the fragments inserted in pPLa2311 was determined by studying the electrophoresis patterns obtained after cutting these plasmids with *Sma*I. Vector pPLa2311 has one *Sma*I site whereas the 4000-base fragment contains four of these sites. Three out of four recombinant plasmids yielded fragments of 3300, 2000, 1000 and 300 bases. The remaining one gave fragments of 4100, 1300, 1000 and 300 bases (not illustrated). Plasmids with the former *Sma*I digestion pattern were designated pGp82 and the other pGp81. After digestion with both *Sma*I and *Eco*RI simultaneously, pGp81 and pGp82 gave a pattern consistent with the oriantation of the 4000-base *Eco*RI fragment shown in Fig. 1. These results indicate that the *tufA* gene of pGp81 is located in the sense orientation with regard to the P_L promoter, whereas pGp82 contains this gene in the opposite orientation.

Media and Chemicals

Rich medium (LC) and minimal medium (VB) were prepared as described previously [1]. The antibiotic kirromycin was kindly supplied by Dr R. Beukers (Gist-Brocades N.V., Delft, The Netherlands). Ampicillin and kanamycin were obtained from Brocacef N.V. Tryptone, yeast extract and agar were from Difco.

Isolation and Purification of Plasmid DNA

For the isolation of plasmid DNA, 40-ml cultures were grown with rotary shaking at 37 °C in LC medium supplemented with kanamycin and/or ampicillin (final concentration 50 µg/ml). To amplify the plasmid DNA, chloramphenicol (final concentration 170 µg/ml) was added, according to the procedure of Clewell [17]. Plasmid DNA was isolated by the cleared lysate technique described by Birnboim and Doly [18] and purified by CsCl/ethidium bromide buoyant density gradient centrifugation.

Transformation and Selection of Transformants

Bacteria were transformed according to the procedure of Lederberg and Cohen [19]. Strains were grown in LC medium to an A_{560} of 0.2. Cells (10 ml) were centrifuged, washed in 7 ml of ice-cold magnesium chloride (100 mM), resuspended in 5 ml of ice-cold calcium chloride (100 mM) and kept at 0 °C for 20 min. They were sedimented and resuspended in 1 ml 100 mM $CaCl_2$ (0 °C). After 15 min 2–5 µg of plasmid DNA was added. After 30 min at 0 °C the temperature was raised to 42 °C for 2 min, whereafter the mixture was kept at 0 °C for 15 min. Subsequently the cells were inoculated in 10 ml of rich medium and permitted to grow for 1 h at 37 °C. They were concentrated by centrifugation, resuspended in 1 ml fresh medium and plated on selective medium.

RESULTS

Plasmids Harbouring tufA *or* tufB

Shibuya et al. [16] reported the cloning of an *Eco*RI fragment derived from λ*fus*3 DNA harbouring *tufA* into the *Col*E1 derivative plasmid RSF2124. The plasmid obtained was called $pTuA_1$ and contains the C-terminal portion of the *fus* gene (EF-G), the intercistronic region between *fus* and *tufA* and the complete structural gene for *tufA* [20]. This cloned *tufA* gene is only weakly expressed in a cell-free system due to a reported lack of the natural promoter [16]. In kirromycin-resistant cells of *Escherichia coli* K12 it is also poorly expressed [21] (and this paper). In order to compensate for the reported lack of the natural promoter, we have introduced the 4000-base *Eco*RI fragment of $pTuA_1$ into the unique *Eco*RI site of the vector pPLa2311 [12]. This vector was originally constructed by Remaut et al. [12] and contains

