λ *bar* minigene-mediated inhibition of protein synthesis involves accumulation of peptidyl-tRNA and starvation for tRNA

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Expression of the bacteriophage λ two-codon, AUG AUA, *barI* minigene (*bar*⁺) leads to the arrest of protein synthesis in cells defective in peptidyl-tRNA hydrolase (Pth). It has been hypothesized that translation of the *bar*⁺ transcript provokes premature release and accumulation of peptidyl-tRNA (p-tRNA). Inhibition of protein synthesis would then result from either starvation of sequestered tRNA or from toxicity of accumulated p-tRNA. To test this hypothesis and to investigate the cause of arrest, we used a coupled *in vitro* transcription–translation system primed with DNA containing *bar*⁺ and the β -lactamase-encoding gene of the vector as a reporter. The results show that expression of *bar*⁺ minigene severely inhibits β -lactamase polypeptide synthesis by Pth-defective extracts and partially inhibits synthesis by wild-type extracts. Fractions enriched for Pth, or a homogeneous preparation of Pth, prevented and reversed *bar*⁺-mediated inhibition. A mutant minigene, *barA702*, which changes the second codon AUA (Ile) to AAA (Lys), was also toxic for Pth-defective cells. Expression of *barA702* inhibited *in vitro* polypeptide synthesis by Pth-defective extracts and, as with *bar*⁺, exogenous Pth prevented inhibition. Addition of pure tRNA^{Lys} prevented inhibition by *barA702* but not by *bar*⁺. Expression of *bar*⁺ and *barA702* led to release and accumulation of p-tRNA^{Ile} and p-tRNA^{Lys} respectively but *bar*⁺ also induced accumulation of p-tRNA^{Lys}. Finally, *bar*⁺ stimulated association of methionine with ribosomes probably as fMet-tRNA^{fMet} and the accumulation of methionine and isoleucine in solution as peptidyl-tRNA (p-tRNA). These results indicate that minigene-mediated inhibition of protein synthesis involves premature release of p-tRNA, misincorporation of amino acyl-tRNA, accumulation of p-tRNAs and possibly sequestration of tRNAs.

Keywords: *in vitro* minigene expression/peptidyl-tRNA accumulation/peptidyl-tRNA hydrolase/protein-synthesis inhibition/tRNA starvation

Introduction

Peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29), an enzyme essential for *Escherichia coli* viability, cleaves the ester linkage of peptidyl-tRNA (p-tRNA) to yield peptides and tRNA. It has been proposed that the natural substrates for Pth are p-tRNAs released from ribosomes (Atherly and Menninger, 1972; Menninger, 1976). A missense mutation in the gene encoding this enzyme, *pth(ts)*, causes temperature-sensitive bacterial growth (Atherly and Menninger, 1972; García-Villegas *et al.*, 1991). Upon shift to non-permissive temperatures, the mutant shows accumulation of p-tRNA, arrest of protein synthesis and cell death. It has been suggested that this arrest may result from toxicity of the p-tRNA and/or from starvation of the critical tRNA sequestered as p-tRNA (Atherly and Menninger, 1972). The latter possibility is supported by the observation that the thermosensitivity of the *pth(ts)* mutant is suppressed by overproduction of tRNA^{Lys} (Heurgué-Hamard *et al.*, 1996), the tRNA which accumulates the fastest as p-tRNA upon temperature increase (Menninger, 1978). An excess of tRNA^{Lys} would also favor accurate translation of the Lys codons, reducing the rate of misincorporation with a consequent decrease in the formation and dissociation of erroneous p-tRNA.

Both *pth(ts)* and another mutation, *pth(rap)*, prevent the vegetative growth of bacteriophage λ under conditions which allow abundant growth of the mutant bacteria (Guzmán and Guarneros, 1989; García-Villegas *et al.*, 1991). Phage mutations, designated *bar*, that grow well on the *pth* mutant cells, map to three loci in the genome: *barI*, at the phage attachment site *attP*; *barII* within the *ssb* gene; and *barIII* near the immunity region (Guzmán and Guarneros, 1989). Plasmid constructs that express a λ DNA segment containing either the *barI* or *barII* loci cannot be maintained in *pth* mutants (Guzmán *et al.*, 1990). Expression of wild-type *bar* sequences from the constructs causes a general arrest of protein synthesis and is toxic for these bacteria (Pérez-Morga and Guarneros, 1990). It has been suggested that wild-type *bar* expression kills the mutants because it leads to excessive dissociation of p-tRNA, which then accumulates and inhibits synthesis (Hernández *et al.*, 1997). Subcloning of these sequences showed that a construct expressing only a 21 bp sequence of the *barI* region in a transcript context is not maintained in the *pth(rap)* mutant (Guzmán *et al.*, 1990). Inspection of the 21 bp nucleotide sequence and 5'-nearby sequences in the λ *bar*⁺ region revealed the presence of a minigene: a DNA segment whose transcripts would contain a Shine–Dalgarno-like sequence appropriately spaced for translation from the sequence AUG AUA UAA. Furthermore, the λ *barII* region contains a Shine–Dalgarno-like sequence preceding the same two-codon ORF but terminated at UGA. We have proposed that toxicity involves translation

