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dissertation entitled

Molecular characterization of the phage exclusion and the local effect caused by the interaction of an e14 encoded protein, Lit, with the gol site from phage Lambda 23

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D degree in Microbiology

Major professor

Date Sep. 2, 1993

MOLECULAR CHARACTERIZATION OF THE PHAGE EXCLUSION AND THE LOCAL EFFECT CAUSED BY THE INTERACTION OF AN e14 ENCODED PROTEIN, LIT, WITH THE gol SITE FROM PHAGE T4 GENE 23.

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1993

ABSTRACT

MOLECULAR CHARACTERIZATION OF THE PHAGE EXCLUSION AND THE LOCAL EFFECT CAUSED BY THE INTERACTION OF AN e14 ENCODED PROTEIN, LIT, WITH THE gol SITE FROM PHAGE T4 GENE 23.

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The Lit protein generated by the cryptic prophage e14 can exclude bacteriophage T4 at a late stage , particularly when the Lit protein is overproduced by an up-promoter mutation or expressed from a multi-copy plasmid. This exclusion is due to cleavage of translation elongation factor Tu caused by the interaction of the Lit protein with a short sequence, gol, within the T4 major head protein gene 23. The proteolytic reaction occurs in a region highly conserved among all the prokaryotic EF-Tus and analogous eukaryotic EF-1αs. The Lit protein contains a Zn-dependent protease motif and its proteolytic activity requires the function of the gol region.

In vitro complementation assays performed by mixing extracts of cells in which the gol region has been transcribed and translated with extracts of cells overproducing Lit protein successfully cleave EF-Tu (43 kD)and generate the 37 kD fragment. Moreover, the addition of the purified Gol oligopeptide (29 amino acids long) to the " Lit " cell extracts also cause the cleavage of EF-Tu in vitro, indicating that the Gol peptide is the activator of the Lit protease activity.

In addition to blocking translation, the interaction between gol and Lit also converts the gol region into a transcription terminator. Unlike the translation block, this transcription termination (we refer to it as the local inhibition) occurs even if not all of the cellular EF-Tu is cleaved. Interestingly, in the absence of Lit protein,

the gol sequence actually enhances transcription, suggesting that the normal role of gol is to be an anti-pausing site that enhances the transcription of gene 23.

To My Parents

and

The One Whose Existence I Believe

ACKNOWLEDGMENT

I would first like to thank Dr. Larry Snyder, my mentor who provided the financial support and intellectual environment for my graduate work and who also gave me plenty of patience and encouragement along the way. I am also grateful for the handy suggestions and thoughtful attentions received from my guidance committee Drs. Lenny Robbins, Richard C. Schwartz, Wendy Champness and Michael Bagdasarian.

It has been a great fun to work in Giltner Hall with lots of neat people, and I wish to thank with particular fondness Dr. Shu-Chih Chen, Dr. Julius Jackson, Sue Dager, David Aceti, Angel Lake and Natalie Moore.

I would like to thank Greg Velicer, my dear friend, for his friendship and help to proofreading the manuscript with a great dear of effort.

And lastly, there are my parents, Mr. Yang-Chang Yu and Mrs. Ar-Jue Chiang-Yu , whom I inherited my genes from and who gave me tons of support and love.

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Introduction

The use of host-parasite systems derived from bacteriophage T4 and its host Escherichia coli has enabled scientists to reveal many essential principles that form the basis of modern molecular biology. Historic examples include the proofs that deoxyribonucleic acid is the genetic material (Hersey and Chase 1952), that ribonucleic acid is the template for proteins (Brenner et al 1961), and that each triplet is sequentially used as a codon for polypeptide chain synthesis (Crick et al 1961).

Studies in this field have also brought some surprising and interesting discoveries that revealed diversity in gene regulatory mechanisms and explored each classic concept in a deeper and broader manner. For instance, the discovery of T4 introns led researchers to analyze the self-splicing catalytic activity of an RNA molecule, and the nature of a mobile intron, which often requires a endonuclease encoded by the intron itself (Bell-Pedersen and Belfort, 1991)(review see Belfort, 1992). This discovery challenged past beliefs that prokaryotic genomes do not contain introns and that introns do not code for proteins. Another example is the characterization of phage T4 Gene 60 which demonstrated an additional mode of regulation at the level of translation (Weiss and Dunn 1990). This event is called ribosomal programmed hopping: ribosomes can somehow bypass a segment of an open reading frame and continue elongation at a certain codon downstream, the precise action involving a special context around the hopped gap. These unusual findings received further recognition as similar mechanisms were gradually discovered in other genes and organisms (Ramirez, et al., 1992, Marshalland Lemieux, 1991, Muscarella, 1993).

Being one of the largest viruses with a coding capacity of almost 200 genes, the extent to which bacteriophage T4 depends on the apparatus of its host Escherichia coli is perhaps surprising. Some phage genes are apparently dispensable for growth in a laboratory strain, but under circumstances imposed by host mutants, particular genes of the nonessential group become important. Thus, although T4 normally relies on the host gene products, phage encoded proteins can compensate for the host mutations. Phage development also requires repeated interactions between phage and host to maintain the accuracy of performance. To determine what host genes are involved, and how they interact with the phage genome to control the cascade of phage gene expression, mutations in the host are screened for their ability to restrict phage growth without affecting the host's own viability; i.e. host mutants for which no plaques are formed in the bacterial lawn. Presumably, the products of mutated genes that interact with phage determinants have either lost their positive regulatory roles or have negative effects during phage development. The participating phage sites or gene products can be identified by selecting suppressor mutations in the phage that can overcome the block and form plaques in the test strain (Cooley, et al. 1979).

Using the genetic approach described above, Champness *et al* (1982) isolated E. coli mutants they called lit, because they cause a late inhibition of T4 gene expression (later changed to lit(con)). The lit(con) mutations were mapped to 25', between purB and fadR loci on the E. coli K12 genome, and were later shown to be in e14, a cryptic DNA element. The dominance of lit(con) mutations allowed Kao and Snyder to clone the lit allele in a multi-copy plasmid. Nucleotide sequencing data showed that all 5 independently isolated lit(con) mutants have the same CG-TA transition in the up-promoter region of the putative lit ORF. This transition, presumably increasing resemblance to a promoter consensus sequence, causes a higher transcription level of the gene. Subclones containing the

wild type lit gene in a multi-copy plasmid are also phenotypically Lit(con). Overproduction of RNA transcripts and of the Lit protein were confirmed by northern hybridization experiments and minicell-labeling assays respectively, the latter done in cells transformed with appropriate plasmid-borne lit constructs. The 1.8 kb EcoRV subclone sequenced contains 2 ORFs with a 4 nt gap in between; their coding capacity, deduced from the nucleotide sequences, is about 34kD and 17 kD respectively. Results of deletion analysis performed by me indicated that only the 34 kD protein is essential for the lit phenotype. Two lines of evidence suggested that gpLit is a membrane -bound molecule; the majority of Lit protein labelled in maxicells was found in an inner membrane fraction, and a computer protein hydropathy analysis of pLit implied 2 possible transmembrane segments with high hydrophobic density (Kao and Snyder 1988). The connection between the membrane-association property and the Lit(con) phenotype, which was later proven to relate to translation elongation factor Tu cleavage, remains a mystery.

The e14 element that contains the lit gene was first discovered by W. C. Hill as a circular DNA molecule that was found to excise after UV irradiation and reintegrate back at its original position. It is believed that e14 is a defective prophage descended from a lambdoid ancestor that gradually lost phage identity except for a tail-fiber like gene similar to that of Mu, P2 and lambda (Barreiro and Haggard-Ljungquist, 1991. Sandmeier and Arber, 1992.), and a putative integrase gene related to that of ϕ21 (Schneider and Champbell, personal communication). It also carries a modified cytosine restriction determinant mcrA (Hiom and Sedgwick, 1991) and sfiC, a cell-division inhibitor gene (Maguin, 1986). The SOS independent excision and reintegration is accomplished by an enzyme encoded by the fragment and the specific att site in the E.coli genome. When hosts acquired this cryptic prophage, and why it is maintained are not clear. Cells cured of e14 by UV irradiation produce no Lit protein and have no apparent defect. They can

only be distinguished from Lit(con) cells because they allow wild type bacteriophage T4 to propagate and form plaques (Brody, et al. 1988; Hill, et al. 1989)..

The exclusion phenomenon of T4 caused by the overproduction of gpLit is due to a global inhibition in T4 true late gene expression and expression of those early genes that are normally made continuously in late stage; this was shown in pulse-chase labeling experiments to monitor phage protein synthesis during multiplication. The inhibition can be suppressed by a T4 phage mutation that mapped within a 40 nt region at the first quarter of T4 major head protein gene 23, one of the true late genes (Bergsland *et al.*, 1990). This wild type region was designated as gol, since mutations in this region allow phage to grow on lit. To avoid confusion, whenever I mention the Lit(con) phenotype or gol activity, I mean the phenotypic properties of this global inhibition. Champness and Snyder (1984) later found that subclones of the wild type gol region from gene 23 are unable to transform lit(con) cells because expression of this fragment is sufficient to trigger a global effect similar to the abortive phage infection caused by gpLit. The use of this transformation assay enabled Bergsland *et al* (1990) to define the minimum gol sequence necessary for its activity and to scan mutations that can abolish the gol effect. Kao and Snyder (1988) took advantage of the same assay and discovered that the global inhibition caused by gol-Lit interaction acts at the translation level. Transcription and translation of the gol+ region, a minimal size of 75nt, is alone responsible for this global tanslational effect in Lit-containing cells, and no other phage genes are involved. Conclusive evidence for this was obtained from study of a plasmid clone, pUC84PZ1, that contains the gol sequence fused to the lacZ gene of a pUC vector in the Gene23 reading frame. In this plasmid, Gol activity is controlled by the IPTG-inducible lacZ promoter. All cellular protein synthesis was shut off soon after IPTG was added to cells. This

IPTG inducible inhibition is dramatically diminished if the construct translates the gol region out of Gene23's reading frame, for example in the -1 frame of pUC12PZ1. This observation also suggested involvement of the gol polypeptide. However, we couldn't rule out the possibility that it is the act of translation, rather than the product, that is required. For example, the translation-coupling ribosomes may follow the transcription apparatus , prevent nascent gol RNA from folding into an appropriate secondary structure, and consequently provoke translation shutdown.

The partial gol RNA sequence has a striking resemblance to lambda nut sites, which are utilized by the anti-terminator N protein to read through downstream terminators. This resemblance consists of a box A sequence and a boxB-like hairpin structure. Interestingly, the mutations that inactivate gol activity all map in the nut-like site. Furthermore, a computer simulation program showed that the entire gol RNA (PvuII- I 75 nt fragment) can form a stable stem-loop structure, with a ΔG of -35.5 Kcal, suggesting that this secondary structure may exist in vivo (Y.-T. N. Yu and L. Snyder). The intriguing interconnection between possible gol secondary structures and their functions is discussed in one of the manuscripts included in this dissertation.

In addition to the severe global inhibition of all cellular translation, the gpLit protein also causes a local inhibition that affects gene expression at the gol site. This local inhibition occurs in the presence of the normal, low amount of gpLit which occurs in the wild type (usually referred to as lit^+) and does not kill the cells. The local effect was characterized by Bergsland and Snyder by using plasmid constructs carrying a gol-lacZ fusion. In JM101lit^0, a strain that lacks the gene, α–complementation can be achieved by the plasmid that translates both gol and lacZ in frame and exhibits blue color in X-gal agar plates (X-gal is subtract for β–galactosidase). But in JM101lit^+, which has the same genetic background

but a functional lit gene, the transformants are white, indicating that no functional LacZ product is made. A similar effect was also observed in lit(con) cells transformed with a -1 frame clone that alters completely the gol polypeptide but retains the same gol RNA sequence. Based on these results, Bergsland *et al* proposed that gol RNA, but not a Gol peptide, is essential for the local inhibition.

The failure of α–complementation has two possible explanations; the fusion protein carrying the Gol polypeptide at its N-terminus can not function in the presence of gpLit; or transcription or translation of the hybrid segment is prematurely terminated before getting to the reporter gene. In an attempt to differentiate between them, Rajiv Gulati (Bergsland et. al., 1991) did S1 nuclease mapping to examine the gol-lacZ RNA transcripts, and I made a gol-CAT hybrid plasmid that is transcribed from the lacZ promoter and translates gol in the -1 frame and the chloramphenicol acetyltransferase gene from a separate ribosome binding site. Preliminary results showed that there were fewer intact transcripts protected in presence of Lit protein, and the transcription fusion clone transformed Lit^+ cells to chloramphenicol sensitive instead of to chloramphenicol resistant as in lit^0. From these observations, we hypothesized that the local effect is due to premature termination of transcription. However, this model is further adjusted in one of the manuscripts presented here.

My main interest was to characterize the molecular basis of the global and local inhibitions caused by the gol-Lit interaction. First, I investigated how translational inhibition happens at the onset of the gol-Lit interaction; what key factor required for translation is affected in the gol-Lit mediated global inhibition, and how the defect influences translation activity. Second, for the local inhibition, the main questions to be answered were: How does premature transcription termination occur? What is the significance of gol sequence and structure in the

above phenomenon? How is the local inhibition related to the global effect caused by the same factors?

This thesis includes 4 main chapters. Chapter 1 reviews modes of gene regulation in bacteriophage T4, mechanisms of the known exclusion systems, current understanding of translation elongation factor Tu which relates to the molecular basis for the global inhibition, and published material on some systems of antitermination that possess similarity to the local effect caused by the gol-Lit interaction. Chapter 2, 3 and 4 are written as manuscripts for publication. Chapter 2 and Chapter 3 present results to suggest that cleavage of elongation factor Tu is, at least in part, responsible for the global translational deficiency in the gol-Lit mediated abortive infection system and demonstrate that the activity of an EF-Tu encoded by lit protease can be assessed in in- vitro experiments. Chapter 4 documents the evidence that helps us comprehend the local inhibition. The experiments presented addressed the involvement of gol RNA structure and the cleaved EF-Tu product in the premature termination of transcription during local inhibition, and the possible anti-pausing effect of the gol sequence in the absence of gplit.

CHAPTER 1

LITERATURE REVIEW

Overview of Bacteriophage T4

As one of the largest coliphages, bacteriophage T4 contains 160 kb of DNA and close to 200 genes and is characterized by an icosahedral head filled with linear double stranded DNA and a tail through which DNA is ejected into its host. The development of this obligate lytic virus is a well orchestrated process, including alteration of host apparati for its own use, transcriptional and translational gene regulation, and delicate protein-protein interactions during virion assembly. One unusual feature of the phage T4 genome is that it contains hydroxymethylcytosine dCMP instead of cystosine as in host DNA. This genetic feature is important for phage multiplication as discussed later.

Infection of E.coli by T4 is initiated by probing specific host surface receptors (LPS and ompC in E. coli K strains). This mission relies on phage T4 tail fiber distal protein gp37, together with gp38, which comprise a "host-range cassette" functional unit (Snyder and Wood, 1989). Several events occur during the first minute after T4's entry into the host. The biosynthesis of host macromolecules (DNA, RNA, and proteins) quickly ceases. Then, the host RNA polymerase (RNAP) begins to transcribe phage early genes from promoters with similarity to host consensus sequences. The early phage proteins include nucleases, which are responsible for the degradation of the cytosine containing host genome, and regulatory proteins such as RegA, which serves as a translational repressor for several phage early genes at a later stage, and phage encoded specific replication and transcription components. Within three minutes post-infection, the alteration and modification of host RNA polymerase core enzyme through the ADP ribosylation of alpha units of RNAP by gpalt and gpmod phage proteins and binding of T4 encoded 11.4 kD (RpbA) occurs. This confers increased affinity of RNAP for phage specific sigma factor gp55, which is required for recognition of the late promoters (Williams, et al. 1987; Malik and Goldfarb, 1988).

In T4 infected cells the theme of transcription regulation can be divided into pre-replicative and post-replicative periods. During the pre-replicative period, which spans from the time of infection to the onset of DNA replication about 5 minutes later, genes are transcribed from the early and middle promoters. The early transcription units contain IE genes (proximal to the promoter) and DE genes (distal to the promoter) messages in long polycistronic molecules separated by potential terminator sequences. Transcriptional readthrough at terminator sites within the T4 intercistronic region is required for synthesis of the promoter distal gene products. It should be noted that the early and middle transcriptional units overlap to a great extent. Under circumstances of polarity effects, RNA synthesis in the DE region could be initiated from a middle promoter within the IE-DE junction. Unlike the early promoters, the T4 middle promoters contain a unique sequence centered at -30 and utilize T4 modified RNA polymerase and a particular transcriptional factor gpmotA. The binding of gpmotA on a T4 middle promoter to facilitate the RNAP function was also demonstrated in vitro by Hinton (1991).

The post-replicative genes are mainly the bacteriophage true late genes which encode proteins for virion components. Their promoters display a totally different sequence without typical TATA boxes or -35 sequences. They are selectively recognized by RNAP coenzyme and a late gene sigma factor encoded by T4 gene 55. Efficient initiation at these promoters also requires enhancement by three T4 encoded DNA polymerase accessory proteins,(gp44, gp62, and gp45) bound to distal mobile enhancer sites. The enhancer exhibits the unique characteristic that it is not sequence specific but a structural break at the non-transcribed DNA strand. Activation by these DNA replication proteins also needs the function of an RNA polymerase bound co-factor protein which is encoded by T4 gene 33. Herendeen and Geiduschek (1991) have indicated that this transcriptional activating signal is communicated between its enhancer and a T4

late promoter by a DNA-tracking mechanism. It is noted that concurrent DNA replication is normally required to activate T4 late transcription. However, transcription of a plasmid borne late gene is independent of replication in vivo and in vitro suggesting an alternative pathway for the activation. The studies from both Geiduschek's and Zoguff's laboratories suggest the idea that the competence for late transcription results from negative torsional stress of the region in the late promoter, which can be generated by host DNA gyrase on a plasmid or by viral replication.

In addition to the transcriptional mechanism that controls phage gene expression, some T4 genetic clusters are also subject to regulation at the post-transcriptional level. The RNA processing, control of translation, and cleavage modification of the protein complex ultimately determines the degree of biological activity for individual phage-encoded enzymes and structural proteins. Multiple enzymatic activities that catalyze the cutting and trimming of precursor RNA molecules, chain extension and base modification are essential for maturation of T4 tRNA species. The majority of the enzymes involved are host encoded (RNase P, RNase 3, RNase PEP, tRNA nucleotidyltranferase, etc.). Those enzymes perform similar functions in uninfected E. coli.

Ribonucleolytic activity was also found to be involved in the function of messenger RNA. Cleavage within the Shine-Delgarno sequence of phage T4 motA and ORF 2 by the product of the T4 regB gene was proposed to regulate expression of messenger RNA transcripts. Gold and coworkers (Ruckman, et al. 1989) have identified additional processing sites within other T4 ribosome binding regions, including two sites in the polycistronic frd transcript, which encodes difyhydrofolate reductase. Mutations that impaired the messenger RNA processing resulted in overproduction of frd protein. Introduction of cloned copies of regB into chromosomes of the mutant phage can restore messenger RNA processing

capacity. All known processing sites of mRNA lie within a similar sequence suggesting a common processing pathway.

Two types of specific translational control are being extensively studied in phage T4, repression of translation of several phage early genes by the product of phage encoded regA and the mechanism of autogenous regulation. Karam and colleagues (Robert, et al. 1987) have demonstrated that T4 regA protein acts as a translation repressor to regulate synthesis of a set of phage induced early proteins. Those proteins include nucleotide synthesis enzymes (gpcd, gp1, gp42, and gp56), DNA polymerase accessory proteins (gp45, gp44, and gp62), rIIA, rIIB and regA itself. The purified regA protein was shown to bind specifically to target mRNA near the initiation codon AUG, and therefore to exclude ribosomes from binding (Unnithan, et al. 1990). Unlike other translational repressor regulated genes, the regA binding domain of all the unlinked transcriptional clusters did not possess sequences in common. Further analysis of mRNA binding by filtration and nuclease protection assays suggested that AUG initiator is necessary but not sufficient to determine regA recognition. A proper distance between ribosome binding sites and the AUG codon also serves as an important factor for regA binding (Liang et al. 1988).