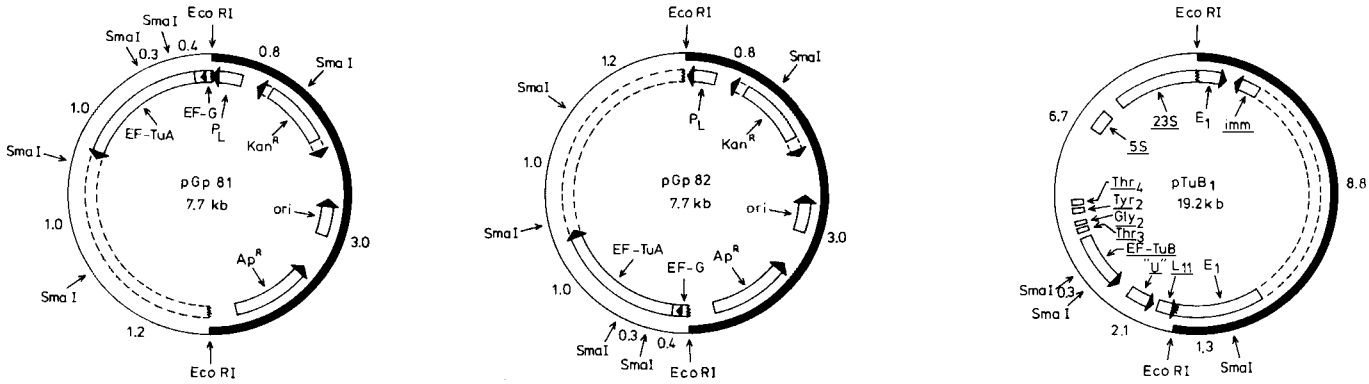

Fig. 1. *Genetic maps of plasmids pGp81, pGp82 and pTuB*$_1$. The heavy lines represent vector DNA, the remaining parts DNA fragments from *E. coli* harbouring *tufA* or *tufB*. The *Eco*RI and *Sma*I sites are indicated at the outer ring. The distance between the restriction endonuclease cleavage sites are given in 10^3 bases (kb). The arrows of the inner ring indicate the direction of transcription. The jogged ends indicate the ligation sites of vector and *E. coli* DNA fragments. The bacterial genes of pTuB$_1$ are: a part of *rrnB*, the complete tRNA-*tufB* transcription unit (*Thr*4, *Tyr*2, *Gly*2, *Thr*3 and EF-TuB), the gene coding for an unknown protein ('U'), and a part of *rplK* (L11). pGp81 and pGp82 contain the entire structural gene for EF-TuA, the intercistronic region between *tufA* and *fus* and the C-terminal encoding part of the *fus* gene. Restriction analysis revealed that pGp81 contains this fragment in the sense and pGp82 in the opposite orientation with regard to the P_L promoter. Detailed information about the vector DNA of pGp81 and pGp82 has been given by Remaut et al. [12]. The construction of pTuB$_1$ has been described by Miyajima et al. [22] and information about the *Eco*RI fragment carrying the *tufA* gene has been given by Shibuya et al. [16]

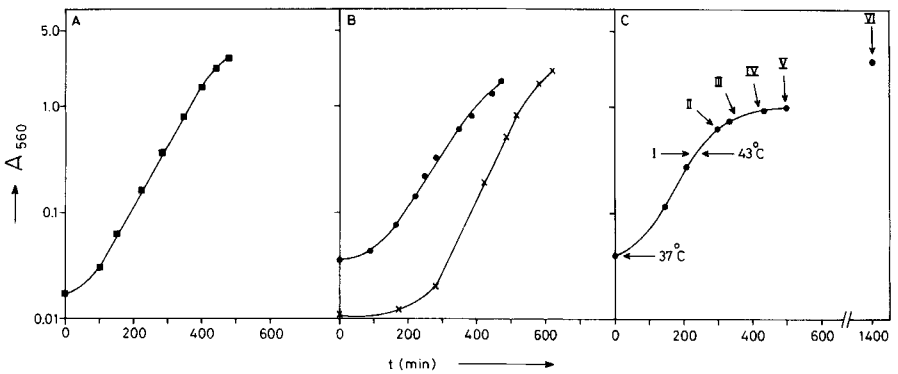

Fig. 2. *Growth of* E. coli *K12 strain LBE 12020,A*$_S$*B*$_O$ *carrying different plasmids*. (A) pcI; (B) pGp82 (lower curve), pcI and pGp81 (upper curve); (C) pcI and pGp81. Cells were grown in liquid culture (LC) at 37 °C under rotary shaking. The media were supplemented with ampicillin and/or kanamycin at a final concentration of 50 µg/ml. The culture of (C) was grown at 37 °C until an absorbance at 560 nm of about 0.3 was reached. At that time the temperature was shifted to 43 °C. Samples of this culture were withdrawn at the times indicated (I, II, etc) and their intracellular EF-Tu and EF-Ts contents were determined (see Table 4) as described in the preceding paper [7a]

the powerful major leftward promoter (P_L) of phage λ. The *Eco*RI site is located about 150 base pairs downstream of the P_L promoter. Two plasmids designated pGp81 and pGp82, harbouring *tufA* in both orientations, were obtained. In order to control transcription of the plasmid-borne *tufA*, a second plasmid compatible with pGp81 and pGp82 was introduced. This plasmid, designated pcI(857), bearing a cI_{ts} gene of phage λ, codes for a temperature-sensitive repressor. Transcription from P_L can be switched off at low temperature in the presence of this cI_{ts} gene.

A recombinant DNA molecule constructed from the *Col*E1-derivative plasmid RSF2124 and an 8900-base *Eco*RI segment from transducing phage $\lambda rif^d 18$, carrying the *tufB* gene, was described by Miyajima et al. [22]. This plasmid was designated pTuB$_1$ and contains a part of *rrnB*, four tRNA genes, *tufB*, the gene coding for an unidentified protein ('U'), and a part of *rplK* (L11) (see Fig. 1). This plasmid permits a good expression of *tufB* both in a cell-free system [22] and in cells of the kirromycin-resistant mutant LBE 2012,A$_R$B$_O$ [21] (compare also Table 2 of this paper). Although the copy number of pTuB$_1$ in the transformants was about 20, the rate of EF-Tu synthesis was not appreciably increased [21].

We have made use of these plasmids to raise the intracellular amount of EF-Tu and have studied the effect on the expression of chromosomal *tufB*. In order to prevent recombination between cloned and chromosomal *tuf* genes the *recA*$^-$ allele of strain KA 273 was crossed into mutant and wild-type strains (see Table 1).

