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Conserved Residues Asp16 and Pro24 of TnaC-tRNA^{Pro} Participate in Tryptophan Induction of *tna* Operon Expression▽

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In *Escherichia coli*, interactions between the nascent TnaC-tRNA^{Pro} peptidyl-tRNA and the translating ribosome create a tryptophan binding site in the ribosome where bound tryptophan inhibits TnaC-tRNA^{Pro} cleavage. This inhibition delays ribosome release, thereby inhibiting Rho factor binding and action, resulting in increased *tna* operon transcription. Replacing Trp12 of TnaC with any other amino acid residue was previously shown to prevent tryptophan binding and induction of *tna* operon expression. Genome-wide comparisons of TnaC amino acid sequences identify Asp16 and Pro24, as well as Trp12, as highly conserved TnaC residues. Replacing these residues with other residues was previously shown to influence tryptophan induction of *tna* operon expression. In this study, in vitro analyses were performed to examine the potential roles of Asp16 and Pro24 in *tna* operon induction. Replacing Asp16 or Pro24 of TnaC of *E. coli* with other amino acids established that these residues are essential for free tryptophan binding and inhibition of TnaC-tRNA^{Pro} cleavage at the peptidyl transferase center. Asp16 and Pro24 are in fact located in spatial positions corresponding to critical residues of AAP, another ribosome regulatory peptide. Sparsomycin-methylation protection studies further suggested that segments of 23S RNA were arranged differently in ribosomes bearing TnaCs with either the Asp16Ala or the Pro24Ala change. Thus, features of the amino acid sequence of TnaC of the nascent TnaC-tRNA^{Pro} peptidyl-tRNA, in addition to the presence of Trp12, are necessary for the nascent peptide to create a tryptophan binding/inhibition site in the translating ribosome.

The *tna* degradative operon of *Escherichia coli* specifies two proteins, one responsible for tryptophan (Trp) degradation and one participating in Trp transport (6). Transcription of the *tna* operon of *E. coli* is regulated by two mechanisms, catabolite repression and inhibition of Rho factor-mediated transcription termination (7, 18, 24). Synthesis of the operon's leader peptidyl-tRNA appears to create a free Trp binding/inhibition site in the ribosome translating *tnaC* (3, 14). Binding of Trp to the translating ribosome induces ribosome stalling/pausing at the mRNA Rho binding site. This reduces Rho factor binding and action, thereby reducing transcription termination in the leader region of the operon (13, 15). When excess Trp is not present, transcription of the *tna* operon is prematurely terminated in the leader region of the operon by Rho factor action (15, 18, 24). In the presence of high, inducing levels of Trp, Rho termination is prevented, and transcription continues into the *tna* operon's two structural genes (15, 17). When the ribosome translating wild-type *tnaC* reaches its stop codon, a free Trp molecule is bound to the ribosome. This Trp molecule prevents peptidyl transferase activity (5) by inhibiting the action of release factor 2 (RF2). Inhibition of hydrolysis of the peptidyl-tRNA results in ribosome stalling at the TnaC stop codon. The stalled ribosome temporarily blocks the Rho factor-binding site on *tna* mRNA, preventing Rho binding and function (13). Trp action has been shown to depend on the presence of a Trp residue at position 12 (W12) of TnaC-tRNA^{Pro} (5, 11, 14). It is thought that W12 of TnaC-tRNA^{Pro} alters the positions of several nucleotides of 23S rRNA in the 50S ribosomal exit tunnel, contributing to the creation of a free Trp binding site in the peptidyl transferase center. Here, bound Trp inhibits RF2 action, preventing ribosome release (3, 5).

In addition to TnaC, there are other examples where nascent peptides can inhibit ribosome function (2, 8, 19, 21, 22). The mechanisms of inhibition appear to differ in different cases (2, 5, 8, 21). However, for each example it has been suggested that interaction of some specific residues of the nascent peptide with the ribosomal translational machinery plays a role in inhibition of ribosome function. Thus, in eukaryotes, expression of the arginine-specific carbamoyl-phosphate synthase small subunit is regulated by the cellular concentration of arginine (Arg) (16). As with Trp and TnaC-tRNA^{Pro}, Arg inhibits translation termination during synthesis of a small leader peptide designated the arginine attenuator peptide (AAP). A recent paper identifies a conserved motif in the AAP peptide sequence that could be essential for peptide function; some residues in this motif are required for Arg inhibition of ribosome function (16). This motif contains a conserved aspartic acid residue (D12 in *Neurospora crassa*) which has been shown to be important for translation inhibition by Arg (9, 25). Much like W12 of the TnaC peptide, D12 of AAP is located 13 residues from the codon where the ribosome is stalled by the presence of Arg (Fig. 1). In view of the similarities between TnaC and AAP, it is presumed that a common mechanism may be used in both, involving ribosome recognition of specific critical amino acids of the peptidyl-tRNA, leading to binding of an amino acid that inhibits ribosome function.

