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Translation elongation factor Tu cleaved by a phage-exclusion system

(prophage e14/protease/T4 phage)

YUEN-TSU NICCO YU AND LARRY SNYDER*

Department of Microbiology, Michigan State University, East Lansing, MI 48824-1101

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ABSTRACT **Bacteriophage T4 multiplies poorly in *Escherichia coli* strains carrying the defective prophage, e14; the e14 prophage contains the *lit* gene for late inhibitor of T4 in *E. coli*. The exclusion is caused by the interaction of the e14-encoded protein, Lit, with a short RNA or polypeptide sequence encoded by *gol* from within the major head protein gene of T4. The interaction between Lit and the *gol* product causes a severe inhibition of all translation and prevents the transcription of genes downstream of the *gol* site in the same transcription unit. However, it does not inhibit most transcription, nor does it inhibit replication or affect intracellular levels of ATP. Here we show that the interaction of *gol* with Lit causes the cleavage of translation elongation factor Tu (EF-Tu) in a region highly conserved from bacteria to humans. The depletion of EF-Tu is at least partly responsible for the inhibition of translation and the phage exclusion. The only other phage-exclusion system to be understood in any detail also attacks a highly conserved cellular component, suggesting that phage-exclusion systems may yield important reagents for studying cellular processes.**

Resident prophages, plasmids, and transposons often help their host by excluding infecting phages. Well-known examples include the exclusion of phages by the *rex* gene products of λ prophage and exclusion of bacteriophage T7 and related phages by the *pif* gene product(s) of the F plasmid. In all these exclusions, one or more nonessential proteins expressed by the resident element somehow recognize that the cell has been infected by a phage and kill the cell, thereby preventing the spread of the phage to other cells that harbor the DNA element. For a review of phage-exclusion systems, see ref. 1.

The DNA element e14, a defective prophage that is integrated in the isocitrate dehydrogenase (*icd*) gene of *Escherichia coli* and contains the *lit* gene for T4 late gene expression (2), partially excludes T-even phages such as T2, T4, and T6. The exclusion is due to an e14-encoded protein Lit that, when overproduced, promotes a severe inhibition of cellular translation late in T-even phage infection (3, 4). The inhibition requires an interaction between the Lit protein and probably either the RNA or polypeptide encoded by a short region of only about 75 bp in the major head protein gene of T-even phages (4). The inhibition of translation occurs when the major head protein gene including this region begins to be transcribed and translated late in infection. We call this short region the *gol* region because it was the first defined by *gol* mutations, which allow the phage to grow on lit. In this report, we show that translation elongation factor Tu (EF-Tu) is cleaved during the phage exclusion. Apparently, the Lit protein is a specific protease that is activated by the polypeptide or RNA encoded by the T4 *gol* region to cleave EF-Tu, thereby causing the inhibition of translation. The EF-Tu is cleaved very close to the site of EF-Tu methylation in a sequence that has been highly conserved throughout evolution.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains were derived from *E.coli* strains JM101 and W3110 *lacI*^{q} *recA*, which have been described (4). To construct isogenic derivatives of these strains, one of which lacks e14 and so has no Lit protein and the other of which has an excess of Lit protein, we first cloned a kanamycin-resistance (Kan^R) cassette between the *Bam*HI sites in the large *Hin*dIII fragment of e14 in the plasmid pAG2 obtained from Charles Hill (5). We then crossed this clone into e14 in the chromosome using a *polA* temperature-sensitive mutation as described (6). The e14 locus had a *lit*(Con) mutation (where Con = constituitive), which is an "up-promoter" mutation causing the overproduction of Lit protein (3). A low percentage of the recombinants still retained the *lit*(Con) mutation, and such a strain was selected and used as a donor for P1 transduction to move the *lit*(Con) mutation into W3110 *lacI*^{q} *recA*$^-$ and JM101 selecting for Kan^R. The e14 prophage can be transduced into a RecA$^-$ recipient because e14 encodes its own integrase (5). The isogenic Lit^o derivatives were obtained by UV irradiating the LitCon Kan^R strains and selecting a kanamycin-sensitive (Kan^S) derivative that simultaneously had lost the LitCon phenotype and so presumably had been cured of e14. The plasmid pUC84PZ1 described previously (4) has a 159-bp DNA fragment including the *gol* region cloned into a derivative of pUC8 such that the *gol* region is transcribed from the *lac* promoter and translated in the gene *23* frame from the *lacZ* ribosome initiation site. The plasmid pACRV9-KAN was derived from pACRV9 (4) by cloning a Kan^R cassette into the *Eco*RI site in the *cat* gene (encoding chloramphenicol acetyltransferase) of pACYC184. It has the wild-type *lit* gene cloned in the *tet* gene of pACYC184, so it will be expressed from its own promoter. The plasmid pTA9 was obtained from David L. Miller and has the *tufA* gene (encoding EF-Tu) cloned in such a way that it will be transcribed from its own promoter as well as the *lac* promoter in these cells.

