The Gene Encoding the Elongation Factor P Protein Is Essential for Viability and Is Required for Protein Synthesis*

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Elongation factor P (EFP) is a protein that stimulates the peptidyltransferase activity of fully assembled 70 S prokaryotic ribosomes and enhances the synthesis of certain dipeptides initiated by N-formylmethionine. This reaction appears conserved throughout species and is promoted in eukaryotic cells by a homologous protein, eIF5A.

Here we ask whether the *Escherichia coli* gene encoding EFP is essential for cell viability. A kanamycin resistance (Kan<sup>R</sup>) gene was inserted near the N-terminal end of the *efp* gene and was cloned into a plasmid, pMAK705, that has a temperature-sensitive origin of replication. After transformation into a recA<sup>+</sup> *E. coli* strain, temperature-sensitive mutants were isolated, and their chromosomal DNA was sequenced. Mutants containing the *efp-Kan<sup>R</sup>* gene in the chromosome grew at 33 °C only in the presence of the wild-type copy of the *efp* gene in the pMAK705 plasmid and were unable to grow at 44 °C. Incorporation of various isotopes in vivo suggests that translation is impaired in the *efp* mutant at 44 °C. At 44 °C, mutant cells are severely defective in peptide-bond formation. We conclude that the *efp* gene is essential for cell viability and is required for protein synthesis.

The most important catalytic function of the ribosome is the synthesis of peptide bonds. A variety of approaches have been used to deduce the components that comprise this catalytic center. The results of *in vitro* reconstitution studies, photochemical cross-linking of substrates, and mutagenesis of conditionally lethal or antibiotic-resistant phenotypes have implicated domain V of the 23 S rRNA as well as proteins L2, L3, and L4 as the minimum components of this active center (1–6).

A surprising finding is that the *in vitro* reconstituted peptidyltransferase cannot condense all aminoacyl-tRNA template combinations (7). This anomaly is reflected in the fact there is a subsite on domain V of the 23 S rRNA that is specific for hydrophobic amino acids (2). In retrospect, it has been known for more than two decades that puromycin, which is one of the most common substrate analogues used to study this reaction, favors peptide-bond synthesis (8). Substitution of the aromatic residue of puromycin by that of other amino acids distorts this structure and drastically impairs peptide-bond synthesis (9). This specificity is reflected in the 50 S catalyzed “fragment” reaction that has been used to deduce the components of the peptidyltransferase catalytic center.

Reconstitution studies as well as photoaffinity labeling experiments indicate that several proteins of the 50 S particle enhance peptide-bond synthesis. The assembled peptidyltransferase in the 70 S ribosome catalyzes peptide bonds at a higher rate than does the peptidyltransferase of the 50 S subunit, but does not efficiently condense nonaromatic amino acids (7, 10). In addressing this issue, we asked whether proteins that stimulate reconstitution of translation from homogeneous translation factors enhance the condensation of several amino acids. A soluble protein, EFP,<sup>1</sup> indeed stimulates the rate of peptide-bond synthesis on 70 S ribosomes and, together with components of the 70 S, may be involved in restoring the ability to condense several amino acids to the peptidyltransferase (10–12).

EFP stimulates peptide-bond synthesis by 70 S ribosomes between fMet-tRNA<sub>Met</sub> and analogues of various aminoacyl-tRNAs. For example, the K<sup>+</sup> for cytidyl(3′-5′)-[2′(3′)-O-L-aminocacyladenosine (CA)-Gly] is enhanced 50-fold, whereas that for CA-Phe is essentially unaltered by EFP (10). EFP may modulate the efficiency of protein synthesis by controlling the rate of synthesis of certain peptide bonds. There are 800–900 molecules of EFP per *E. coli*, or about 0.1 to 0.2 copy per ribosome, suggesting that EFP may function catalytically in the cell (13).

