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Evidence of *bar* Minigene Expression and $tRNA_2^{Ile}$ Sequestration as Peptidyl-$tRNA_2^{Ile}$ during Lambda Bacteriophage Development

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Lambda bacteriophage development is impaired in *Escherichia coli* cells defective for peptidyl (pep)-tRNA hydrolase (Pth). Single-base-pair mutations (*bar*$^-$) that affect translatable two-codon open reading frames named *bar* minigenes (*bar*I or *bar*II) in the lambda phage genome promote the development of this phage in Pth-defective cells (rap cells). When the *bar*I minigene is cloned and overexpressed from a plasmid, it inhibits protein synthesis and cell growth in rap cells by sequestering $tRNA_2^{Ile}$ as pep-$tRNA_2^{Ile}$. Either $tRNA_2^{Ile}$ or Pth may reverse these effects. In this paper we present evidence that both *bar*I and *bar*II minigenes are translatable elements that sequester $tRNA_2^{Ile}$ as pep-$tRNA_2^{Ile}$. In addition, overexpression of the *bar*I minigene impairs the development even of *bar*$^-$ phages in rap cells. Interestingly, tRNA or Pth may reestablish lambda phage development. These results suggest that lambda *bar* minigenes are expressed and $tRNA_2^{Ile}$ is sequestered as pep-$tRNA_2^{Ile}$ during lambda phage development.

Bacteriophage lambda is unable to grow vegetatively in *Escherichia coli* (rap) mutants defective in peptidyl (pep)-tRNA hydrolase (Pth) activity (8, 9, 11). Phage mutants that grow readily in the defective bacteria are affected in regions of the lambda genome named *bar*. One of these, *bar*I, is located at the phage attachment site, *attP*, and another one, *bar*II, is located within the *ssb* gene (9). Overexpression of *bar* regions in plasmid vectors causes growth inhibition of Pth-defective *E. coli* cells (8, 10, 20). Analogous constructs carrying lambda mutant *bar* regions are nontoxic (10, 26). The nearly identical *bar*I and *bar*II sequences harbor minigenes, which are DNA segments whose transcripts contain a Shine-Dalgarno sequence appropriately spaced for translation from either AUG AUA UAA (*bar*I) or AUG AUA UGA (*bar*II) sequences. Overexpression of *bar* minigenes under limiting Pth activity in vivo and in vitro leads to the accumulation of formylmethionyl-Ile-$tRNA_2^{Ile}$ (pep-$tRNA_2^{Ile}$), and purified preparations of Pth protein or $tRNA_2^{Ile}$ are able to reverse minigene-mediated inhibition of protein synthesis in vitro (12, 25).

Minigene-mediated cell toxicity achieved in Pth-defective cells by the use of multicopy plasmids differs from lambda phage exclusion, in which both *bar*I and *bar*II minigenes must be transcribed to reduce phage development (9). Both the *bar*I and *bar*II regions are parts of the lambda left operon and are transcribed by the protein RNA polymerase antitermination complexes which are initiated at p_L (3, 23). Thus, the mutations *sex*1 of lambda, which results in a phage defective in the p_L promoter (21), and *nut*L44, which prevents transcript elongation beyond the transcription terminator t_{L1} (22), develop successfully in Pth-defective cells. On the contrary, those mutations that enhance the expression of *bar* regions inhibit lambda phage development. The mutations *int*C266, which causes constitutive transcription from the p_I promoter across the *attP* site (24), and *cro*27, which results in a phage defective in the repression of p_L (7), make these phages unable to develop in Pth-defective cells. Therefore, the stringency of lambda phage exclusion in Pth-defective cells depends on the degree of transcription through the *bar*I and *bar*II regions (9).

The data presented in the present paper indirectly suggest that the *bar* minigene regions are expressed during lambda phage development. Additionally, the capacity of Pth or $tRNA_2^{Ile}$ to promote lambda phage development in Pth-defective cells indicates that $tRNA_2^{Ile}$ is probably sequestered as pep-$tRNA_2^{Ile}$. We argue about the role of these observed conditions as part of a potentially interesting interaction between the phage and the host, which could be involved in a type of mini-open reading frame (ORF)-mediated translational regulation of gene expression.