The biosynthesis of DNA polymerase gp43 in bacteriophage T4 is autogenously regulated at the translational level. gp43 represses its own translation by binding to its RNA transcript 5' to the AUG codon at a 36-40 nt region that includes the Shine-Delgarno sequence and the putative RNA stem loop structure (Andrake, et al. 1991). Many mutations that either disrupt the stem or substitute the base in the loop segment diminished binding of purified gp43 in vitro. Tuerk and Gold (1990) have shown that gene 43 mutations that derepress gp43 synthesis do not necessarily affect the rate of replication indicating that the replicative and autoregulatory functions of gp43 are independent

The production of ssDNA binding protein gp32, which is utilized in replication, repair and recombination, is also subject to a similar mode of autogenous repression (Krassa, et al. 1991; McPheeters, et al. 1988). In vitro studies suggest that autoregulation of gp32 occurs by its own product specifically binding to its mRNA at a highly structured pseudo-knot near the 5' end. Nucleation of gp32 binding through the pseudo-knot is thought to be crucial for cooperative binding of gp 32 to a largely unstructured region that overlaps the initiation codon, therefore blocking formation of translation initiation complex. gp32 contains a zinc binding subdomain to which homology was found in a variety of retroviruses and plant viruses. Shamoo and Konigsberg (1991) have demonstrated that gp32 loses its autogenous regulation ability upon removal of the zinc binding motif, but retains the ability to bind ss DNA. Together with the evidence that deletion of the pseudo-knot from gp32 mRNA abolished autogenous repression, the authors proposed that the zinc binding motif of gp32 plays an important role in directing the binding of gp32 at the RNA pseudo-knot region.

Post-translational control of protein function was studied in depth with the proteolytic processing of protein precursors in phage T4. Examples are the maturation of proteins that compose the phage virion. Assembly of the phage virion occurs on the host inner membrane about 15 minutes post-infection. The majority of constituents are produced by T4 late genes. The head and tail are assembled in two separate pathways. The process of assembly was summarized both in Kao's and Bergsland's theses. However, I would like to emphasize our current understanding of one of the capsid components, gp23, which is intimately related to my research.

gp23 is the major head protein of phage capsid. Each mature head contains more than 1000 copies of gp23 and each cell produces about 200 progeny. Therefore, one can imagine that codon usage in gene 23 has to be optimal to keep

up with the demand. In SDS polyacrylamide gels, gp23 electrophoreses at two different positions, 53 kD and 48 kD. The 53 kD precursor form of the gp23 is assembled into the shell (the outermost layer of prohead). After the formation of the prohead, a protease (T4 PPase) encoded by gene 21 recognizes a unique secondary conformation of the prohead precursor as the cleavage signal and cleaves nears the N terminal end (Hinterman, 1992). This process results in rearrangement of the prohead and expansion of the inner head cavity (Steven, et al. 1992). The phage genome that is replicated in multi-chromosome concatemers is then measured and cleaved by an ATP dependent DNA nuclease derived from a variant of gp23 as one headful length in the mature head (Xue and Black, 1990).

It is an interesting notion that gp23 possesses both enzymatic and structural functions and that the role of the protein is determined by the variant derived by the proteolytic process. Rao and Black (1985) have observed that the DNA packaging enzyme (also called capsizyme gp23**) results from the N terminal processing found in the mature capsid protein gp23* as well as truncation at the C terminal end. Lack of the consensus gp23 cleavage site at the C terminus suggests that the cleavage is unlikely to be due to the gp21 proteinase. The observation that amino acid substitutions affecting the C terminal cleavage exhibited a new gene 23 mutant phenotype, defective DNA filled heads supports the idea that the cleavage product of gp23 directly acts in the DNA packaging mechanism.

The temporal interconnection of prophage maturation and DNA packaging by a single polypeptide, gp23, is an intriguing phenomenon. Many questions arise such as: How are the alternative proteolytic processes involving two different proteases determined? Does the location of the gp23 molecule on the head structure influence the decision? Is it possible that gp 23 near the bottom entrance of the head may possess a distinct conformation that can be recognized by the C-

terminus specific protease? Identification and characterization of the protease acting on the gp23 C terminus would aid in understanding these puzzles.

Molecular Basis for Phage Exclusion

Extrachromosomal DNA elements, such as plasmids and integrated prophages, are able to protect their hosts from infecting phages in a number of ways. Basically, these defense systems can be classified into two major categories: superinfection exclusions and abortive infections. The former deals with the phenomenon that includes: conferring inhibition of early gene transcription and replication in superinfecting phage carrying a homologous immunity region (Lu, 1989); preventing successful injections of viral DNA from the adsorbed phages into the cytoplasm (Kliem, 1989); and altering surface receptors, thus hindering phage adsorption. Overall, the consequence of this type of exclusion allows hosts to survive viral attacks. Less well understood are those systems of abortive infection that are characterized by an initial event that usually takes place in a productive infection, followed by the occurrence of cellular dysfunction, that consequently kills the host and prevents the production of phage progeny and their spread into the bacterial population.

The employment of plasmid clones carrying genes that can provoke a defect similar to abortive infection enables researchers to probe the molecular basis for each system. Reviews of our current understanding of some exclusion systems have recently been published (Snyder and Kaufmann, 1993; Molineux, 1991). Here, I would like to concentrate on two notable exclusion systems of bacteriophage T4, with emphasis on their molecular themes: the exclusion of T4 rII mutants by the rex genes of E. coli lambda lysogens and by the prr gene product of a cryptic DNA prophage identified in the clinical isolate CT196. Both mediate physiological defects but in different ways: the former interferes with

membrane potential and the latter attacks a specific tRNA, thus impairing translation.

The rex exclusion: The phenomenon of restricting T4 rII mutants by an E. coli lambda lysogen, first discovered by Benzer in 1955, was later developed into a powerful genetic system for mapping mutations, uncovering the nature of the genetic code, and a variety of other important contributions. Compared to the glory of its applications, the phenomenon itself did not receive very much attention until the 1980s (Toothman and Herskowitz, 1980). This abortive infection requires the synthesis of two lambda prophage gene products, RexA and RexB. The RexA protein, being on average hydrophilic, is thought to be a cytoplasmic protein, whereas RexB is extremely hydrophobic and is associated with the inner membrane (Parma et al. 1992). The structural similarity between RexB and some ion channels, and the dependence of restriction on the presence of monovalent cations, led to the hypothesis that RexB may form an ion channel that selectively transports monovalent cations (Sekiguchi, 1966). The influx of ions might depolarize the cytoplasmic membrane, causing loss of internal ATP, termination of cellular metabolism, and blockage of phage development.

On the basis of the observation of requirement of RexA for the exclusion, Parma *et al* have proposed a two component regulatory model where RexA is a cytoplasmic sensor that detects the status of infecting phage and then passes the signal to membrane bound RexB, destroying the membrane potential. The ratio of RexA to RexB appears to be important for the loss of membrane potential. Snyder and McWilliams (1989) have observed that overproduction of RexA over RexB resulted in a defect in uninfected cells similar to that seen after rII mutant infection of lambda lysogens. This effect does not exist in the situations of overproduction of both RexA and RexB or of RexA alone. Moreover, overproducing RexB over Rex A, does not abort the lytic growth of rII mutants (Parma 1992). According to

the model, this evidence suggests that it takes more than 1 molecule of RexA sensor to activate the function of RexB, to mediate the exclusion pathway, in response to phage infection.

This severe physiological defect can be prevented by the presence of both rIIA and rIIB gene products of phage T4. The rIIA and rIIB functions are normally non-essential, although it has been suggested that they may play a role in DNA replication (Manoil et. al. 1977). The relationship between rex and rII is quantitative; and plasmid clones of rex A and B not only restrict rII mutants, but also T4 wild type and other unrelated phages as well (Shinedling, et al. 1987). Mutations that can substantially alleviate exclusion of T4 by rex map in the T4 motA gene that activates transcription of T4 middle gene promoters. The delayed T4 DNA replication of motA mutants was thought to promote the escape from abortive infection, but the detailed mechanism is not clear.

If the two component model proposed by Parma *et al* holds, then one major question is how the signal which is apparently manifested in the absence of rII proteins potentiates the sensor protein RexA to activate the putative ion channel RexB. Several suggestions have been made. As described above, an excess of RexA to RexB can provoke a phenotype similar to abortive infection in uninfected cells, so one hypothesis focuses on the absence of rII protein somehow unbalancing the ratio of RexA to RexB and consequently leading to abortive infection. Alternatively, RexA might be potentiated by some sort of modification to activate RexB in response to the infection by rII mutants.

With the significant progress toward unraveling the underlying mechanisms of abortive infection, hopefully it will not be long before we reach the stage of full understanding. Furthermore, the knowledge might lead to interesting new insights of host-parasite interactions.

The prr exclusion: T4 mutants lacking polynucleotide kinase and RNA ligase can't form plaques on E.coli strains derived from CT196. The restriction depends on the function of a cryptic DNA element, prr, located at 29 min. on the bacterial chromosome (Snyder and Kaufmann, et al. 1987). If prr is transduced into other strains, it will confer the same phenotype. Underlying prr restriction is the specific manifestation of a T4 induced RNA ribonuclease, which cleaves preexisting host lysine tRNA 5' to the wobble position of the anticodon loop(Kaufmann, 1985).. The reaction generates 2':3'-phosphorus and 5'-OH termini (Amistsur, et al. 1987). The damaged tRNAs can be repaired by T4 polynucleotide kinase and RNA ligase in Wild-type phage but not in pnk- and rli- mutants (Abdul, 1984; Kaufmann, 1985). Translational inhibition by depleting $tRNA_{\underline{lys}}$ accounts for the abortive infection in prr-containing cells.

The RNA ribonuclease has been renamed anticodon nuclease and found to be encoded by the E. coli prr locus. Nucleotide sequences and mutational analysis of a cloned prr region indicate that the prr region is composed of four tandem ORFs designated prrA-D, and that only prrC is responsible for anticondon nuclease activity. Intriguingly enough, the other ORFs prrA, prrB, and prrD are homologous to type-1 restriction-modification genes hsdM, hsdS and hsdR, respectively (Amistsur, et al. 1992; Linder, et al. 1992). The interaction between prr and hsd may couple and mutually enhance protection at the DNA and RNA levels.

Activity of prrC-encoded nuclease can only be induced upon phage infection, implying the association of phage genetic elements with the enzymatic activity. Suppresser mutations that can inhibit prr restriction were isolated and mapped in the rII to gene52 of T4 DNA, a region called *stp*. The suppressors defined an open reading frame of 29 codons (Chapman, et al. 1988). This small polypeptide appears to stimulate the prrC-encoded anticodon nuclease. Amistsur

and Kaufmann (1989) have successfully demonstrated cleavage of lysine tRNA in an in vitro assay by adding synthetic Stp polypeptide to an extract made from prr-containing cells. However, Stp with a missense mutation will not activate the cleavage. The result indicates that Stp is a very specific activator for prrC enzymatic function.

Studies emerging from a subclone containing only prrC indicated that prrC-encoded product appears to cleave cellular tRNA without phage infection. To ensure cellular safety, the expression of prrC definitely has to be tightly regulated in uninfected cells. The safety policy seems to be endowed by the flanking HSD sequences, for a clone carrying the whole prr region retains the latency of prrC function, that can only be activated by the Stp peptide. From these observations, Kaufmann and coworkers (Levitz, et al. 1990) proposed that the anticodon nuclease latency might be due to the masking of the core enzyme PrrC by the flanking HSD elements, and the Prr ribonuclease could be activated indirectly by the binding of Stp polypeptide to remove the putative masking agents. Recent evidence showed that activation of PrrC nuclease also depends on an endogenous DNA besides Stp, ATP and GTP. Stp function can be substituted by a small, heat-stable E. coli factor which only exists in non-prrC containing cells. This implies that PrrC might function in circumstances other than phage infection, and we might predict these systems may be controlled by different conditions.

The current model of Prr-Stp mediated abortive infection bears some features similar to our gol-Lit exclusion system: Firstly, they both involve a small polypeptide that functions as an activator of a particular enzyme. The Gol polypeptide of about 25 amino acids long activates the EF-Tu protease, Lit. Secondly, the molecular mechanism in both cases lead to depletion of translational components; EF-Tu in gol-Lit and $tRNA_{lys}$ in Stp-PrrC mediated exclusion. It is possible that interaction of the host and phage determinants works in a similar

fashion in both systems. Hopefully, the forthcoming investigation of one will have input into understanding the other in detail and vice- versa.

Properties of Translation Elongation Factor Tu

In the course of my research, one of the major discoveries regarding gol-Lit mediated exclusion is the cleavage of elongation factor Tu. Knowledge of EF-Tu is therefore important for understanding the physiological basis of the abortive infection.

EF-Tu encoding genes and their regulation: EF-Tu, as the most abundant protein in E. coli, is encoded by two nearly identical, unlinked, genes, tufA and tufB (Jacobson and Rosenbusch, 1976; Weiljland, et al. 1992; Young and Zurano, 1981). The only difference in their products is the residue at the C terminus: Gly and Ser, respectively. The majority of cellular EF-Tu is made from the tufA gene, which is distal to, but cotranscribed with, two ribosomal proteins (rpsL, rpsG) and EF-G (fus) as part of the str operon. The str operon is located at 72 min. on the genetic map (Shibuya, et al. 1979). The expression of tufA does not appear to be subject to feedback control by S7, the autogenous regulatory protein identified for the str operon (Zengel, et al. 1984). Moreover, an interesting feature of the tufA gene is that the translational efficiency of tufA mRNA is significantly higher than that of other gene transcripts in the same operon. The high molar concentration of EF-Tu relative to EF-G is attributable to this difference.

The tufB gene, located at 88 min., is the last gene of the thrU operon that also contains four tRNA genes and is under the additional control of a vicinal weak secondary promoter. The thrU operon is particularly interesting since its transcript functions both as structural RNA and as a messenger RNA. This arrangement suggests that the polycistronic transcript undergoes extensive processing to generate the four tRNAs and the tufB message. The synthesis of EF-

Tu from this region in vitro is autogenously regulated by the intracellular content of EF-Tu B (van der Meide, et al. 1983). More recently, Nilsson reported that transcription of the thrU operon is stimulated by a transactivation mechanism during rapid growth, and contains a cis-activating sequence, designated UAS (Upstream Activating Sequence) (Nilsson, 1990; Bosch et al. 1990). This stimulatory effect depends on a UAS-binding element with characteristic similarity to FIS (Factor for Inversion Stimulation) protein. In early log phase, the cellular level of FIS is maximal and drops about 70 fold when cells go from exponential growth to stationary phase. These results indicate that it is the FIS-dependent transactivating mode that enables the cell to respond properly and efficiently to environmental signals. The copurification of FIS with EF-Tu in my work raises the question whether communication between these two factors determines the expression of tufB. Interestingly, this UAS-FIS regulatory mechanism also works on other operons including the tRNA(tyrT) operon and the rRNA (rrnB) operon. For the latter, a regulatory role of EF-Tu is also suggested by my work.

Although there is no functional difference between TufA and TufB, inactivation of tufA by virtue of Mu insertion makes the cell sick whereas inactivation of tufB has little effect on cell growth. Moreover, unlike tufA, tufB expression is under control of growth rate and the stringent response, as are most rRNA and tRNA operons. Conceivably, these differences are due to specific transcriptional modulation of their promoter activity. The FIS-dependent transactivation mechanism may be responsible for this.

Function and structure of EF-Tu: EF-Tu is responsible for bringing amino-acid charged tRNAs to the ribosome during the peptide elongation cycle, where interaction of EF-Tu with its targets, tRNAs and ribosome, is promoted by binding of an allosteric effector GTP. The codon-anticodon interaction triggers GTP hydrolysis and causes the release of GDP bound EF-Tu from the ribosome.

The dissociation allows formation of a new peptide bond between the N-terminus of the aa-tRNA in the A site and the C terminus of the peptidyl-tRNA in the P site. GDP·EF-Tu is in an "off" state. To regenerate functional EF-Tu, a GDP/GTP switch is catalyzed by an exchange factor EF-Ts, and the resulting GTP·EF-Tu repeats the cycle.

In addition to elongating peptide chains, EF-Tu is one of the host-encoded components in RNA phage Qβ replicase(Blumenthal and Garmichael 1979). Its possible role in rRNA synthesis has also been suggested but not yet defined (Travers et al.,1983; Haseltine, 1972). In fact, the observation that EF-Tu is methylated in response to starvation conditions (Young and Bernlohr 1991), which can concurrently provoke stringent control in ribosome production, indirectly echoes a possible regulatory function in transcription. It has been reported that the combination of an EF-TuA/B mutant can suppress a number of nonsense and frameshift mutations, implying a proof-reading ability in EF-Tu (Vijgenboom et al., 1985).

The single polypeptide of EF-Tu, 393 amino acids long, can fold into three discrete domains (Arai, et al. 1980). The first 200 residues, composed of a six-stranded β sheet surrounded by six α helices, is the GTPase domain. This is the most thoroughly characterized portion of the entire sequences (it is also the first G-domain crystal structure identified.) and EF-Tu is the best understood guanine nucleotide-binding protein. Domain 2 (aa190-aa297) contains a six-stranded antiparallel β sheet, forming a hydrophobic pocket . Domain 3, located at the C-terminal region, is a small β barrel (Jurnak, F. 1985). The middle and C-terminal domains are needed for enhanced affinity that is needed for productive interaction with the tRNA, EF-Ts, and ribosomal components. Extensive studies of EF-Tu's its G-domain reveal striking similarity of sequence and three-dimensional structure to that of the eukaryotic oncogene ras p21 protein (Parmeggiani, 1987; Valencia,

et al. 1991). Several point mutations introduced in EF-Tu show the near-identity of the GTPase mechanism with p21. These structural and functional analogies in the G-domain have also been extended in part to other G-binding proteins, including adenylate kinase (Chen et al. 1990) and chemotaxis protein CheY (Chen et al. 1990), by comparison of crystal structures.

The two post-translational modifications known to happen to EF-Tu are acetylation at the N-terminal serine and methylation at lysine residue 56 (Ames, 1979). Interesting enough, the methylation site is only 4 amino acids upstream of the gol-Lit initiated EF-Tu cleavage site (residue 59-60) which is identified in my research (Yu and Snyder, 1993). This region also contains two trypsin sensitive sites (residues 40 and 58) and usually disappears in crystallized EF-Tu. Its accessibility to a variety of enzymes suggests that the region may be on the surface. The result of a computer search of all the EF-Tus and the analagous EF-1αs of eukaryotes showed that the 5 amino acids (R. G. I. T. I), covering the Lit and trypsin cleavage sites, at position 58-62 of E. Coli EF-Tu, are totally conserved during evolution. From these observations, we speculate that this conserved region may play a regulatory role. Perhaps methylated EF-Tu and the cleaved EF-Tu fragment resulting from different environmental signals (starvation for methylation, and phage infection for cleavage), have similar effects on the cellular regulatory networks. With this hope, my research may provide a new avenue to fully understand the role of this most prominent protein.

Transcription Antitermination in E. coli

Transcription antitermination, which allows readthrough of otherwise efficient terminators by a regulatory protein, provides an important mechanism for regulating gene expression. "Action-at-a-distance" is the hallmark of antiterminators, such as phage λ gene N and Q products (Friedman and Gottesman 1983; Roberts 1988) and, probably, the Tat protein of human immunodeficiency

virus HIV (Cullen 1990, 1993; Cunnery, et al. 1990). Unlike λ Q protein which, by binding to DNA in the -35 to -10 region, modifies RNA polymerase at an early elongation pause site (Yarnell and Roberts, 1992), the λ pN and the HIV Tat protein probably recognize a particular site on nascent RNA transcripts, designed as *nut* for pN and *tar* for Tat antiterminator (Nodwell and Greenblatt, 1991; Cunnery et al. 1990).

The studies emerging from genetic analysis and in vitro reconstitution assays indicate that, in bacteriophage λ, processive transcription antitermination in early genes requires the direct interaction of N and a cellular cofactor NusA. The interaction enhances the recruitment of three other host factors NusB, NusG to subsequently modify RNAP to a terminator-resistant form (Mason and Greenblatt 1991; Linn and Greenblatt, 1992; Roberts 1993). The late-gene antitermination system in contrast needs only the Q protein, the λ *qut* site and E. coli NusA (Barik and Das 1990).

The *nut* site that is targeted by pN has a distinct structure (Lazinski, et al. 1989). It is comprised of characterized boxA sequences and a region of dyad symmetry (boxB) with an undefined boundary between them. Cloning experiments by deCrombrugghe and coworkers (1979) demonstrated that the *nut* site is functionally separable from the cognate promoter. Furthermore, the nut site allows pN to suppress multiple terminators located thousands of base pairs away. The genetic dissection of *nut* elements by several mutations and hybrid *nut* sites between close relatives of λ, such as phages p21 and p22 , indicate that BoxB is essential and is the locus of specificity, while BoxA promotes the specificity by facilitating cognate pN binding to BoxB. A remarkable discovery regarding *nut* function is that, the same elements can be converted from an antiterminator into a strong terminator by a phage HK022 protein (Nun, Oberto and Weisberg, 1989). The similarities between Nun-mediated termination and N-mediated

antitermination suggest that the two processes have biochemical steps in common (Robert, et al 1987).