Previous studies using comprehensive mutagenesis analyses suggested that residues Asp16 (D16) and Pro24 (P24) of TnaC

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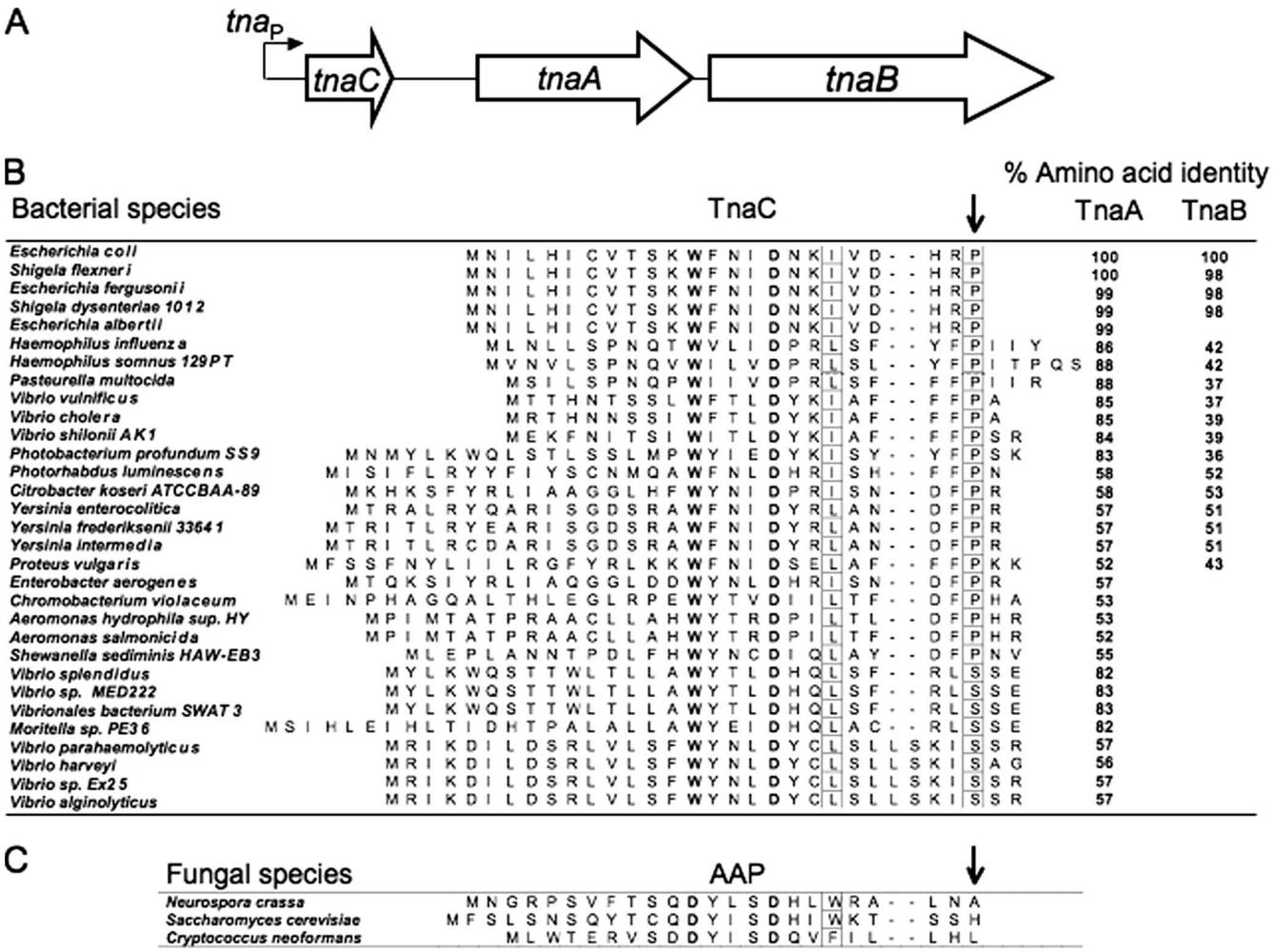

FIG. 1. Organization of the *tna* operon of *Escherichia coli* and alignment of the amino acid sequences of the TnaC peptides of different bacterial species. (A) Diagram of the general organization of the coding regions of the *tna* operon of *Escherichia coli*. The *tna* operon consists of a catabolite-repressible regulated promoter (*tnap*) (15), the *tnaC* coding region, and two structural genes, *tnaA*, encoding tryptophanase, and *tnaB*, encoding a low-affinity Trp permease. (B) TnaC peptide sequences predicted for different bacterial species were aligned and compared using a ClustalW program (see Materials and Methods). Most of the bacterial species shown are within the *Gammaproteobacteria* group. Twelve are in *Enterobacteriaceae*, 11 are in *Vibrionaceae*, 3 are in *Pasteurellaceae*, 2 are in *Aeromonadaceae*, 1 is in *Shewanellaceae*, and 1 is in *Moritellaceae*. Only one species shown, *Chromobacterium violaceum*, is in the *Betaproteobacteria* group. The percents identity of residues in tryptophanase (TnaA) and in the Trp permease (TnaB) to those in these proteins of *E. coli* (100%) are shown to the right of the corresponding TnaC peptides. (C) Alignment of three AAP peptide sequences. The bold letters in both alignments correspond to residues completely conserved at their respective positions. Letters inside boxes identify residues at positions where there is appreciable conservation. The arrows indicate the position of ribosome stalling following synthesis of TnaC of *E. coli* (15) or of AAP of *Neurospora crassa* (9), *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* (16).

may be optimal for *tna* operon induction by Trp (10, 18). However, no studies were performed to determine whether changing these residues affects induction by preventing Trp binding to the ribosome or acts by some other mechanism, e.g., interfering with Rho action. In the studies described in this paper, we performed in vitro analyses to examine the effects of D16 and P24 amino acid substitutions on Trp binding to the translating ribosome and on the ability of Trp to inhibit puromycin or sparsomycin binding and action on the ribosome. These two residues are highly conserved in TnaC from various bacterial species. Our findings suggest that D16 and P24, as well as Trp12, are essential for creating the Trp binding site in the translating ribosome at which bound Trp inhibits the peptidyl transferase reaction. Our analyses also suggest that these residues influence the locations of specific residues of 23S rRNA of the translating ribosome.