Expression of Plasmid-Borne tufA *in Strains of* E. coli *Resistant to the Antibiotic Kirromycin*

Restriction analysis (cf. Materials and Methods) showed that pGp81 contains *tufA* in the sense orientation with regard to the P_L promoter whereas pGp82 contains this gene in the opposite orientation (Fig. 1). Transformants harbouring pGp81 are unable to survive on selective plates at temperatures ranging over 28 – 37 °C in the absence of the plasmid pcI in contrast to transformants harbouring pGp82 which do survive under these conditions. This indicates that transcription of the cloned *tufA* initiated at the P_L promoter is incompatible with growth. In the presence of the repressor the steady-state growth rate of LBE 12020 transformed with pGp81 is only slightly reduced as compared to that of the

parental strain (Fig. 2). The same holds true for cells transformed with pGp82 but lacking pcI. It should be noted, however, that cells from LBE 12020 carrying both pcI and pGp81 or pGp82 show an extended lag phase which is extremely prolonged in semi-rich medium (casamino acids medium, see [1]). Expression of the plasmid-borne *tufA* was studied in two kirromycin-resistant strains: LBE 12021,A_RB_O and PM 1455,A_R. Since sensitivity to the antibiotic dominates resistance [23], expression in the transformants is revealed by a change in phenotype. The data presented in Table 2 illustrate that pGp81 and pGp82 are expressed in both strains. This indicates that transcription of the plasmid-borne *tufA* can proceed independently of the P_L promoter, presumably from a secondary promoter of the *tufA* gene. There is accumulating evidence that in addition to the major promoter of the *str* operon, *tufA* probably has a secondary promoter located approximately 120 base pairs from the start codon of *tufA* [20, 24, 25].

Table 2 shows that expression of $pTuA_1$ in LBE 12021,-A_RB_O and PM 1455,A_R is hardly detectable. By contrast expression of pGp81 and pGp82 in these strains is much more efficient. Since transcription of *tufA* on all three plasmids is assumed to occur from the same secondary promoter, the expression of *tufA* in the genetic environment of $pTuA_1$ is reduced for unknown reasons.

Table 2. *Minimal kirromycin concentrations causing growth inhibition in various transformed and parental strains of* E. coli *K12*
Transformed cells were grown at 37 °C in liquid broth (LC) containing 50 µg/ml kanamycin and/or 50 µg/ml ampicillin. Parental strains were grown under identical conditions in the absence of the antibiotics. Cells (2×10^6) were plated on VB agar [1] which contained 1 mM EDTA (to make cells permeable for kirromycin), 1% glucose, 0.5% casamino acids, varying concentrations of kirromycin, and kanamycin and/or ampicillin at a final concentration of 50 µg/ml (in the case of transformants)

Transformed and parental strains	Kirromycin concn
	µg/ml
LBE 11001,A_SB_O	< 20
LBE 12021,A_RB_O	> 4000
PM 1455,A_R	> 4000
LBE 12021,A_RB_O, $pTuA_1$	> 4000[a]
PM 1455,A_R, $pTuA_1$	> 4000[a]
LBE 12021,A_RB_O, $pTuB_1$	< 20
PM 1455,A_R, $pTuB_1$	< 40
LBE 12021,A_RB_O, pcI, pGp81	< 20
PM 1455,A_R, pcI, pGp81	< 40
LBE 12021,A_RB_O, pGp82	< 20
PM 1455,A_R, pGp82	< 30

[a] Some growth retardation.

The Concentration of EF-Tu in Cells Carrying pGp81, pGp82 or $pTuA_1$

After having established the expression of the plasmid-borne *tufA in vivo*, the intracellular EF-Tu concentrations were determined in transformants and parental strains with an immunological assay which has been described in the preceding paper [7a]. Cells carrying pGp81 or pGp82 were grown at 37 °C in the presence of both ampicillin and kanamycin. In the case of $pTuA_1$ only ampicillin was included in the medium. Table 3 and Fig. 3 show that transformants

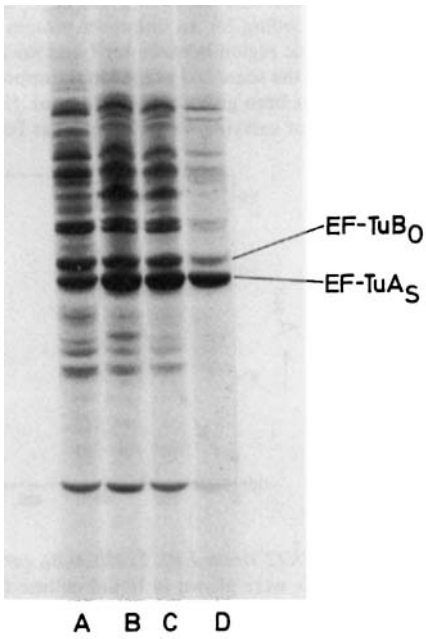

Fig. 3. *Isoelectric focusing gel electrophoresis of high-speed supernatant fractions of strain LBE 12020,A_SB_O carrying different plasmids.* 100000 × *g* supernatant protein fractions were prepared as described in the preceding paper [7a] and submitted to isoelectric focusing gel electrophoresis on 3% $NaDodSO_4$/polyacrylamide gels [27]. Gels were stained with Coomassie brilliant blue. Gels A, B and C contain approximately 50 µg of protein derived from transformants carrying pcI, pGp82, and pGp82 together with pcI, respectively, cultured at 37 °C. Gel D contains 25 µg protein derived from induced cells harbouring pGp81 and pcI. These cells were harvested at growth stage IV as illustrated in Fig. 2C