Fig. 1. Linear map of pFGbar constructs. *bla* is the gene encoding β -lactamase; *oLpL*, λ leftward operator-promoter fragment corresponding to co-ordinates 35 535–35 713 in the λ map (Daniels *et al.*, 1983); *bar* is a *Bam*HI (*B*)-*Hind*III (*H*) DNA fragment comprising nucleotides 27 976–27 480 in the λ map and including λ *bar*⁺ and *t*_L transcription-terminator; *ori* replication origin of pBR322 (distances are not drawn to scale). The sequence below the map shows a segment of *bar*⁺-region transcript which includes *bar*⁺ minigene. The Shine-Dalgarno-like sequence is in bold, the two-codon ORF is underlined and the stop codon is in italics. The base substitutions *bar*101 and *bar*A702 are indicated.

of the *bar*⁺ transcripts because they associate with ribosomes and are released by run-off translation. It was also suggested that *bar*⁺ transcript-translation leads to 'drop-off' and, under limiting Pth activity, to accumulation of p-tRNA (Ontiveros *et al.*, 1997). This drop-off could result either from misincorporation of aminoacyl-tRNA or from a natural propensity of *bar*⁺ messengers to pause at the stop codon by a defect in translation termination (for a review see Hernández *et al.*, 1997). Under the so-called 'ribosome editor' hypothesis, amino acids inappropriately incorporated into the growing polypeptide chain lead to preferential dissociation of p-tRNA from the ribosomes (Menninger, 1977). It has been shown that dissociation of non-cognate p-tRNA happens more readily with tRNAs carrying short peptidyl chains than longer ones (Gast *et al.*, 1987). On the other hand, to our knowledge, there is no published evidence supporting a defect in termination for faithful translation of messengers containing two or a few sense codons.

In these experiments, we have used a cell-free transcription-translation system to investigate the mechanism of minigene-mediated inhibition of protein synthesis. From the results we conclude that ribosome drop-off of p-tRNA is a common event in minigene transcript-translation and that translation inhibition involves p-tRNA accumulation and aminoacyl-tRNA misincorporation.

Results

Expression of λ *bar*⁺ minigene inhibits protein synthesis in cell-free extracts

The expression of *bar*⁺ from plasmids is lethal to *pth*(rap) mutant cells (Guzmán *et al.*, 1990). Lethality may result from a translational defect because it correlates with inhibition of protein synthesis, whereas RNA synthesis remains unaffected (Pérez-Morga and Guarneros, 1990). To elucidate further the mechanism of inhibition, we used a DNA-dependent cell-free system for protein synthesis. We prepared S30 extracts from *pth*(rap) or wild-type cells and conducted assays primed by plasmid constructs containing *bar*⁺ and *bla* genes (Figure 1) as described in Materials and methods. The results showed that the construct containing

Fig. 2. λ *bar*⁺ minigene expression inhibits protein synthesis *in vitro*. *In vitro* reactions (total volume 50 μ l) contained the indicated plasmids (Vector, pFG93; *bar*⁺, pFGbar⁺ carrying λ *bar*⁺ region; *bar*101, pFGbar101) and S30 extracts from either *pth*(rap) mutant, or wild-type cells as indicated. Reactions were stopped after 60 min at 37°C, and the products were analyzed by SDS-PAGE and autoradiography as described in Materials and methods. The migration position of β -lactamase protein in the gel is indicated.

bar⁺ did not express β -lactamase protein (Bla) in *pth*(rap) extracts and severely inhibited Bla synthesis in wild-type extracts (Figure 2, lanes 6 and 3). The construct carrying the *bar*101 mutation, a 1 bp substitution which changes the AUG start codon to AUA, is not toxic for Pth-defective cells (Guzmán *et al.*, 1989). This construct directed Bla synthesis efficiently, as did the plasmid vector, both with wild-type and *pth*(rap) extracts (Figure 2, compare lanes 4 and 7 with lanes 2 and 5). As estimated by Northern blot assays, we found comparable concentrations of *bla* RNA and *bar* RNA, both in *bar*⁺ or *bar*101 reactions (data not shown). These *in vitro* results reproduce the basic features of *in vivo* protein-synthesis inhibition mediated by expression of *bar*⁺.