Preparation of Cell Extracts and S30 and S150 Supernatants and *in Vitro* Translation. The extracts were prepared from cells grown in 1% tryptone containing 1.0% NaCl, 0.5% glucose, and 50 μg of ampicillin and 50 μg of kanamycin (Kan) per ml. When OD_{625} reached 0.3, the cells were divided into two 500-ml cultures, and isopropyl thiogalactoside (IPTG) was added to 5 mM to one of the cultures for 20 more min. The cells were collected, resuspended in 4 ml of resuspension buffer, and lysed in a French pressure cell. The S30 and S150 supernatants were prepared and the *in vitro* translations were performed as described (7) with a few minor

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Abbreviations: EF, elongation factor; Kan, kanamycin; Kan^R and Kan^S, Kan resistance or sensitivity; IPTG, isopropyl thiogalactoside.

*To whom reprint requests should be addressed.

modifications. No RNA and no ribonucleoside triphosphates except GTP and ATP were added to the *in vitro* reactions. The reaction mixtures contained 200 μl of supernatant (100 μl of S30 + 100 μl of S150) in a total volume of 300 μl. Aliquots of 70μl were withdrawn and precipitated with 3 ml of 5% CCl_3COOH in the cold. The precipitates were heated to 100°C for 5 min, centrifuged, resuspended in 0.1 ml of 2% KOH, and reprecipitated with 3 ml of 5% CCl_3COOH before collection on WF/A Whatman filters for counting.

Antibody Precipitations. Cells (10 ml) were grown in M9 medium containing all 20 amino acids except methionine and 50 μg of ampicillin per ml to OD = 0.4 at 30°C. The proteins were labeled by adding 4 μl of [^{35}S]methionine (1200 Ci/mmol; 1 Ci = 37 GBq) for 10 min and chased with 10 mM unlabeled methionine for 10 min. Depending on the experiment, either the cells were infected with CsCl-purified T4 phage at a multiplicity of infection (moi) of 10 or IPTG was added to 5 mM to induce transcription and translation of the *gol* region. After 30 min, the cells were collected by centrifugation and resuspended in 100 μl of H_2O. They were lysed, and the antibody precipitations were performed as described (8).

Phage One-Step Growth Experiment. *E. coli* JM101 with and without the Lit protein (see bacterial strains) and with and without the *tufA* clone in pTA9 were grown at 30°C in 1% tryptone/1% NaCl/0.5% glucose to midlogarithmic phase, and IPTG was added for 20 min before infection. CsCl-purified T4 phage were added at a moi of 10, and 1-ml aliquots were taken to add to ice for SDS/PAGE. Aliquots were also taken to add to saline/$CHCl_3$ and were diluted to plate with indicator bacteria to determine the phage yield.

Other Methods. SDS/PAGE was by the method of Laemmli (9). The cleavage fragment of EF-Tu was electroeluted onto nitrocellulose and sequenced by the Michigan State University Macromolecular Facility using Edman degradation.