$tRNA_2^{Ile}$ or Pth may alleviate lambda *bar*I and *bar*II minigene-mediated cell growth inhibition in Pth-defective cells. Expression of lambda bacteriophage *bar*I and *bar*II minigenes from plasmid constructs inhibits protein synthesis and cell growth in Pth-defective cells (26). In addition, the *bar*I minigene expressed in vitro accumulates pep-$tRNA_2^{Ile}$ under limiting Pth activity (12). $tRNA_2^{Ile}$ supplementation of Pth-defective cells reverses *bar*I-mediated cell growth inhibition. Thus, we extended these investigations to the lambda *bar*II minigene to ascertain whether the behavior of *bar*II parallels that of *bar*I.

E. coli C600 *c*I857 or Pth-defective C600 *c*I857 (rap) cells were transformed with any of the following plasmids containing wild-type or mutant minigenes under the p_L promoter (17, 26): pFGbarI (bearing the wild-type *bar*I minigene), *bar*101 (with an AUG to AUA substitution at the first codon of the *bar*I mini-ORF), pCMbarII (containing the *bar*II minigene), and pCMbar205 (with a base pair substitution from AUA to AUG at the second codon of the *bar*II mini-ORF). For Pth

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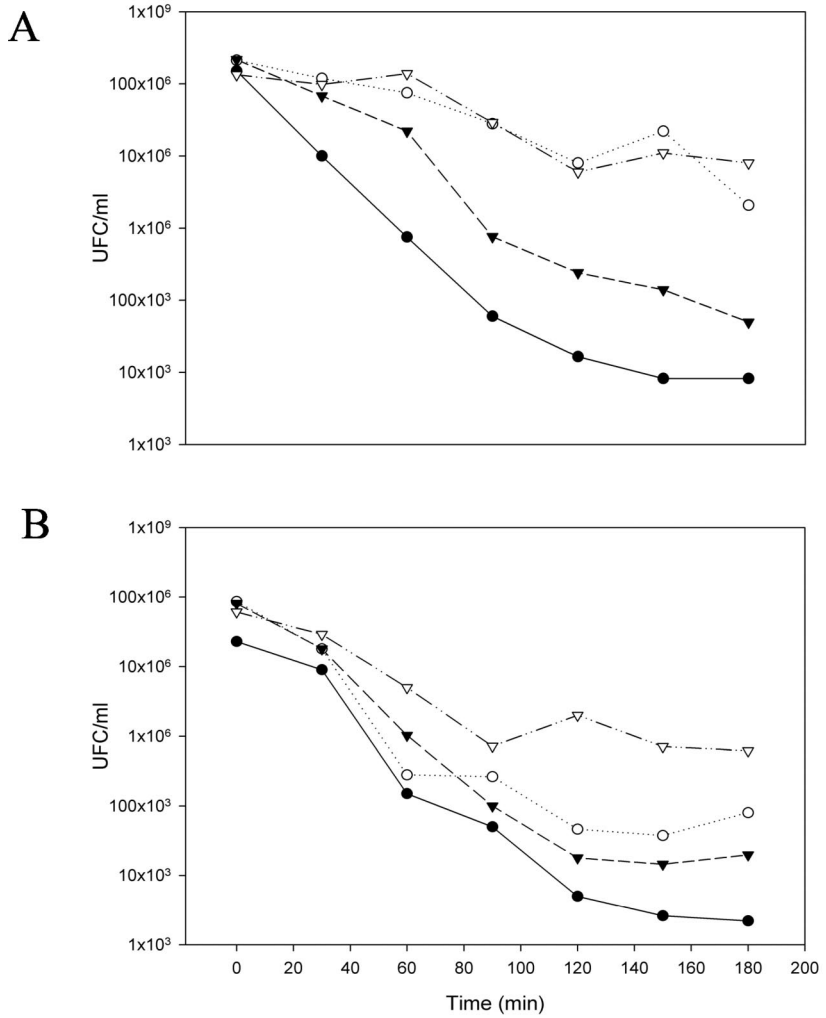

FIG. 1. Effect of Pth or $tRNA_2^{Ile}$ on the viability of *bar*I or *bar*II minigene-expressing Pth-defective cells. The cells transformed with either pFGbarI or pFGbarII and cotransformed with pPth or pIle2 (containing the Pth or $tRNA_2^{Ile}$ gene, respectively) were grown on LB medium containing ampicillin at 32°C to an optical density at 600 nm of 0.4 and shifted to 43°C for *bar* transcription derepression. At the indicated intervals, samples were taken to measure viable bacteria at 32°C on plates with LB medium containing ampicillin. (A) ●, pFGbarI + pACYC; ▼, pFGbarII + pACYC; ○, pFGbarI + pPth; ▽, pFGbarII + pPth; (B) ●, pFGbarI + pArg4; ▼, pFGbarII + pArg4; ○, pFGbarI + pIle2; ▽, pFGbarII + pIle2.