The requirements for peptide-bond and ester-bond formation stimulated by EFP have been studied with fMet-tRNA<sub>Met</sub> bound to 30 S subunits and native or reconstituted 50 S subunits. EFP functions in both peptide and ester-bond synthesis promoted by the peptidyltransferase (12, 14, 15). The 50 S particle’s L16 (or its N-terminal fragment) are required for the EFP-mediated synthesis of peptide bonds, whereas L11, L15, and L7/L12 are not required in this reaction, suggesting that EFP may function at a different ribosomal site than most other translation factors (15).

To obtain the sequence of EFP as well as to examine whether it represents an essential cellular function, we have cloned and sequenced the gene encoding this protein (16). The *efp* gene sequence is unique and is represented only once in the *E. coli* chromosome. The EFP protein has been overexpressed, purified to homogeneity and crystallized (17).

To learn whether the gene encoding EFP is essential, we introduced a kanamycin marker in the early coding region of the *E. coli* *efp* gene and cloned the interrupted gene into the pMAK705 plasmid. pMAK705 contains a temperature-sensitive origin of replication. A homologous recombination proce-

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<sup>1</sup>The abbreviations used are: EFP, elongation factor P; CA, cytidyl(3′-5′)-[2′(3′)-O-L-aminocacyladenosine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reactin; X-gal, 5-bromo-4-chloro-3-indoly1 β-D-galactoside; bp, base pair(s); kb, kilobase pair(s).
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Strains and DNAs—pMAK705 (5.6 kb) was a gift from Drs. Sidney R. Kushner and Valerie Maples, University of Georgia. The host strain, *E. coli* JM101, is recA−. The temperature-sensitive cloning vector, plasmid pMAK705, is a mutant isolate of pSC101 (called pH01) (18). The temperature-sensitive restriction enzyme sites were used to replace the *efp-KanR* gene in the *E. coli* chromosome with the mutant copy of the gene. We show that integration of the *efp-KanR* gene into the *E. coli* chromosome results in a lethal phenotype indicating that the *efp* gene is indeed essential for growth.

MATERIALS AND METHODS

**Plasmid and Genomic DNA Isolation—**Plasmid DNA was isolated by the method of Birnboim and Doly (19). To isolate genomic DNA, the *E. coli* JM101 cells (1 × 10^10/ml) were washed with 1 × SSC twice and were suspended in 1 × SSC supplemented with 27% sucrose. After incubation with lysozyme (10 mg/ml) at 37 °C for 20 min, SDS was added to a final concentration of 1% and the reaction was incubated at 60 °C for 10 min. Promase (1 mg/ml final) was then added and the solution was incubated at 37 °C for 7 h. After the addition of an equal volume of phenol, the mixture was rotated gently (20–30 min), then spun at 3000 rpm for 10 min. After two phenol extractions, DNA was dialyzed three times with 1 × SSC. The buffer was changed every 12 h. Plasmid DNA was removed from chromosomal DNA by agarose gel electrophoresis. The chromosomal DNA was isolated by electroelution.

**PCR and Sequence Analysis of Amplified DNA—**High performance liquid chromatography-purified oligonucleotide primers were provided by the Hospital for Sick Children Biotechnology Service Center (University of Toronto). Oligo EF1 is 5'-CGTATTCACCCAGAGCGGTTA-3', which is complementary to a 20-bp sequence of the *efp* coding region. Oligo EF2 is 5'-AGCAACGATTTTCGTGCTGG, which is complementary to the sequence upstream of the N-terminal end of the kanamycin resistance (aminoglycoside 3'-phosphotransferase) gene. *Kan* 3 is 5'-GAGATTTTGAGACACAACGTGG, which is complementary to the C-terminal end of the *efp* gene.