RESULTS

Extracts of Inhibited Cells Are Inactive for Translation *in Vitro*. Infection of e14 Lit protein-containing cells by phage T4 causes a severe inhibition of translation because of the interaction of the RNA or polypeptide encoded by the T4 *gol* region with the Lit protein. If extracts of the infected cells are inactive for translation, it may be possible to determine the cause of the inhibition. To avoid possible complications due to phage infection, we can mimic the effect of phage infection using constructs like that shown in Fig. 1. In this construct, the *lit* gene of e14 has been cloned in the multicopy plasmid pACYC184 so that it will be expressed from its own promoter. The *gol* site of T4 has been cloned in the compatible vector, pUC8, so that it will be transcribed from the *lac* promoter and translated in the gene *23* frame from the *lacZ* ribosome initiation site. When IPTG is added to cells containing both plasmids, the transcription and translation of the T4 *gol* region will commence, and the severe inhibition of translation will ensue (4). To make the extracts as similar as possible, we grew the cells illustrated in Fig. 1 and divided the culture in half just before adding IPTG to one of the two subcultures. After a 20-min induction period to allow transcription and translation of the *gol* region, the cells were concentrated and lysed, and radioactive methionine was added to measure incorporation into acid-insoluble polypeptides. We observed the extracts of the cells to which IPTG had been added to be significantly less active for *in vitro* translation than the parallel culture (data not shown).

There are many possible causes for the relative inactivity of the extracts of the inhibited cells. An inhibitor of translation could have been generated. Alternatively, the ribosomes or one of the soluble factors (e.g., tRNA or initiation factors or EFs) could be inactivated somehow. To distinguish these possibilities, we further fractionated the extracts by differential centrifugation. We prepared S30 supernatants as described in *Materials and Methods*. These supernatants retained everything required for translation but lost their activity when they were dialyzed overnight in the cold, presumably because the endogenous mRNA was degraded. We also prepared S150 supernatants by centrifuging part of each S30 supernatant before dialysis. The S150 supernatants were not dialyzed. They should lack ribosomes but have all of the soluble factors, including mRNA required for translation.

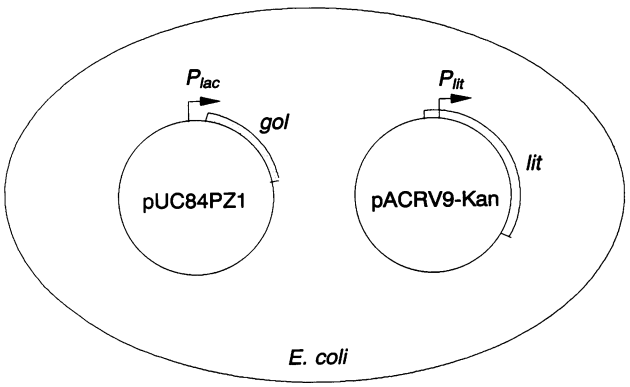

FIG. 1. The "imitation infected cell" used in these experiments. The *E. coli* W3110 *lacI^q recA* cells have been cured of e14 and so lack the normal chromosomal source of Lit protein. They contain two compatible plasmids as described in text. One plasmid expresses the *lit* gene of e14 constitutively from its own promoter; while the other plasmid has the *gol* region of T4 gene *23* cloned so that its transcription is induced by IPTG and it will be translated in the gene *23* frame from the *lacZ* ribosome initiation site.

To determine the effect of the supernatants on translation, we mixed the supernatants from inhibited and uninhibited cells in the *in vitro* translation assay. Addition of the S150 supernatant from the uninhibited cells stimulated incorporation of amino acids by the S30 supernatant from uninhibited cells (Fig. 2 *Upper*). In contrast, addition of S150 supernatant from inhibited cells did not stimulate incorporation. We conclude that some difference in the S150 supernatants is responsible for the inhibition of translation.