MATERIALS AND METHODS

Computational search for *tnaC* genes in various bacterial species. Potential TnaC peptides were identified by examining the predicted amino acid sequence encoded by a short open reading frame (ORF) located immediately upstream of the *tnaA* gene of different bacterial species. The structure of the *E. coli tna* operon was used for reference in this search (Fig. 1A). Initially, a standard computational search was performed to identify genes orthologous to *tnaA* of *E. coli* in *Eubacteria*. This search was performed with the BLAST web server hosted by the National Center for Biotechnology (NCBI). The search was performed using the tryptophanase amino acid sequence of *E. coli* and the protein-protein Basic Local Alignment Search Tool (BLASTp) program (1). The ORF for each presumed tryptophanase protein was identified and a putative transcriptional

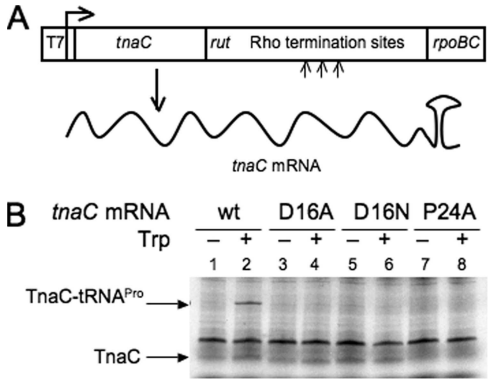

FIG. 2. Analysis of the accumulation of TnaC-tRNA-ribosome-mRNA complexes upon Trp addition in vitro. (A) Diagram of PCR-derived DNA fragments used for preparation of mutant mRNAs for TnaC-tRNA-ribosome complex synthesis and isolation. The template DNA contained a T7 promoter and the entire *tna* operon leader region, with (i) the coding region for the 24-residue TnaC leader peptide; (ii) a *rut* site, which as RNA is the site used for Rho factor binding; and (iii) multiple optional sites of Rho-dependent transcription termination, the initial segment of the *tnaA* gene, and the *rpoBC* terminator sequence. (B) Translation in vitro was performed using the *tnaC* mRNAs indicated in each lane, using cell extracts prepared from the strain A19 RNase I$^-$. The accumulation of TnaC-tRNA peptidyl-tRNA was monitored by the incorporation of [^{35}S]methionine into the TnaC peptide in the absence (−) or presence (+) of 1 mM Trp. The final products of the reactions were resolved, and the dried gel was exposed to an X-ray film (see Materials and Methods). The positions of TnaC-tRNA^{Pro} and the TnaC peptide are shown with arrows. A prominent contaminant band, located just above the TnaC band, is generally observed. wt, wild type.

promoter was designated using a neural network promoter prediction server (23). Finally, a search of potential ORFs between the designated transcriptional promoter and the start codon of the tryptophanase ORF was performed to identify the corresponding *tnaC* gene, using the ORF finder web server hosted by NCBI. Each *tnaC* ORF was used to predict its presumed corresponding peptide product. The amino acid sequences of the predicted TnaC peptides were compared using a ClustalW web server hosted by the European Bioinformatics Institute.

Mutagenesis of a plasmid-borne *tnaC* gene and synthesis of *tnaC* mRNAs. Directed mutations were introduced at different positions in the *tnaC* gene present in pGF25-00, an ampicillin-resistant plasmid bearing the *tna* promoter and the initial segment of the *tna* operon leader region. The plasmid contained the wild-type *tna* operon 5′ regulatory leader region, followed by sequences specifying the *rpoB* transcriptional terminator (Fig. 2A). Nucleotides in *tnaC* of pGF25-00 were changed using a QuikChange mutagenesis PCR kit (Stratagene) and a specific complementary pair of deoxyoligonucleotide sequences, as previously reported (5); for changing the residue Asp16 to Ala, the deoxyoligonucleotide sequences used were 5′-CTCAAAATGGT TCAATATTGCCAACAAAATTGTCGATCACC-3′ and 5′-GGTGATCGA CAATTTTGTTGGCAATATTGAACCATTTTGAG-3′; for changing the residue Asp16 to Asn, the deoxyoligonucleotide sequences used were 5′-CT CAAAATGGTTCAATATTAACAACAAAATTGTCGATCACC-3′ and 5′-GGTGATCGACAATTTTGTTGTTAATATTGAACCATTTTGAG-3′; and for changing the residue Pro24 to Ala, the deoxyoligonucleotide sequences used were 5′-GTCGATCACCGCGCTTGATTTGCCTTC-3′ and 5′-GAAG GCAAATCAAGCGCGGTGATCGAC-3′. *tnaC* mutant plasmids were used as templates for amplification of a DNA segment by use of PCR standard procedures. The resulting DNA fragments contained a T7 transcriptional promoter, the region between the *tna* +1 transcriptional start site and the *tnaA* start codon, followed by the *rpoB* transcription terminator sequence (Fig. 2A). These DNA fragments, containing mutant *tnaC* genes, were used to synthesize mRNAs with a T7 maxi RNA synthesis kit (Promega). The transcription reactions were performed with or without 0.7 mM biotin 16-UTP (Roche) in the presence of 7 mM of each of the ribonucleoside triphosphates. The synthesized *tnaC* mRNAs and the biotinylated *tnaC* mRNAs were purified as previously described (5).