Pth prevents λ *bar*⁺-mediated protein synthesis inhibition

If *bar*⁺-mediated protein-synthesis inhibition were caused by accumulation of p-tRNA as a consequence of defective Pth (Pérez-Morga and Guarneros, 1990; Ontiveros *et al.*, 1997), the addition of exogenous Pth activity should prevent inhibition. To test this prediction as well as the possible involvement of another factor, we fractionated wild-type S30 extracts and assayed for *in vitro* *bar*⁺-mediated inhibition. Previous investigations have shown that Pth activity partitioned between the S150 supernatant and the ribosomal wash (RW) (Menninger *et al.*, 1970; Brun *et al.*, 1971). We prepared S150, RW fractions and 70S ribosomes from wild-type and Pth-defective cells as described in Materials and methods. Addition of S150 or RW fractions from wild-type cells to *in vitro* transcription-translation mixtures prepared from Pth-defective cell extracts, restored protein synthesis inhibited by *bar*⁺ expression (Figure 3A, lanes 7 and 8). Cellular fractions derived from a *pth*(rap) mutant were less effective than the respective fractions from wild-type cells in restoring Bla synthesis (Figure 3A, lanes 4 and 5). The amount of Pth antigen in the wild-type and *pth*(rap) fractions was comparable (Figure 3B), as shown by Western blot. Therefore, the difference in the restorative capacity could be accounted for by the wild-type and mutant Pth-specific

Fig. 3. Pth prevents λ *bar*-mediated inhibition of protein synthesis. (A) *In vitro* reaction mixtures contained the indicated plasmids, S30 extracts from *pth(rap)* mutant cells and 80 μ g protein of cell fractions (RW, ribosomal washing; S150, 150 000 g supernatant; 70S, washed ribosomes) or 0.5 μ g of purified Pth as indicated. After 1 h incubation at 37°C the products were processed and autoradiographed as described in Materials and methods. The migration position of β -lactamase protein is indicated. (B) Pth protein content in 70S, S150 and RW fractions estimated by immunoblot assay. Fractions were electrophoresed, blotted onto nitrocellulose membranes and Pth-antigen detected using a rabbit polyclonal serum against Pth as described in Materials and methods. Purified Pth was introduced as a control. Protein concentration was determined by densitometry from a standard curve with known concentrations of purified Pth in the same immunoblot.

activities. To show that Pth was the active component in protein synthesis restoration, we purified the Pth protein to homogeneity (Kösel *et al.*, 1970). Pure Pth prevented protein synthesis inhibition by λ *bar*⁺ expression (Figure 3A, lane 9). We found a direct correlation, in the range of 2–40 ng/ μ l, between Pth concentration and its capacity to prevent *bar*⁺ inhibition (not shown). These results suggest that Pth-mediated restoration of protein synthesis is derived from its hydrolytic activity on free p-tRNA, which in turn implies that *bar*⁺-mediated inhibition of Bla synthesis is caused by the release and accumulation of uncleaved p-tRNA(s).

λ *bar*⁺ minigene transcript is translated

From the nucleotide sequence of λ *bar*⁺ we would expect that abortive translation termination of its transcripts would yield fMet-Ile-tRNA^{Ile}_{AUA}. To test this prediction, we monitored the incorporation of [³⁵S]methionine into translation intermediates directed by the *bar*⁺ constructs with unlabeled isoleucine as the only additional amino acid. Therefore, the [³⁵S]methionine-containing TCA-precipitable material would presumably be tRNA-bound intermediates and not polypeptides (see Materials and methods). Using sedimentation through sucrose gradients,

we analyzed the distribution of [³⁵S]methionine TCA-precipitable products. Expression of the *bar*⁺ construct promoted the association of [³⁵S]methionine with ribosomes and its incorporation into the soluble fractions (Figure 4B). The mutant *bar101* construct with the poor initiation codon AUA in the miniORF (Shinedling *et al.*, 1987), failed to mediate detectable association of methionine with ribosomal fractions (Figure 4A). As the absence of cold isoleucine from the reaction mixtures did not affect the association of [³⁵S]methionine to the ribosomal fraction (data not shown), the ribosome-associated methionine was probably fMet-tRNA^{fMet}. Thus, the incorporation of [³⁵S]methionine seems to depend on efficient translation initiation of the minigene mRNA. Other mRNAs encoded in the constructs, including that of the *bla* gene, did not affect incorporation even in the absence of inhibition by a *bar101* mutation (Figure 4A). The loss of [³⁵S]-methionine-containing material, by alkali treatment of the fractions, is consistent with the notion that it was bound to tRNA (Figure 4E). Addition of purified Pth to the *in vitro* reaction prevented the accumulation of [³⁵S]methionine-containing TCA-precipitable material in the ribosomal fractions and reduced its concentration in the upper soluble fractions (Figure 4C). The addition of puromycin to the *in vitro* reactions released [³⁵S]methionine from the ribosomal fraction (Figure 4D). Puromycin mimics an amino acyl-tRNA in the ribosomal A-site, and acts as an acceptor of the peptide or fMet-tRNA derivatives in the P-site releasing peptidyl-puromycin or fMet-puromycin (Nierhaus and Wittmann, 1980). Since fMet-tRNA^{fMet} may be on the ribosomes, as argued above, the product of this reaction is probably fMet-puromycin. In the presence of [³H]isoleucine and unlabeled methionine as the only amino acids in the reaction, most of the TCA-insoluble ³H-label was incorporated into the soluble fraction (Figure 5A). Addition of Pth to these reactions significantly reduced the ³H-label incorporated in solution (Figure 5B). Based on these results we suggest that translation of the *bar*⁺ transcript yields fMet-Ile-tRNA^{Ile}_{AUA} which is released and accumulates in the soluble fraction. As the concentration of free tRNA^{Ile}_{AUA} becomes limiting it causes fMet-tRNA^{fMet} to pause at the P-site on the ribosome.