Table 3. *Intracellular amounts of EF-Tu and EF-Ts in transformants of LBE 12020,A_SB_O carrying different multicopy plasmids*
Bacterial cultures were grown to the mid-log phase at 37 °C in rich medium (LB) containing 50 µg/ml kanamycin (LBE 12020, pcI) or 50 µg/ml ampicillin (LBE 12020, $pTuA_1$) or in the presence of both antibiotics (LBE 12020, pcI, pGp81 and LBE 12020, pGp82). The contents of EF-Tu and EF-Ts were determined in crude bacterial extracts as described in the preceding paper [7a]

Strain	Medium	Growth rate	EF-Tu content	EF-Tu content	EF-Ts content
			nmol/mg protein	%	nmol/mg protein
LBE 12020,A_SB_O, pcI	LC	1.1	2.34	10.1	0.35
LBE 12020,A_SB_O, pcI, pGp81	LC	0.9	3.96	17.0	0.34
LBE 12020,A_SB_O, pGp82	LC	1.0	4.10	17.6	0.34
LBE 12020,A_SB_O, $pTuA_1$	LC	1.2	2.21	9.5	0.36

carrying pGp81 or pGp82 contain an almost twofold EF-Tu level as compared to the parental strain. This increase appeared to be quite specific as the levels of EF-Ts (Table 3) and ribosomes (not illustrated) remained unaltered. Transformants harbouring $pTuA_1$ showed no demonstrable increase in EF-Tu content (Table 3) in accordance with the low expression of the plasmid-borne *tufA* (the former section).

The opposite orientations of the 4000-base *Eco*RI fragment in pGp81 and pGp82 have no effect on the total EF-Tu level assayed in cells cultured at 37 °C (Table 3). This indicates that transcription is not initiated at the P_L promoter under these conditions.

The Thermo-Inducible Expression of the Cloned tufA *Gene*

To determine whether transcription of the cloned *tufA* fragment of pGp81 was under the control of the P_L promoter, bacteria were grown at 37 °C in L-broth supplemented with kanamycin and ampicillin (final concentrations 50 µg/ml) to a density of approximately 3×10^8 cells/ml. The temperature was subsequently shifted to 43 °C. At various times thereafter, samples were taken and analyzed for EF-Tu and EF-Ts content. Due to inactivation of the λ repressor the EF-Tu content reached values up to 30% of total cellular protein (see Fig. 3 and Table 4) within 2 h after the temperature shift. Prolonged incubation at 43 °C did not lead to a further increase in EF-Tu content. That this increase is rather specific and restricted to EF-Tu can be seen in Table 4, showing that the EF-Ts level remained virtually constant under these conditions. Soon after the temperature shift the growth rate started to decline (Fig. 2C). After about 24 h at 43 °C (stage VI of Fig. 2C) cells had lost substantial amounts of EF-Tu. EF-Ts showed a far less pronounced decrease (Table 4). When cells transformed with pGp82 were also submitted to a temperature shift-up their EF-Tu level increased from 17% to 22% of the bacterial protein (not shown). The additional EF-Tu found in pGp81 transformants at 43 °C may thus be ascribed to transcription from the P_L promoter.

The Overproduction of EF-TuA Leads to a Decrease in Chromosomal EF-TuB Content in vivo

In order to study the effect of an elevated EF-Tu level on the expression of *tufB*, the intracellular EF-TuA and EF-TuB_O contents of transformants of LBE 12020,A_SB_O growing exponentially at 37 °C were determined as described in the preceding paper [7a]. The parental strain of these transformants codes for an altered *tufB* gene product (EF-TuB_O) which enables separation of wild-type EF-Tu and EF-TuB_O by isoelectric focusing. The results (Table 5) show that overproduction of EF-TuA leads to a specific reduction of the amount of EF-TuB_O but does not alter the intracellular amount of EF-Ts (not illustrated here).

This reduction requires some comment. It may be realized that EF-TuB_O in the transformants has to be assayed in the presence of the strongly accumulated EF-TuA which makes high demands upon the separation of the two EF-Tu species. For reasons which have remained unclear so far, about 10% of the EF-TuA appears in the EF-TuB position after isoelectric focusing. This spillover of EF-TuA contributes substantially to the EF-Tu amount assayed in the B position. If this is taken into account, the EF-TuB_O content of pGp81 transformants of LBE 12020,A_SB_O is 0.48 nmol/mg protein, if not it is 0.78 nmol/mg protein. For pGp82 transformants these two values are 0.48 and 0.80 nmol/mg protein.

Table 4. *Intracellular amounts of EF-Tu and EF-Ts in transformants of LBE 12020,A_SB_O carrying both pcI and pGp81 during different stages of growth after a temperature shift up from 37 °C to 43 °C*
The growth curve is illustrated in Fig. 2C. The cells were harvested at the times indicated in this figure (I, II, etc.). The experimental conditions are described in the legend to Fig. 2 and in the preceding paper [7a]

Growth phase (from Fig. 2 C)	EF-Tu content		EF-Ts content
	nmol/mg protein (%)		nmol/mg protein
I	3.85	(16.6)	0.33
II	4.36	(18.7)	0.29
III	6.88	(29.6)	0.31
IV	6.92	(29.8)	0.29
V	6.59	(28.3)	0.30
VI	1.90	(8.2)	0.23

Table 5. *Intracellular amount of EF-TuB_O in a strain of* E. coli *K12 (LBE 12020,A_SB_O) carrying different plasmids*
Culture conditions were as described in the legend to Table 3. The cells were harvested at the mid-log phase and assayed for EF-TuB_O content as described in the preceding paper [7a]

Strain	Medium	Growth rate	EF-TuB_O content
			nmol/mg protein (%)
LBE 12020,A_SB_O pcI	LC	1.1	0.96 (4.1)
LBE 12020,A_SB_O pcI, pGp81	LC	0.9	0.63[a] (2.7)
LBE 12020,A_SB_O pGp82	LC	1.0	0.65[a] (2.8)

[a] See comments in the text.