supplementation, cells were transformed with pGREC (harboring the *E. coli pth* gene) (G. Rosas-Sandoval, unpublished results), and for $tRNA_2^{Ile}$/$tRNA_4^{Arg}$ supplementation, cells were transformed with plasmids pDC952 and pI289, which were derived from pACYC184 (2) by cloning the $tRNA_4^{Arg}$ and $tRNA_2^{Ile}$ genes, respectively (4). For clarity, plasmids pGREC, pDC952, and pI289 are designated in this paper as pPth, pArg4, and pIle2, respectively. Transcription through the *bar* minigene was derepressed at 43°C via a thermosensitive lambda *c*I repressor in a cryptic prophage in C600 rap cells (see reference 26). The effect of Pth or $tRNA_2^{Ile}$ on the viability of C600 *c*I857 rap cells transformed with either *bar*I or *bar*II minigene-containing plasmids was monitored for 120 min. The results (Fig. 1A) revealed that Pth reversed *bar*II minigene-mediated cell growth inhibition, as has previously been reported for *bar*I (26). Under the conditions tested, $tRNA_2^{Ile}$ had a moderate but significant effect on cell growth restoration (Fig. 1B). pArg4 containing the $tRNA_4^{Arg}$ gene or pACYC184 where these tRNA genes were cloned had no effect (data not shown). These results suggest that both *bar*I and *bar*II minigenes sequester $tRNA_2^{Ile}$ as pep-$tRNA_2^{Ile}$. Therefore, both minigenes are translatable entities when they are cloned and expressed outside of their context.

Translation of minigenes that sequester $tRNA_2^{Ile}$ as pep-tRNA2Ile reduces the development of mutant lambda bacteriophages in Pth-defective cells. Bacteriophage lambda is unable to grow in *E. coli* mutants defective in Pth activity (8, 9, 11). Phage mutants in which the translatability of *bar*I or *bar*II minigenes is impaired increase their capacity to grow in Pth-defective cells (9). These antecedents, together with the above observations, suggest that *bar* minigenes are expressed and that the activity of Pth is required during lambda phage development. To further analyze the role of these elements in lambda development, we artificially exacerbated their effect by a con-

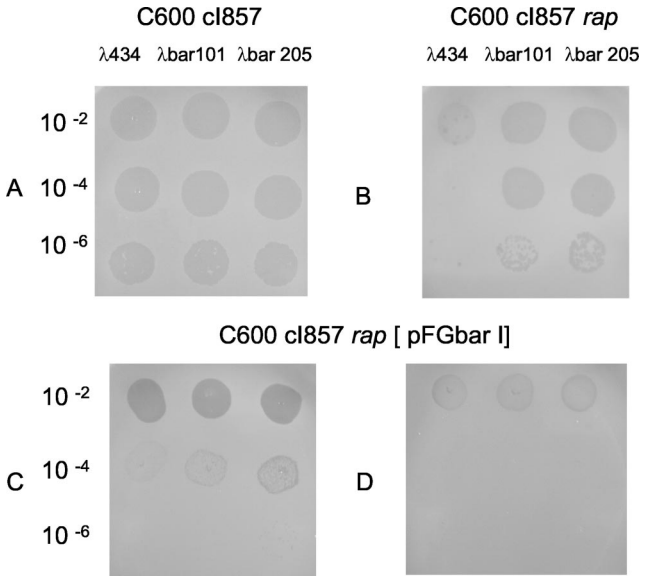

FIG. 2. tRNA-sequestering minigenes impair the development of mutant lambda phages in Pth-defective cells. Dilutions of the indicated phages were spotted on cell lawns of C600 or Pth-defective C600 cells. (A) Wild-type C600 *c*I857 cells incubated at 42°C; (B) C600 *c*I857 rap cells incubated at 42°C; (C) *bar*I minigene-expressing C600 *c*I857 rap cells incubated at 32°C; (D) *bar*I minigene-expressing C600 *c*I857 rap cells incubated at 42°C.