Several observations indicate that transcription antitermination also regulates ribosomal (rrn) gene expression. The detailed mechanism is not clear, but a number of features are shared by the rrn and λ systems (Suzanne et al 1984). First, a version of the boxA and boxB elements, although reversed, is found in the highly conserved bacterial rrn operons (Moorage 1986). However, unlike λ *nut* sites, the BoxA sequences are both necessary and sufficient for antitermination. Secondly, the productive antitermination also requires ribosomal protein S10 and cellular Nus proteins, such as NusB (Squires et al 1993). No cellular analog of N protein has been identified thus far. In vitro reconstitution using purified proteins and templates containing rrn *nut*-like sites failed to completely read through downstream terminators, suggesting the requirement of additional not-yet-characterized cellular factors.

The gol site that is essential for cleavage of EF-Tu in Lit-containing cells, not only looks like the antitermination nut site, but, from my work, may actually function as one by enhancing transcription through the region in the absence of Lit protein. In local inhibition, the gol sequences can then be converted to a transcriptional terminator presumably by the cleaved form of EF-Tu or by other un-identified factors. Further investigation of this gol-mediated effect may provide new insights toward a full understanding of the molecular nature of transcription antitermination.

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CHAPTER 2

TRANSLATION ELONGATION FACTOR TU CLEAVED BY A PHAGE EXCLUSION SYSTEM

KEY TERMS (E14/PROTEASE/T4 PHAGE)

Submitted to Proc. Nat. Acad. Sci. USA

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ABSTRACT **Bacteriophage T4 multiplies poorly in *Escherichia coli* strains carrying the defective prophage, e14. The exclusion is caused by the interaction of an e14 encoded protein, Lit, with a short RNA or polypeptide sequence, *gol*, from within the major head protein gene of T4. The interaction between Lit and *gol* causes a severe inhibition of all translation, and prevents the transcription of genes downstream of the *gol* site in the same transcription unit. It does not, however, 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 T7 and related phages by the *pif* gene product(s) of the F plasmid. In all these exclusions, a nonessential protein or proteins expressed by the resident element somehow recognizes that the cell has been infected by a phage and kills 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 (1).

The DNA element e14, a defective prophage integrated in the isocitrate dehydrogenase (*icd*) gene (2), partially excludes Teven 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 Teven phage infection (3,4). The inhibition requires an interaction between the Lit protein and probably either the RNA or polypeptide from a short region of only about 75 base pairs in the major head protein gene of Teven 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 first defined by *gol* mutations that 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 which is activated by the polypeptide or RNA from 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 JM101 and W3110 lacIq *recA*, which have been described (4). To construct isogenic derivatives of these strains, one of which lacks e14 so has no Lit protein and the other of which has an excess of Lit protein, we first cloned a kanamycin resistance (Kn^R) cassette between the *BamHI* sites in the large *HindIII* 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*(TS) mutation as described previously (6). The e14 had a *lit*(Con) mutation, 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 LacIq *recA*$^-$ and JM101 selecting for Kn^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 Kn^R strains and selecting a Kn^S derivative which had simultaneously lost the LitCon phenotype so presumably had been cured of e14. The plasmid pUC84PZ1 described previously (4) has a 159 b.p. 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 Kn^R cassette into the *EcoRI* site in the *cat* gene 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 (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, 1.0% NaCl, 0.5% glucose, 50 μg/ml ampicillin, 50 μg/ml kanamycin. When the $O.D._{625mu}$ reached 0.3, the cells were divided into two 500 ml cultures, and IPTG was added to 5 mM to one of the cultures for 20 more minutes. The cells were collected, resuspended in 4 ml resuspension buffer and lysed in a French Pressure Cell. The S30 and S150 supernatants were prepared and the *in vitro* translations performed as described (7) with a few minor modifications. No RNA and no ribonucleoside triphosphates except GTP and ATP were added to the *in vitro* reactions. The reaction mixtures contained 200 μl supernatant (100 μl S30 + 100 μl S150) in a total volume of 300 μl. Aliquots of 70 μl were withdrawn and precipitated with 3 ml 5% trichloroacetic acid (TCA) in the cold. The precipitates were heated to 100°C for 5', centrifuged, resuspended in 0.1 ml 2% KOH, and reprecipitated with 3 ml 5% TCA before collecting on WF/A Whatman filters for counting.

Antibody Precipitations. 10 ml of cells were grown in M9 medium plus all 20 amino acids except methionine plus 50 μg/ml ampicillin to an O.D. = 0.4 at 30°C. The proteins were labeled by adding 4 μl of ^{35}S methionine (1200 Ci/mmole) for 10' and chased with unlabeled methionine (10 mM) for 10'. Depending on the experiment, the cells were either infected with CsCl purified T4 at an M.O.I. of 10, or IPTG was added to 5 mM to induce transcription and translation of the gol region. After 30 minutes, the cells were collected by centrifugation and resuspended in 100 μl H_2O. They were lysed and the antibody precipitations 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 midlog phase and

IPTG was added for 20 minutes before infection. CsCl purified T4 were added at an M.O.I. of 10 and 1 ml aliquots were taken to add to ice for SDS-PAGE electrophoresis. Aliquots were also taken to add to saline plus $CHCl_3$ and diluted to plate with indicator bacteria to determine the phage yield.

Other Methods. SDS-PAGE electrophoresis 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 T4 causes a severe inhibition of translation due to the interaction of the RNA or polypeptide from 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 figure 1. In this construct, the *lit* gene of e14 has been cloned in the multicopy plasmid pACYC184 such that it will be expressed from its own promoter. The *gol* site of T4 has been cloned in the compatible vector, pUC8, such 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 cartooned in figure 1 and divided the culture in half just before adding IPTG to one of the two subcultures. After 20 minutes induction, to allow transcription and translation of the *gol* region, the cells were concentrated and lysed as described in Methods, 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, initiation or elongation factors, could be inactivated somehow. To distinguish these possibilities, we further fractionated the extracts by differential centrifugation. We

prepared S30 supernatants as described in 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 the soluble factors including mRNA required for translation.

To determine the effect of the supernatants on translation, we mixed the supernatants from inhibited and uninhibited cells in the *in vitro* translation assay (see Methods). Addition of the S150 supernatant from the uninhibited cells stimulated incorporation of amino acids by the S30 supernatant from uninhibited cells (see Fig. 2A). 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 has been 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. The results are shown in figure 2B. A major protein band of 43 kDa is missing from the induced supernatants, and it is replaced by a smaller band of about 37 kDa. This is the only reproducible difference we have detected between the inhibited and uninhibited extracts. In the experiment shown in Fig. 2B, another polypeptide of about 75 kD appears to be missing from the supernatants of the inhibited extracts. In other inhibited extracts, this polypeptide is present and is probably a protein that sporadically sediments during the preparation of the supernatants.

From its size and abundance, we suspected that the 43 kDa protein is EF-Tu, which is being 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 figure 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 precipitates the 43 kDa EF-Tu protein and more of this protein precipitates in cells with the overproducing clone. In the cells in which the *gol* region had been induced in the presence of Lit protein, much less of the 43 kDa protein precipitates, and a second polypeptide of 37 kDa also precipitates (Lanes 9-11). We conclude that the 43 kDa protein that is being cleaved is EF-Tu, and that the 37 kDa polypeptide is one of the cleavage products.

The induction of the cloned *gol* region in the presence of Lit protein mimics in many ways the infection of Lit protein containing cells by T4 phage suggesting that EF-Tu would also be cleaved after T4 infection. To test this, we labeled cells with S^{35} methionine and chased with cold methionine before infecting them with T4 as in Methods. After 30 more minutes we lysed the cells and precipitated the EF-Tu with anti EF-Tu as before. After infection of the non-Lit protein-containing cells only one protein, the 43 kDa EF-Tu protein precipitated. However, after infection of Lit protein-containing cells, the 37 kDa cleavage fragment also precipitated (data not shown). Therefore, EF-Tu is also cleaved after T4 infection of Lit protein containing cells. In contrast EF-Tu was not cleaved after infection by T4 with a *gol* mutation. This was expected since *gol* mutations allow T4 to multiply in Lit protein containing cells. Presumably, they change the *gol* peptide or RNA so it can no longer activate the cleavage of EF-Tu.

The experiment above was performed with cells that contain abnormally high levels of Lit protein. It is of interest whether EF-Tu is also cleaved after

infection of *E. coli* K12 containing normal levels of Lit protein. When *E. coli* containing an integrated copy of wild type e14 is infected by T4, EF-Tu is also cleaved (data not shown). However, in this case, only about 50% of the EF-Tu is ever cleaved, even at late times. Nevertheless, phage production is significantly delayed (data not shown). Apparently, normal levels of Lit protein can cleave a significant part of the cellular EF-Tu and inhibit, but not totally block, phage development.

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 block to 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 figure 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 multi-copy plasmid, then not all the EF-Tu might be cleaved after T4 infection. If depletion of EF-Tu due to inactivation by cleavage is 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 is somehow interfering with phage production, or another protein required for T4 development is also being cleaved, then phage production under these conditions should not be normal. From figure 4A, it is apparent that cells with the *tufA* clone have about three times the normal amount of EF-Tu (compare lanes 10-12 to lanes 4-6); and even though more EF-Tu is cleaved after T4 infection, judging by the amount of cleavage fragment (lanes 7-9), a supernormal amount still remains (compare lanes 7-9 to lanes 1-3). The remaining uncleaved EF-Tu does support

some phage production (Fig. 4B) indicating that the depletion of intact EF-Tu is partially responsible for the block in phage production.

While the experiment shown in Fig. 4B supports the conclusion that depletion of EF-Tu is partially responsible for the block in T4 production, it also suggests it may not be the only contributing factor. The excess of EF-Tu causes the cells to grow more slowly and delays phage production (Fig. 4B), complicating the interpretation of the results somewhat. 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 it is not makes us entertain other possibilities such as that the cleaved form is inhibitory, or that some other protein besides EF-Tu is being cleaved. 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 has 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··· (see Figure 5). A comparison of this sequence with the known sequence of *E. coli* EF-Tu (10) revealed the cleavage occurs between glycine 59 and isoleucine 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 from the *gol* region of T4, a short sequence within gene 23, the major head protein gene. The cleavage occurs between glycine 59 and isoleucine 60 in a highly conserved region of EF-Tu. The cleavage apparently inactivates EF-Tu for translation because translation is completely blocked if all 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 if supernormal amounts of EF-Tu remain uncleaved.

At present, we do not know the mechanism of cleavage of EF-Tu. We have only observed the cleavage when both the e14 Lit protein and the T4 *gol* region are present and the T4 *gol* region is both transcribed and translated. The Lit protein contains a motif characteristic of zinc dependent proteases (Yu and Snyder, to be published); indicating that it is probably the protease. If so, the Lit protease must be activated by something generated from the transcription and translation of the *gol* region after T4 infection. Note that the *gol* RNA or peptide could possibly activate the Lit protease not by binding to it but rather by binding to EF-Tu, changing its conformation and thereby making it susceptible to cleavage. Our preliminary searches have revealed no similarities between the gol RNA and peptide and tRNA, EF-Ts or other proteins known to bind to EF-Tu. It is also possible that the Lit protein is not the protease, and that another protease is being activated, or that EF-Tu cleavage is due to autoproteolysis, stimulated somehow by the *gol*-Lit interaction. In this respect, it may be relevant that EF-Tu is often cleaved during purification and/or crystallization (16,17) giving rise to fragments of approximately the same size as those induced by *gol*-Lit. It is of interest whether this apparently spontaneous cleavage bears any relationship to the *gol*-Lit

induced cleavage or whether this region is merely unusually sensitive to proteolysis. We have recently observed the cleavage of EF-Tu *in vitro* in experiments in which 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 us to determine, unambiguously, 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 considering 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 arginine 44 and arginine 58 (14), 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*. On the question of whether this cleaved form of EF-Tu retains activity, different methods

have yielded apparently contradictory results. For example, by their methods, Wittinghofer *et al.* (14) found that the EF-Tu of *E. coli* cleaved by limited trypsin digestion, retains the ability to stimulate polyphenylalanine synthesis in response to poly U. However, Gulewicz *et al.* (15) found that the cleaved form of the closely related EF-Tu of *Thermus thermophilus* has lost this ability although it retains the ability to form a ternary complex with GTP and tRNA. Such seemingly contradictory results make it difficult to predict what activities would be retained by the *gol*-Lit cleaved EF-Tu *in vivo*.

At present, it is not known if the interaction of *gol* and Lit is activating the cleavage of other proteins besides EF-Tu. The five amino acid sequences surrounding the cleavage site of EF-Tu is shared by EF-G and a similar sequence exists in SelB, the specific EF-Tu for selenocysteine tRNA. In the absence of any knowledge of the sequence and structural requirements for the cleavage, it is difficult to predict what other *E. coli* proteins can be cleaved. If the components of the cleavage reaction can be purified, other proteins can be tested. Until then, the possibility remains that cleavage of another protein required for translation could be at least partially responsible for the inhibition of translation.

Even if depletion of intact EF-Tu sufficiently explains the inhibition of translation, from Figure 4 it apparently is not the only cause of the block in phage production. When EF-Tu is overproduced, so not all 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 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 what consequence of the *gol*-Lit interaction is causing the local inhibition. It may be due to the cleaved form of EF-Tu, or the cleavage of an, as yet unknown, other 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 (*c.f.* 11, 13). As mentioned, the two most sensitive sites for trypsin cleavage of the native protein are just upstream of the *gol*-Lit promoted cleavage (14) which also suggests that this region is exposed on the surface of the molecule. This region may also play a regulatory role. The EF-Tus of many bacteria including *E. coli* are known to be methylated in response to starvation conditions (18,19). The one site of methylation in *E. coli* is the lysine at position 56 (10), only four amino acids upstream of the cleavage site. It is of interest 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. The EF-Tus of bacteria (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 (20). 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 (20, and Figure 5). This same five amino acid sequence has also been conserved in the EF-Gs of all organisms (called EF-2 in eukaryotes) even though the flanking sequences have diverged (20). It will also be of interest to determine which elongation factors from other organisms can serve as substrates for the protease.

The only other phage exclusion to be understood in any detail, the exclusion of T4 by the *prr* element in a clinical isolate of *E. coli*, shows

remarkable similarity to the exclusion of T4 by e14. In both exclusions, a small T4 encoded polypeptide seems to activate an endogenous enzyme to attack a highly conserved cellular constituent. In the case of the *prr* exclusion, the host lysine tRNA is cleaved (c.f. 21). This system will even cleave the human lysine tRNA (G. Kaufmann, pers. commun.). Because of the specificity of these phage exclusions for highly conserved cellular components, they may be useful reagents for studying cellular processes in many organisms.

ACKNOWLEDGMENTS

We thank David L. Miller and Robert Bernlohr for their generous gifts of anti EF-Tu antibodies and David L. Miller for a clone of *tuf*A. We also thank Allen Nicholson and Richard Schwartz for their advice on the antibody precipitation experiments; Nanette Guyer for help with strain construction; and Joseph Leykam of the Michigan State University Macromolecular Facility for his help with the N terminal polypeptide sequencing. This work was supported by a grant from the NSF to L.S. and was submitted as part of the requirement for the Ph.D. by Y.-T.Y.

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Figure Captions

Figure 1. The "imitation infected cell" used in these experiments. The *E. coli* W3110 *lacIq recA* cells have been cured of e14 so lack the normal chromosomal source of Lit protein. They contain two compatible plasmids as described in Methods. 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.

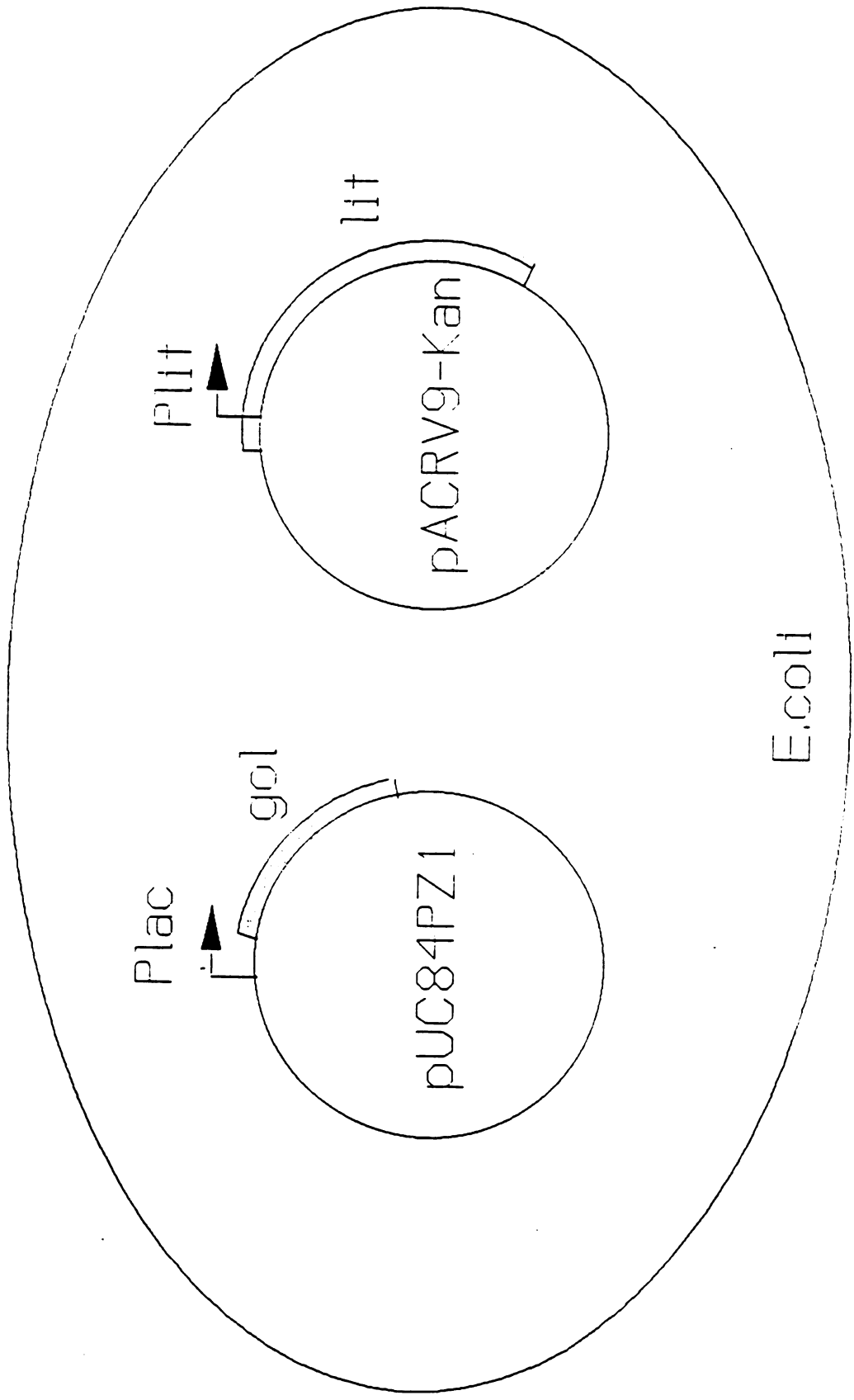
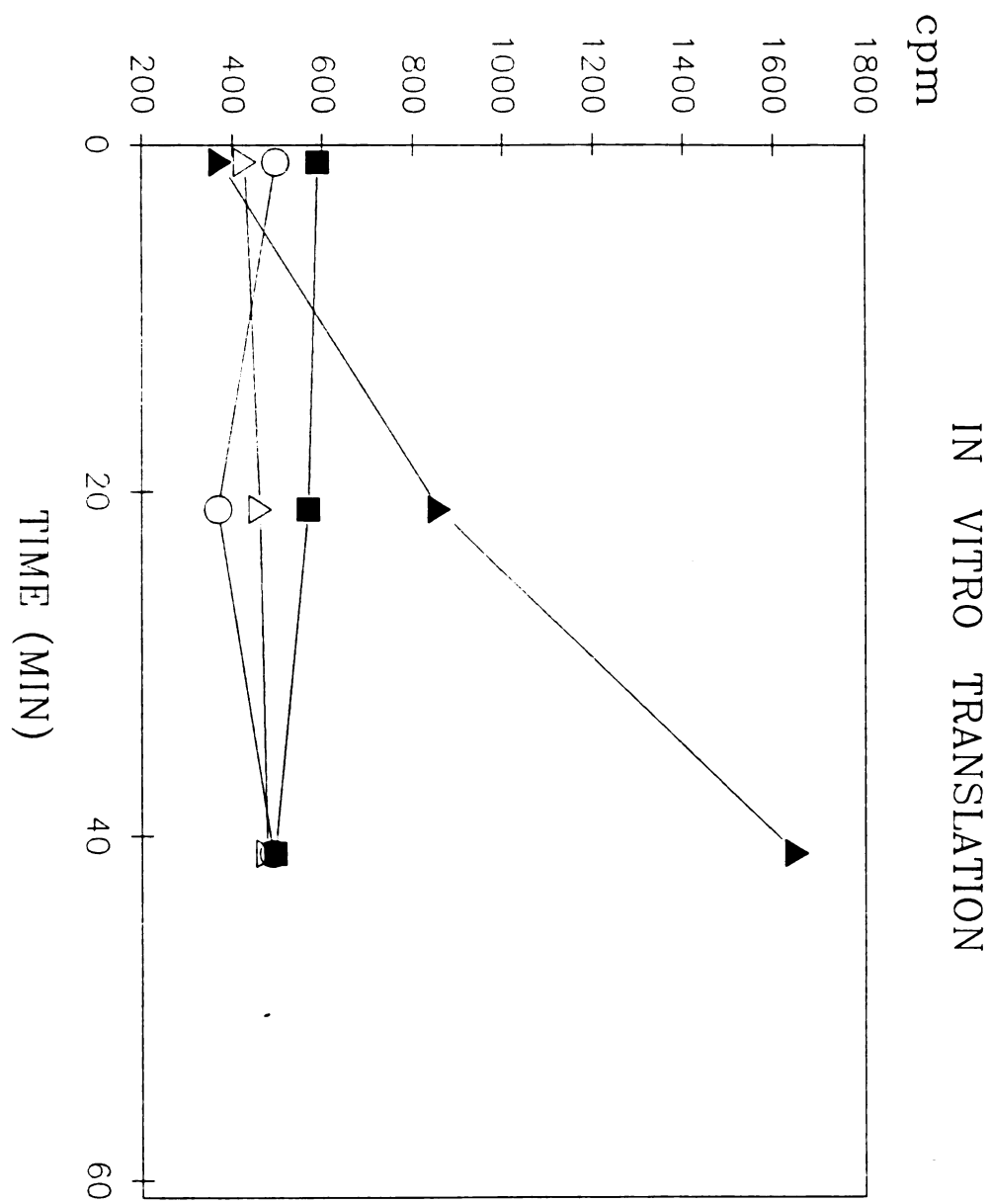