Expression of Plasmid-Borne tufB *in Strains of* E. coli *Resistant to the Antibiotic*

Expression of *tufB* on $pTuB_1$ in transformants of LBE 12021,A_RB_O and PM 1455,A_R is illustrated in Table 2, showing a change in phenotype from kirromycin resistance to sensitivity. These data confirm earlier results reported by Miyajima and Kaziro [21] for the strain LBE 2012,A_RB_O.

The Intracellular Concentrations of EF-TuA, EF-TuB, and EF-Ts in E. coli *Cells Carrying Plasmid $pTuB_1$ or $pTuB_O$*

As mentioned in the introduction, the plasmid $pTuB_1$ harbours the entire tRNA-*tufB* transcription unit. $pTuB_O$ differs from $pTuB_1$ only be the replacement of the wild-type *tufB* by the mutant $tufB_O$ gene. Transformation of cells of the A_SB_O type with $pTuB_1$ enables monitoring of the expression of the chromosomal *tufB*, transformation of these cells with $pTuB_O$ monitors expression of the chromosomal *tufA* and transformation of cells of the A_SB_S type with $pTuB_O$ monitors expression of the plasmid-borne *tufB*. Previously [7a] we showed that transformation with either plasmid raised the intracellular EF-Tu content of LBE 12020,A_SB_O to the same extent. This increase in EF-Tu was not accompanied by an increase in EF-Ts.

Fig. 4A illustrates the results after transforming LBE 11001,A_SB_S with $pTuB_O$, Fig. 5A results after transforming LBE 12020,A_SB_O with the same plasmid. Transformant and

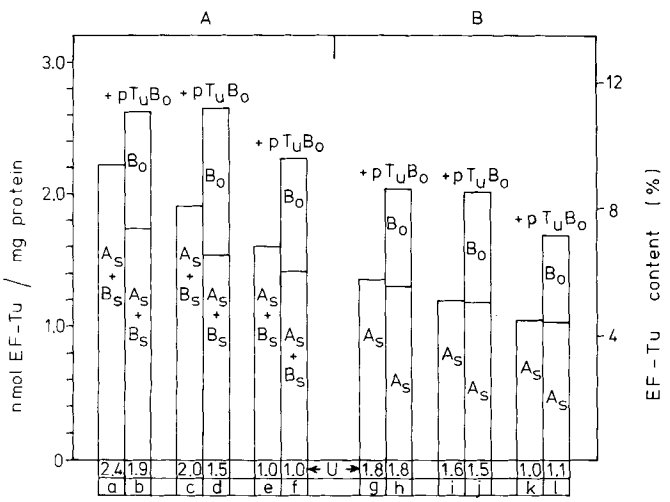

Fig. 4. *Intracellular concentrations of chromosome-encoded and plasmid-encoded EF-Tu with and without plasmid pTuBo.* (A) Intracellular concentrations of chromosome($A_S + B_S$)-encoded and plasmid(B_O)-encoded EF-Tu in strain LBE 11001,A_SB_S with (b, d and f) and without (a, c and e) plasmid $pTuB_O$. Cells were grown in rich (a, b), semi-rich (c, d) and minimal (e, f) medium under conditions described in [7a]. The growth rates (U, doubling/h) are indicated underneath the bars. (B) Intracellular concentrations of chromosome(A_S)-encoded and plasmid(B_O)-encoded EF-Tu in strain PM 1505,A_S with (h, j and l) and without (g, i and k) plasmid $pTuB_O$. The cells were cultured in rich (g, h), semi-rich (i, j) and minimal medium (k, l) under conditions described in [7a]. The growth rates (U, doubling/h) are indicated underneath the bars

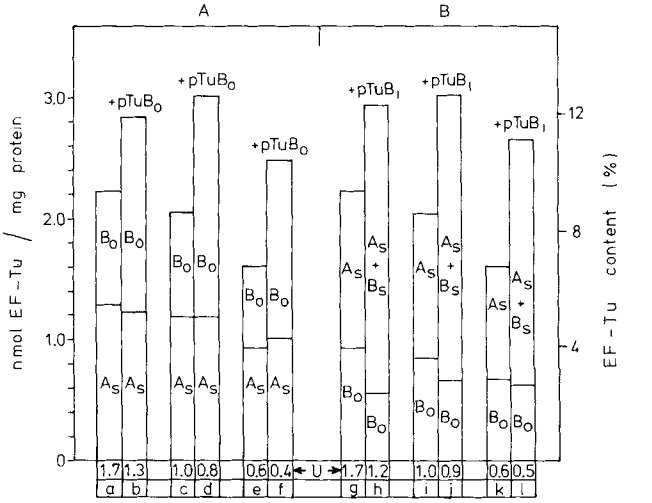

Fig. 5. *Intracellular concentrations of the EF-Tu products of chromosome and plasmid-encoded EF-Tu species in strain LBE 12020,A_SB_O transformed with $pTuB_O$ (a–f) and $pTuB_1$ (g–l).* Cells were grown in rich (a, b, g, h), semi-rich (c, d, i, j) and in minimal (e, f, k, l) medium under conditions described in [7a]. The growth rates (U, doubling/h) are indicated underneath the bars