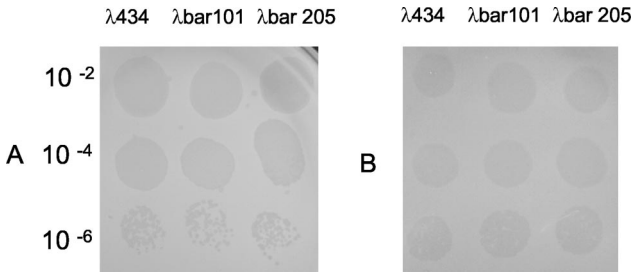

FIG. 3. Pth or $tRNA_2^{Ile}$ may enhance phage development in Pth-defective cells. Dilutions of the indicated phages were spotted on cell lawns of Pth-defective C600 cells and incubated at 42°C. The cells were additionally transformed with pPth containing the Pth gene (A) and with pIle2 containing the $tRNA_2^{Ile}$ gene (B).

trolled expression of the *bar*I minigene from a multicopy plasmid in wild-type or rap cells under conditions where cell growth is not apparently affected.

Lysates of λ *lac trp* W205 *red*114 *imm*434, *bar*101, and *bar*205 bacteriophages, which are λ derivatives able to grow at 32°C, were prepared (9, 10). Phage dilutions from lysates containing the same titer (~10 μl) were spotted on cell lawns prepared by pouring 2.5 ml of soft tryptone broth with 100 μl of *bar* minigene-expressing C600 or Pth-defective C600 cells over a Luria-Bertani (LB) medium plate.

The lambda *bar*101 or *bar*205 mutants that were affected in only one of the minigenes were able to grow in Pth-defective cells, although not as efficiently as in wild-type cells (Fig. 2B). This may be due to the fact that they do not demand as much $tRNA_2^{Ile}$ as wild-type lambda. Accordingly, the development of lambda *bar*101 or *bar*205 mutants was dramatically reduced when the limited $tRNA_2^{Ile}$ levels were further exhausted by expressing the $tRNA_2^{Ile}$-sequestering *bar*I minigene (Fig. 2D). Importantly, the development of both mutant and wild-type phages is also inhibited in Pth-defective cells under conditions where cell growth is not affected (Fig. 2C). These results indicate that the degree of lambda phage development depends on the Pth cell activity and $tRNA_2^{Ile}$ cell levels and on the translatability of *bar* minigenes.

Pth or $tRNA_2^{Ile}$ restores lambda bacteriophage development in Pth-defective cells. The reduced lambda phage development in Pth-defective cells suggests that *bar* minigenes may produce pep-$tRNA_2^{Ile}$ which may not readily be hydrolyzed by the low Pth activity levels. Therefore, Pth supplementation of Pth-defective cells should restore the cells' capacity to support lambda phage development. Phage development in Pth-defective cells supplemented with Pth was comparable to that in wild-type cells (Fig. 2A and 3A). If *bar*I-mediated reduction of phage development in Pth-defective cells were caused by starvation of free $tRNA_2^{Ile}$ sequestered as pep-$tRNA_2^{Ile}$, supplementing the cells with $tRNA_2^{Ile}$ should also reestablish lambda phage development. As expected, wild-type phage development in Pth-defective cells was further enhanced by supplementing $tRNA_2^{Ile}$ (Fig. 3B). pArg4 containing the $tRNA_4^{Arg}$ gene or pACYC184 in which Pth or the tRNA genes were cloned had no effect (data not shown). Optimal phage development was promoted by $tRNA_2^{Ile}$ supplementation, presumably because the size of the $tRNA_2^{Ile}$ cell pool is increased.

The results presented in this paper suggest that *bar* regions are translated during lambda phage development. In addition, the promoting activities of Pth and $tRNA_2^{Ile}$ in lambda phage development strongly indicate that $tRNA_2^{Ile}$ is sequestered as pep-$tRNA_2^{Ile}$.

Indirect evidence of the translatability of the *bar* regions stems from previous work and the results presented in Fig. 1A, in which experiments the *bar*I and *bar*II regions are overexpressed by the use of multicopy plasmid constructs. Under these circumstances, minigenes become toxic in Pth-defective cells. Even though the *bar*I and *bar*II regions cloned in the constructions used in this work differ broadly in their nucleotide sequences, except for the ORF and a 6-bp tract beyond the termination codons, they show the same properties of cell growth inhibition and growth restoration by Pth or $tRNA_2^{Ile}$.