In vitro translation assays and isolation of TnaC-tRNA-ribosome-*tnaC* mRNA complexes. In vitro translation assays using [^{35}S]methionine were performed as previously described (5). Cell-free translational competent extracts were prepared from a derivative of strain A19 RNase I$^-$ (*trpR ΔlacZ ΔtrpEA2 tnaA bgl*::Tn*10*) (15). In the experiments described in the legend to Fig. 2, the in vitro translation reactions were performed using 2 μg of *tnaC* mRNA per 50 μl of a reaction mixture. In the experiments described in the legends to Fig. 3 to 5, the isolated complexes examined were obtained from in vitro translation reactions performed using 2 μg of biotinylated *tnaC* mRNA per 50-μl reaction mixture.

Isolated TnaC-tRNA-ribosome-*tnaC* mRNA complexes were used in the experiments described in the legends to Fig. 3 to 5. Cell extracts were pretreated with an anti-RF2 antiserum to remove all the RF2 (12); ~40-μl volumes of the treated extracts were then used in 250-μl in vitro translation reaction mixtures. Two different reactions with biotinylated *tnaC* mRNAs were carried out: (i) one in the presence of unlabeled amino acids, in the experiments whose results are shown in Fig. 4A, and (ii) one in the presence of [^{35}S]methionine, for the experiments whose results are shown in Fig. 3, 4B, and 5. After the translation reactions were completed, stalled ribosomes bound to biotinylated mRNAs were isolated using streptavidin paramagnetic beads and an electromagnetic field, as previously described (5). The isolated complexes were resuspended in 200 μl of a buffer solution containing 35 mM Tris-acetate (pH 8), 10 mM magnesium acetate, 175 mM potassium glutamate, 10 mM ammonium acetate, and 2 mM dithiothreitol (3). Importantly, the final solutions containing the isolated complexes were examined the same day they were isolated.

Puromycin-tryptophan competition assays. For the experiments whose results are shown in Fig. 3 and 5, 20-μl volumes of isolated complexes were mixed with different concentrations of puromycin in the presence or absence of 2 mM Trp.

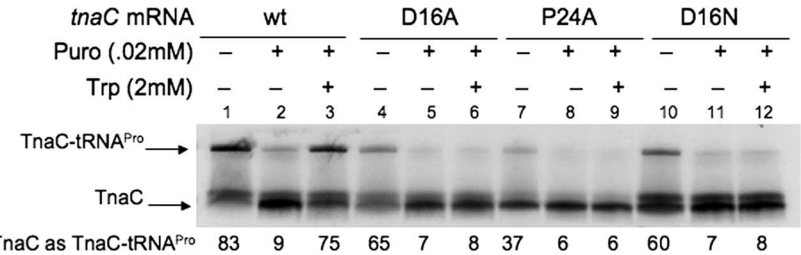

FIG. 3. Competition assays between puromycin and Trp, using isolated TnaC-tRNA-ribosome complexes. In vitro translation reactions were performed with [^{35}S]methionine to label the TnaC peptides, as indicated in Fig. 2. The cell extracts employed were pretreated with an anti-RF2 antiserum, and biotinylated *tnaC* mRNAs were used in each reaction. The TnaC-tRNA-ribosome-mRNA complexes were isolated from the in vitro translation reactions by using streptavidin beads, and the isolated complexes were initially incubated with (+) or without (−) 2 mM Trp at 37°C for 5 min. Later, the complexes were incubated with (+) or without (−) 0.02 mM puromycin (Puro) at the same temperature for 10 additional minutes (3) and then the reactions were stopped. The final products of the reaction were resolved, and their accumulation was determined by measuring radioactivity (see Materials and Methods). The percentage of the TnaC as TnaC-tRNA was calculated by dividing the cpm in each TnaC-tRNA band by the combined cpm in the TnaC-tRNA and TnaC bands. Note that there was less TnaC-tRNA detected in all the mutant extracts in the absence of puromycin and Trp. wt, wild type.