Plac
gol
pUC84PZ1
Plit
lit
pACRV9-Kan
E.coli

Figure 2. A defect in translation by supernatants from induced cells correlates with the absence of a 43 kD polypeptide. Panel A: Incorporation of ^{35}S methionine into acid insoluble material with different mixtures of supernatants. (-♦-) S30 uninduced + S150 uninduced; (-Δ-) S30 uninduced + S150 induced; (-O-) S30 uninduced; (-■-) S30 induced. Panel B: SDS polyacrylamide gel electrophoresis of the proteins in the supernatants of Panel A. Lane 1: S30 supernatant extract of induced cells. Lane 2: S30 supernatant of uninduced cells. Lane 3: S150 supernatant of induced cells. Lane 4: S150 supernatant of uninduced cells. The 43 kD polypeptide that is missing from the supernatants of the inhibited extract as well as the polypeptide which replaces it are indicated by the arrows. As discussed in Results, the other difference between the two extracts at about 75 kDa is not reproducible and is probably due to a protein that sporadically sediments in the preparation of the supernatants.

Panel A


IN VITRO TRANSLATION
cpm
200
400
600
800
1000
1200
1400
1600
1800
0
20
40
60
TIME (MIN)

Panel B

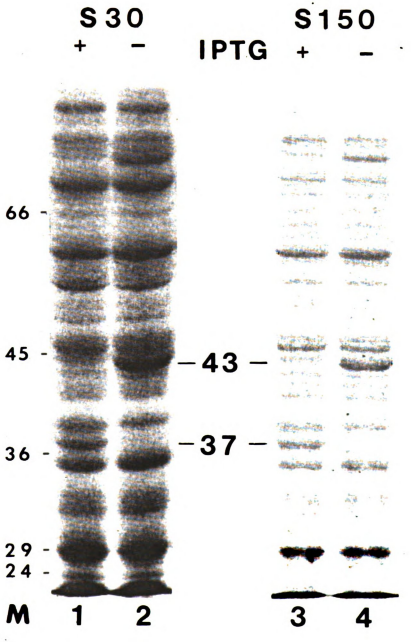


Figure 3. Antibody precipitation of EF-Tu in inhibited and uninhibited cells. The cells were those in figure 1 except they were derived from *E. coli* JM101 and the *lit* gene was overexpressed from e14 in the chromosome because of an up-promoter mutation rather than from pACRV9. Shown is an autoradiogram of the dried gel prepared as in figure 2. Lanes 1-3: Cells without Lit protein or pUC84PZ1 but containing the plasmid, pTA9, that overproduces EF-Tu. Lanes 4-7: Induction of pUC84PZ1 in cells with no Lit protein. Lanes 8-11: Induction of pUC84PZ1 in cells with Lit protein: The antibody precipitations in lanes 1, 5 and 9 had three times as much antiserum as those in lanes 2, 6 and 10. Lanes 3, 7, 11: one preparation of nonimmune serum. Lanes 4, 8: a different preparation of nonimmune serum. The nonimmune serums were added at the higher concentration.

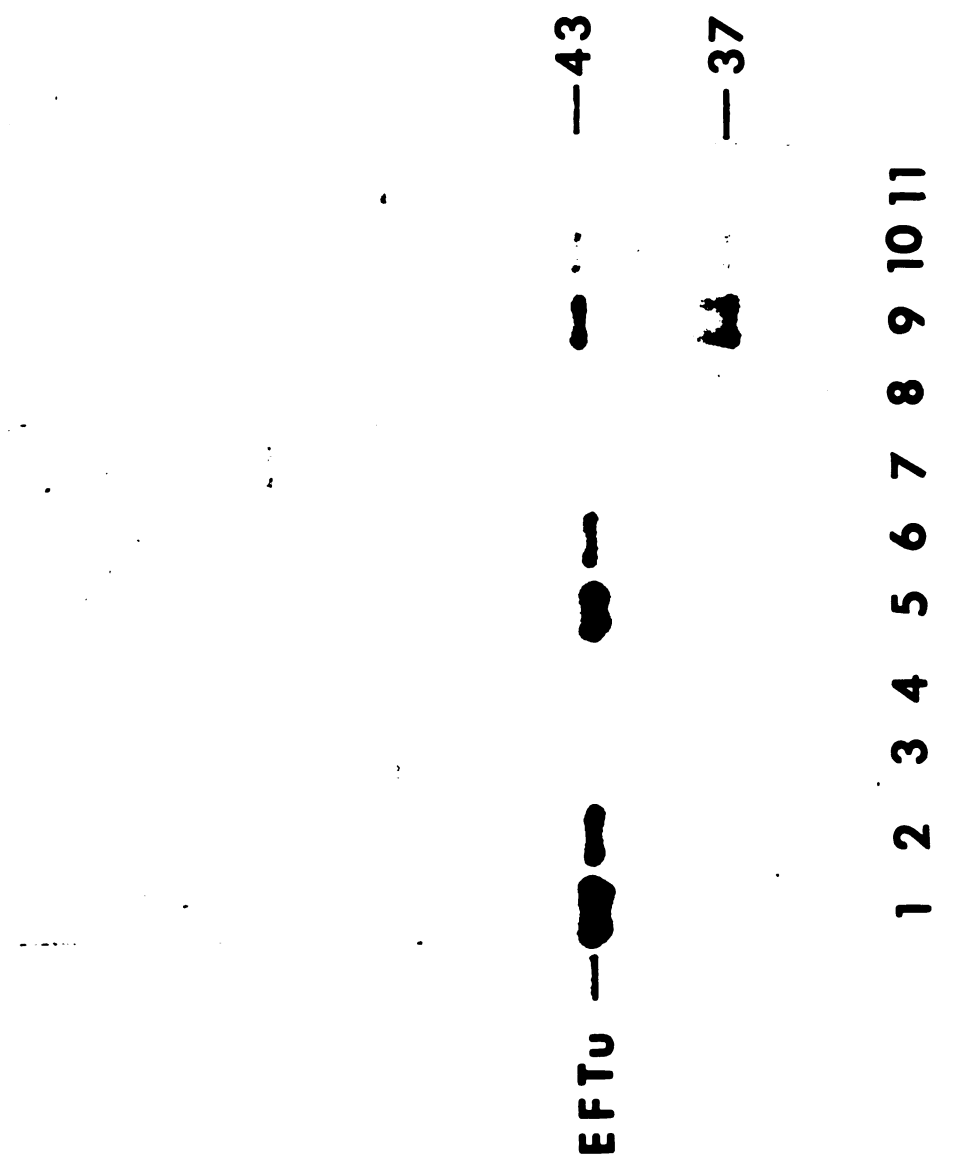
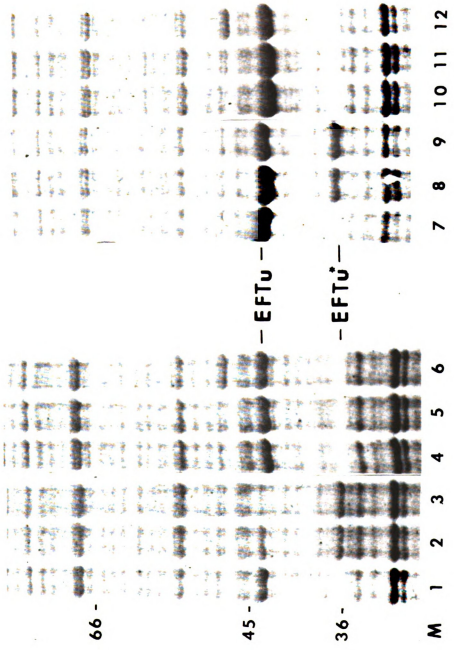

EFTu
43
37
1 2 3 4 5 6 7 8 9 10 11

Figure 4. Multiplication of T4 in cells with an excess of EF-Tu. The cells were the same as those in figure 3, but some of the cells had an excess of EF-Tu because they contained the plasmid pTA9 with the *tufA* gene. Panel A: Commassie 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. Lanes 4-6: Cells with no Lit protein and normal amounts of EF-Tu. Lanes 7-9: Cells with Lit protein and excess EF-Tu. Lanes 10-12: Cells with no Lit protein and excess EF-Tu. Times after infection are 0', 20', 40' for each set. Panel B: One step growth experiment of T4 in cells from Panel A. (-♦-) no Lit protein, normal amounts of EF-Tu; (-∇-). no Lit protein, excess EF-Tu; .(-O-) Lit protein, excess EF-Tu; (-●-) Lit protein, normal EF-Tu.

Panel A



Panel B

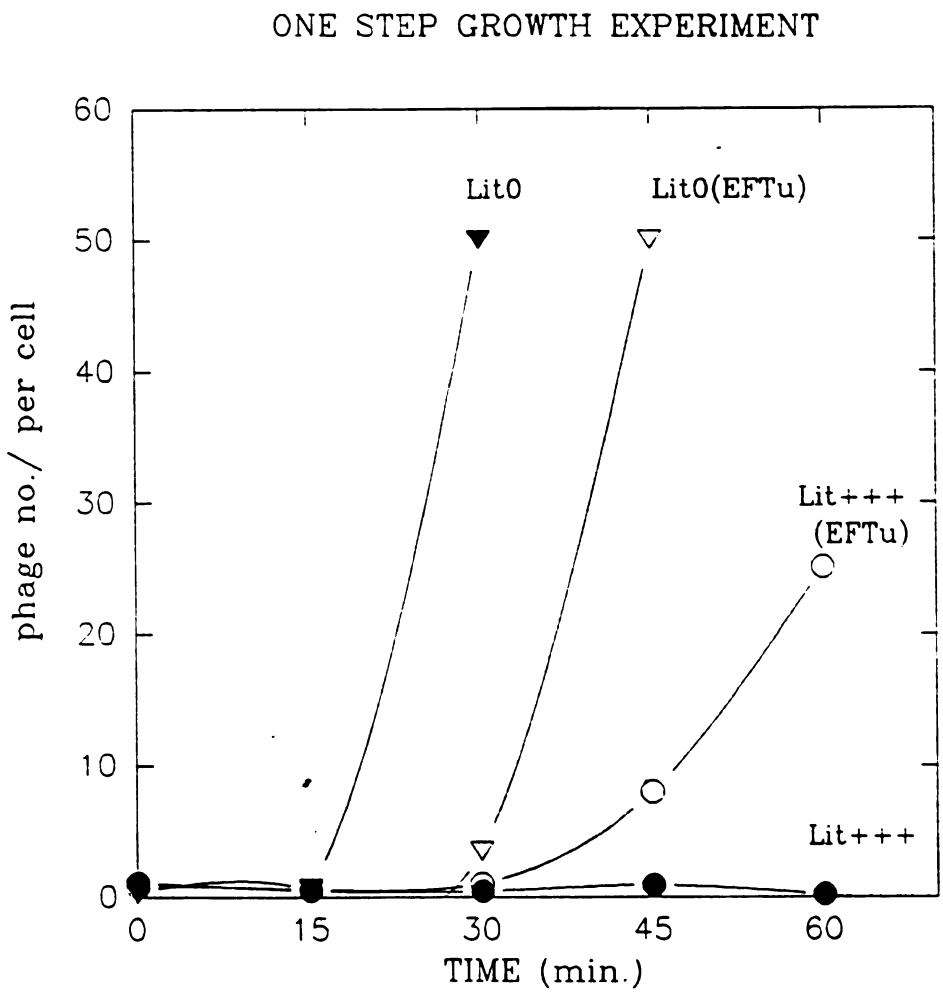
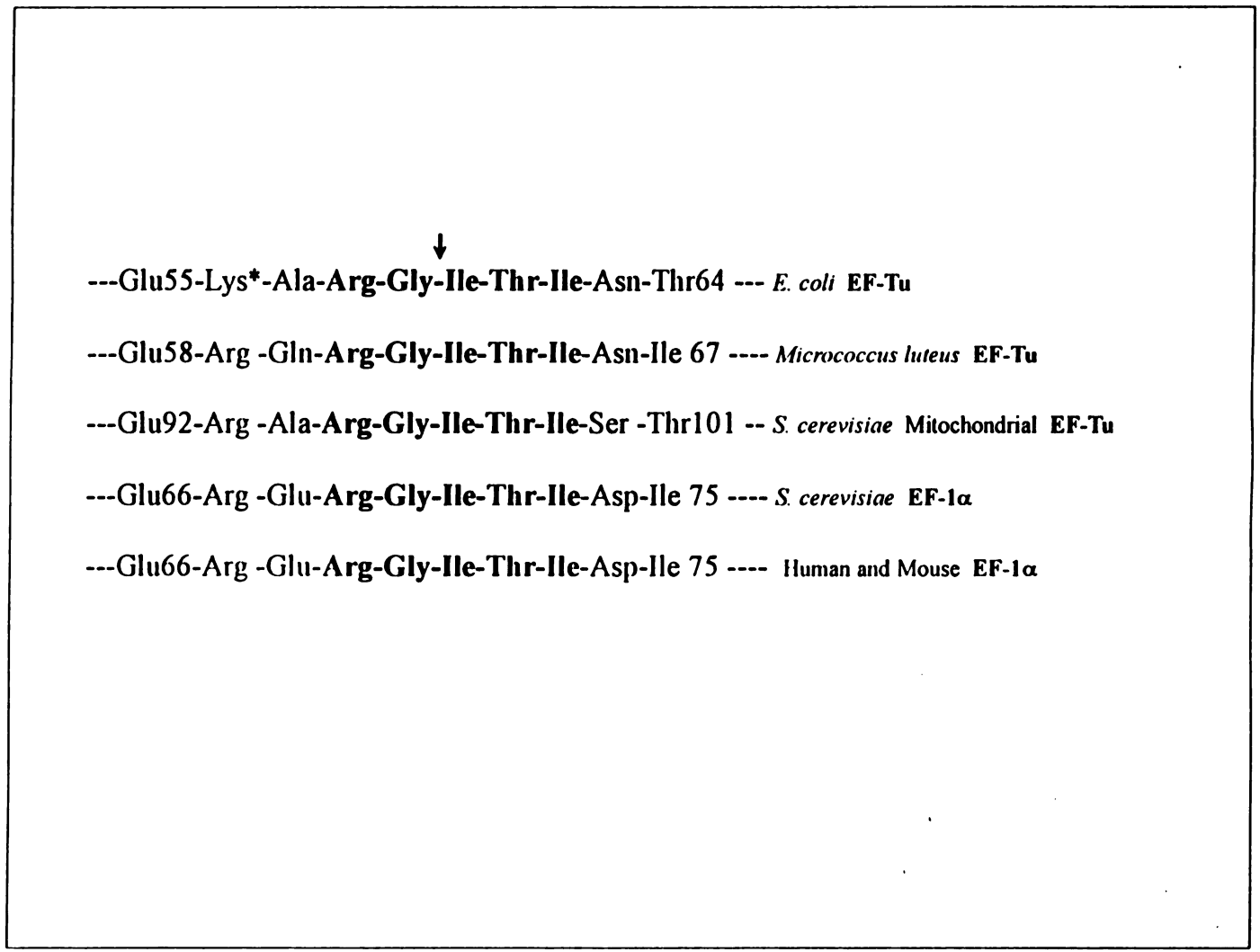

ONE STEP GROWTH EXPERIMENT
phage no./ per cell
60
50
40
30
20
10
0
0
15
30
45
60
TIME (min.)
Lit0
Lit0(EFTu)
Lit+++
(EFTu)
Lit+++

Figure 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 highlighted. The asterisk shows the lysine that is methylated in *E. coli* in response to starvation conditions. The sequences were obtained from Gene Probe.


---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

CHAPTER 3

ACTIVATION OF THE E14 ENCODED LIT PROTEASE BY A 25 AMINO ACID OLIGOPEPTIDE FROM THE T4 GOL SITE

Abstract

Cleavage of translation elongation factor EF-Tu has been shown to occur in an exclusion phenomenon of E. coli phage T4 caused by a cryptic prophage e14 encoded protein, pLit(5, 16). The cleavage results from the interaction between Lit protein and a short sequence, gol. The gol sequence has a minimum length of 75 base pairs within T4 gene 23. The cleavage of EF-Tu apparently inhibits all cellular and phage translation. To determine the roles of factors that might be involved in the proteolytic reaction, in vitro assays were performed . Here we report that the Escherichia coli translation elongation factor EF-Tu can be cleaved after mixing two cell free extracts, one from cells containing a Lit recombinant plasmid, another containing a gol fusion plasmid. A computer search for the function of the Lit protein revealed a possible zinc-dependent metalloprotease motif at sequences close to the middle of the protein. RNase treatment of the gol extract prior to mixing did not abolish proteolytic activity, suggesting that the Gol peptide rather than gol RNA is required for cleavage of EF-Tu. Also adding in vitro synthesized gol RNA did not activate the protease. In contrast, adding the synthesized Gol polypeptide did activate the cleavage. Our current hypothesis for the proteolytic mechanism is that the Lit protein functions as a protease and the Gol peptide works as an activator for gpLit's catalytic activity.

Introduction

Bacteriophage T4, one of the largest viruses, is composed of a circular double strand DNA genome close to 160 kb long with the capacity of 200 genes. It propagates in a majority of E. coli laboratory strains, but hosts sometimes acquire the ability to exclude infecting phages by functions exerted from resident plasmids or prophage. The exclusion mechanisms usually provoke cellular dysfunction that

lead to a cessation of all macromolecular synthesis, terminating phage development, thus preventing phage progeny from spreading into the bacterial population. Exclusion of phage T4 rII mutants and other related phage by the rex genes of lambda, and exclusions of T7 by the pif of F plasmid are classic examples and documented (1, 2). But the detailed mechanisms are still not fully understood.

The cryptic prophage e14, which is located at 25 minutes of E. coli K strain genome, also excludes phage T4 at late stage of phage infection, particularly when one of its gene products Lit is overproduced, either by an up-promoter mutation or expression from a high copy number plasmid (4). The Lit polypeptide has a molecular weight of 30 kD and is believed to be an inner membrane protein by virtue of its copurification with a cellular inner-membrane fraction (3). It causes a severe blockage of translation late in T4 infection, owing to its interaction with a short region, gol, about 75 bp long and 300 bp downstream from the T4 gene 23 ribosome binding site (6). The Gene 23 encodes the phage T4 major head protein and is only expressed during the late stage of development.