Toxicity (cell growth and protein synthesis inhibition) in Pth-defective cells is the result of tRNA sequestration as pep-tRNA during *bar* minigene overexpression from multicopy plasmids. This situation differs from the exclusion of lambda phage where both *bar*I and *bar*II presumably must be transcribed and translated to block phage development in Pth-defective cells. In addition, our data indicate that $tRNA_2^{Ile}$ is also sequestered as pep-$tRNA_2^{Ile}$. However, we have been unable to detect pep-$tRNA_2^{Ile}$ in total cell extracts by a Northern blot assay (16) because the concentration of $tRNA_2^{Ile}$ and presumably the corresponding pep-$tRNA_2^{Ile}$ levels produced in the cell are very low (6, 13). In this way, lambda phage development may be impaired in Pth-defective cells, because the scarce $tRNA_2^{Ile}$ is promptly sequestered as pep-$tRNA_2^{Ile}$, and this in turn is not readily hydrolyzed by the limiting Pth activity.

The pep-tRNA accumulated may provoke protein synthesis inhibition per se (1) and/or deplete the levels of tRNA under a critical concentration incompatible with phage development

and/or cell protein synthesis. Since an excess of specific tRNA in vitro (12) or in vivo (25) suppressed protein synthesis inhibition (12) and restored phage development in Pth-defective cells (Fig. 3B), the latter inference is more plausible.

We infer that minigene expression and pep-$tRNA_2^{Ile}$ production should occur during normal phage development in wild-type cells. However, as soon as pep-$tRNA_2^{Ile}$ is produced, it is hydrolyzed by normal Pth activity, and phage development is not impaired. It is feasible to attain high pep-tRNA levels in wild-type cells by expressing *bar*I from a pUC-based vector (J. G. Valadez, unpublished results). This plasmid occurs in about 10-fold more copies per cell than the pBR322-based vector (15) used to do the experiments in this work. An uncontrolled overexpression from this derivative is lethal even in wild-type cells. In addition, *bar*I overexpression using wild-type cell extracts also inhibits protein synthesis, albeit less stringently than with Pth-defective cell extracts (12). However, in these cases an exaggerated overexpression of a minigene or even a gene may compete with other genes for the translational machinery, leading to an unspecific inhibition of protein synthesis irrelevant for the $tRNA_2^{Ile}$-sequestering mechanism proposed in the present work (14). Thus, the amount of pep-$tRNA_2^{Ile}$ produced by wild-type lambda phage in wild-type cells or by mutant *bar*I or *bar*II phage in Pth-defective cells should not overcome the capacity of Pth activity to hydrolyze it. Accordingly, when the $tRNA_2^{Ile}$ pool was artificially reduced by overexpressing the $tRNA_2^{Ile}$-sequestering *bar*I minigene, the development of mutant *bar*I or *bar*II phage was also impaired in Pth-defective cells (Fig. 2D). These results are the basis of the argument that minigene-mediated toxicity or phage exclusion in Pth-defective cells depends on both the level of Pth activity and minigene expression. The suggestion that these phenomena are related to the $tRNA_2^{Ile}$-sequestering mechanism and the expression of AUA-containing *bar* minigenes is also supported, at least in the plasmid system, by the fact that the change of the rare AUA to the common synonymous AUU codon renders the *bar*I minigene nontoxic in Pth-defective cells (18; R. Cruz-Vera, unpublished results).

Experiments performed in vitro have shown different parameters affecting minigene toxicity, including the nature of the translational signals (Shine-Dalgarno sequence, initiation codon, stop codon, and last sense codon), pep-tRNA drop-off, pep-tRNA hydrolysis rate by Pth, and minigene recycling (12, 19, 25). Minigenes contain the necessary signals for translation; however, their toxicity is always associated with an inefficient translational termination and pep-tRNA release from the ribosome. This could be due in part to ribosome pausing at the rare AUA codon and to the proximity of the initiation and stop codons (5, 12).

A computer program designed to recognize potentially translatable short ORFs in prokaryote genomes identified 118 possible minigenes in lambda DNA. However, *bar*-like minigenes (toxic in Pth-defective bacteria) represented only 10% of the identified clones (18). Among these minigenes, *bar*I and *bar*II contribute greatly to the reduced lambda phage developing capacity in Pth-defective cells, as the mutations that affect the translatability of any of these minigenes indicate. However, their role in lambda biology is yet to be determined. Lambda phage might have evolved the *bar* minigene system for a fine translational downregulation of lambda genes containing the rare ATA codon. In fact, a computer analysis shows a high frequency of ATA-containing genes in the regulatory region of the lambda genome (F. de la Vega, unpublished results). Alternatively, this system might also be a general mechanism to inhibit host translation of ATA-containing genes, since the translation of *bar* minigenes poses an unusual demand on the cellular pool of $tRNA_2^{Ile}$.

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