**FIG. 1.** A, position of the relevant restriction enzyme sites within the polyclonal-site region of the plasmids pMAK705 and M13 pBluescript. There are about 50 nucleotides between the EcoRI and the SacI or *KpnI* II sites within this region. The relevant restriction enzyme sites of the *efp-KanR* and of the *efp* gene and their size are also shown. B, restriction enzyme patterns of two plasmid isolates derived from cells carrying the interrupted *efp* gene. Lanes 1 and 14 have calibrated DNA molecular size ladders. Lanes 2–4 have the EcoRI digests of M13 pBluescript and of the pMAK705 plasmids isolated from mutants 5 and 13, respectively; lanes 5–7 have the SacI/KpnI digests of the M13 pBluescript and of the pMAK705 plasmids isolated from mutants 5 and 13, respectively. Lanes 8–10 have the KpnI digests of the M13 pBluescript and of the pMAK705 plasmids isolated from mutants 5 and 13, respectively. The two upper bands in the EcoRI digests of the plasmids isolated from the mutants (lanes 3 and 4) are identical to those obtained with pMAK705 (not shown). The faint 600-bp EcoRI fragment isolated from the mutants contains the C-terminal 500 bp of the *efp* gene. The pMAK705 vector did not harbor the 800-bp SacI/KpnI *efp* fragment present in both pMAK705 plasmids isolated from the mutants. C, detection by PCR of amplification products from chromosomal DNA. The EF1 and EF2 primers were annealed to the chromosomal DNA isolated from the wild-type or from the mutants harboring the *efp* gene interruption and were treated as described under "Materials and Methods." The reactions were run on 1% agarose gels and were stained with ethidium bromide. The size scale on the 100-bp ladder is on the first lane. Lane 2 shows the PCR product (0.6 kb) from the wild-type strain (JM101). Lanes 3–6 show the PCR product (1.9 kb) isolated from chromosomal DNA derived from colonies 5, 11, 12, and 13 harboring the *efp* gene interrupted by the *KanR* gene. The PCR product produced with the *KanR* primers (see "Materials and Methods") corresponds to 1.3 kb (not shown).
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covered in Saran Wrap, and exposed to Kodak X-Omat AR film for at least 2 h, where necessary with intensification screens.

After PCR amplification, the DNA was purified by agarose gel electrophoresis. Dideoxy DNA sequencing was determined by the method of Sanger et al. (20). Sequencing was performed after hybridizing the appropriate clone to DNA probes using DNA Sequanase (Stratagene).

Recombinant DNA Methods—Transformation of E. coli and agarose gel electrophoresis were performed as described previously (21). Restriction endonuclease enzyme digestions and ligations were carried out as outlined by the manufacturers (Life Technologies, Inc. and New England Biolabs).

Construction of an efp Gene Mutant—The N-terminal end of the efp gene has a unique EcoRI restriction site (16) that was used to insert the kanamycin resistance gene. For convenience, the efp-KanR fragment was first ligated into the KpnI/SacI site of M13 plBluescript. After transformation of competent JM101 cells, the plasmid was isolated once again from lac clones that were kanamycin- and ampicillin-resistant. Aliquots of the plasmids were treated with KpnI/Sacl or EcoRI, and the restriction digests were analyzed electrophoretically on 1% agarose gels. The fragment carrying the efp-KanR gene was isolated electrophoretically and was cloned into the KpnI/SacI site of plasmid pMAK705. The pMAK705 plasmid, carrying the interrupted efp gene, was transformed into JM101 cells at 33 °C in LB broth. After transformation was completed, integration of the plasmid was selected by overnight growth at 44 °C in LB medium in the presence of chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml). Twenty-four white colonies (co-integrates) were selected and inoculated into 100 ml of LB broth containing chloramphenicol and kanamycin, and allowed to grow overnight at 33 °C so that a second recombination event could take place resulting in the resolution of the plasmid from the chromosome. Two more cycles of growth were carried out by inoculating 100 ml of an overnight culture into 100 ml of fresh medium. Single colonies were isolated by plating dilutions of the overnight culture on LB media containing 50 μg/ml of chloramphenicol and 50 μg/ml of kanamycin (22). The plate in C has the M13 plBluescript plasmid (upper half) and the M13 plBluescript plasmid with the efp gene insert (lower half). The plate in D has the pUC18 plasmid (upper half) or the pUC18 plasmid with the 0.8-kb efp gene insert (lower half).