EF-Tu Was Cleaved in the Inhibited Extracts. As part of an effort to determine the difference between the inhibited and uninhibited extracts, we electrophoresed the proteins in the S30 and S150 supernatants on SDS/polyacrylamide gels and stained the proteins. A major protein band of 43 kDa was missing from the inhibited supernatants and was replaced by a smaller band of about 37 kDa (Fig. 2 *Lower*). This is the only reproducible difference we detected between the inhibited and uninhibited extracts. In the experiment shown in Fig. 2 *Lower*, another polypeptide of about 75 kDa appeared to be missing from the supernatants of the inhibited extracts. In other inhibited extracts, this polypeptide was present and probably was a protein that sporadically sedimented during the preparation of the supernatants.

From its size and abundance, we suspected that the 43-kDa protein is EF-Tu, which was cleaved, and the 37-kDa polypeptide is one of the cleavage products. To prove this, we performed an antibody precipitation experiment. The results are shown in Fig. 3. In this experiment, the proteins were radioactively labeled and the *gol* region was induced in the presence and absence of Lit protein. After lysis, the proteins were precipitated with specific antibodies directed against EF-Tu. As a control, we included cells that contain an overproducing clone of *tufA*, a gene for EF-Tu. As expected, the antiserum specifically precipitated the 43-kDa EF-Tu

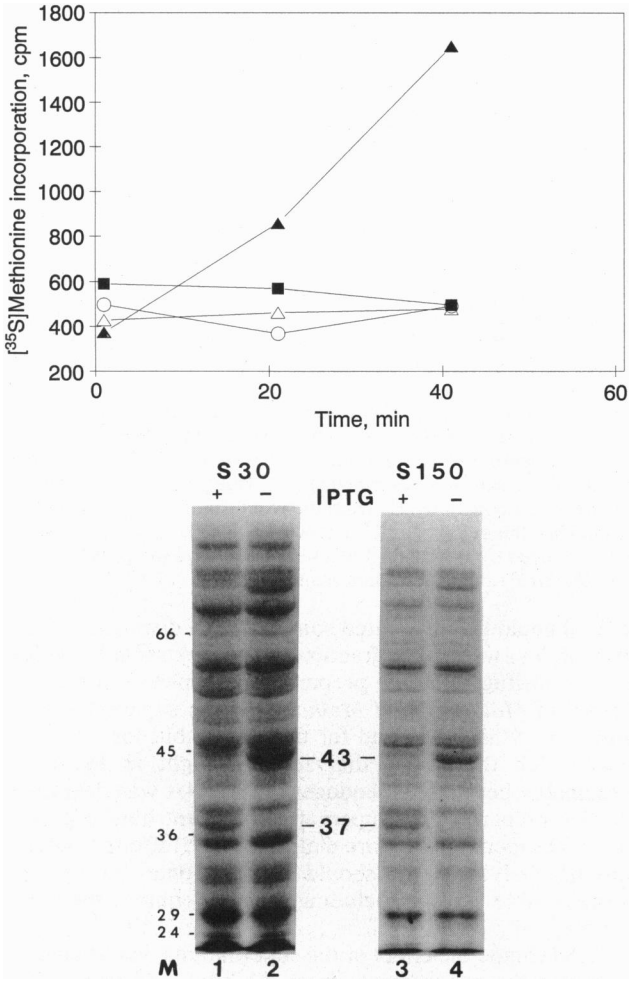

FIG. 2. A defect in *in vitro* translation by supernatants from induced cells correlates with the absence of a 43-kDa polypeptide. (*Upper*) Incorporation of [^{35}S]methionine into acid-insoluble material with different mixtures of supernatants. ▲, S30 uninduced and S150 uninduced; △, S30 uninduced and S150 induced; ○, S30 uninduced; and ■, S30 induced. (*Lower*) SDS/PAGE of the proteins in the supernatants of *Upper*. Lanes: 1, S30 supernatant extract of induced cells; 2, S30 supernatant of uninduced cells; 3, S150 supernatant of induced cells; 4, S150 supernatant of uninduced cells. The 43-kDa polypeptide, which is missing from the supernatants of the inhibited extract, and the 37-kDa polypeptide that replaces it are indicated. As discussed in *Results*, the other difference between the two extracts is a band at about 75 kDa which is not reproducible and probably is due to a protein that sporadically sediments in the preparation of the supernatants.