We recently reported that the translation deficiency in the phage exclusion system , caused by the interaction of gpLit with the inducible gol activity (RNA or peptide), is at least partly due to cleavage of translation elongation factor Tu (5: Yu and Snyder submitted). Partial amino acid sequencing data from the N terminus of the major 37 kD cleaved fragment revealed the location of the cleavage site to be in between glycine 59 and isoleucine 60, which is an unusual sequence for protein hydrolysis. There are a few interesting features around the cleavage site context. First, a computer database search indicated that the surrounding 5 amino acids including the target site is the second longest stretch of identity, beside the one within the GTP binding motif ,in all the prokaryotic EF-Tus and their eucaryotic counterparts, EF-1αs (GCG database). Secondly, one of the two trypsin sensitive sites in the native form of EF-Tu is located just one

residue ahead of the Lit-gol mediated cleavage site (13). Furthermore, EF-Tu of E. coli is known to be methylated under starvation conditions and the only lysine residue found to be methylated is located at position 56 only 4 amino acids away from the gol-Lit mediated cleavage site (14,15). The intriguing interconnection between cleavage and methylation is also discussed in this report.

The evidence obtained from genetic and molecular experiments indicated that the gol-Lit interaction somehow triggers a specific proteolytic reaction to attack the essential protein EF-Tus. The consequence of the cleavage is blockage of translation; thus abortion of the phage infection. Subjects remaining to be resolved include how does activation occurs; what the roles of Lit and gol are; and which protease is involved. In order to approach those questions, in vitro assays were undertaken to characterize the proteolytic reaction. In this paper we demonstrate the cleavage of EF-Tu in vitro and propose potential roles for the Lit protein and the gol sequence in the catalytic reaction.

Materials and Methods

Bacteria, phages, and plasmid constructs: Table 1 summarizes the relevant features and references of the bacterial strains and plasmids used in our experiments. Crossing a kan resistance gene into e14 was carried out by the means of restricting ColE1 plasmid in a polA(ts) strain as described(16). The kan resistance cassette obtained from Strategene Inc. was cloned into the BamH1 site of the pAG plasmid (obtained from Dr. Charles Hill) carrying an e14 fragment (17). The recombinant clone cannot replicate in a polA(ts) strain at 42^0C, therefore hosts can only acquire the kan resistant trait through homologous recombination at the e14 region. Kan resistance was then transduced by P1 phage from the kan^r-lit^+ polA donor to JM101 lit^+ and JM101 lit(con) respectively by the method of Miller (18). To make an isogenic lit^0 strain, a JM101lit(con) kan^r

strain 609 was cured of e14 by UV irradiation, selecting for kan^S and testing for the loss of the lit(con) phenotype by cross-streaking with wild type T4. A lacZ fusion plasmid, pUC84PZ1, translates the gol region in the frame of T4 gene 23, and exhibits the gol phenotype which cannot transform lit(con) cells due to blockage of cellular translation. pTTQ18lit contains the entire lit gene oriented clockwise downstream of the tac promoter at the polylinker site of pTTQ18. The Lit protein was made from its own promoter and the ribosome binding site. pBlue75gol transcribes the 75 bp gol sequence from the T7 promoter of pBluescript (the vector is obtained from a hybrid clone given by Dr. J. Dogdson). pET11b-S transcribes and translates gol-lacZ fusion RNA from the T7 promoter of pET11 (Novagen).

Media and culture conditions: E. coli strains were usually grown in LB media (1% tryptone, 1% NaCl, 0.5% yeast extract). In protein labeling experiments, cells were grown in M9 media with 0.4% glucose plus 20 amino acids except L-methionine. For making cell free-extracts, cells were grown in tryptone broth (1% tryptone, 1% NaCl, and 50 ug/ml vitamin B1) supplemented with 0.4% glucose. When desired, antibodies were used in the following concentrations: ampicillin, 50 ug/ml; kanamycin, 50 ug/ml; IPTG (BMB)was added to 1 mM to induce the lacZ promoter.

Plasmid purification: All plasmids used in experiments were purified by Qkit obtained from Qiagen. The restriction enzymes used to construct recombinant clones were obtained from BMB.

In vitro cleavage assay: S30 cell extracts were made according to the procedure described in the previous paper(5). Substrate proteins were prepared from crude extract of S35-methionine labeled cells. Cells cured of e14 (lit0) were transformed with a tufA clone, pTA9, to express increased levels of Tu protein from a inducible promoter. The transformants were grown in M9+B1 media to

OD=0.4, then induced with IPTG to 1mm and labeled with radioactive S35-methionine for 10 min. Cells were washed 1x with cold saline and resuspended in resuspension buffer. The labeled crude proteins were then extracted by freezing and thawing 5x. Assays were performed at 30^0 water bath by mixing equal volume of substrate proteins and S30 from inhibited and uninhibited cells. Samples were taken at various time points and analyzed in 12.5% SDS Page. The results were visualized by autoradiography with Kodak X-ray film.

In vitro complementation assay: To prepare cell free extract, the isogenic strains JM101lit^0 and JM101lit(con) harboring pUC84PZ1 and pTTQ18lit respectively, were grown in 50 ml of tryptone broth, supplemented with ampicillin at 37^0C overnight. The overnight cultures were then diluted into 250 ml of fresh media and grown at 30^0 until an $OD_{625m\mu}$ of 0.4, and IPTG was added to 1 mM to induce gol activity from the lacZ promoter. 30 minutes after induction, the cells were centrifuged 5K for 10 minutes and washed once with cold saline solution and resuspended in 3 ml of resuspension buffer (10mM Tris-acetate, pH 8.2, 60 mM KCl, 14 mM MgoAc, to which 1 mM DTT was added fresh). The cells were then sonicated for three cycles and centrifuged 10K for 10 minutes to remove cell debris. The supernatant was then aliquoted and quickly frozen in acetone and dry ice before storing in a -70^0C freezer. The in vitro complementation assays were done by mixing the two cell extracts, one made from cells overproducing Lit protein, the other made from cells carrying a gol fusion clone. Each cell extract was used separately as a control. All the in vitro cell extract assays were performed in a 30^0C water bath, and appropriate amounts of samples were withdrawn at different time points, and the reaction was stopped by an addition of equal volumes of 2x loading buffer. The samples were boiled for 3 minutes and applied to 12.5% polyacrylamide SDS PAGE and electrophoresed 16 hours at 80V (19). Chemically synthesized Gol polypeptides from Chiron inc. are dissolved in

distilled water to make up a stock solution of 2uM. 30 ul of "Lit" cell extract is mixed with a various amount of Gol polypeptide and the mixture is incubated for 30 min. and processed as described above.

Computer Aide analysis: The functional domain of gpLit was searched by the " Motif " program of the Wisconsin GCG protein analysis package. The homology search of the cleavage site in EF-Tus and EF-1α was done according the database of the Gene probe program.

Results

Cleavage of EF-Tu in an inhibited S30 cell extract: Blockage of cellular translation by the gol-lit initiated exclusion system requires two major factors: one is production of the Lit protein, and the other is gol region expression in the gene 23 frame. The use of a recombinant plasmid with the gol sequence expressed under control of the lacZ promoter enabled us to investigate the biological cellular defect without dealing with the complexity of phage infection. In a previous paper we have presented evidence that a S30 cell fraction made from cells harboring both lit and gol clones in two compatible vectors failed to incorporate radioactive-labeled amino acid in in vitro translation experiments. The translational defect of the inhibited extract (IPTG-induced) was correlated with cleavage of the predominant protein EF-Tu, which is responsible for bringing aa-tRNAs to ribosomes during polypeptide synthesis.

This observation led to a way of testing the specific protease function in the S30 cell fractions. In order to distinguish added EF-Tu from the already existing proteins in the preparation, and to differentiate the relative levels of proteolysis in between the inhibited and uninhibited extracts, the substrate proteins were radioactive-labeled in cells containing a tufA plasmid, which expresses an excess of EF-Tu. An equal amount of the labeled proteins were added to the inhibited and

uninhibited S30 extracts respectively. In the autoradiogram of Fig1 (compare Lane 1 and 5) , we saw only about one half of as much intact EF-Tus in the inhibited cell extract compared to the uninhibited one, but no difference in the amount of other proteins. Thus, the EF-Tu protease was solely in the induced (inhibited) extract. We could not detect the cleavage fragment. Perhaps it is unstable under these conditions.

Of course, one can argue that the dimunition of EF-Tu might have resulted from the cessation of cellular translation instead of from a gol-Lit activated proteolytic function. To eliminate this possibility, a similar assay was done with a chloramphenicol-treated cell extract, in which the translation process was severely blocked. The result showed no reduction in the level of EF-Tu (shown in Fig 2). Therefore it is unlikely that EF-Tu cleavage is the consequence of translational deficiency, otherwise we should expect to see a similar effect when translation is blocked in other ways, such as the chloramphenicol experiment.

Characterization of the EF-Tu protease: The use of this assay system enabled us to characterize this enzyme in a few general aspects. First, we found that catalytic activity of the protease was impaired in an alkaline condition but not affected in an acidic one. At pH=10, there was no obvious difference in the amount of EF-Tu substrate proteins after normalizing it with the amount of protein loaded. (Fig 3A), whereas in the buffer of pH=4.8 the reduction of EF-Tu occurred normally in the inhibited S30(Fig 3B). Secondly, in an attempt to examine the possibility that the enzymatic function activated by gol-Lit interaction is via a phosphorylation mechanism, we treated the S30 extracts with phosphatase to counteract a possible kinase, prior to mixing with the labeled substrate. The preliminary result showed that the treatment did not abolish the proteolytic reactions, implying the unlikeness of phosphorylation involvement (Fig 3C). It should be noted that, unlike the in vivo assays, all of the experiments presented

above only allowed us to detect the protease activity by the fact of decreased amount of intact EF-Tu (43kD) but not by the appearance of the cleaved fragment (37kD), which we usually can detect in vivo. This could be explained by its instability in the incubated cell extracts as mentioned above.

. **Cleavage of EF-Tu can be generated in an in vitro complementation assay:** To further characterize the EF-Tu proteolytic event and learn more about the roles of gol and Lit , we devised a simple in vitro complementation system. Instead of using purified components, the in vitro assay was performed by mixing two cell-free extracts; one provides Lit protein from the pTTQ18lit transformed Lit(con) cells and the other provides the gol function from the isogenic Lit^0 strain carrying a pUC84PZ1 clone expressing the gol region . The results shown in figure 4 indicated that the EF-Tu did get cleaved by mixing both extracts (Lane2), but not by either one alone(Lane4 and Lane6). Interesting enough, in this complementation experiment we not only observe the reduction of intact EF-Tu but also can detect the cleaved fragment at the position of 37kD. So far we have no explanation for this difference.

An independent assay showed that the reaction happened very rapidly. We saw the presence of the cleaved fragment about 2 min. after mixing both extracts . The result supported the idea that EF-Tu cleavage is the cause of translation dysfunction instead of vice versa. From Commassie Blue stained gels we detected no other proteins being cleaved than EF-Tu (data not shown). However, we cannot rule out the possibility that some minor proteins are subject to the same protease. The specificity of EF-Tu was tested on its substrate eukaryotic counterpart EF-1α (about 53 kD) using the same in vitro mixing assay plus purified EF-1α from rabbit reticulocytes (obtained from Dr. William C. Merrick). The EF-1α failed to be cleaved impling the specificity of EF-Tu protease, even though these two share

extensive homology in the target site microenvironment. Alternatively, some modification of EF-1α may prevent it from being cleaved.

Which is the protease? Lit protein or gol?: A key challenge is to identify the EF-Tu protease. Overproduction of either the gpLit or gol sequence (RNA or/and peptide)alone did not cause cleavage of EF-Tu in cells. The proteolytic reaction depends on the presence of the two components, gol and pLit. Therefore it is reasonable to propose that one of the key factors actually exerts enzymatic function, while the other activates it somehow.

A computer search for a functional motif of Lit protein revealed that pLit possesses a common pattern of primary structure (shown in Fig 5) which is also shared by a family of neutral zinc-dependent metallopeptidases composed of at least 22 members. This suggested that Lit is the EF-Tu protease. However, to convince ourselves that the protein itself is indeed directly involved as an enzyme, the purification of the Lit protein and evaluation of its catalytic role in vitro is necessary.

Gol polypeptide is required for the proteolytic reaction: If Lit does play a protease role, it can not function unless a cofactor is provided from extracts made of the isogenic lit^0 strain with a "gol" clone in it. From analysis of several frame shift and nonsense mutations in that region, it is clear that, to exhibit its maximum effect, translation of the region in the same frame as in gene 23 is crucial. These results suggest that the gol peptide sequence is the active component. Nevertheless, we cannot rule out the possibility that the action of translation itself instead of the product causes the effect of gol. For example, the translating ribosome could release activity by opening up the secondary structure of gol RNA.

The likelihood of RNA vs. polypeptide activity was tested by a RNase-treating the gol extract. The rational behind this experiment is that if the gol RNA molecule is responsible for the activation, we might expect that removal of RNAs

from the extract by RNase digestion would prevent the activation of the protease. Fig 6 (Lane8) showed that pre-RNase incubation of the "gol" cell extract, thereby eliminating RNA species prior to mixing with the "Lit" cell extract, still retained the proteolytic activity. The reduction of RNAs resulting from RNase treatment in "gol" extract was significant compared to the proteolytic activity (data not shown). Further experiments showed that addition of full length gol RNA made from a T7 system, to the "Lit" extract, failed to exhibit the catalytic activity (data not shown). These two lines of evidence argued against the RNA model but favored the idea that polypeptide made from the gol region could be the key activator. Recently, in a attempt to confirm the role of Gol polypeptide, we added the chemically synthesized Gol oligopeptide (29 amino acid long made from Chiron Inc.) to the "Lit" extracts to investigate its function in EF-Tu proteolysis. In Fig 7, Lane 3-6 is shown an SDS PAGE gel of the proteins after adding various amount of Gol polypeptide to the "Lit " extracts. More than 95% of the EF-Tu was cleaved even in the presence of the lowest concentration (Lane 3, about 0.025uM) of the Gol peptides. The proteolytic reaction also generated the 37 kD fragment with the same mobility as the cleavage fragment in control experiment in Lane 1, obtained by mixing the "Lit" and "gol" cell extracts. This dramatic effect is detected neither in "Lit " extracts alone (Lane 2) nor in the presence of 1% non-ionic detergent NP40 (Lane 7). The result clearly indicates that Gol polypeptide is the activator for the function of EF-Tu protease.

Characterization of gol mutations: The minimal nucleotide length of gol for its activity encodes a 25 aa long polypeptide. The sequence does not have any functional motifs, nor does it contain highly charged residues. In fact, the molecule is quite hydrophobic, with 9 nonpolar groups out of 25 . Fig 7 shows the wild type gol peptide sequence and several mutant sequences with a single base substitution in nucleotide sequences that abolished the gol phenotype, by the criterion of

whether a plasmid construct containing the sequence was capable of transforming Lit(con) cells . Based on whether their mutations can be rescued in phages to overcome Litcon phenotype we can classify them into two major classes, those that can not be crossed back into phage T4 to cause the gol mutant phenotype and those that can. The first class includes six independently isolated mutations in four different positions where changes are apparently lethal to the phage. For example, NTG1 and MTD 8 both created a stop codon in gene23 that would be lethal because the gene 23 product is required for T4 maturation. The rest of the group may either generate an unacceptable amino acid or the substitution can not inhibit its wild type effect in the context of a full length gp23 molecule. The class II mutations overcome the gol effect and allow the recombinant phage to grow in lit(con) cells; presumably the amino acids are not essential for gene23 function. We assume that the T4 phages carrying this type of non-lethal mutation prevent the cleavage of EF-Tu. So far we have only tested two of them, gol6B and MTD16; both change a methionine into threonine and isoleucine respectively and fail to cleave EF-Tu (data not shown).

Discussion

In this paper, we demonstrated cleavage of EF-Tu in vitro and complementation of the protease activity with the mixture of the " Lit " and the "gol" cell extracts. We also presented evidence that the Gol polypeptide activates the proteolytic function of Lit protein to cleave EF-Tu. The mode for activating the protease will not be fully understood until experiments with purified components are done. However, the activity itself holds equal interest as a highly specific action on translation elongation factor Tu, which has at least 70% homology in protein sequences with its functional counterparts in all prokaryotic and some lower eukaryotic species.

EF-Tu, a single polypeptide chain of molecular weight 43 kD, is the most abundant of cellular proteins, about 5% of the total (7). When bound to GTP, EF-Tu is in the "on" state to carry amino acid-tRNAs to the ribosome A site during protein synthesis and is released from the ribosome-RNA complex following GTP hydrolysis. Its abundance makes it the most prominent component of E. coli. In addition to elongating polypeptides, EF-Tu has other functions, including serving as one of the four subunits in RNA phage Qβ replicase (8). A regulatory role in rRNA synthesis has also been proposed but the specific mechanism has not yet been characterized (9,10).

As the best known ubiquitous guanosine nucleotide binding protein with counterparts in both prokaryotes and eucaryotes, EF-Tu has attracted much attention from researchers, particularly for the investigation of function-structure relationships. Its 393 aa peptide chain is folded into three distinct domains. The N terminal GTP binding domain (residues 1-200) has structural features common to a family of ras oncogene p21 proteins. The comparison of primary amino acid sequences, homology and tertiary structural similarities between EF-Tu and H-ras p21 has been well described (11,12).

The cleavage site (residues 59-60), together with the preferential trypsin cleavage site (residues 58-59) and methylation site (residue 56) reflect that in the three dimensional structure of EF-Tu the cleaved region is probably exposed on the surface. This prediction is also supported by a computer aided simulation of EF-Tu protein structure, which indicates this region has a high water accessibility..

Our results suggested that EF-Tu may exist in two forms and one is subject to proteolysis. From Figures 1, 4 and 6, the low mobility of remaining EF-Tus might be due to modification. One of the known modifications in EF-Tu is methylation and the only known methylation site is Lys_{56} which is four amino acids away from the cleavage site. Our preliminary methylation results also

indicated that the H3 methylated EF-Tu did run slower than the S35 labeled EF-Tu (data not shown). Following current thought about the influence of methylation in EF-Tu proteolysis, it is possible that the addition of methyl groups on residue 56 lysine may reduce the accessibility of incoming proteases to the target site, thus conferring resistance to cleavage (the influence of methylation does affect the rate of trypsin degradation in EF-Tu) (21). The best way to prove this is to isolate the remaining EF-Tus from the gel and use mass-spectrometry to examine the methylation status of the protein. This hypothesis, if true, can also help account for the failure of the protease to cleave EF-1α, since it is highly methylated when purified from rabbit reticulocytes. But one thing that needs to be reconciled is the dilemma based on the recent result that the majority of EF-Tu did get cleaved (Fig 7) by adding the purified Gol into the same "Lit" extracts in vitro. It is possible that an excess of Gol oligopeptides can overcome the hindrance caused by methylation However, more experiments need to be done to elucidate this point.

The observation that some amount of intact EF-Tu was still present in the in vitro assays are consistent with what we knew from previous studies that EF-Tu can somehow retain resistance to the gol -induced protease when cells were grown in poor conditions, such as in minimal M9+B1 labeling media. Apparently, there is a correlation between cell physiological conditions and the resistance of EF-Tu to protease. This discrepancy between them could be explained by the EF-Tu methylation which was discussed above. Several researchers have observed that the level of EF-Tu methylation increased in cells grown in media depleted of carbon , nitrogen or phosphorus source, and cells of different developmental stages such as in mycelia form of the fungus *Mucous racemosus* but not in the spores. The vitro extract assay which exhibits EF-Tu protease activity may enable us to characterize the EF-Tu of cells in different growth environments and developmental stages by the criterion of its sensitivity to cleavage.

The EF-Tu proteolysis by the potential protease gpLit and its gol peptide cofactor not only reveals the molecular mechanism of phage exclusion but also provides an excellent system to exploit the foundations of protein-protein interactions for an oligopeptide activated catalytic reaction. A short peptide activating an enzyme is not a novelty. Mangel and Anderson (22) have reported that a proteinase activity of human Adenovirus (AD2) requires two cofactors. One of them was found to be a 11 aa long oligopeptide with high negatively charged density. The sequence was present at the carboxyl terminal of pIV, the 250 aa precursor protein to a virion component. The details of the activation mechanism were not discussed . It is possible that these two systems may work in a similar fashion.