RESULTS

The gene replacement method used to delete the efp gene was adapted from that of Hamilton et al. (22). The method makes use of a temperature-sensitive pSC101 replicon to facilitate gene replacement (18). A mutant strain is created when the gene fragment of interest is mutated i.e. by deletion, site-directed mutagenesis or interruption by another gene, and then cloned into the replicon. This replicon, carrying the mutated gene, can then be transformed into an appropriate host. Homologous recombination can then occur between the wild-type gene on the chromosome and the homologous sequences of the mutated gene carried on the temperature-sensitive plasmid. After transformation is completed, it is possible to select for integration of the plasmid into the chromosome at 44 °C. If these co-integrates are then grown at 33 °C, a second recombination event takes place resulting in the resolution of the plasmid from the chromosome. Depending on where the second recombination event takes place, the chromosome will either have undergone a gene replacement or retained the original copy of the gene. This technique will establish if a gene is essential.

Evidence that the efp Gene Was Replaced by the efp-KanR Gene in the E. coli Chromosome—Twenty-four colonies that grew at 33 °C but not at 44 °C in kanamycin were isolated as described under “Materials and Methods” to determine if the plasmid that was present contained the mutant copy or the chromosomal copy of the gene. The plasmids isolated from colonies that grew at 33 °C in kanamycin were subjected to restriction enzyme analysis. Fig. 1A shows the position of the relevant restriction enzyme sites within the polyclonal site region of the pMAK705 plasmid harboring the efp-KanR gene as well as the relevant restriction sites of the efp and efp-KanR genes. For convenience the efp-KanR gene was cloned into the SacI/KpnI site of M13 plBluescript. The SacI/KpnI fragment was excised and cloned into the corresponding SacI/KpnI site of pMAK705. pMAK705 has unique KpnI and SacI sites within this region. The kanR and efp genes, on the other hand, have no KpnI or SacI sites and the kanR gene has no EcoRI sites. The efp gene has an EcoRI site close to the 5’-terminus of the coding region. A second EcoRI site occurs in the pMAK705 polyclonal site. Therefore, EcoRI was used to detect the kanR gene and KpnI/SacI was used to detect the (0.8 kb) efp gene. The coding sequence of the efp gene is 0.6 kb; but the 0.8-kb region har-
boring the upstream region was cloned into pMAK705. Fig. 1B shows the restriction enzyme analysis of the plasmid isolated from two typical mutant clones where the \textit{efp-KanR} gene in pMAK705 was replaced by a gene of identical size to that of \textit{efp}.

Plasmid DNA isolated from 23 of the 24 colonies had the same restriction pattern. The inserts exhibit the restriction enzyme pattern expected of the \textit{efp} gene, further suggesting that the pMAK705 plasmid isolated from these temperature-sensitive clones now harbors the wild-type \textit{efp} gene and not its interrupted version.

The presence of the mutant \textit{efp} in the \textit{E. coli} chromosome was verified by PCR. Total DNA was isolated from wild-type and mutant cells. PCR was then performed on each sample, using primers specific to the 3'- and 5'-regions of both the \textit{efp} and the \textit{KanR} genes. A PCR fragment of the wild-type DNA primed with the probes complementary to the \textit{efp} gene has the size expected (0.6 kb) of the \textit{efp} gene (Fig. 1C, lane 2). However, the PCR of the chromosomal DNA from three mutant clones with the kanamycin insert showed a 1.9-kb band (Fig. 1C, lanes 4–6), which corresponds to the \textit{efp-KanR} gene. The insertion of the kanamycin gene into the \textit{E. coli} chromosome was also confirmed by the sequencing of the PCR products using primers complementary to the kanamycin gene (data not shown).