parental strains were grown under steady-state growth conditions at varying rates. The EF-Tu contents of the parental cells increased with the growth rate. After transformation of LBE 11001, A_SB_S a maximum EF-Tu level of about 11.3% of the bacterial protein was recorded at growth rates of 1.9 and 1.5 doublings/h (Fig. 4). Subtracting the EF-TuB_O content from the total amount of EF-Tu, yielded the sum of the two chromosomal products EF-TuA_S and EF-TuB_S. As can be seen from Fig. 4, transformation causes a drop in the total amount of these EF-Tu species as compared to that of the parental strain. This drop varies with the growth conditions and reaches a maximum of about 20% at the highest growth rate. Previously [7a] we found that at growth rates exceeding 0.8 doubling/h the intracellular concentrations of EF-TuA, EF-TuB, EF-Ts and the ribosomes increased with the growth rate in a constant molar ratio. Since transformation was also accompanied by a drop in growth rate the question must be considered here whether the drop in the total expression of the two chromosomal *tuf* genes is the consequence of this growth retardation rather than that of the transformation-induced increase of total intracellular EF-Tu. Assays of EF-Ts contents of these cells (not illustrated here) and those of the transformants of LBE 12020,A_SB_O studied in Fig. 5 and in [7a] did not reveal any changes in EF-Ts content relative to that of the parental cells. We conclude, therefore, that the reduction in total chromosomal EF-Tu recorded in Fig. 4 is a specific *tuf* gene dosage effect. Fig. 5 demonstrates that this reduction concerns EF-TuB only since that of EF-TuA remains unaltered.

In order to study the effect of an elevated EF-Tu level on the expression of chromosomal *tufB* directly, cells of LBE 12020,A_SB_O were transformed with $pTuB_1$ (Fig. 5B). The transformation-induced increase amounted to 30–60% of total EF-Tu originally present in the parental strain depending on the nutritional conditions. EF-Tu derived from the plasmid-borne *tufB* and that from the chromosomal *tufA* migrated to the same position during isoelectric focusing whereas the chromosome-encoded EF-TuB_O moved to a different position due to its difference in isoelectric point (compare also Fig. 3). As can be seen in Fig. 5, expression of chromosomal *tufB* is suppressed when the total level of EF-Tu increases as a result of transformation. This suppression becomes less pronounced at lower growth rates. That it cannot be ascribed to the growth retardation observed, is concluded again from the lack of any change in EF-Ts and ribosome content (compare [7a]).

The data presented in Fig. 4 and 5 were obtained by the combined use of specific immunoelectrophoresis assays of EF-Tu in crude bacterial extracts and the separation of the electromeric EF-Tu isomers in the $100000 \times g$ supernatants as described [7a]. Virtually the same ratios between the electromeric isomers were found when EF-Tu was isolated from transformants and parental cells, purified to homogeneity by affinity chromatography and submitted to isoelectric focusing (Fig. 6). These findings and those of Fig. 4 and 5 place our conclusion, that elevation of the intracellular level of EF-Tu suppresses the expression of *tufB* but not that of *tufA*, on a firm experimental basis.

The Expression of Plasmid-Borne tufB *in Cells Lacking an Active Chromosomal* tufB *Gene*

To study the effect of lowering the intracellular EF-Tu level on the expression of *tufB*, we made use of the plasmid $pTuB_O$ which was introduced into strains PM 1505,A_S and PM 1455,A_R. These strains carry an inactive chromosomal *tufB* gene due to the insertion of bacteriophage Mu DNA into the coding part of the latter gene. As mentioned in the introduction, their intracellular EF-Tu level is reduced by approximately 40% as compared to that of the wild-type strain [1].

Fig. 4B shows that transformants of PM 1505,A_S contain approximately 20–25% less EF-TuB_O than transformants of LBE 11001,A_SB_S. Apparently the inactivation of the chromo-