Our level of understanding of the phage T4 exclusion phenomenon by a cryptic DNA element E14 encoded Lit protein is already sufficient to draw a clear and reasonable connection between the host-parasite gene interaction and the occurrence of cellular dysfunction and tie into the observable abortive infection phenotype. What remains to be answered are many questions of fundamental importance: Is any specific secondary structure involved? When does EF-Tu get cleaved, since it usually associated with other translation factors (GTP, GDP, aa-tRNAs, EF-Ts and ribosomal components)? What is the significance of EF-Tu methylation when cells encounter stressful conditions? Does the cleaved form of EF-Tu retain any functions? Is it possible that the 37kD cleaved product plays a similar role to methylated EF-Tu, since phage infection is a dreadful circumstance to cells? We are not sure that every question will have defined answers, but they definitely give scope for imagination.

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Table

Table 1: The characteristics and references of bacterial strains, plasmid constructs and phage mutants used in this acticle.

STRAIN		
JM101	(lac, pro) thi, supE, lit+, traD36, proAB, LacIZ M15	Yanisch-Perron etal 1985
608	*JM101* lit+, Kan resisitance cassette in e14 BamH1 site	this work
609	*JM101* lit(con) , overproduction of pLit	this work
610	*JM101* lit0, cured of e14 by UV irradiation	this work
JM109 λ (DE3)	*JM109 ,E.coli*K carry λ DE3 with T7 RNA polymerase under LacUV5 promoter.	Promaga Inc.
PLASMID		
pUC84PZ1	159 bp clone of wild type gol region from pst1site to 1 deletion in frame lacZ fusion.	Bergsland, K.,etal
pTTQ18lit	2.3 kb EcoRV of intact lit gene cloned in pTTQ18 under trp\ lacZ promoter.	this work
pBlueScript	EcoR1-HindIII gol region of pUC84PZ1 cloned into EcoR1-HindIII sites of pBlueScriptKS. Synthisize gol RNA from T7 promoter.	this work
pET11BSau3A	Sau3A fragment containing gol region from pUC84PZ1 cloned in BamH1 site of pET11B(Novagen) to made gol -partial lacZ fusion RNA.	this work
PHAGE		
T4gol 6B	Spontaneously isolated mutant whose mutation allows phage grow in lit(con) strains.	Champness,C and Snyder,L.

Figure Captions

Fig 1: Cleavage of EF-Tu in the inhibited S30 extract. The S35-labeled protein substrate were added to an equal volume of inhibited S30 (Lanes 1, 2, 3, 4) and uninhibited S30 (Lanes 5, 6, 7, 8) and incubated at 30^0 water bath. Samples were taken at different time points, 10' (Lanes 1 and 5), 30' (Lanes 2 and 6), 50' (Lanes 3 and 7), and 70' (Lanes 4 and 8). The samples were applied to 12.5% SDS Page, and the autoradiogram was visualized with X-ray film.

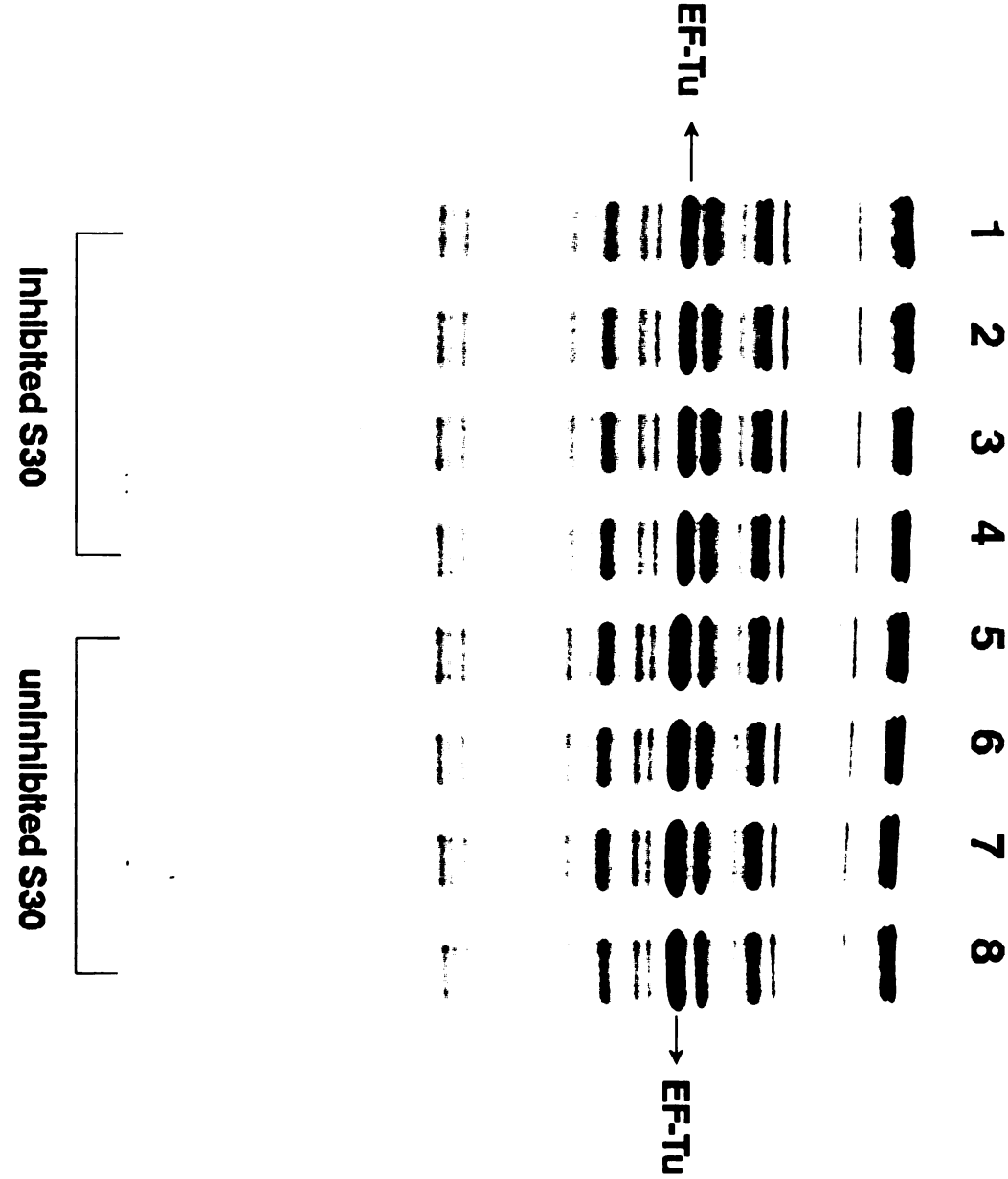

1 2 3 4 5 6 7 8
EF-Tu
EF-Tu
Inhibited S30
uninhibited S30

Fig2: Chloramphenicol-treated cell extract did not cleave EF-Tu. Cells harboring both pTTQlit and pUC84PZ1 (strain #582) were grew to OD=0.4 and treated with chlorramphenicol to 300ug/ml to block translation. The cell extract was made by the method of sonication and used in the in vitro assay (see material and method). Lane 3 and 4 showed the autoradiogram of substrate proteins taken from the mixture at 20 (Lane 3) and 40 min. (Lane 4). Controls were run side by side in Lanes 1 and 2 with cell extract made from un-induced (uninhibited) cells by the same procedure.

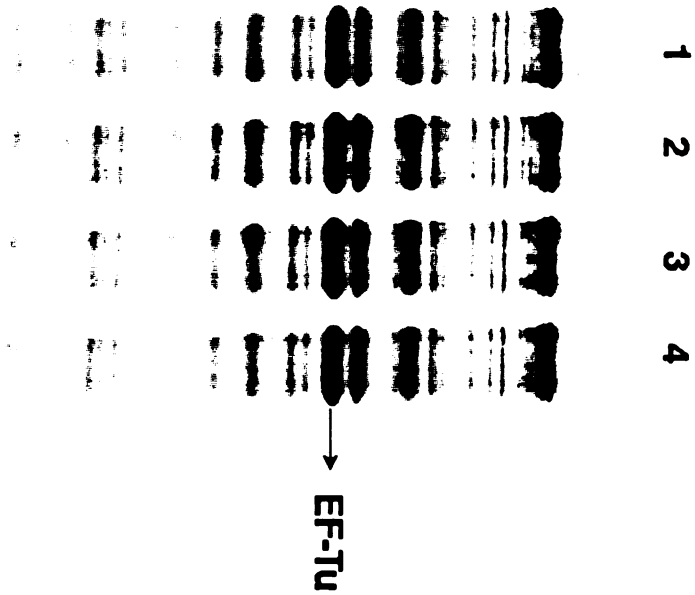
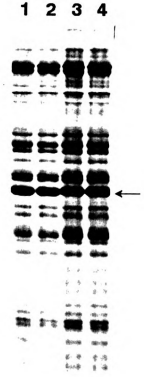
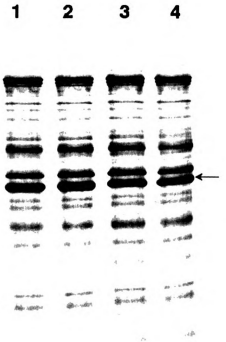

1
2
3
4
EF-Tu

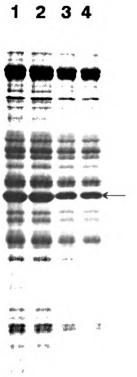
Fig 3: Effect of buffer pH value and phosphatase on the EF-Tu proteolytic activity. Panel A showed autoradiogram of substrate proteins in pH=8.2 (Lanes 1 and 2) and in pH=10 (Lanes 3 and 4) at different time points 0 (Lanes 1 and 3) and 30 min. (Lanes 2 and 4). Panel B compared the effect in pH=8.2 (Lanes 1 and 2) and in pH=4.8 (Lanes 3 and 4). Panel C demonstrated the substrate proteins in phosphatase-treated cell extracts (Lanes 3 and 4) and untreated control (Lanes 1 and 2). Phosphatase was added to the IPTG-induced #582 cell extract in vitro to 300u/ml. The control was done with the un-induced #582 in the absence of phosphatase.



A



B



C

Fig 4: Coomassie Blue stained SDS PAGE of in vitro cell extracts assay. Samples were taken at different time points, o' (Lanes 1, 3, 5) and 20' min. (Lanes 2, 4, 6,) during incubation. Lanes 1 and 2: shows the proteins of the mixed cell extracts containing "Lit" and "gol". Lanes 3 and 4: "gol" cell extract only. Lanes 5 and 6: "Lit" cell extract only. Arrows show the position of EF-Tu and EF-Tu cleaved fragment (EF-Tu*). The presence of cleavage product is indicated by the star (*) along the lanes of the gel. The molecular weight markers were shown on the left site of the gel.

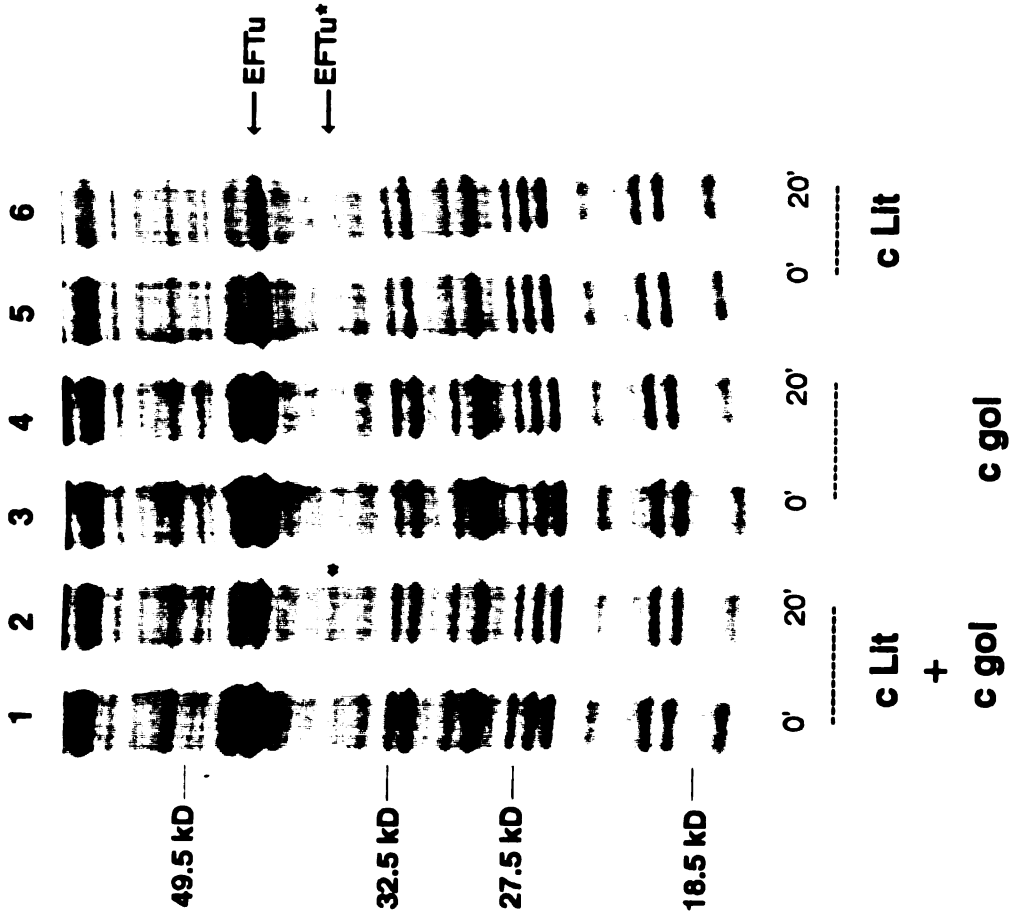

EFTu
EFTu*
1
2
3
4
5
6
0'
20'
0'
20'
0'
20'
c Lit
+
c gol
c Lit
c gol
49.5 kD
32.5 kD
27.5 kD
18.5 kD

Fig 5: The Zinc-binding protease motif of Lit protein. The majority of zinc-dependent metallopeptidases share a common pattern of primary sequence highlighted in the above. The sequences of Lit protein detected by the pattern lie from position 162-171, ILHHEISHVV. From the study of thermolysin, that belongs to this family, two of the zinc ligands are histidines that are very close together in the sequences. The glutamic acid following the histidine dimer acts as a nucleophile and promote the attack of a H_2O molecule. A signature pattern including the two histidines and the glutamic acid is sufficient to detect the proteases of this family.

MOTIFS from: Littrn.pep

Mismatches: 0 January 26, 1993 11:33 ..

Littrn.Pep Check: 4067 Length: 297 ! REFORMAT of: Littrn.Pep check: 4067 from

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Zinc_Protease (S,T,A,I,V)x2HE(L,I,V,M,F,Y,W)-(D,E,H,R,K,P)Hx(L,I,V,M,F,Y,W,Q)
(I)x(2)HE(I)-(D,E,H,R,K,P)Hx(V)
157: CAIAW ILHHEISHVV LQHPL

****
* Neutral zinc metallopeptidases, zinc-binding region signature *
****

- Thermostable thermolysins (EC 3.4.24.4), and related thermolabile neutral proteases from various species of Bacillus.
- Mammalian neutral endopeptidase (EC 3.4.24.11) (NEP) (enkephalinase).
- Mammalian extracellular matrix metalloproteinases (EC 3.4.24.7) (known as matrixins) [3]: MMP-1 (interstitial collagenase), MMP-2 (72 Kd gelatinase), MMP-9 (92 Kd gelatinase), MMP-7 (pump-1), MMP-8 (neutrophil collagenase), MMP-3 (stromelysin-1), MMP-10 (stromelysin-2), and MMP-11 (stromelysin-3).
- Angiotensin-converting enzyme (EC 3.4.15.1) (dipeptidyl carboxypeptidase I) (ACE) the enzyme responsible for hydrolyzing angiotensin I to angiotensin II. There are two forms of ACE: a testis-specific isozyme and a somatic isozyme which has two active centers [4].
- Extracellular zinc-metalloprotease from Serratia.
- Secreted proteases B and C from Erwinia chrysanthemi.
- Extracellular elastase from Pseudomonas aeruginosa (gene lasB).
- Extracellular proteinase proA from Legionella pneumophila [5].
- Immune inhibitor A from Bacillus thuringiensis (gene ina) [6]. Ina degrades two classes of insect antibacterial proteins, attacins and cecropins.
- Cell surface protease (surface glycoprotein gp63) from various species of Leishmania.
- Extracellular neutral metalloprotease from Streptomyces cacaoi.
- Astacin, a crayfish endoprotease.
- Meprin A, a mouse kidney brush border metalloendopeptidase [7].
- PABA-peptide hydrolase, a human intestine membrane protease [7].
- Bone morphogenic protein 1 (BMP-1), a protein which induces cartilage and bone formation and which expresses metalloendopeptidase activity [7] and a Drosophila homolog, the dorsal-ventral patterning tolloid protein.
- Hemorrhagic metalloproteinases from snakes venom [8].
- Sea urchin hatching enzyme (envelysin) (EC 3.4.24.12) [9]. A protease that allows the embryo to digest the protective envelope derived from the egg extracellular matrix.
- Mammalian aminopeptidase N (EC 3.4.11.2).
- Escherichia coli aminopeptidase N (gene pepN).

- BP-1/6C3 antigen [10], an early B-lineage cells phosphorylated cell surface glycoprotein which is most probably a zinc metallopeptidase. It may play a role in regulating growth and differentiation of early B-lineage cells.
- Kell blood group glycoprotein [11], a major antigenic protein of human erythrocytes. The Kell protein is very probably a zinc endopeptidase.

- Leukotriene A-4 hydrolase (EC 3.3.2.6), is the enzyme responsible for the hydrolysis of an epoxide moiety of LTA-4 to form LTB-4. It has been shown [12] that this enzyme binds zinc and is capable of peptidase activity.

Fig 6: Coomassie Blue stained SDS PAGE of RNase-treated cell extracts. Samples were taken at different time points, 0' (Lanes 1, 3, 5, 7,) and 25' (Lanes 2, 4, 6, 8,). Lanes 1 and 2 : positive control of EF-Tu cleavage by mixing "Lit" and "gol". Lanes 3 and 4: negative control of EF-Tu cleavage by incubating "Lit" alone. Lanes 5 and 6 :Pre-incubate "gol" 30' prior to mixing with "Lit", serving as a parallel control for Lanes 7 and 8. Lanes 7 and 8: "gol" was pre-incubated with RNase (0.1u\ ul) for 30' prior to mixing with "Lit". The activity of EF-Tu protease is reflected by the presence of EF-Tu* shown by the star (*) signs along lanes of the gel.

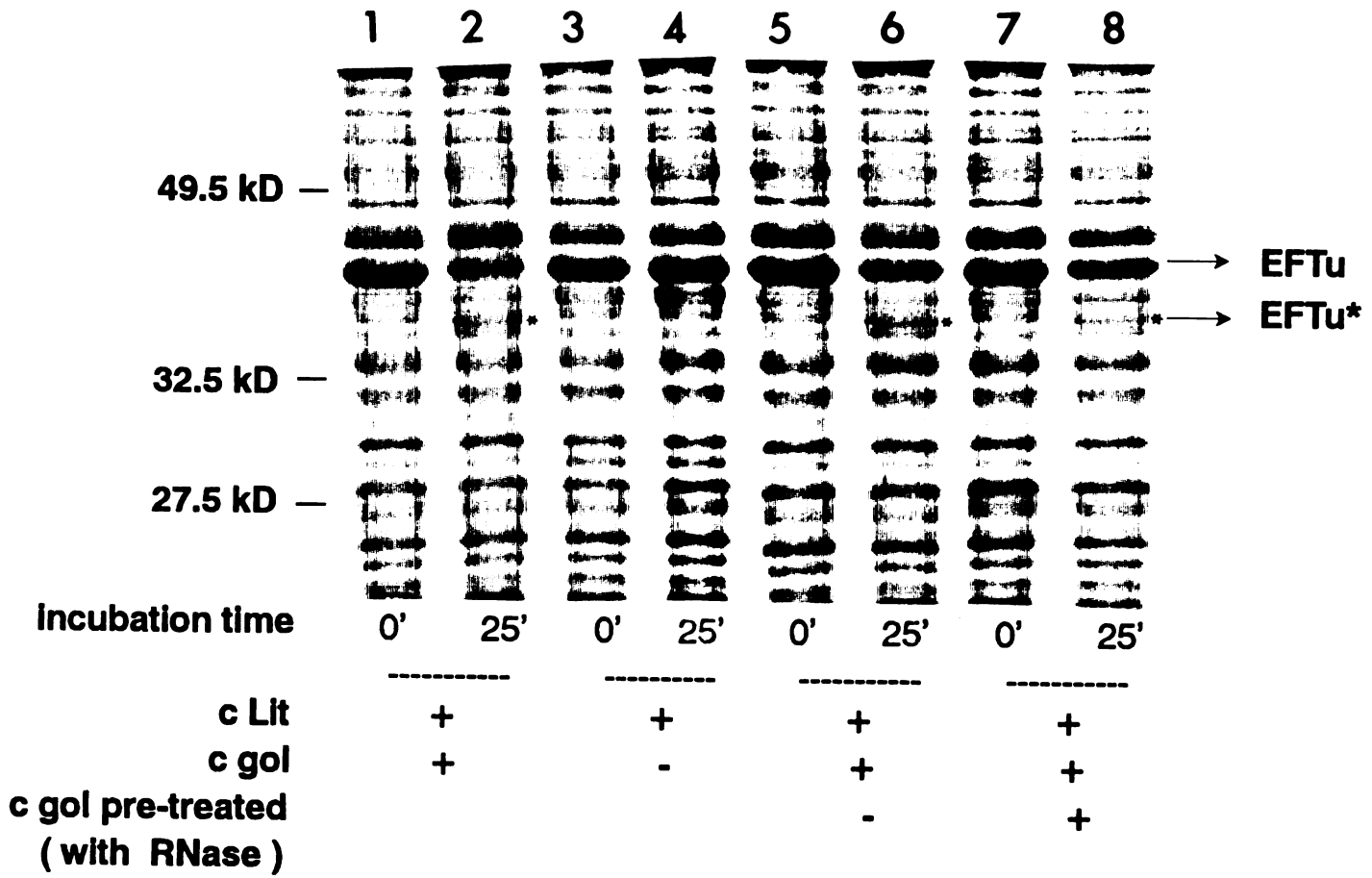
1
2
3
4
5
6
7
8
49.5 kD
32.5 kD
27.5 kD
EFTu
EFTu*
Incubation time
0'
25'
0'
25'
0'
25'
0'
25'
c Lit
c gol
c gol pre-treated
(with RNase)
+
+
+
-
+
+
-
+
+
+
+
+

Fig 7: Activation of the EF-Tu protease by the Gol polypeptide. Lane 1 shows cleavage of EF-Tu by mixing the "Lit" and "gol" cell extracts serving as a positive control. Lane 2 contains the "Lit" extracts alone. Lanes 3-6 indicate cleavage of EF-Tu and generation of the cleavage fragment (indicated by * signs) by adding various amounts of purified Gol peptide: 0.5x (Lane 3), 1x (Lane 4), 2x (Lane 5) and 5x (Lane 6), respectively. Lane 7 contains the "Lit" and "gol" extracts and 1% NP40. 1x of the Gol petide is equal to 0.05uM . The position of the intact EF-Tu (43 kD) is indicated by arrows.