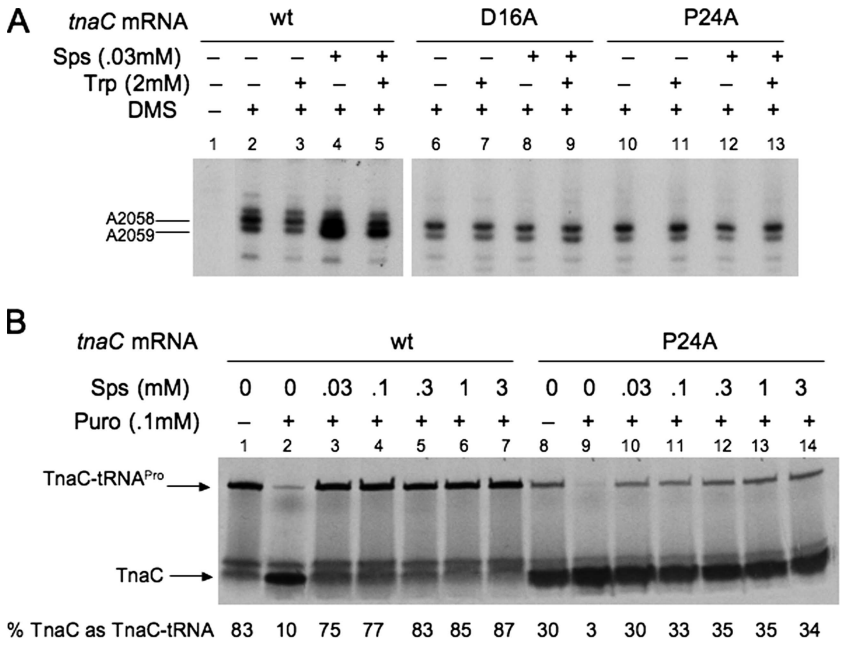

FIG. 4. Action of the antibiotic sparsomycin on different isolated TnaC-tRNA-ribosome-mRNA complexes. (A) Complexes isolated as indicated in Fig. 3 were prepared using the biotinylated *tnaC* mRNA designated for each panel. The isolated complexes were preincubated for 5 min with (+) or without (−) 0.03 mM sparsomycin (Sps) at 37°C. They were then incubated for five additional minutes at the same temperature in the presence (+) or absence (−) of 2 mM Trp. Finally, the mixtures were exposed to the methylating reagent DMS to modify the A2058 and A2059 adenine nucleotides of 23S rRNA. A sample was incubated in the absence of DMS as a methylation control (lane 1). The rRNA isolated from the final mixtures was used to perform primer extension analyses (see Materials and Methods). Horizontal lines mark the positions for the adenines at positions 2058 and 2059. wt, wild type. (B) ^{35}S-Met-labeled, isolated complexes containing a wild-type or P24A mutant mRNA were incubated with the concentration of sparsomycin (Sps) indicated in each lane for 5 min at 37°C. Then, 0.1 mM puromycin (Puro) was added (+) or not (−) to the mixes and the final solutions were incubated for an additional 10 min. The final products of the reaction were resolved by electrophoresis, and the percentage of TnaC as TnaC-tRNA remaining in each reaction mixture was calculated as indicated for Fig. 3.

The TnaC transfer reaction from TnaC-tRNA to the added puromycin was stopped by adding a loading buffer containing 100 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 30% glycerol, and 0.03% bromophenol blue. The products of the reaction were resolved by electrophoresis in 10% Tris-Tricine polyacrylamide gels, and the gels were dried before exposing them to X-ray films (3). The bands corresponding to TnaC-tRNA and TnaC were excised from the dried gel, and their radioactivity (in counts per minute [cpm]) was determined using a scintillation counter. The percentage of the total TnaC peptide observed that was TnaC-tRNA was calculated to determine the stability of the peptidyl-tRNA during the assays, as indicated in Fig. 3.

Sparsomycin-tryptophan competition assays. In the experiments whose results are shown in Fig. 4, 50-μl samples of isolated complexes, as well as isolated complexes containing 0.03 mM sparsomycin, were incubated for 5 min at 37°C. Then, some samples were mixed with 2 mM Trp and all samples were incubated for an additional 5 min at the same temperature. Methylation-protection assays were performed with the final mixtures as previously described (3). The solutions were mixed with 1/150 (vol/vol) of the methylation agent dimethyl sulfate (DMS) and incubated at room temperature for 10 min. The methylation reaction was stopped by adding 25 μl of a solution containing 1 M Tris-HCl (pH 8) and 1.4 M 2-mercaptoethanol. The final reaction solution was mixed with 325 μl of 1 mM EDTA, total RNA was extracted using phenol plus chloroform, and the RNA was precipitated with 2 volumes of ethanol. Approximately 2 μg of total RNA was used in primer extension analyses by reverse transcription (AMV; Gibco) with 5′-^{32}P-labeled deoxyoligonucleotide primers. An oligonucleotide sequence (5′-CTATCCTACACTCAAGGCTC-3′) complementary to nucleotides 2102 to 2122 of 23S rRNA was used to detect modified nucleotides in 23S rRNA.

RESULTS AND DISCUSSION

Conserved residues in TnaC peptide sequences. Previous mutagenesis/expression analyses with the TnaC leader peptide

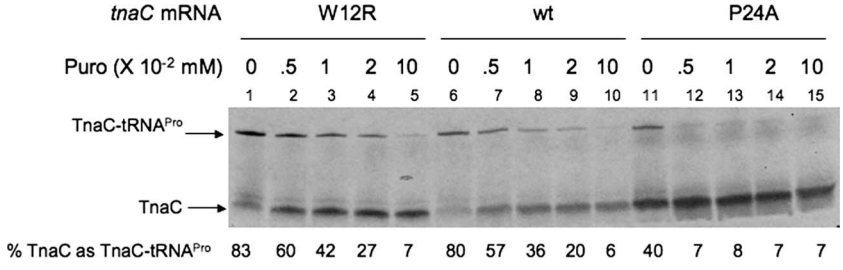

FIG. 5. Stability of the TnaC-tRNA of different TnaC-tRNA-ribosome-mRNA complexes in the presence of puromycin. Isolated biotinylated complexes free of RF2 were prepared as indicated in Fig. 3. The complexes were incubated in the absence of Trp with different concentrations of puromycin for 10 min at 37°C. The percentage of the TnaC remaining as TnaC-tRNA was calculated as indicated for Fig. 3. The positions of TnaC-tRNA^{Pro} and of TnaC are marked with horizontal arrows. wt, wild type.