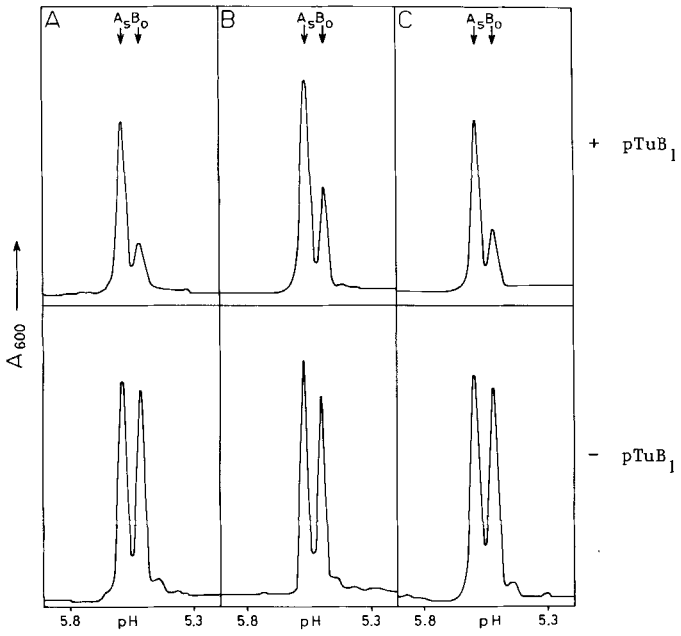

Fig. 6. *Scanning profiles of isoelectric focussing gels loaded with EF-Tu isolated and purified to homogeneity by affinity chromatography from transformant (LBE 12020,A_SB_O, $pTuB_I$) and parental strain in three different media.* For experimental details see [7a]. The letters above the peaks signify the two EF-Tu species with different isoelectric points present in the EF-Tu preparations. (A), (B) and (C) refer to EF-Tu preparations from cells cultured in rich medium (LC), semi-rich medium (casamino acids) and minimal medium (glucose), respectively

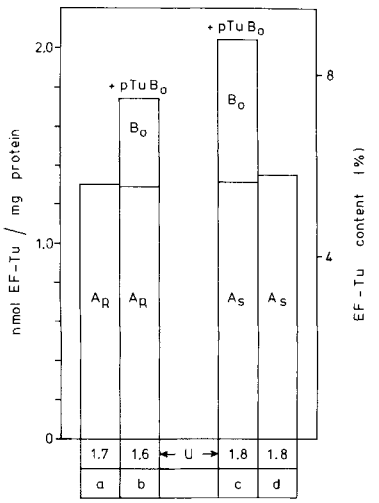

Fig. 7. *Intracellular concentrations of chromosome(A_R or A_S)-encoded and plasmid(B_O)-encoded EF-Tu species in two strains of* E. coli *K12 lacking an active chromosomal* tufB *gene.* The amounts of chromosome and plasmid-derived EF-Tu species were assayed in strain PM 1455 (a, b) and PM 1505 (c, d) with (b, c) or without (a, d) plasmid $pTuB_O$. The cells were cultured in rich medium (LC) in the absence (without plasmid) or presence (with $pTuB_O$) of ampicillin at a final concentration of 50 µg/ml. The growth rates (U, doubling/h) are indicated underneath the bars

somal *tufB* by Mu DNA insertion has reduced the expression of the plasmid-borne *tufB*. This reduction is even more pronounced (60%) in transformants of the strain PM 1455,A_R as is illustrated in Fig. 7. Note that transformation of neither PM 1505,A_S nor that of PM 1455,A_R is accompanied by a decrease in the growth rate. This contrasts with the significant retardation of growth in cells harbouring two active chromosomal *tuf* genes when transformed with either $pTuB_O$ or $pTuB_I$ (see Fig. 4, 5 and 7). We come back to these results in the Discussion.

DISCUSSION

The major conclusion from the present investigation is that an elevation of the intracellular EF-Tu level causes suppression of the expression of the chromosomal *tufB* gene. This elevation has been achieved by transforming *Escherichia coli* cells with multicopy plasmids harbouring either *tufA* or *tufB*. Although suppression of the expression of the chromosomal *tufB* gene is observed in both cases, the elevation of the EF-Tu level after transformation with the former plasmid is much higher than after transformation with the latter. Also the growth behaviour of the transformants differs significantly. A strong retardation of growth followed by a complete stop of cell division is observed when the transcription of the plasmid-borne *tufA* from the P_L promoter on pGp81 is initiated by thermal inactivation of the temperature-sensitive λ repressor. Growth, however, is already considerably affected when the λ repressor is not inactivated and transcription from the P_L promoter is blocked. Transcription of the plasmid-borne *tufA* then occurs from an RNA polymerase binding site, which, according to various authors [20, 24, 25], is located in the C-terminal region of the *fus* gene and has been cloned together with *tufA*. Transcription from this site results in a 75% increase in EF-Tu content (in rich medium) and transformants cultivated under these conditions (37 °C) show an extended lag phase in their growth. In semi-rich medium this lag is prolonged to such an extent that growth is virtually abolished. In rich medium (LC), however, transformants start to grow after the lag, whereafter they reach a steady-state growth rate which does not differ from that of cells transformed with pcI only (Fig. 3). Apparently pGp81 and pGp82 transformants are able to overcome an initial block but when they reach the logarithmic phase their growth is hardly diminished.

Consequences of transformation with $pTuB_I$ or $pTuB_O$ are much less dramatic. Transformed cells do not have an extended lag phase. Their steady-state growth rate is, compared to that of the parental strain, diminished. The maximum EF-Tu level represents approximately 12.5% of the bacterial protein which contrasts to that of cells transformed with a plasmid carrying *tufA* (17%). It should be recalled that $pTuB_I$ and $pTuB_O$ harbour the entire tRNA-*tufB* transcription unit, including the elements controlling the expression of this unit. Miyajima and Kaziro [21] reported that the rate of EF-Tu synthesis in transformants was not appreciably increased although the copy number of $pTuB_I$ was about 20. These authors suggested the presence of a regulatory mechanism that maintains the normal EF-Tu level in the cell. This is in agreement with the regulatory role we ascribe to the EF-Tu protein itself. Our experiments show that transformation with $pTuB_I$ or $pTuB_O$ does elevate the intracellular EF-Tu level in our strains albeit to a limited extent. This elevation (about 25%) is sufficient to reveal a significant suppression of the expression of *tufB* (about 40% at the highest growth rate). This makes $pTuB_I$ and $pTuB_O$ better instruments for studying the regulation of the expression of *tufB* than pGp81 and pGp82, carrying *tufA*. The latter plasmids raise the EF-Tu level too much, even at 37 °C, which complicates the accurate estimation of *tufB* expression and causes

a complex cell response as is evident from the growth behaviour.