tRNA^{Lys} prevents protein-synthesis inhibition by the mutant *barA702*

If *bar*⁺-mediated protein-synthesis inhibition were caused by starvation of free tRNA^{Ile}_{AUA} sequestered as p-tRNA^{Ile}_{AUA}, adding excess tRNA^{Ile}_{AUA} to the reaction mixtures should reverse the inhibition. However, this is technically a difficult experiment to do because the tRNA^{Ile}_{AUA} is a very scarce isoacceptor, not easily available in pure form (Muramatsu *et al.*, 1988). We therefore took advantage of a mutation in *bar*⁺, *barA702*, in which the AAA (Lys) codon, cognate to an abundant isoacceptor, substitutes the AUA at the second position in the *bar*⁺ ORF (J.G. Valadez *et al.*, in preparation; Ikemura, 1985; Dong *et al.*, 1996). Expression of *barA702*, like *bar*⁺, was toxic to *pth(rap)* cells (not shown) and prevented Bla synthesis in reactions prepared with *pth(rap)*-cell free extracts (Figure 6, lane 3). As predicted, the addition of a purified tRNA^{Lys} preparation restored synthesis of the *bla* gene product (Figure 6, lanes 4 and 5). The addition of non-cognate

Fig. 4. Distribution of [^{35}S]methionine-containing products along the ribosomal profile of samples from *in vitro* bar^+ primed reactions. *In vitro* reactions carried out as described in Materials and methods with [^{35}S]methionine and cold isoleucine as the only amino acids added to the reaction, were directed with: (A) pFG $\text{bar}101$; (B) pFG bar^+ ; (C) pFG bar^+ in the presence of 10 ng/ μl of Pth; (D) pFG bar^+ further incubated with 1 mM puromycin at 37°C for 10 min; (E) pFG bar^+ . 50 μl samples from the *in vitro* coupled transcription-translation reactions were layered onto a 5–40% linear sucrose gradient containing 50 mM Tris-HCl, 3.5 mM Mg(Ac) $_2$, 60 mM NH $_4$ Cl, 0.6 mM 2-mercaptoethanol and run as described in Materials and methods. Fractions (300 μl) were collected, and aliquots (10 μl) were drawn to measure absorbance at 260 nm and cold TCA-insoluble radioactivity. Gradient fractions from (E) were treated with 1 M NaOH for 10 min at 37°C before TCA precipitation. Absorbance at 260 nm (●); ^{35}S -activity (○).

tRNAs did not reverse the effects of barA702 expression (Figure 6, lanes 6 and 7), nor did the addition of tRNA $^{\text{Lys}}$ to mixtures primed by bar^+ (AUA) minigene (not shown). Addition of tRNA $^{\text{Lys}}$ at various times up to 60 min after reaction initiation by the barA702 construct, restored Bla protein synthesis (data not presented). This result suggests that the accumulated p-tRNA $^{\text{Lys}}$ (Table I) is not toxic for translation initiation as proposed (Atherly, 1978) but it could be a competitive inhibitor of tRNA $^{\text{Lys}}$. Inhibition by barA702 was also prevented by exogenous Pth to the same degree as Pth reversed the effect of bar^+ regardless of whether Pth was added at the beginning of the reaction (compare Figure 6, lane 8 with Figure 3A, lane 9) or up to 30 min after initiation (not shown). This is consistent with the recycling of tRNAs from p-tRNAs generated during barA702 inhibition.

***bar* expression causes p-tRNA accumulation**

In an attempt to test the assumption that unsuccessful translation of the bar^+ transcript leads to accumulation of fMet-Ile-tRNA $^{\text{Ile}}_{\text{AUA}}$, we assayed directly for the presence of p-tRNA upon *in vitro* minigene expression. Reactions were primed with plasmid constructs carrying either bar^+ or barA702 minigenes in the presence of all 20 amino acids, and the p-tRNA was measured in ribosomal and

soluble fractions. The data in Table I showed, relative to bar101 controls, that bar^+ and barA702 expression induced accumulation of p-tRNA $^{\text{Ile}}$ and p-tRNA $^{\text{Lys}}$ in solution, respectively, whereas no accumulation of control p-tRNA $^{\text{His}}$ was observed in any case. Interestingly, bar^+ expression, which does not encode lysine, accumulated p-tRNA $^{\text{Lys}}$ but barA702 did not accumulate p-tRNA $^{\text{Ile}}$. In both bar^+ and barA702 -inhibited reactions p-tRNAs were found in the soluble fractions. The significant values for accumulation of p-tRNA were above the average acceptor activity for each of the three amino acids assayed for the soluble and ribosomal fractions from control reactions without priming plasmid (Table I, lines 1 and 2).