of *E. coli* have shown that residues in addition to W12 are needed for induction (10, 18). To extend these findings, we compared the predicted sequences of the TnaC leader peptides of other bacterial species, searching for conserved amino acid residues. This search was performed using standard computational resources (see Materials and Methods). Comparisons of these TnaC peptide sequences revealed several conserved residues. These genome comparisons are summarized in Fig. 1B. Thirty-one strains and species of bacteria contained a *tnaC* ORF upstream of the *tnaA* gene. However, unlike for *tna* operon organization in *E. coli*, not all of these organisms contained a permease gene (a putative *tnaB* gene) following *tnaA*. The lengths of the predicted TnaC peptides varied, ranging from 23 to 36 residues per peptide. The amino-terminal segment was highly diverse, both in size and in amino acid content, whereas the carboxyl-terminal segment was highly conserved. These observations support previous findings with TnaC of *E. coli*, where additions, deletions, and replacements in the carboxyl-terminal portion, unlike changes in the amino-terminal segment, had significant effects on free-Trp-induced ribosome stalling (14).

Alignment of predicted TnaC amino acid sequences revealed one absolutely conserved residue in addition to W12, the Asp residue at position 16 (D16) (Fig. 1B). Two semiconserved residues were also evident, Leu at position 19 (L19) and Pro at position 24 (P24) (Fig. 1B). As was observed when W12 was replaced by other residues, replacing Asp16 with glutamine prevented *tna* operon induction by Trp (10). It was also observed that replacing L19 with threonine or asparagine reduced *tna* operon induction (10) and that changing P24 to threonine or serine also reduced induction (18). These observations support the importance of the W12 residue of TnaC in peptide function and suggest that along with W12, other residues of TnaC are involved in optimizing the Trp induction process. Several 23S rRNA nucleotides and L22 ribosomal protein residues have also been shown to be required for Trp induction. Thus, adding an extra adenylate in the 750 region, replacing A752 or U2609 of 23S rRNA with other nucleotides, or changing the K90 residue of the L22 protein reduced or eliminated Trp binding to the ribosome and prevented Trp inhibition of the function of the peptidyl transferase center (4). We also compared the 23S rRNA nucleotide sequences and the L22 protein sequences from bacteria containing a *tnaC* gene. The results revealed that these 23S rRNA nucleotide sequences and L22 protein residues, essential for Trp induction in *E. coli*, are conserved (not shown). The finding that key TnaC and ribosomal residues essential for induction are conserved in different species suggests that these organisms may use the same mechanism in recognizing the key features of the TnaC peptide that create a Trp binding site in the ribosome at which bound Trp inhibits the peptidyl transferase reaction.

In *E. coli*, Trp inhibits translation termination by inducing ribosome stalling at the *tnaC* mRNA stop codon (5, 12). In these stalled complexes, the translating ribosome contains the TnaC-tRNA^{Pro} peptidyl-tRNA (5). The last residue of the nascent TnaC peptide, P24, is putatively located in the peptidyl transferase center of the ribosome (Fig. 1B) (5). W12 of this stalled peptidyl-tRNA is located in the narrow region of the ribosome exit tunnel (5). It has been shown that the spacing between these two residues, W12 and P24, is essential for Trp inhibition in TnaC (14). In the alignment in Fig. 1B, with the exception of the bottom four *Vibrio* species, W12 is always separated from P24 by 12 residues, and the second conserved residue, D16, is invariably separated from P24 by 8 residues. Similar arrangements are evident in the fungal regulatory peptide AAP (Fig. 1C) (16). Thus, the spacing between the conserved D12 and D16 residues in AAP (*Neurospora crassa*) relative to the stalled ribosome position induced by arginine is comparable to the spacing between W12 and D16 of TnaC of *E. coli* relative to the TnaC stalling position (Fig. 1C). These comparisons suggest that conserved residues in TnaC and those of the AAP probably play somewhat similar roles during inhibition of the peptidyl transferase reaction by the corresponding free amino acid.