protein, and more of this protein precipitated in cells with the overproducing clone (Fig. 3, lane 1). In the cells in which the *gol* region had been induced in the presence of Lit protein, much less of the 43-kDa protein precipitated, and a second polypeptide of 37 kDa also precipitated (Fig. 3, lane 5). We conclude that the 43-kDa protein that was cleaved is EF-Tu and that the 37-kDa polypeptide is one of the cleavage products. In a similar experiment, we found that EF-Tu was cleaved after wild-type T4 infection of Lit protein-containing cells, but not if the infecting T4 had the *gol 6B* mutation, making it likely that the cleavage of EF-Tu is at least partially responsible for the inhibition of translation and the phage exclusion in the presence of excess Lit protein. T4 also multiplied more poorly in cells containing a wild-type copy of e14 than in cured cells, and about 50% of the cellular EF-Tu was eventually cleaved under these conditions (data not shown). Apparently, EF-Tu is normally cleaved after infec-

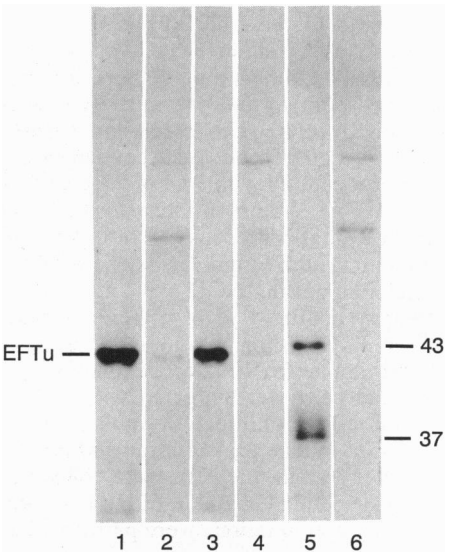

FIG. 3. Antibody precipitation of EF-Tu in inhibited and uninhibited cells. The cells were those in Fig. 1 except that they were derived from *E. coli* JM101 and the *lit* gene was overexpressed from e14 in the chromosome because of an up-promoter mutation rather than from pACRV9. An autoradiogram is shown of the dried gel prepared as in Fig. 2. Lanes: 1 and 2, cells without Lit protein or pUC84PZ1 but containing the plasmid pTA9, which overproduces EF-Tu; 3 and 4, induction of pUC84PZ1 in cells with no Lit protein; 5 and 6, induction of pUC84PZ1 in cells with Lit protein; 2, 4, and 6, controls with nonimmune serum.

tion of *E. coli* K-12 strains, most of which contain e14, but the exclusion is only partially effective, at least under these laboratory conditions.

Is Depletion of EF-Tu Solely Responsible for the Inhibition of T4 Production? Depletion of EF-Tu through inactivation by cleavage would be sufficient to explain the inhibition of translation and the blockage of phage production, since EF-Tu is required for translation. However, this may not be the only contributing factor. Some other protein may also be cleaved, or the cleavage fragment may be inhibitory. To begin to investigate these possibilities, we performed the experiment shown in Fig. 4. The rationale behind this experiment is as follows. If cells contain an excess of EF-Tu because they have a clone of the *tufA* gene in a multicopy plasmid, then not all of the EF-Tu might be cleaved after T4 infection. If depletion of EF-Tu due to inactivation by cleavage were solely responsible for the inhibition of phage production, then, under these conditions of an excess of EF-Tu, T4 production should be normal. If, however, the cleaved form of EF-Tu were somehow interfering with phage production or if another protein required for T4 development were also cleaved, then phage production under these conditions should not be normal. Fig. 4 *Upper* shows that cells with the *tufA* clone had about 3 times the normal amount of EF-Tu (compare lanes 10–12 with lanes 4–6); and even though more EF-Tu was cleaved after T4 infection, a supernormal amount still remained, judged by the amount of cleavage fragment (lanes 7–9) (compare lanes 7–9 with lanes 1–3). The remaining uncleaved EF-Tu did support some phage production (Fig. 4 *Lower*), indicating that the depletion of intact EF-Tu is partially responsible for the block in phage production.