The suppression of *tufB* expression is a specific effect of the rise in EF-Tu level rather than the result of growth retardation. This is concluded from the intracellular contents of EF-Ts and ribosomes which remain unaltered after transformation. This effect on *tufB* expression *in vivo* is in agreement with results previously obtained *in vitro* [7a] which demonstrated that addition of relatively small amounts of EF-Tu to a coupled transcription/translation system programmed with $pTuB_I$ DNA inhibited the synthesis of EF-Tu significantly. They are also in line with our observation that a single-site mutation of *tufA* alters the expression of *tufB*, which was the first indication that EF-Tu itself is involved in the regulation of *tufB* [1]. Furthermore, they are in agreement with reports by Gausing [10, 11] who observed an enhanced expression of *tufB* in an *E. coli* strain carrying an inactive *tufA* gene.

Recently, Zengel and Lindahl [26] also studied the effect of overproduction of EF-Tu after transformation with plasmids carrying *tufA* or *tufB*. These authors failed to observe any reduction of the synthesis rate of either *tufA* or *tufB* under minimal growth conditions. Their results do not necessarily contradict our results. Under comparable growth conditions the expression of the *tufB* gene in our transformants is only marginally affected (compare Fig. 4 and 5).

The question may be raised at which level EF-Tu may control the expression of *tufB*. A regulatory function at the level of transcription may not be excluded but it is attractive to consider a control function at a post-transcriptional level similar to that suggested for the regulation of the expression of ribosomal protein genes (compare Discussion in [7a]). An analogous mechanism for the regulation of *tufB* expression would imply that the main part of the EF-Tu population is taken up in ternary complexes with aminoacyl-tRNA and GTP, while only EF-Tu not complexed with aminoacyl-tRNA would exert its regulatory action.

Lowering of the Intracellular EF-Tu Concentration

Insertion of bacteriophage Mu DNA into chromosomal *tufB* resulted in a complete elimination of EF-TuB from the cell and in a 40% decrease of the total EF-Tu content. Previously [1] we have demonstrated that the expression of *tufA* remains unaltered despite this reduction in the intracellular EF-Tu level. The present data (Fig. 4) show that, after introduction of *tufB* via the vector $pTuB_O$, the expression of this plasmid-borne gene is suppressed as compared to that in transformants harbouring two intact chromosomal *tuf* genes. In the light of the experiments discussed in the previous section, suppression rather than stimulation may seem somewhat surprising. It suggests that EF-Tu is not the only cellular component involved in the regulation of *tufB* expression.

Since insertion of Mu DNA occurred in the coding part of chromosomal *tufB*, the expression of the tRNA genes cotranscribed with *tufB* presumably is not affected. It is conceivable, therefore, that the inactivation of chromosomal *tufB* has resulted in a decrease in the EF-Tu/tRNA ratio. It has been pointed out already that wild-type cells maintain their EF-Tu and tRNA at a 1:1 molar ratio. Possibly the cell corrects deviation from this ratio by suppressing *tufB* expression but the mechanism underlying such an suppression can only be a matter of speculation. One speculation is that an excess of aminoacyl-tRNA, not taken up in ternary complexes EF-Tu · GTP · aminoacyl-tRNA, is responsible for the suppression. In agreement with this assumption is the stronger suppression in cells (PM 1455,A_R) producing an EF-TuA which binds aminoacyl-tRNA less efficiently than wild-type EF-TuA [7a] (Fig. 7). A notable further observation is that transformation did not affect the growth rate of cells harbouring an inactivated chromosomal *tufB* (PM 1505,A_S and PM 1455,A_R) (compare Fig. 4 and 7). The possibility exists that this also must be ascribed to the relative excess of tRNA over EF-Tu. In the reverse situation, evoked by transformation of cells harbouring two active chromosomal *tuf* genes, with *tufA* or *tufB*-carrying plasmids, changes in the growth rate were conspicuous. Under these conditions it is the elevated EF-Tu level which suppresses *tufB* expression. It may be relevant in this respect that *tufB* is cotranscribed with four upstream tRNA genes. Presumably the expression of the latter genes is also affected by raising the EF-Tu level. When such an increase becomes too high, as is the case after transformation with pGp81 and pGp82, cells may suffer from a serious lack of specific tRNAs. When the increase is moderate, which is the case after transformation with $pTuB_I$ and $pTuB_O$, this lack may be expressed in a reduced growth rate but not in an extended lag phase. Moreover, $pTuB_I$ not only elevates the intracellular EF-Tu but that of the four specific tRNAs as well. Finally, when cells with a relative excess of tRNA over EF-Tu (PM 1455,A_R and PM 1505,A_S) are transformed, no deficiency of a specific tRNA is to be expected and growth is not affected (Fig. 4 and 7).

Obviously further analyses, particularly of the intracellular tRNAs and of the primary transcripts of the tRNA-*tufB* transcription unit are needed to obtain a deeper insight into the regulation mechanism of this interesting transcription unit.

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