Role of conserved residues in the ribosome in *tna* operon induction by Trp. *tna* operon expression depends on competition between two molecular events: (i) Trp induction of ribosome stalling at the *tnaC* stop codon and (ii) Rho factor binding, causing transcription termination (13). It has been proposed that changes in the TnaC peptide have their primary effect by reducing Trp induction of ribosome pausing (14, 18). To analyze the roles of the conserved residues in TnaC, in vitro expression was examined with mutant *tnaC* mRNAs; these mutant mRNAs contained changes in the codons that are normally conserved. These results are summarized in Fig. 2B. As expected, changes induced in the absolutely conserved residue D16 by Ala or Asn eliminated inhibition of translation termination by Trp (not shown). Thus, during translation of *tnaC* (D16) mutant mRNA, unlike translation of wild-type *tnaC* mRNA, there was no detectable TnaC-tRNA^{Pro} evident in the presence of Trp (Fig. 2B, compare lane 2 with lane 4 or 6). Also, no detectable TnaC-tRNA^{Pro} was detected following replacement of residue D16 with Glu or Trp (not shown). Similarly, changes in the semiconserved P24 residue of TnaC also prevented the accumulation of the corresponding TnaC-tRNA in the presence of Trp. Previous in vitro assays using P24S and P24A *tnaC* mutant genes expressed from DNA plasmids indicated that residue P24 is important for Trp induction of ribosomal pausing (14). We confirmed these findings by using mRNAs in which the D16 codon was replaced by an Ala codon (Fig. 2B, compare lane 2 with lane 8); other changes in which D16 was replaced by Ser, Leu, Cys, Val, or Lys had the same negative effect as the Ala replacement (not shown). These results indicate that the conserved and semiconserved residues D16 and P24 are important for Trp induction of ribosome pausing. As controls, two mutants bearing changes in nonconserved TnaC residues were also tested: I15, located between the W12 and D16 residues, and K18, between the D16 and P24 residues. Mutant mRNAs containing *tnaC*(*I15A*) or *tnaC*(*K18A*), like wild-type *tnaC* mRNA, led to the accumulation of their corresponding TnaC-tRNA^{Pro} peptidyl-tRNA in the presence of Trp (not shown). These results suggest that only specific residues in the TnaC peptide are involved in the inhibition of the ribosome function in the presence of Trp. Thus, residues D16 and P24, as well as W12, appear to be important for Trp induction of ribosome pausing.

How do these residues and their mutant replacements affect Trp inhibition of TnaC-tRNA cleavage? Changes in residue W12 of TnaC have been shown to reduce or eliminate the ability of added Trp to inhibit the hydrolysis of TnaC-tRNA by

RF2 and the TnaC transfer reaction from TnaC-tRNA to puromycin (12). Puromycin, like RF2, interacts in the ribosomal A site, suggesting that the primary target of Trp is altering some feature of the peptidyl transferase center (3, 4). Competition experiments between Trp and puromycin were performed to determine if Trp was still capable of binding to the ribosome and inhibiting puromycin action, with D16A or P24A mutant templates. These assays were performed using isolated RF2-deficient, stalled-ribosome complexes containing *tnaC* (D16 or P24) mutant mRNAs (see Materials and Methods). The results obtained are shown in Fig. 3. Initially we observed that the changes D16A and P24A appreciably reduced the percentage of TnaC recovered as TnaC-tRNA in isolated mutant complexes. Normally, with isolated wild-type complexes, between 80 and 85% of the TnaC is recovered as TnaC-tRNA. But with D16A and, more significantly, with P24A, this level was reduced to 64% and 37%, respectively (Fig. 3, compare lane 1 with lane 4, 7, or 10). Despite the lower percentage of peptidyl-tRNA recovered, after purification we observed that the remaining mutant peptidyl-tRNAs were as stable as the wild-type peptidyl-tRNA under the reaction conditions employed (not shown). We believe that residual trace RF2 activity in our anti-RF2 pretreated cell extracts may be responsible for hydrolysis of some of the TnaC-tRNA in the mutant complexes (see below). Nevertheless, recovery of some mutant peptidyl-tRNAs after purification allowed us to perform Trp-puromycin competition assays. As expected, the wild-type and mutant TnaC-tRNAs in ribosome complexes lacking RF2 were able to transfer their TnaC peptide to puromycin (Fig. 3, lanes 2, 5, 8, and 11). Addition of Trp inhibited the TnaC transfer reaction from TnaC-tRNA to the added puromycin in complexes containing wild-type TnaC-tRNA^{Pro} but did not inhibit the transfer reaction in complexes with mutant TnaC-tRNA (Fig. 3, compare lane 3 with lanes 6, 9, and 12). Thus, the mutant changes examined appear to affect Trp binding and/or position in the translating ribosome.