While the experiment shown in Fig. 4 *Lower* supports the conclusion that depletion of EF-Tu is partially responsible for the block in T4 production, it also suggests that it may not be the only contributing factor. The excess of EF-Tu caused the cells to grow more slowly and delayed phage production (Fig. 4 *Lower*), complicating the interpretation of the results some-

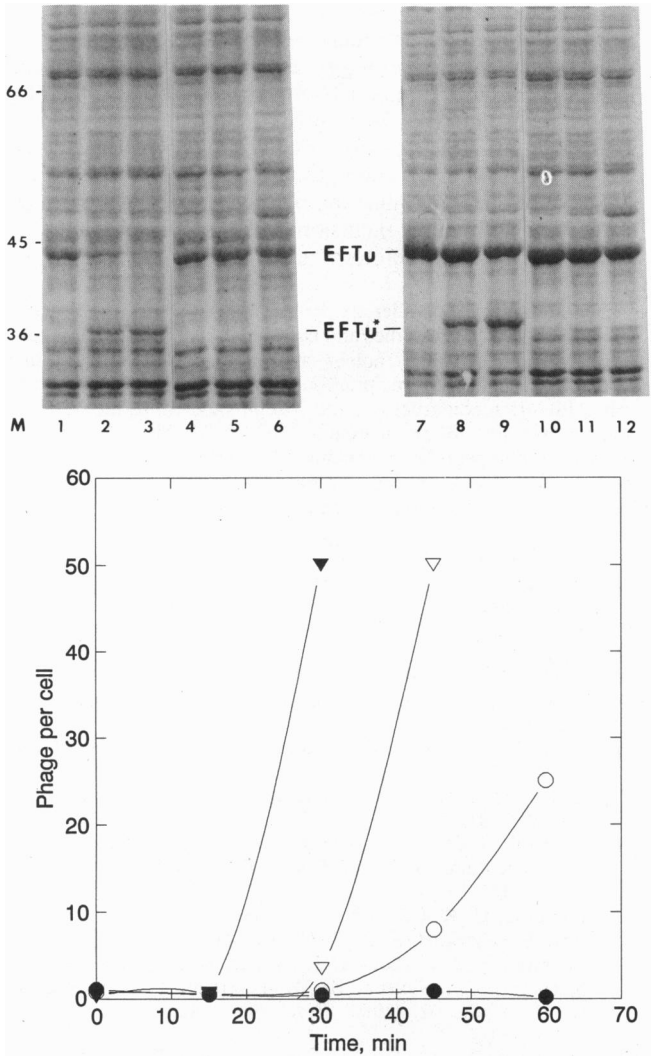

FIG. 4. Multiplication of T4 in cells with an excess of EF-Tu. The cells were the same as those in Fig. 3, but some of the cells had an excess of EF-Tu because they contained the plasmid pTA9 with the *tufA* gene. (*Upper*) Coomassie blue-stained SDS/PAGE gels of proteins from cells infected by T4. Lanes: 1–3, cells with Lit protein and normal amounts of EF-Tu; 4–6, cells with no Lit protein and normal amounts of EF-Tu; 7–9, cells with Lit protein and excess EF-Tu; 10–12, cells with no Lit protein and excess EF-Tu. Times after infection are 0, 20, and 40 min for each set. (*Lower*) One-step growth experiment of T4 in cells from *Upper*. ▼, No Lit protein and normal amounts of EF-Tu; ▽, no Lit protein and excess EF-Tu; ○, Lit protein and excess EF-Tu; ●, Lit protein and normal EF-Tu.

what. Nevertheless, if depletion of intact EF-Tu were solely responsible for the inhibition of phage production, we might expect production in the presence of excess EF-Tu and Lit protein to be at least as high as phage production in cells with excess EF-Tu but without Lit protein. That this was not the case makes us entertain other possibilities such as inhibition by the cleaved form or cleavage of some other protein besides EF-Tu. More experiments are required to resolve these issues.