Effect of \textit{efp} Gene Replacement on Protein Synthesis and on Cell Growth—If the \textit{efp} gene is essential, the cells containing the \textit{efp-KanR} chromosomal copy and the wild type copy of the gene on the plasmid will appear conditionally lethal under these conditions and should be kanamycin resistant at the permissive temperature. The reason is that the mutant gene on the chromosome can no longer be complemented by the wild-type gene on the plasmid since the pMAK705 does not replicate at 44 °C. Cells containing the altered chromosomal copy of the \textit{efp} gene on the pMAK705 plasmid were grown at 44 °C in LB broth in the absence of chloramphenicol in attempt to cure the plasmid from the cell. Indeed, mutant strains grew well at 33 °C but did not grow well or at all at 44 °C (Fig. 2, A and B). This suggested that the \textit{efp} gene is essential for cell viability.

To verify further if the inserted \textit{efp} gene on the pMAK705...
plasmid is essential for growth, we attempted to substitute this plasmid with pUC18 or M13 pBluescript which harbor a ampicillin resistance marker. JM101 is ampicillin-sensitive, but transformation of JM101 cells with these plasmids results in growth in ampicillin (data not shown). JM101 cells harboring the efp-KanR gene and pMAK705 carrying the wild-type efp gene were transformed at 33 °C with the pUC18 or M13 pBluescript plasmids with or without the efp gene insert. Transformation of JM101 cells harboring the efp-KanR gene by M13 pBluescript or pUC18 does not result in growth at 44 °C (Fig. 2, C and D, plates 1 and 2, upper half). Cells grow very well on ampicillin at 44 °C only when the 0.8-kb fragment containing the efp gene coding sequence was cloned into either M13 pBluescript or pUC18 (Fig. 2, C and D, plates 1 and 2, lower half). Thus, the presence of the efp gene in these plasmids is indeed essential for growth.

To establish whether the efp gene affects translation in vivo, we grew one of the mutant strains at 33 °C in the presence of kanamycin and shifted the temperature to 44 °C after the cells had entered the logarithmic phase of growth. Cells harboring the efp gene on the pMAK705 plasmid have a generation time of 30 min at 33 °C, whereas cells from the wild-type JM101 strain double every 24 min. Growth of cells from the mutant strain begins to slow almost immediately when the temperature is shifted to 44 °C. In contrast, the wild-type strain continues to grow at 44 °C (Fig. 3A).

After 60 min of growth at 33 °C, the JM101 cells harboring the efp-KanR gene were shifted to 44 °C and were incubated with [35S]methionine, [3H]thymidine, or [3H]uridine. Control cultures labeled with [3H]thymidine or [3H]uridine show that DNA replication and transcription proceed at an equal rate in JM101 or in the JM101 cells harboring the efp-KanR mutation (Fig. 3, B and C). The [35S]methionine continues to be incorporated into protein by the JM101 wild-type strain after the shift in temperature to 44 °C (data not shown). JM101 cells harboring the efp-KanR gene incorporate [35S]methionine efficiently at 33 °C, but incorporation of [35S]methionine stops when the cultures are shifted to 44 °C (Fig. 3D).

Cells from the control and mutant strain were grown at 33 °C and were shifted to 44 °C (see Fig. 3A). The cells were harvested and were used to partially purify the EFP protein. This was done to remove inhibitors from the extracts. A single step of purification on QAE-Sepharose sufficed to accomplish this. As shown in Fig. 4, the ability of the extracts to stimulate peptide bond synthesis is markedly decreased when the cells are grown at 44 °C to deplete them of EFP. In contrast, the control cells exhibit the expected level of fMet-puromycin synthesis. Taking the activity of EFP recovered from the wild-type strain as 100%, only 20% of the activity is recovered from the strain harboring the interrupted EFP gene. These experiments therefore show that the mutant strain is indeed defective in the EFP function and that depletion of EFP from the cell results in an abrupt cessation of growth.