Discussion

We have shown that expression of bacteriophage λ bar^+ minigene *in vitro* leads to accumulation of p-tRNA and inhibition of protein synthesis. Presumably, p-tRNAs dissociate from ribosomes by a defect in translation termination and accumulate when Pth activity is limited. These data confirm and extend the notion, based on *in vivo* results, that translation of λ bar^+ mRNA is essential for inhibition of protein synthesis. In fact, bar^+ transcripts seem to act as true messengers because they associate with

Fig. 5. Distribution of [^3H]isoleucine-containing products along the ribosomal profile of samples from *in vitro* bar^+ primed reactions. *In vitro* reactions carried out as described in Materials and methods with [^3H]isoleucine and cold methionine as the only amino acids added to the reaction, were directed with: (A) pFG bar^+ ; (B) pFG bar^+ in the presence of 10 ng/ μl of Pth. The gradients were done as described in Figure 4.

Fig. 6. tRNA $^{\text{Lys}}$ and Pth prevent barA702 mediated inhibition of protein synthesis. *In vitro* transcription-translation reactions prepared with Pth-defective S30 extracts as described in Materials and methods, were primed with pFG barA702 DNA except lane 1 (primed with pFG bar101) and lane 2 (with pFG bar^+). Where indicated, 5 or 10 μg of specific tRNA(s) or 0.5 μg of Pth were added to the reaction. The migration position of β -lactamase protein in the SDS-PAGE gel is indicated.

ribosomes and dissociate by run-off translation (Ontiveros *et al.*, 1997). *In vitro* inhibition is stronger with *pth*-defective cell-free extracts than it is with wild-type extracts, presumably because they accumulate p-tRNA faster (Figure 2, compare lanes 6 and 3). The hydrolysis of p-tRNA suppresses inhibition since preparations enriched for Pth restored protein synthesis, and a direct correlation has been found between pure Pth

Table I. Accumulation of specific p-tRNA upon *in vitro* bar expression

Plasmid	Fraction	% p-tRNA		
		Ile	Lys	His
None	S	0.5	0.1	0.1
	R	0.1	0.1	0.2
bar101	S	4.1	3.8	3.1
	R	0.7	0.8	0.9
bar^+	S	10.5	8.0	2.2
	R	1.8	0.8	0.1
barA702	S	2.7	40.1	3.6
	R	0.1	2.4	0.6

The percent of peptidyl-tRNA in solution (S) or in the ribosomal (R) fractions was estimated as the accepting activity for each of the indicated amino acids relative to the total accepting activity of the *in vitro* reactions as described in Materials and methods. Reactions were performed in the presence of all 20 amino acids. The values are averages of two independent determinations.

concentration and its restoring capacity (Figure 3; data not shown).

Free tRNA sequestration as uncleaved p-tRNA or direct toxicity by p-tRNA have been suggested as the possible causes of lethality in the thermosensitive mutant of *E. coli* $\text{pth}(ts)$ at the restrictive temperature (Atherly and Menninger, 1972; Menninger *et al.*, 1973; Menninger, 1976). Overproduction of tRNA $^{\text{Lys}}$, the isoacceptor which accumulates the fastest as p-tRNA upon temperature upshift, overcomes lethality (Menninger, 1978; Hergu-Hamard *et al.*, 1996), indicating that the growth limitation of the mutant could be due to tRNA $^{\text{Lys}}$ starvation or to a rise in the rate of misincorporation and a consequent increase in the formation and dissociation of erroneous p-tRNA.

Improperly terminated translation of bar^+ mRNA, AUG AUA UAA, is expected to yield fMet-Ile-tRNA $^{\text{Ile}}$ _{AUA}. Our *in vitro* results show that the accumulated material in the soluble fraction could include such p-tRNA: it contains methionine (Figure 4B); it is susceptible to alkaline degradation (Figure 4E); its accumulation is prevented by Pth (Figure 4C); it contains [^3H]isoleucine (Figure 5); and its tRNA is specific for isoleucine (Table I). Mutant minigene barA702 , which carries AAA (Lys) as a second codon, is toxic for *pth*-deficient cells and inhibits protein synthesis in the cell-free system (J.G. Valadez, unpublished results and Figure 6). Likewise, a construct carrying barA702 may have promoted the accumulation of [^3H]lysine-containing p-tRNA $^{\text{Lys}}$ because Pth treatment released the ^3H -label and lysine accepting activity (Table I; data not shown). Neither bar^+ nor barA702 mediate incorporation of ^3H -labeled p-tRNA to the ribosomal fractions suggesting that little, if any, p-tRNA remains bound to ribosomes (Figure 5). Expression of bar^+ constructs promoted association of formyl-[^{35}S]Met-tRNA $^{\text{fMet}}$ to ribosomes (Figure 4B). As [^{35}S]methionine was released by puromycin treatment (Figure 4D), it is likely that the fMet-tRNA $^{\text{fMet}}$ resided in the ribosomal P-site (Nierhaus and Wittmann, 1980). Such a situation would be expected by pausing of ribosomes at the AUA codons upon starvation for free tRNA $^{\text{Ile}}$ _{AUA} during bar^+ mRNA translation.