Locating the Cleavage Site of EF-Tu. To locate the site of cleavage of EF-Tu, we isolated the 37-kDa cleavage fragment from gels and had the N terminus sequenced in our Macromolecular Facility. If the N terminus had been cleaved off, the N-terminal sequence should be different from that of intact EF-Tu and the sequence should reveal the exact site of cleavage. The N-terminal sequence of the cleavage fragment was determined to be N-Ile-Thr-Ile-Asn-Thr . . . (Fig. 5). A

↓

---Glu55-Lys*-Ala-**Arg-Gly-Ile-Thr-Ile**-Asn-Thr64 --- *E. coli* EF-Tu

---Glu58-Arg -Gln-**Arg-Gly-Ile-Thr-Ile**-Asn-Ile 67 ---- *Micrococcus luteus* EF-Tu

---Glu92-Arg -Ala-**Arg-Gly-Ile-Thr-Ile**-Ser -Thr101 -- *S. cerevisiae* Mitochondrial EF-Tu

---Glu66-Arg -Glu-**Arg-Gly-Ile-Thr-Ile**-Asp-Ile 75 ---- *S. cerevisiae* EF-1α

---Glu66-Arg -Glu-**Arg-Gly-Ile-Thr-Ile**-Asp-Ile 75 ---- Human and Mouse EF-1α

FIG. 5. A comparison of the sequences of amino acids in EF-Tu (EF-1α in eukaryotes) from the region of cleavage. The arrow shows the site of cleavage. The identical amino acids on either side of the cleavage site are in boldface type. The asterisk shows the lysine that is methylated in *E. coli* in response to starvation conditions. The sequences were obtained from Gene Probe.

comparison of this sequence with the known sequence of *E. coli* EF-Tu (10) revealed that the cleavage occurs between Gly-59 and Ile-60. This cleavage site is also consistent with the relative sizes of the intact EF-Tu and the cleavage product. The entire EF-Tu has 393 amino acids, and a cleavage at amino acid 60 would remove about 15% of the protein, leaving a polypeptide of about 37 kDa. Note that there could be other cleavage sites in the extreme N terminus of the protein, but the smaller cleavage fragments would not be detected by our method.

DISCUSSION

In this paper we have shown that EF-Tu is cleaved following T4 infection of cells containing the defective prophage e14. The cleavage requires the interaction between the e14-encoded Lit protein and the polypeptide or RNA encoded by the *gol* region of T4, a short sequence within gene *23*, the major head protein gene. The cleavage occurs between Gly-59 and Ile-60 in a highly conserved region of EF-Tu. The cleavage apparently inactivates EF-Tu for translation because translation is completely blocked when all of the EF-Tu in the cell is cleaved. However, depletion of EF-Tu is probably not solely responsible for the block in T4 phage development, since T4 development is not normal even when supernormal amounts of EF-Tu remain uncleaved.

At present, we are not certain of the mechanism of cleavage of EF-Tu. However, the Lit protein is probably the protease because it contains the motif characteristic of zinc-dependent proteases (Y.-T.N.Y. and L.S., unpublished data). Also, it is probably the short polypeptide encoded by the *gol* region that activates the cleavage, since the T4 *gol* region must be translated for the activation. We recently observed the cleavage *in vitro* when crude extracts of cells in which the *gol* region had been induced were mixed with crude extracts of cells containing Lit protein. This assay should allow unambiguous determination of the function of the various components of the *gol*–Lit-induced cleavage of EF-Tu.