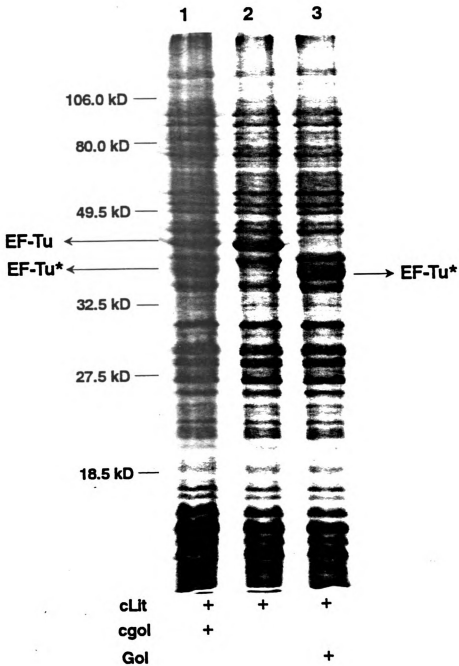
1
2
3
106.0 kD
80.0 kD
49.5 kD
EF-Tu
EF-Tu*
32.5 kD
27.5 kD
18.5 kD
EF-Tu*
cLit + + +
cgol +
Gol +

Fig 8: The amino acid sequences of wild type gol and its mutants: All the mutants were selected by the criterion of their plasmid constructs capable of transforming Lit (con) cells (indicated by "+" sign). The peptide sequence (represented by single letter) is deduced from the nucleic acid sequences of the PvuII- BalI fragment of gol region and mutations are identified by DNA sequencing data. MTD8 and NTG1 single base substitution generated a stop codon at residues C and Q, respectively. The dashed line represents the same amino acid residue. Class I includes mutations that can't be crossed back into the phage to give the gol mutant phenotype. Class II contains mutations that can be crossed back into phage and allow phage grow in Lit(con) cells.

		CLASS	Lit(con)
wt gol	A V M G M V R R A I P N L I A F D I C G V Q P M N		-
MTD8	----------(TGA)	I	+
NTG1	----------(TAG)	I	+
NTG2	----------E----------	I	+
NTG6	----------K----------	I	+
NTG10	----------N----------	I	+
2AP13	----------T----------	I	+
NTG4	----------S----------	II	+
MTD16	----------I-----	II	+
gol6B	----------T-----	II	ND

CHAPTER 4

MOLECULAR BASIS FOR THE LOCAL INHIBITION CAUSED BY GOL-LIT INTERACTION AND THE ROLE OF THE T4 PHAGE GOL SITE

Abstract

Transcriptional termination in the T4 gol site, blocking the expression of downstream genes, occurs in the presence of a wild-type, low amount of the Lit protein. This gol-Lit mediated local inhibition requires translation of the gol region for its full activity. However, the gol region, which contains a nut-like antitermination sequence, enhances the level of transcription in the absence of Lit, suggesting that the function of the gol sequence maybe exert an antipausing effect and increase the transcription of gene23. Here, we also propose a model to correlate the function and structure of the gol sequences.

Introduction

The cryptic prophage e14 (1) is integrated within the icd gene (2) located at 25' of the Escherichia coli K chromosome. The cryptic prophage generates a protein , Lit, (3) that can inhibit bacteriophage T4 late gene expression at the level of translation, particularly when it is overproduced by an up-promoter mutation or made from a multiple-copy plasmid (4, 5). The inhibition is due to the interaction of Lit with a short sequence , gol, within the phage major capsid protein Gene23 (6). Transformation of Lit-overproducing cells with a plasmid , expressing the gol region in the Gene23 frame, can generate a global translational defect similar to the phage exclusion. No other T4 sequences are required .

The minimal length of gol region was determined to be a 75nt fragment located about 300nt downstream of the Gene23 ribosome binding site (4). We have shown in the previous paper (7, Yu and Snyder submitted) that the gol-Lit interaction somehow caused cleavage of translational elongation factor Tu (27), thereby blocking cellular translation. In addition to this dramatic global effect, Bersgland et al (4) also observed another effect of the gol-Lit interaction. Transformants of a gol-LacZ construct that block translation in a Lit-

overproducing strain (Litcon) are normally blue on X-gal plates in the absence of Lit protein, whereas in the presence of a wild-type low amount of Lit protein, the transformants are white, indicating no functional LacZ is made. We referred to this as local inhibition since only genes downstream of the gol region and expressed from the same promoter are affected. The previous study (Bergsland et al 1990) suggested that the local inhibition acts at the level of transcription and only requires wild-type sequences of gol RNA.

In this report, we focus on reevaluating the molecular mechanism of this local effect and illustrate the unexpected discovery that gol region has a significant enhancement effect for downstream gene expression in the absence of Lit protein. Discussions of the possible linkage between the apparent transcriptional termination of the local inhibition and the putative antitermination effects in the same region are also included here.

Materials and Methods

Baterial strains and media: The strains used in this work Jm101, JM101lit^0, JM101lit$^+$ and litcon were described in the precious paper (8, Yu and Snyder in preparation). W3110Iq (#539) is a strong lacIq recA E.coli K strain (9). Unless otherwise mentioned, Transformants for the IPTG induction were grown in Tryptone Broth (1% tryptone, 1%NaCl, 50ug/ml vitamin B1) supplemented with the appropriated antibiotics. Supplements for media were at the following concentration: ampicillin (Amp), 50ug/mi; chloramphenicol(Cm), 25ug/ml; tetraclycin,12.5ug/ml; IPTG, 1mM; X-gal, 50ug/ml. All the experiments were performed in a 30^{0}C water bath or incubator.

DNA manipulations and plasmid constructs: All the endonucleases used for plasmid cloning were obtained from BMB and used with the buffer and conditions recommended by BMB. DNA fragments of interest were isolated from

agarose gels by the method of squeezing and thawing followed by several cycles of phenol extraction followed by ethanol precipitation. The DNAs were resuspended in H_2O. The vector M13mp18 and mp19 were obtained from BMB (10). The plasmid pSP64 was a gift of Dr. Richard Schwartz (11). The pTA9 that contains an EF-Tu structural gene under control of lac, T7 and its own promoters was a generous gift of Dr. David Miller (12). The construction of the plasmids used in this article are described as the following. The pUC84PZ1(4) has a 159 bp Pst1- 1 (HindIII linker) fragment of the gol region cloned into pUC84, that has a 4 bp filled in BamHI site. The pUCgol-cat contains a pvuII-Xho1 75 bp fragment of the gol region from pUC12PZXR (4) at the Sma1 and Sal1 sites and a CAT gene at the HindIII site of pUC8 vector. The pUCUAAgol-cat is like pUCgol-cat, in that it contains the same gol region and CAT segments but has a 4bp filled in EcoRI site that generates a UAA ochre stop codon upstream of the gol region. All three plasmids described above translate the gol region in gene23's frame and CAT in the Cat ribosome binding site (RBS) from the same transcript made from lac promoter. The pUC 12-cat served as a control without the gol fragment. The M13PZ1 contains the PZ1 fragment from pUC84PZ1 cloned into EcoR1 and HindIII sites of M13mp18 vector. With the same scheme, MTD16 that has a single-base substitution in the gol region was cloned into M13mp18 to make M13MTD16(8). Both the M13 constructs transcribed the hybrid genes from a lac promoter and confer α-complementation when the phages plate on the E. coli male strains Jm101 lit^0. The pSP64PZ1-JD has a EcoRI-HindIII PZ1 fragment cloned at the opposite orientation at the HindIII and EcoR1 sites and a 110 bp chicken histone gene segment at the Pst1 site of pSP64. The pACYCtufA(R) and pACYCtufA(W) were made by cloning the PvuII segment from the plasmid pTA9 into the Sma1 site of the Kan resistance cassette in a pACYC derivative that contains the Kan^r cassette (obtained from Pharmacia) in the EcoR1 site of

pACYC184. The R and W stand for clockwise and counterclockwise transcription of tufA relative to the Kan^r gene's promoter, respectively.

Plating with M13-gol hybrid phages:M13PZ1, M13MTD16 along with the vector M13mp18 were introduced into the male E. coli strains via transfection of competent cells (13). Transfectants were plated on LB plates with 3ml of top agar(1% tryptone, 1%NaCl, 0.5% yeast extracts, 0.7% agar). A single plague was isolated by toothpick and incubated in 5 ml pre-diluted culture. The hybrid M13 particles were partially purified 6-8 hours post- infection by reserving the supernatant of centrifugation at 5K for 5min. The M13 RF forms left in the cell pellets were isolated and tested for inserts. The appropriate E .coli male strains (lit^0, lit^+, and litcon) grown to OD=0.5. 3 ml of top agar supplemented with IPTG,X-gal and 0.5ml culture of indicator strains were plated on LB plates and spotted with various dilution of the M13-gol hybrid phages, and the plates were incubated at 34^0-35^0C.

RNA protection with S1 nuclease: The RNA probe that can hybridize the PZ1 segment was labeled uniformly by the method of in vitro transcription (14)., using a Sal1-linearized template of pSP64PZ1-JD and SP6 RNA polymerase. Cellular RNA samples were extracted by a modification of the method of Young and Furano (15). A 10ml culture of the pUC84PZ1 transformants was induced by IPTG for 20 min. and lysed in corex tubes containing 2.5 ml lysis buffer (1M NaCl, 1%SDS and 50mM EDTA pH 8) and incubated in a boiling water bath. The lysate was cooled down to room temperature and phenol- extracted twice and ethanol precipitated before dissolving the pellets in 0.3 ml H_2O. The cellular RNA samples were hybridized overnight with an excess of the labeled probe at 55^0C in 30 ul hybridization buffer (70% formamide, 1mM EDTA, 0.5M NaCl, and 20mM Tris-HCl pH8). The hybrids were chilled on ice and digested for 1 hour at 37^0C with S1 nuclease(BMB) in 300ul S1 buffer (7% formamide, 0.25M NaCl, 30mM

Na-acetate pH 4.5 and 1mMZn-sulfate) (16). The ethanol-precipitated pellets were resuspended in sample buffer and applied to 10% polyacrylamide, 7M urea gels. The gels were then dried and exposed to a Kodak X-ray film.

CAT ELISA assay: Transformants containing various plasmids were innoculated into 5 ml fresh tryptone media supplemented with ampicillin and grown until OD=0.4. Aliquots of cultures were taken and washed once with 0.25M Tris-HCl pH 7.5 , then lysed by freezing and thawing. The cell extracts were then quickly frozen using a dry ice / ethanol bath and stored at -70^{0}C. Protein concentrations of each sample were determined by the method of Dradford (17). The amount of CAT in each cell extract was measured by a CAT ELISA assay kit (BMB) following the procedures recommended by the supplier. The final CAT concentration was normalized to the total cellular protein .

Results

The use of M13-gol constructs to detect local inhibition: Instead of analyzing the gol-induced inhibition with the gol-LacZ fusion clone of the pUC vector, it is more convenient to monitor the gol-Lit initiated effects using the gol site cloned in an M13 vector. If the entire gol region, the EcoRI-HindIII fragment of pUC84PZ1, is cloned into the polylinker site of filamentous phage vector, M13mp18, the fusion construct will transcribe and translate the gol region fused to lacZ gene as in the pUC system. This strategy had at least two advantages over the previous assay: It allowed us to screen a large quantity of samples for the desired gol mutations, and that may help to identify nucleotides essential for the global and the local effects or both; Additionally, we can directly sequence mutations of interest without extra cloning procedures.

M13 phage particles containing the proper construct were prepared and spotted on various indicator strains (Table 1). Plates were supplemented with

IPTG and X-gal to test α-complementation of β-galactosidase. The M13-gol hybrid phage generated blue plaques in cells cured of e14 (no Lit produced, Lit^0), whereas in the wild-type cells (Lit^+) the plaques were completely white, and no plaques were formed in the Lit-overproducing strain (Litcon). The M13mp18 vector served as a control producing blue plaques in all of the tester strains regardless of the presence or absence of Lit protein. The result agreed with the expectation of the local inhibition exhibited in Lit^+ and the global effect in Litcon. M13 phages have a poor plating efficiency at 30^0, at which the gol-Lit mediated inhibitions were usually detected . However, this plating assay works well at temperatures of 34^0-35^0.

We isolated several gol mutants and localized their changes within the gol region. One of the mutants, MTD16, generated by a MutD strain , was cloned into M13 and used in this plating assay as a control to show the depression of the local and global inhibition by a single- base mutation. It produced equally blue plaques in all of the indicators. The single-base mutation of MTD16 changes residue methionine into isoleucine, which is shown in Fig8 of Chapter 3.

The local inhibition occurs at the level of transcription: It is difficult to interpret the cause of blockage of gene expression in a translational fusion, such as pUC84PZ1, in our previous study. Inhibition of the LacZ expression could be at the level of transcription or translation. In order to further differentiate these possibilities, a construct pUC75gol-cat containing the CAT (chloramphenicol acetyltransferase) gene at the HindIII site downstream of gol was made. This plasmid transcribes through the gol region into the CAT gene from an inducible lacZ promoter, and translates CAT protein independently from its own ribosome binding site. If the blockage caused by gol-Lit interaction occurs at the level of transcription, one would expect that no complete messages would be made through

the reporter CAT gene, and consequently cells would be sensitive to the antibiotic, chloramphenicol (Cm).

Transformants of pUC75gol-cat did fail to grow on Cm+IPTG plates in the presence of a wild-type amount of Lit, but made colonies comparable to the vector control, pUC-cat, on plates supplemented with ampicillin (Table 2). This result is significant for two reasons. First, the failure of plating on Cm+IPTG is indeed due to the local blockage of the CAT gene expression downstream of the gol region, not the global effect , otherwise one would see a similar phenotype on the ampicillin plates. Secondly, according to the rationale described above, the data suggested that the local inhibition is at the level of transcription.

This conclusion received further support from the result of a RNA protection assay. I used a uniformly labeled probe, in which the first 180 bp is complementary to the gol transcript of the EcoRI-HindIII fragment in pUC84PZ1 and the last 110 bp is chicken histone gene specific, to detect the length and the amount of protected transcripts from total RNA extracts of the appropriate transformants. If transcription is not affected by the function of Lit, one should expect a similar pattern of RNA protection regardless of the presence of Lit. The data in Fig1 showed that 20' after IPTG addition to induce transcription of pUC84PZ1, the amount of message protected by the anti-gol probe decreased proportionally to the increase of Lit protein in the corresponding transformants (Lane 2, 3, 4). The length of the protected RNA was virtually the same among all 3 types of host (Lit^0, Lit^+, Litcon), except that a low concentration of S1 nuclease revealed a smear of shorter species protected in Litcon cells (Lane 8) as well as in Lit^+ (Lane 9) with a weaker signal. Those shorter bands were not found in the control (Lane 5), that contained the labeled probe and a excess of tRNAs. This result suggested that the appearance of the shorter species was due to protection

of the truncated transcripts in Lit-containing cells rather than to the incomplete digestion of the un-hybridized probes.

Translation of the upstream gol region is crucial for the transcriptional blockage: The transcriptional termination in the gol region could be due to a polarity effect of blocking translating ribosomes upstream. To examine whether the putative transcriptional termination in the gol region was due to block of translation possibly by the gol-Lit interaction, thereby exposing a termination signal for RNA polymerase, an ochre stop-codon was generated 5' approximal to the gol region. The logic behind this is that the UAA construct should arrest ribosome at the EcoRI site and cause a polarity effect in transcription, if a ρ-dependent terminator exists in the gol region or beyond. As the data in Table 2 show, pUCUAAgol-cat transformed Lit^+ cells were chloramphenicol resistant, indicating the CAT enzyme was made. Apparently, the entire gol RNA doesn't act like a terminator in the course of transcription, otherwise the transformants wouldn't grow on Cm+IPTG plates.

In addition, the Lit protein didn't cause the same local inhibition in the stop-codon construct as in the translatable equivalent plasmid pUCgol-cat, illustrating that translation of the gol region is necessary for the function of Lit to block message synthesis to the downstream CAT gene.

To further quantitatively analyze the CAT reporter gene expression, we used a CAT ELISA assay to measure the actual amount of CAT products in the transformants of the various CAT constructs (Fig 3). There was no distinct difference between CAT production of the vector pUC-cat in the absence and presence of Lit protein, whereas, in the presence of Lit, pUCgol-cat produced only 0.3% of CAT that was made in Lit^0 cells and 2% of that found in absence of the gol region (pUC-cat). Generally speaking, the results obtained from the ELISA assay were in agreement with the plating assay.

Gol sequence stimulates gene expression in the absence of Lit protein: Two additional observations emerged from the data of the Lit^0 transformants of pUCgol-cat and the UAA construct. In the absence of Lit protein , pUCgol-cat made almost 5 fold more CAT protein than the vector control (pUC-cat). The elevating effect was apparently abolished when translation of the gol region was blocked by a up-stream stop codon of the pUCUAAgol-cat. In fact, CAT production of the UAA exhibited a significant decrease compared to that of the vector control. Possible explanations for the decrease are that the context surrounding the cat ribosome binding site could interfere with the untranslated gol structure, or that RNA polymerase, which has to travel further, could be partially terminated in the gol region.

We hypothesize that the enhanced CAT production resultes from an increased rate of transcription by the presence of the upstream gol region. Of course one could argue that the increase might be due to the function of an additional promoter in the gol construct instead of a stimulating effect of gol in transcription. However, if most of the transcription is coming from the lacZ promoter, then, in the absence of IPTG, we should expect less Cat to be synthesized. To improve the experiment, we have transformed a strong lac Iq strain W3110, cured of e14, with the same set of plasmids to monitor the amount of Cat before and after IPTG induction (Fig 3). The addition of IPTG to induce the lacZ promoter did cause a significant increase in CAT synthesis of all of the CAT constructs , implying that the increased transcription utilized the Lac promoter (comparison of Cat in Fig 3, -IPTG vs. +IPTG). The low amount of CAT produced in pUCUAAgol-cat, in which the gol sequence is virtually the same as in the pUCgol-cat, also argued against the possibility that this region contains an additional promoter.