If translation termination proceeded normally, fMet-Ile-tRNA $^{\text{Ile}}$ _{AUA} would efficiently be cleaved into fMet-Ile and

tRNA^{Ile}_{AUA}. We do not know the extent to which this termination occurs, but substantial levels of defective termination are indicated by the accumulation of p-tRNA^{Ile} in the ribosome-free fraction of *in vitro* reactions (Table I). The nature of the p-tRNA depends upon the minigene expressed: *bar*⁺ provokes accumulation of p-tRNA^{Ile}, whereas *barA702* leads to build up of p-tRNA^{Lys} (Table I). This indicates that the last sense codon, before the stop in the minigene message, determines the specificity of the accumulated p-tRNA. However *bar*⁺, which does not contain lysine codons, also mediated accumulation of p-tRNA^{Lys} (Table I). The 'ribosome editor' hypothesis proposes that inappropriate p-tRNAs, resulting from erroneous incorporation of aminoacyl-tRNA, preferentially dissociate from the ribosome (Menninger, 1977). Upon starvation for tRNA^{Ile}_{AUA}, sequestered as p-tRNA^{Ile}_{AUA}, Lys-tRNA^{Lys} would compete for binding at AUA codons, misincorporate lysine, and dissociate as inappropriate fMet-Lys-tRNA^{Lys}. This, in turn, may lead to starvation for tRNA^{Lys}. The fact that *barA702* did not promote misincorporation of isoleucine (Table I), might be explained by a lack of misreading of the AAA codon by the tRNA^{Ile}_{AUA}. tRNA^{Lys}, specific for lysine codons AAA and AAG, is a fairly abundant tRNA, (Ikemura, 1991; Dong *et al.*, 1996), thus *bar*⁺-mediated inhibition is not only specific for scarce isoacceptors cognate to rare codons. A significant proportion of tRNA^{Lys} accumulates as p-tRNA upon *barA702* expression (40%, Table I) and an additional supply of tRNA^{Lys} prevents inhibition caused by *barA702* minigene (Figure 6, lanes 4 and 5). These features are compatible with inhibition being caused by starvation for tRNA^{Lys} (for a model, see Hernández *et al.*, 1997). Although our data do not exclude the possibility of competitive inhibition by p-tRNA^{Lys} during translation initiation (Atherly, 1978) they are better explained assuming tRNA starvation.

We do not know why p-tRNA, generated upon translation of minigene transcripts, does not terminate normally but is abortively released from the ribosome instead. In the absence of *bar*⁺, *in vitro* translation is competent in polypeptide termination. This is inferred from the synthesis of Bla protein of the expected size (Figure 2, lanes 2, 4, 5 and 7). Therefore, we speculate that the two-codon ORF mRNAs of the minigenes examined here might be inherently defective in translation termination. Ribosomes translating *bar*⁺ mRNA might pause before UAA and eventually release p-tRNA. Prematurely terminated polypeptides are generated during the normal synthesis of proteins albeit at a relatively low frequency. Independent calculations have estimated the frequency of prematurely terminated molecules between 2×10^{-4} to 4×10^{-4} per translated codon. The most likely cause of abortive translation termination are p-tRNA drop-off events (Menninger 1976; Manley, 1978; Caplan and Menninger, 1984; Dong and Kurland, 1995). Studies of M.Ehrenberg and collaborators in Uppsala, Sweden (personal communication) have shown that initiation factors IF1 and IF2 synergistically remove p-tRNA with short polypeptides from the P-site of ribosomes. This is true for di-, tri-, tetra- but not for heptapeptides. The rate of IF-independent dissociation is approximately the same for all studied peptide lengths. A previous proposal implies that the nascent peptide chain

stabilizes the binding of p-tRNA to the ribosome when a critical length is reached (Gast *et al.*, 1987).

In addition to β -lactamase, we have observed inhibition of other reporter proteins upon *in vitro bar*⁺ expression, including Pth whose gene does not contain AUA codons (data not shown). Thus, tRNA^{Ile}_{AUA} starvation cannot be the only cause of protein-synthesis inhibition in these reactions. Other secondary effects may contribute to the inhibition after starvation of specific tRNA, such as depletion of tRNA^{Lys} or of ribosomes complexed with *bar*⁺ mRNA and other translation factors. We are currently studying the relationship between these *in vitro* findings and *bar*⁺-mediated inhibition of general protein synthesis *in vivo*.