Important interactions between TnaC-tRNA and the ribosome essential for Trp induction. With wild-type TnaC-tRNA complexes, the antibiotic sparsomycin, a peptidyl transferase inhibitor, has been shown to compete with Trp for a binding site in the ribosome (3, 4). Changes in the conserved residue W12, as well as changes in exit tunnel nucleotides of 23S RNA, affected the ability of Trp to compete with sparsomycin (3, 4). These findings are in agreement with the observations mentioned above for puromycin, suggesting that Trp acts at the peptidyl transferase center. Interactions between sparsomycin and the ribosome can be detected as changes in the methylation of the 23S rRNA nucleotide adenine 2059 (A2059); protection from methylation is altered by the addition of sparsomycin to wild-type TnaC-tRNA-ribosome complexes (3). Competition experiments between sparsomycin and Trp were therefore performed to analyze the effects of D16 and P24 substitutions in TnaC on Trp binding (Fig. 4A). Methylation-protection assays using DMS were performed with isolated complexes as previously reported (4). As expected, the presence of sparsomycin enhanced methylation of nucleotide A2059 in isolated complexes with wild-type *tnaC* mRNA (Fig. 4A, compare lane 2 with lane 4). However, this enhancement was reduced by the addition of Trp (Fig. 4A, compare lane 4 with lane 5). Unexpectedly, addition of sparsomycin without added Trp to isolated complexes containing a mutant *tnaC* (D16A or P24A) mRNA did not increase methylation of A2059 (Fig. 4A, compare lane 6 with lane 8 and lane 10 with lane 12). Addition of Trp did not have any noticeable effect with this assay (Fig. 4A, compare lane 7 with lane 9 and lane 11 with lane 12). These results were not due to the absence of interaction of sparsomycin with the ribosomes containing mutant TnaC-tRNAs. We found, using different concentrations of sparsomycin, that this antibiotic protects the mutant TnaC(P24A)-tRNA as well as the wild-type TnaC-tRNA from transferring its TnaC peptide to puromycin (Fig. 4B), even at lower ratios of sparsomycin-puromycin (c.a. 1/3) (Fig. 4B, compare lane 9 with lane 10). Thus, sparsomycin and puromycin continue to bind to ribosomes bearing TnaC mutations. Therefore, although we were unable to test for competition between sparsomycin and Trp in methylation experiments with mutant complexes, the results obtained suggest that following interaction of sparsomycin with the ribosome, wild-type TnaC normally displaces nucleotides in some segments of the ribosome exit tunnel, resulting in repositioning of residues A2058 and A2509. Our findings indicate that the D16 and Pro24 residues of TnaC are important for this displacement. It is therefore likely that the positions occupied by these, and perhaps other, amino acid residues of the mutant TnaC peptides in the ribosome are at locations somewhat different from those occupied by residues of wild-type TnaC. Whether this displacement contributes in any way to the creation of the free Trp binding site in the ribosome is unknown.

Peptidyl transferase center activity in the presence of the P24 residue. Increased affinity (or activity) of RF2 for the translating ribosome could explain the reduction in the percentage of TnaC(P24A)-tRNA^{Ala} recovered in our isolated complexes (Fig. 3, lane 7). To examine this possibility, hydrolysis assays were performed using different concentrations of puromycin, an analog of RF2 activity, with isolated ribosome complexes containing *tnaC*(*P24A*) mutant mRNA (Fig. 5). Wild-type and W12R mRNAs were run as controls. The results obtained show that, in the absence of added Trp, higher concentrations of puromycin are needed to transfer the TnaC peptide to this molecule from wild-type TnaC-tRNA^{Pro} and TnaC(W12R)-tRNA^{Pro}, a noninducible control, than from TnaC(P24A)-tRNA^{Ala} (Fig. 5, compare lane 1 with lane 4, lane 6 with lane 9, and lane 11 with lane 12). These results suggest that the presence of the wild-type P24 residue in the TnaC-tRNA^{Pro} peptidyl-tRNA may reduce the affinity of puromycin for the ribosome. Thus, TnaC(P24A)-tRNA^{Ala} appears to increase transfer of the TnaC(P24A) peptide to puromycin; a similar interpretation may be offered for the hydrolysis induced by RF2. This finding could explain the reduction in basal level expression of the *tna* operon (without Trp) that was observed previously for *tna-lacZ* reporter gene constructs containing TnaC P24 mutations (18). Presumably, the presence of P24 in the peptidyl transferase center alters the locations of some 23S nucleotides, influencing the functions of the peptidyl transferase center (20). These changes could contribute to free Trp binding and inhibition of RF2 and ribosome function.

Conclusions. In vitro assays performed with isolated wild-type and mutant TnaC-tRNA-ribosome complexes have identified crucial residues in TnaC: W12, D16, and P24. These residues in TnaC are required for Trp induction of *tna* operon expression. Thus, replacing the conserved wild-type TnaC residues D16 and P24 with other residues (Fig. 1) reduced or eliminated the ability of Trp to inhibit ribosomal peptidyl

transferase activity, as well as eliminating Trp inhibition of puromycin action (Fig. 2 and 3). Our findings suggest that residues D16 and Pro24 may affect TnaC positioning within the ribosomal exit tunnel, altering some amino acid-nucleotide interactions (Fig. 4). Thus, sparsomycin appears to act differently with wild-type and mutant complexes. Furthermore, the presence of P24 in TnaC appears to diminish peptidyl transferase center activity considerably (Fig. 5). This finding is consistent with recent studies showing that of all the amino acids, the presence of a Pro residue of a peptidyl tRNA in the ribosomal P site has the greatest effect on transfer of the peptide to a puromycin molecule (20). Even though Pro24 of TnaC-tRNA presumably reduces peptidyl transferase activity, free Trp binding to the ribosome is essential for induction of ribosome stalling; however, the presence of Pro24 allows Trp binding and induction (Fig. 2B) (14, 17). On the basis of our results and those of previous studies (10, 14, 18), we conclude that the conserved residues W12, D16, and P24 of TnaC probably play an important role in creating a Trp binding site in the ribosome where Trp inhibits the peptidyl transferase reaction. In studies with the AAP-Arg system, conserved residues at the same relevant positions are essential for peptide function (Fig. 1) (16). Both systems appear to employ similar mechanisms, with ribosome activity affected by a short nascent peptide and a free amino acid. Moreover, in both examples the distances between the ribosome stalling position and conserved peptide residues are identical (Fig. 1). Structural studies will be required to establish the precise mechanism of leader peptide and free Trp action.

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