There are many possible mechanisms by which the cleaved form of EF-Tu could affect cellular functions. It may bind tRNA but not function for translation, thereby depleting the available pool of tRNA. Or it may enter the ribosome but not function for translation, thereby clogging up the translation apparatus. Or the effect of the cleaved form of EF-Tu may be indirect. For example, the cleaved form may bind GTP but not recycle it, thereby making it unavailable for other cellular processes, but this seems unlikely when one considers that there is much more GTP in the cell than EF-Tu. Also there seems to be little or no general effect of the cleavage of EF-Tu on transcription (4), which also requires GTP. Finally, the inhibition of translation may be due to depletion of the active form of EF-Tu. This latter explanation is most consistent with the observation that overproducing EF-Tu so that not all is

cleaved has an ameliorating effect on the inhibition of protein synthesis and phage production.

The results of *in vitro* experiments with EF-Tu do not give a clear indication of what properties to expect of the cleaved form of EF-Tu *in vivo*. In the early stages of digestion of the native protein, trypsin cleaves at amino acids Arg-44 and Agr-58 (11), very close to the *gol*–Lit-induced cleavage. The larger fragments produced by these cleavages do not dissociate, although the small fragment of 14 amino acids may be lost. These results suggest that the fragments created by the *gol*–Lit-induced cleavage may not dissociate *in vivo*. However, on the question of what activities are retained by the cleaved form of EF-Tu, different methods give apparently contradictory results (11, 12).

Even if depletion of intact EF-Tu sufficiently explains the inhibition of translation, Fig. 4 shows that it apparently is not the only cause of the block in phage production. When EF-Tu is overproduced so that not all of the EF-Tu is cleaved, T4 production is still retarded. This result might have been predicted from our earlier work, which showed that there is another effect of the interaction of the Lit protein with the *gol* region. In addition to promoting the inhibition of all translation, the Lit protein also prevents the transcription of genes serviced by the same promoter but downstream of the *gol* site. This phenomenon, which we call the "local inhibition" (4) occurs even when not all of the EF-Tu is cleaved. Because it interferes with the transcription of T4 gene *23*, an essential gene, the local inhibition should also cause a delay in phage production. At present, we do not know that consequence of the *gol*–Lit interaction causes the local inhibition. It may be due to the cleaved form of EF-Tu or the cleavage of an as-yet-unknown protein. Further experiments are needed to distinguish these and other possibilities.

The site of cleavage of EF-Tu has some interesting features. The cleavage occurs in what is predicted to be a largely unstructured region spanning the GTP-binding pocket (13, 14). As mentioned, the two most sensitive sites for trypsin cleavage of the native protein are just upstream of the *gol*–Lit-promoted cleavage (11), which also suggests that this region is exposed on the surface of the molecule. This region may also play a regulatory role. The EFs-Tu of many bacteria including *E. coli* are known to be methylated in response to starvation conditions (15, 16). The one site of methylation in *E. coli* is Lys-56 (10), only four amino acids upstream of the cleavage site. It would be of interest to know whether the methylated form of EF-Tu is also cleaved.

Further testimony to the importance of the region of cleavage comes from its evolutionary conservation. Bacteria EF-Tus (called EF-1α in eukaryotes) are among the most highly conserved cellular constituents, and the site of cleavage lies within one of the most highly conserved regions of all (17). In fact, the two amino acids on one side of the cleavage site and the three on the other side are probably the same in all organisms on earth (Fig. 5). EFs designated EF-G (called EF-2 in eukaryotes) also share this five-amino acid sequence (17). A similar sequence also exists in SelB, the specific EF-Tu for selenocysteine tRNA. It should be of interest to determine which EFs from other organisms can serve as substrates for the protease. Furthermore, the only other phage exclusion to be understood is also due to cleavage of a highly conserved cellular component, the lysine tRNA (18), suggesting that phage exclusion systems may provide valuable reagents for cell biology research.

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