Materials and methods

Bacterial strains, plasmids and culture conditions

K-12 *E.coli* strains C600 (*thr1 leuB6 thi1 lacY1 sup E44 tonA21*) and C600 *pth*(rap) (Guzmán and Guarneros, 1989) were transformed with pFgbar plasmids carrying either *bar*⁺ region or *bar101* and *barA702* base substitutions (Ontiveros *et al.*, 1997) and grown with vigorous aeration to log-phase in Luria-Bertani (LB) medium. When necessary, 50 µg/ml ampicillin (Ap) was added. The transcription of *bar* sequences in the plasmid constructs is under the control of the λ pL (Figure 1). Plasmid DNA was obtained by the sodium dodecyl sulfate (SDS) alkali method (Silhavy *et al.*, 1984) and further purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (Sambrook *et al.*, 1989).

Cell-free extracts

The *E.coli* coupled transcription-translation system was prepared essentially as described (Zubay, 1973; Mackie *et al.*, 1990) with minor modifications. Briefly, 3 l of medium containing 5.6 g KH₂PO₄, 28.9 g K₂HPO₄, 10 g yeast extract, 10 mg thiamine and 4 g glucose/l were inoculated with 7.5 ml/l from an overnight culture of C600 or C600 *pth*(rap) and incubated to mid log-phase ($A_{600}=0.4$). Cells were quickly chilled by shaking in ice-water and harvested by centrifugation. The cell pellet was washed twice with buffer A [10 mM Tris-Ac pH 8.0, 14 mM Mg(Ac)₂, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), 60 mM KCl, 6 mM 2-mercaptoethanol] and any dark cell debris removed. The washed pellet was re-suspended in buffer B [10 mM Tris-Ac pH 8.0, 14 mM Mg(Ac)₂, 60 mM KCl, 1 mM dithiothreitol, 50 µg/ml PMSF] to a concentration of 0.25 g/ml. The cell suspension was passed through a French pressure cell (SLM AMINCO Instruments Inc.) at approximately 3500 p.s.i. The resulting lysate was centrifuged for 30 min at 30 000 g. The supernatant was agitated for 80 min at 37°C with 0.15 vol. of pre-incubation mix [0.75 M Tris-Ac pH 8.0, 7.5 mM dithiothreitol, 21.3 mM Mg(Ac)₂, 75 µM each amino acid, 6 mM ATP, 20 mg/ml phosphoenol pyruvate, 50 units of pyruvate kinase (Boehringer Mannheim)]. The S30 was then dialyzed versus three, 2 l changes of buffer C [10 mM Tris-Ac pH 8.0, 14 mM Mg(Ac)₂, 60 mM K(Ac), 1 mM dithiothreitol, 50 µg/ml PMSF] for 3 h/change. S30 was then divided into 300 µl aliquots and stored at -80°C. Optimal Mg²⁺ and K⁺ concentrations were determined for S30 extracts.

S30 fractionation

For the experiments reported in Figure 3, S30 was spun at 45 000 r.p.m. for 4 h in a Beckman SW 50.1Ti rotor to pellet crude ribosomes. The supernatant was the S-150 fraction. The crude ribosomes were re-suspended in 10 mM Tris-HCl pH 7.6, 15 mM MgCl₂, 1.0 M NH₄Cl, 5 mM 2-mercaptoethanol, and incubated on ice for 1 h with occasional agitation. The ribosomes were collected by ultracentrifugation as above, and proteins were precipitated from the supernatant by the addition of 470 mg/ml (NH₄)₂SO₄. This precipitate was collected by centrifugation and re-suspended in 10 mM Tris-HCl pH 7.6, 20 mM NH₄Cl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, and dialyzed overnight against the same buffer. This fraction represents the ribosomal wash (RW). Aliquots of the RW fraction were then quick-frozen and stored at -80°C. The washed ribosomes were re-suspended in 10 mM Tris-HCl pH 7.6, 15 mM MgCl₂, 60 mM NH₄Cl, 5 mM 2-mercaptoethanol, and pelleted again as above. Aliquots were re-suspended in the same buffer, quick-frozen and stored at -80°C. This fraction is called 70S.