Based on the rationale described above, it is likely that the 8-fold increase of CAT in the pUCgol-cat transformants before addition of IPTG resulted from an enhancement of the basal transcription by the presence of the translatable gol region. This result also explains the observation that Lit^0 transformants of pUCgol-cat grew much better on Cm + glucose plates than the cells containing the control alone, obviously the enhanced production of CAT even from the basal transcription of the uninduced Lac promoter supported cell growth on the chloramphenicol plate. The level of CAT found in transformants of the UAA construct was consistently about 6-7 times lower than that of control both before and after IPTG. The RNA structure of the entire gol sequence, that may influence the efficiency of ribosome binding on the neighbor cat gene RBS, could account for the reduction of CAT protein, even through it did not exhibit the phenotype of a transcriptional terminator on the plate assay.

Given the evidence presented above, we concluded that transcription of the Cat gene comes from a single promoter, plac, and that the increase of CAT produced in transformants of pUCgol-cat in the absence of Lit is due to a transcriptional stimulation by the gol sequence. The stimulatory effect also requires translation of the gol region. Interesting enough, translation of the gol region also is an important factor to determine the transcription termination in the local inhibition caused by Lit protein.

Is Cleavage of EF-Tu responsible for the local inhibition ?: When the majority of cellular EF-Tu gets cleaved, a global inhibition of translation occurs (7). We wondered whether cleavage of EF-Tu also takes place in wild-type hosts (Lit^+), that only inhibits the transcription of genes downstream of the gol site. From the comparison of the total protein profile of the isogenic Lit strains (Fig 4) that contained the gol construct, pUC84PZ1, we found that ,40' after IPTG induction, no more than 5% of intact EF-Tu was left in Litcon (Lane 1, 2, 3) but

half of EF-Tu remained intact in the Lit$^+$ cells even after a long period of induction (Lane 5, 6, 7, 8). The data showed that some EF-Tu did get cleaved in the presence of a wild-type amount of Lit protein. Apparently the degree of EF-Tu cleavage is proportional to the amount of Lit produced in the hosts.

From the previous paper(7), we knew that eleavage of EF-Tu is at least a contributing factor for phage exclusion and we have demonstrated that the global defect can be relieved to some extent, in terms of supporting phage growth, by an overproducing tufA clone that encodes EF-Tu. Presumably, the phage can multiply because not all of cellular EF-Tu got cleaved after T4 infection of the EF-Tu-overproducing Litcon host. Therefore, we applied the same philosophy in the situation of local inhibition. If a wild-type host can maintain the same amount of EF-Tu after induction of gol activity as before by the aide of a tufA clone , it would be interesting to see whether the uncleaved EF-Tu can suppress the local inhibition.

We approached this by transforming the Lit+ host with two compatible plasmids, one containing the EF-Tu structural gene in a pACYC184 derivative and the other carrying the gol region in pUC84PZ1. Overproduction of EF-Tu somehow made the cells sick and the degree of sickness was varied from strain to strain. However we found that a clone carrying the tufA gene in the opposite direction of the interrupted kanamycine resistance gene promoter can be tolerated by the hosts and allowed us to pursue the question. If an excess of EF-Tu can prevent the occurrence of the local inhibition in Lit$^+$ when the gol-activity is induced in pUC84PZ1 , then we should expect to see the colonies as blue as in a Lit^0 cell that does not have a local inhibition for functional LacZ synthesis. What we found was that the Lit$^+$ transformants were white on X-gal plates just like the parental strain without a tufA (Table 3). The data demonstrated that the remaining EF-Tu, although more than the normal amount , did not overcome the local

inhibition. Instead ,it suggested that the cleaved form of EF-Tu, which is the only known difference between a Lit^+(tufA) and a Lit^0 host, determines whether the local inhibition takes place in the gol region. If this idea turns out to be true, it also could explain why the incomplete recovery of phage growth occurred in the Litcon hosts containing a tufA clone, since the presence of the cleaved form may cause the local inhibition in Gene23 to block the synthesis of T4 major head protein.This theory also accounts for the observation that wild-type T4 grew at a much lower rate in Lit^+ hosts than in Lit^0 according to their performance in one step growth experiments (Data not shown).

Discussion

In this article, we uncovered the molecular basis of the gol-Lit initiated local inhibition. The inhibition is thought to occur at the level of transcription and somehow prevents RNA polymerase reading through to reporter genes located downstream. The transcriptional termination depends on translation of the gol region. Interestingly, in the absence of Lit protein, we discovered that production of the downstream gene product is highly stimulated by the presence of the gol sequence provided it is translated. We also demonstrated that ,unlike global inhibition , local inhibition occurs even if not all cellular EF-Tu gets cleaved. Overproduction of EF-Tu does not overcome the defect.

To understand the mechanism of the gol-Lit initiated effects, the putative secondary structure of gol RNA sequences could be informative. The entire RNA encoded by the minimal gol fragment ,PvuII-Δ1, can form a long stem-loop structure with a stability of ΔG=-35.1 Kcal (Fig 5). This stable structure could be generated in the transcript when ribosomes are arrested upstream. Additionally, the gol RNA possesses an λ antitermination nut-like site composed of a typical boxA sequence and a boxB hairpin structure. The nut-like sequences located in the

middle of the second part of the long stem are expected to be exposed by a coupling translating ribosome (Fig 5). With the evidence presented in this paper, we postulate that in the absence of Lit protein, the transcription rate of the gol-CAT fusion unit could be enhanced by the unique nut-like site in the gol RNA segment, that can be exposed with movement of translating ribosomes. In other words, the nut-like site may function as a transcriptional enhancer or an antiterminator signal that could be converted into a terminator by the function of Lit.

Of course, one could argue that the increase in CAT production and in the protection of full-length gol message in the absence of Lit may result from an enhanced stability of the transcripts instead of from a stimulation of transcription by the presence of gol in the construct. If so, it would be hard to imagine how the function of Lit destabilizes the gol-containing transcripts. Based on our current knowledge about gol and Lit, the possibility of RNA stability seems unlikely. However, experiments to measure of stability of RNA degradation and protein production have to be done in the future.

The most intriguing phenomenon of the gol-Lit interaction may be the interdependence of the global and local inhibition. All the mutations isolated thus far for their ability to overcome the global effect always suppress the local inhibition. Our preliminary result also showed that mutations in gol that failed to activate Lit protease to cleave EF-Tu retained the stimulating effect in the absence of Lit. A simple explanation for this phenomenon is that the cleaved EF-Tu molecules or some other proteolysis product resulting from the gol-Lit activated proteolysis perhaps recognized the putative antitermination site of gol and converted it into a termination signal, thereby causing the local inhibition. If it is due to the cleaved form of EF-Tu, it may lead to a reasonable speculation,

concerning the general nature of cellular antitermination systems. The intact EF-Tu might play an important role in the function of antitermination apparatuses.

The studies of transcription antitermination of E. coli phage λ (19, 20) and the highly conserved cellular rRNA operons (20, 21, 22) have given insights into the constitution of the antitermination complex and allowed some specific functions to be assigned to the known components. Unlike the λ nut site whose function requires a particular transcription antiterminator pN, there is no known cellular analogy of N protein involved in the antitermination utility of cellular rRNA operons that also carry a version of boxA and boxB substructure(21). The hint of linkage between EF-Tu and the rRNA antitermination came from characterization of EF-Tu in functions other than its traditional role in translation, including the study of its participation in phage Qβ RNA replication (23, 24) and that of being a positive regulator for rRNA synthesis(25, 26). In fact, our observation of the local inhibition that might be due to the consequence of EF-Tu cleavage provides encouraging evidence for this provocative concept. Thus , it is plausible that the cleaved EF-Tu product not only aborts its antitermination effect in transcription of gol but also may compete off the remaining intact antiterminator for it has a stronger affinity for the nut-like site. If a physical binding of the nut-like site does exist, then the binding would block transcription through the gol region. Although we can not detect reduction of 16s and 23s RNA syntheses in cells (pUC84PZ1 in Lit^+)of an overnight plate, we did notice that the colonies became sick after a prolonged period of incubation. The delayed sickness of Lit^+ transformants may be attributed to the inhibition of rRNA transcription by the presence of the EF-Tu cleaved product.

In summary, based on the logic discussed above, the model of EF-Tu's involvement in the gol-Lit induced local inhibition and the cellular antitermination is extremely attractive but lacks solid support. Experiments to directly analyze the

effect of EF-Tu cleavage on antitermination of rRNA operons are emergent. Continuing investigation of the enhancing effect of gol on transcription may also shed some light on general mechanism of transcription antitermination.

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Table

Table 1: Characterization of M13-gol hybrid phages on Lit-containing cell. The hybrids M13PZ1, M13MTD16 prepared as in Materials and Methods harbor a wild-type and a single-base mutant sequence of gol ,respectively. M13PZ1 produces white plagues on Lit^+ cell and no plagues on Lit-overproducing strain (Litcon).

phage\Strain	JM101lit0	JM101lit+	JM101litcon
M13PZ1 (gol+)	Blue Plaque	White Plaque	no Plaque
M13MTD16 (gol-)	Blue Plaque	Blue Plaque	Blue Plaque
M13mp18	Blue Plaque	Blue Plaque	Blue Plaque

Table 2: A transcriptional gol-Cat fusion clone does not confer chloramphenicol resistance in the presence of a wild-type low amount of Lit protein. The host is JM101lit^{+} as in Materials and Methods. The results were recorded after overnight culture in a 30^{0} incubator.

Plate \ Plasmid	pUC8 -75gol - CAT.	pUC12UAA - 75gol-CAT	pUC12-CAT
Ap+G	+++	+++	+++
Cm+IPTG	—	++	+++
Ap+IPTG	+++	+++	+++

Ampicillin: 50ug per ml, Chlorophenical: 25ug per ml, IPTG:0.5mm, G: 0.4% glucose

Table 3 : Overproduction of EF-Tu does not overcome the local inhibition. The color of each transformant is analyzed on X-gal plates supplemented with appropriate antibiotics for ON at 30^0. Overproduction of EF-Tu is obtained by introducing a multi-copy plasmid containing tufA.

Strain\ Plasmid	lit0	lit+	lit0 (tufA)	lit+ (tufA)
pUC84PZ1	Blue	White	Blue	White
pUC12	Blue	Blue	Blue	Blue

Figure Captions

Fig 1: Plasmid constructs of pUC8PZ1 and p64PZ1-JD. pUC8PZ1 shown above contains a 180 bp Pst 1-HindIII fragment of gol fused in frame with lacZ and transcribes from lacZ promoter. p64PZ1-JD shown at bottom contains a gol sequence cloned in the opposite orientation (log) followed by a 110bp chicken Histone specific Pst1 fragment (JD-H). The riboprobe made from p64PZ1-JD in a in vitro transcription system by sp6 polymerase is indicated by a heavy line with a arrow. The putative protected region of hybridized gol RNA is indicated by the two vertical dashed lines. The thin lines beneath pUC8PZ1 represent various length of transcripts from lacZ promoter. Restriction sites are simplified by single letters: H, HindIII; S, Sal1; P, Pst1.

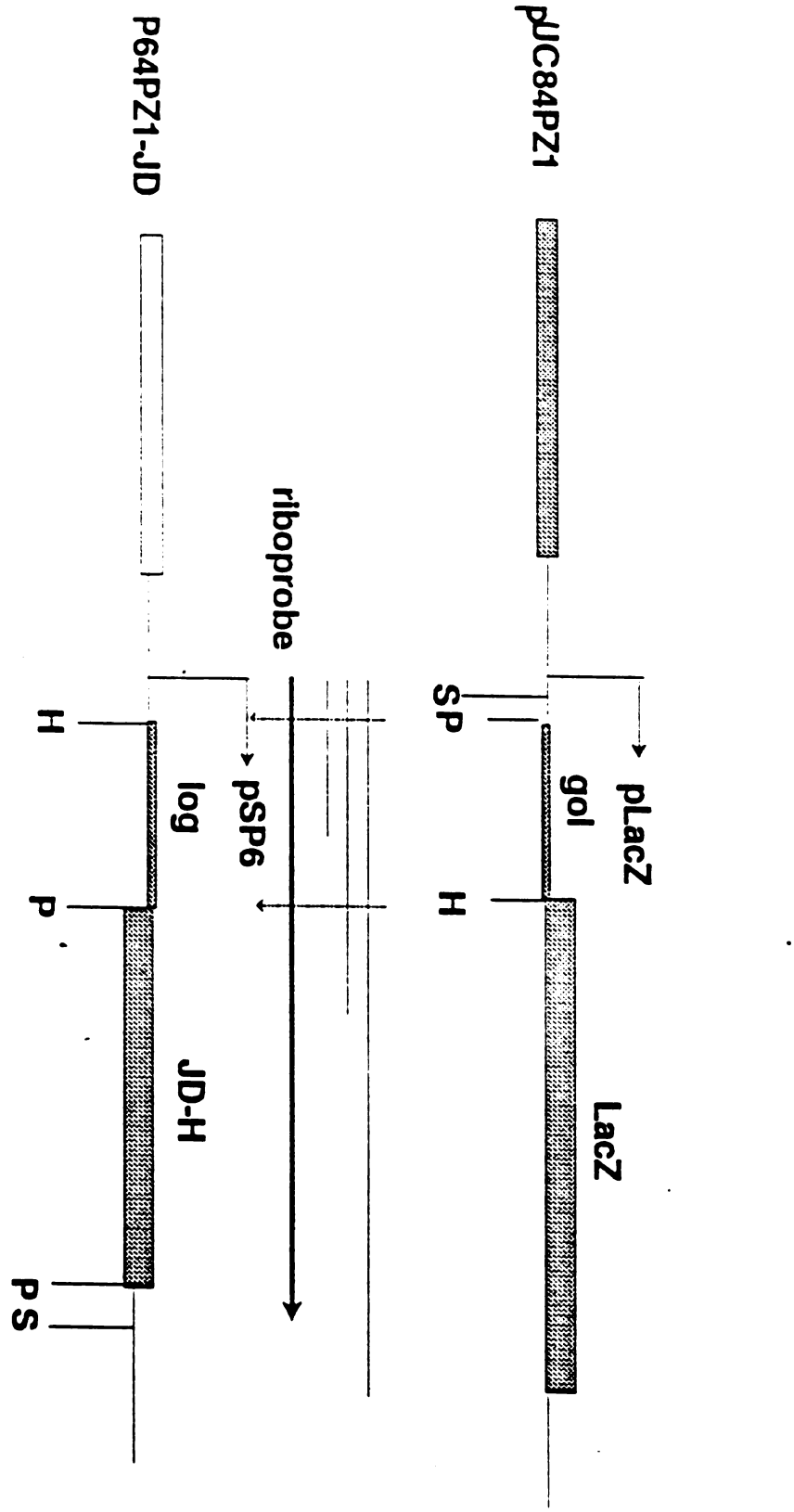
pJC84PZ1
P64PZ1-JD
riboprobe
SP
H
gol
pLacZ
pSP6
log
H
P
LacZ
JD-H
PS

Fig 2: Autoradiogram of the probe protected transcripts. RNA samples made from pUC8PZ1-containing Lit^0 (Lane 4, 10), Lit^+(Lane 3, 9) and Litcon(Lanes 2, 8) were hybridized with the riboprobes of which full length are shown by the first arrow on the left side (Lanes 1, 11). The mixture were then digested with various concentration of S1: 400u/sample (Lanes 8, 9, 10a), 1200u/sample (Lanes 2, 3, 4, 5, 6), and 3200u/sample(Lane 10b). The riboprobe that has been incubated ON alone is shown in Lane 7(without S1 digestion) and Lane 8(with S1). Result of S1 digestion of the tRNA-riboprobe mixture is shown in Lane 5. The amount of tRNA is approximately 10 times the concentration of the RNA sample used. Full-length protection of the 180 bp gol transcripts is indicated by the second arrow from the top on the left-handed side.


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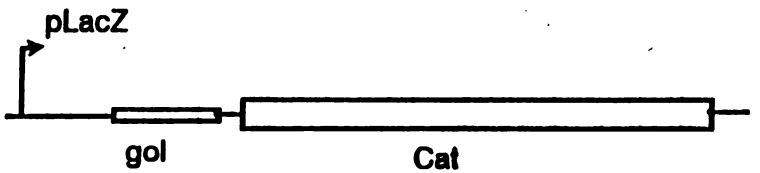
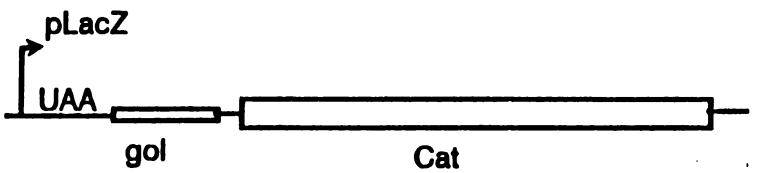
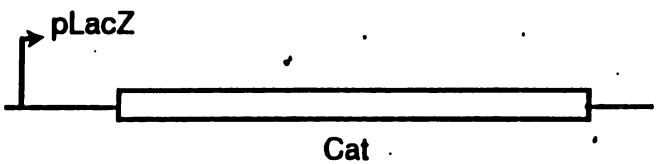
Fig 3: Inhibition of downstream gene transcription by the gol-Lit interaction. The plasmid constructs shown on the left-handed side are used to transform Lit^+ and Lit^0 cells. The downstream Cat enzymes synthesized are determined by the CAT ELISA assays and normalized to total cellular proteins. -Lit and +Lit indicate that the samples are prepared from the Lit^0 transformants and Lit^+ transformants, respectively.

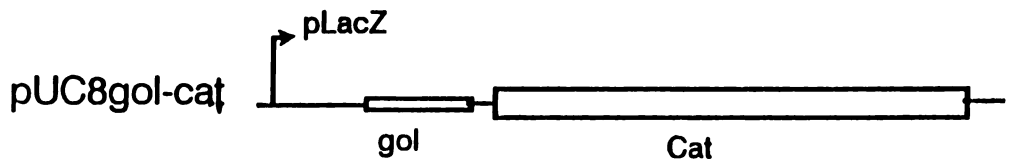
Local inhibition of downstream gene transcription by gol -Lit interaction

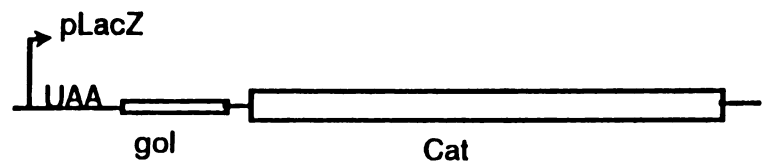
		- Lit	+ Lit
pUC8gol-cat		26.506	0.088
pUC12UAA gol-cat		0.844	0.714
pUC12-cat		5.206	4.452

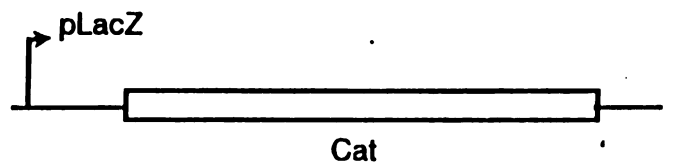
Cat ELISA Assay : one unit = one nanogram of cat enzyme per microgram of protein.

Fig 4: Stimulation of gene transcription by the gol site in the absence of the Lit protein. The plasmids shown on the left-handed side are used to transform Lit^0 cells and the cell extracts made from these transformants ,before ITPG (-IPTG) and after IPTG (+IPTG) are used to determined the Cat enzyme concentrations.

Stimulation of downstream gene transcription by the gol sequence in the absence of pLit



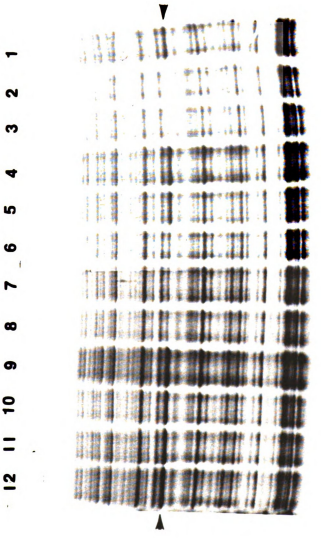




	- IPTG	+IPTG
pUC8gol-cat	5.271	25.954
pUC12UAA gol-cat	0.065	0.531
pUC12-cat	0.571	3.721

Cat ELISA Assay : one unit = one nanogram of cat enzyme per microgram of protein.

Fig 5: EF-Tus get cleaved in the presence of a wild-type, low amount of the Lit protein. pUC84PZ1 is used to transform Litcon (Lanes 1-4), Lit^{+} (Lanes 5-9) and Lit^{0}(Lanes 10-12). The protein contents of the cell samples, made in the absence of IPTG for 60' (Lanes 4, 9), and the presence of IPTG for various time period: 0 ' (Lanes 1, 5, 10); 20' (Lanes 2, 6,' 11); 40' (Lanes 3, 7, 12 and 60' (Lane 8). are visualized in a coomassei-blue stained SDS PAGE. The position of EF-Tu is indicated by arrows.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