**DISCUSSION**

A surprising result has been the demonstration that the genes encoding certain ribosomal proteins can be deleted from the chromosome without an apparent effect on cell viability (23). These observations have rekindled interest in whether other genes involved in translation, such as encoding certain translation factors, are essential. There is now evidence that most initiation, elongation and termination factors are required for cellular growth (24–34). Some of these proteins, for example the termination factor RF3, may be dispensable under certain growth conditions, but not others (35). In addition to these classical translation factors are a set of proteins whose action is either stimulatory or indispensable for translation reconstituted in vitro. Of these proteins, the RRF, which is thought to be involved in disassembly of the termination complex produced by RF1 or RF2 (peptide release factors) is only stimulatory to translation in vitro yet it is encoded by an essential E. coli gene (24). Other proteins involved in the association/dissociation of ribosomes, such as the initiation factor (IF3) and the “rescue” protein, appear to be necessary for proper growth, but their action in vivo can be overcome by ionic conditions that foster the association/dissociation of ribosomal particles (36).

Of particular interest in this regard is a protein, EFP, that has been implicated in formation of the first peptide bond in vitro (10). EFP appears to be the prototype of a set of highly conserved proteins named eIF5A in eukaryotic cells (37, 38). These proteins stimulate the formation of a peptide bond between the 70 or 80 S bound fMet-tRNA<sup>Met</sup> and puromycin which mimics synthesis of peptide bonds. These proteins are not required for formation of initiation complexes and do not stimulate synthesis with poly(U), suggesting that they act on synthesis of the first peptide bond encoded in the native template.

For the case of the *Saccharomyces cerevisiae* counterpart of the EFP protein, the genes are essential for growth. These mutants exhibit certain abnormalities in the pattern of their polysomes compatible with an early defect in translation. However, the eIF5A-depleted cells synthesize protein to about 60–70% of the wild-type cells. It is not known whether leakiness of the transcribed eIF5A gene is responsible for the incomplete shut-off of protein synthesis. Alternatively, eIF5A could be selectively involved in synthesis of certain proteins that are essential for growth (39). In this context, it is of interest that the prokaryotic EFP preferentially stimulates synthesis of certain dipeptides and has substantially less effect on synthesis of other dipeptides (10). EFP does stimulate translation reconstituted with a natural mRNA but this stimulation depends on the presence of other less well characterized proteins.

Here we show by PCR and sequence analysis of the chromosomal and plasmid DNA that the interrupted efp gene replaced the wild-type copy of the chromosomal efp gene. After this gene
exchange occurred, the altered cells survived only in the presence of the wild-type copy of the efp gene that was carried on the temperature-sensitive pMAK705 replicon. At restrictive temperatures, cells were unable to grow because the plasmid could not replicate. We conclude that interruption of the efp gene in the chromosome is lethal to the cell indicating that this gene is essential for growth.

A comparison of the growth and radioactive labeling properties at permissive and nonpermissive temperatures of the mutant harboring the efp gene on the chromosome and the wild-type allele in the plasmid suggests that the efp gene is required for protein synthesis in vivo. Extracts derived from mutant cells shifted during growth from permissive to nonpermissive temperatures lost 80% of their peptide-bond forming activity while the control cells remained unaltered. Since plasmids must segregate before the temperature-sensitive lesion is expressed, these results suggest that the efp gene product is essential for protein synthesis in vivo and involves a reaction directly or indirectly involving peptide-bond formation.

The bulk of the evidence indicates that the efp gene functions in an early event of translation. The sequence of this gene is conserved throughout species and represents an essential function. The failure of the EFP and eIF5A proteins to be expressed, these results suggest that the efp gene product is essential for protein synthesis in vivo and involves a reaction directly or indirectly involving peptide-bond formation.

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