Cell-free synthesis

50 µl reactions were prepared with 2 µg of plasmid DNA in TE (10 mM HCl, pH 8.0, 1 mM EDTA), 20 µl of premix [87.5 mM Tris-Ac pH 8.0, 476 mM potassium glutamate, 75 mM NH₄(Ac), 5 mM dithiothreitol, 20 mM Mg(Ac)₂, 1.25 mM each of 20 amino acids, 5 mM ATP, 1.25 mM each of CTP, UTP, GTP, 50 mM phosphoenol pyruvate, 2.5 mg/ml *E. coli* tRNA, 87.5 mg/ml polyethylene glycol (8000 *M_r*), 2.5 mM cAMP, 50 µg/ml folinic acid] and 15 µl of S30. Radiolabeled proteins were synthesized using a premix lacking methionine. 10 µCi of [³⁵S]methionine (1 170 Ci/mmol) were then added to a final concentration of 2 × 10⁻¹¹ M. Protein was precipitated from the reactions by addition of 4 vol. of acetone followed by centrifugation. The protein pellets were dried and re-suspended in SDS sample buffer prior to gradient polyacrylamide gel electrophoresis (Laemmli, 1970). Immediately following electrophoresis, wet gels were dried and then visualized by autoradiography. To analyze the effect of Pth on protein-synthesis inhibition by λ *bar*⁺ we tested a range of protein concentrations, from 0.01–2.0 µg. A clear effect was seen with 0.01 µg/µl. The Pth used in the assays (specific activity 480 pmol of acetyl-lysyl-tRNA/min/µg of Pth) was purified as previously described (Kössel *et al.*, 1970). The purified protein revealed only a single 21 kDa band when 3 µg of the Pth preparation was electrophoresed in a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie Blue.

Sucrose-gradient centrifugation

A standard reaction mixture (50 µl), chilled to 4°C and in 5% sucrose, was layered on 4 ml of 5–40% (w/v) sucrose gradient composed of 50 mM Tris-HCl, 3.5 mM Mg(Ac)₂, 60 mM NH₄Cl, 0.6 mM 2-mercaptoethanol, and centrifuged in a Beckman SW 50.1 rotor at 27 000 r.p.m. for 2.5 h at 4°C. Fractions of 300 µl were collected from the top of the tube using a density gradient fractionator (Auto Densi-Flow IIC, Buchler Instruments), the absorbance monitored at 260 nm and cold trichloroacetic acid-insoluble radioactivity of each fraction measured using a scintillation counter.

Immunoblots

Gradient (7.5–15%) acrylamide slab gel electrophoresis was performed by the method of Laemmli (1970) with the following modifications. A Mini Protean II electrophoresis unit (Bio-Rad) was used. 30 µg of protein of the S30 fractions S150, 70S and RW were normally applied in each lane. After electrophoresis, the proteins were blotted to nitrocellulose membranes as described by Towbin *et al.* (1979). The membrane was blocked in phosphate-buffered saline (3 mM KCl, 2 mM KH₂PO₄, 10 mM Na₂HPO₄, 250 mM NaCl, pH 7.2) containing 0.25% (w/v) gelatin (Sigma, type 1), and 5 mM EDTA at 37°C for 60 min. Dilutions (1:4000) of the anti-Pth rabbit serum were made in the blocking buffer and incubated with the membrane for 1 h at 37°C. The filter was washed with PBS-T (PBS with 0.05% Tween 20) followed by the addition of a 1:2000 dilution of a goat anti-rabbit-IgG peroxidase conjugate. After 1 h incubation, the filter was washed and incubated with 4-α-chloronaphthol and 0.03% H₂O₂ until sufficient color developed. Pth concentrations from S30 fractions were determined by densitometry from a standard curve with known concentrations of purified Pth in the same immunoblot.

Assay for p-tRNA

50 µl of a standard *in vitro* transcription-translation reaction containing all 20 amino acids was centrifuged at 339 000 *g* for 2.5 h using a Beckman type TLN-120 rotor to sediment ribosomes. The ribosomal fraction was re-suspended in 10 mM Tris-HCl pH 7.6, 15 mM MgCl₂, 1.0 M NH₄Cl and 5 mM 2-mercaptoethanol. p-tRNA was assayed both in the supernatant (soluble fraction) and in the ribosomal fraction as described by Menninger (1976) with the following modifications: after treatment with 10 mM CuSO₄ to hydrolyze amino acyl-tRNA, samples were either assayed directly for amino acid-accepting activity as described below or were pre-treated with Pth (0.25 ng/µl) in 0.2 M NH₄Cl and 0.15 M Tris-HCl pH 7.5, and incubated for 20 min at 32°C. Following ethanol precipitation, the pellet was washed twice with 70% ethanol and re-suspended in H₂O. For accepting activities, 50 µl samples of 0.04 M Tris-HCl pH 7.6, 0.06 M NH₄Cl, 9 mM Mg(Ac)₂, 6 mM ATP, 0.1 mM CTP, 5 mM 2-mercaptoethanol and 0.3 µM of either [³H]lysine, [³H]isoleucine or [³H]histidine (specific activities 82, 113 and 48 Ci/mmol, respectively) were used. The reactions were initiated by adding 1 µl of S-100 and samples were incubated for 30 min at 37°C. The p-tRNA in the soluble or ribosomal fractions was expressed as the percentage of the accepting activity unmasked by Pth treatment (Menninger, 1976).

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