Ribosomal Features Essential for *tna* Operon Induction: Tryptophan Binding at the Peptidyl Transferase Center[▽]

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Features of the amino acid sequence of the TnaC nascent peptide are recognized by the translating ribosome. Recognition leads to tryptophan binding within the translating ribosome, inhibiting the termination of *tnaC* translation and preventing Rho-dependent transcription termination in the *tna* operon leader region. It was previously shown that inserting an adenine residue at position 751 or introducing the U2609C change in 23S rRNA or introducing the K90W replacement in ribosomal protein L22 prevented tryptophan induction of *tna* operon expression. It was also observed that an adenine at position 752 of 23S rRNA was required for induction. In the current study, the explanation for the lack of induction by these altered ribosomes was investigated. Using isolated TnaC-ribosome complexes, it was shown that although tryptophan inhibits puromycin cleavage of TnaC-tRNA^{Pro} with wild-type ribosome complexes, it does not inhibit cleavage with the four mutant ribosome complexes examined. Similarly, tryptophan prevents sparsomycin inhibition of TnaC-tRNA^{Pro} cleavage with wild-type ribosome complexes but not with these mutant ribosome complexes. Additionally, a nucleotide located close to the peptidyl transferase center, A2572, which was protected from methylation by tryptophan with wild-type ribosome complexes, was not protected with mutant ribosome complexes. These findings identify specific ribosomal residues located in the ribosome exit tunnel that recognize features of the TnaC peptide. This recognition creates a free tryptophan-binding site in the peptidyl transferase center, where bound tryptophan inhibits peptidyl transferase activity.

There are several known systems where a nascent peptide can act in *cis* to inhibit the completion of its synthesis or of its release from the ribosome (3, 8, 10, 17, 19, 21). It appears that certain nascent peptidyl-tRNAs, acting either alone or with the aid of an accessory molecule, can promote changes in the ribosomal polypeptide exit tunnel that lead to the prevention of amino acid additions or peptidyl-tRNA cleavage at the peptidyl transferase center.

The leader segment of the *tna* operon transcript contains a coding region, *tnaC*, for a 24-residue leader peptide. Following *tnaC*, there is a short untranslated RNA segment containing sites of Rho factor binding and action (28). This leader regulatory region is followed by the coding regions of two structural genes, *tnaA* and *tnaB* (6). *tnaA* encodes the enzyme tryptophanase, which can degrade tryptophan (Trp) to indole, pyruvate, and ammonia by a reversible reaction. Pyruvate and ammonia may then be used as carbon and nitrogen sources, and indole can function as a volatile signal molecule in quorum sensing and in biofilm formation (22, 29). *tnaB* encodes a Trp permease that transports this amino acid into the cell (6). Initiation of transcription of the *tna* operon is regulated by catabolite expression (6). Transcription of the *tnaA-tnaB* region of the operon is regulated by transcription termination/antitermination in response to ribosome sensing of the operon's anti-termination inducer, Trp, during translation of *tnaC* (28). When the initial ribosome translating *tnaC* reaches the *tnaC* stop codon, Trp, if present at a sufficient concentration, binds

to the translating ribosome and inhibits translation termination (10). This inhibition results in ribosome stalling at the *tnaC* stop codon. The stalled ribosome then blocks the Rho factor binding site in the *tna* transcript, thereby preventing Rho from binding and terminating transcription in the leader region of the operon just downstream from *tnaC*. This blockage of Rho binding permits transcription to continue into the *tnaA* and *tnaB* structural genes (28). In other studies, it was shown that the stalled ribosome is eventually released by the combined action of two proteins, ribosome recycling factor and release factor 3 (13a).

Inhibition of translation termination at the *tnaC* stop codon by Trp results in the retention of uncleaved TnaC-tRNA^{Pro} within the stalled translating ribosome (13). This stalling has been shown to depend on features of the amino acid sequence of the 24-residue TnaC nascent peptide as well as on features of the ribosome (5, 28). Comparison of the TnaC amino acid sequences from different microorganisms revealed the presence of a highly conserved Trp residue at position 12 (Trp12). This Trp residue has been shown to be essential for induction (28). The carboxyl-terminal residue of TnaC, proline (P24), also is conserved and also appears to be essential for Trp-mediated induction (12). Recent findings suggest that residue Trp12 of TnaC may be essential for free Trp binding to the translating ribosome (4). However, specific nucleotides and amino acids that form the peptide exit tunnel in the 50S ribosomal subunit have also been shown to be important for Trp-mediated induction (5). Thus, the nucleotide replacement U2609C, or an adenylate insertion at position 751, in 23S rRNA as well as the K90W replacement in the L22 ribosomal protein reduced or eliminated Trp-mediated induction (5). Cross-linking analyses have demonstrated that Trp12 of TnaC

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TABLE 1. Strains of *E. coli* K-12 and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
W3110	Wild-type strain containing all the <i>rnm</i> operons and the <i>rplV</i> wild-type gene	Our collection
SR-14	$\Delta 7rm \Delta lacZYA \Delta recA prnCsacB$ ptRNA67 (λ <i>tna_p</i> <i>tnaA'</i> - <i>lacZ</i>)	5
Plasmids		
pNK	Wild-type <i>rnmB</i> operon; Amp ^r	19
pNH153	Derived from pNK; has an A insertion at position 751 in the 23S rRNA gene	19
pKKU2609C	Derived from pNK; has a T-to-C replacement at position 2609 in the 23S rRNA gene	9
pNKA752C	Derived from pNK; has an A-to-C replacement at position 751 in the 23S rRNA gene	This study
pNKA752T	Derived from pNK; has an A-to-T replacement at position 751 in the 23S rRNA gene	This study
pL22K90W	Has an <i>rplV</i> gene with a lysine-to-tryptophan codon change at position 90; Amp ^r	5
pGF25-00	Derived from pUC18 and has wild-type <i>tnaC</i> and the <i>rpoBC</i> terminator	13
pGF25-14	Derived from pUC18 and has a noninducible mutant <i>tnaC</i> (<i>Trp12Arg</i>) and the <i>rpoBC</i> terminator	13

is located in the vicinity of residues that occupy the narrow region of the ribosome exit tunnel, suggesting the possibility that Trp12 of TnaC interacts with nucleotides U2609 and A751 and residue K90 of the L22 protein and that these interactions are essential for induction (5). However, it was not known if they affect Trp action at the peptidyl transferase center.

In this study, we examined the effects of these specific ribosomal mutations on Trp action. Using isolated wild-type and mutant ribosome complexes, we determined the ability of added free Trp to inhibit puromycin and sparsomycin action at the peptidyl transferase center. We also examined the effects of the presence of Trp on the protection of key 23S rRNA residues from methylation. Our findings establish that interactions between the TnaC nascent peptide and ribosomal residues in the exit tunnel region create a unique free Trp binding site at which this bound amino acid inhibits peptidyl transferase activity. In the critical mutants examined, the loss of induction appears to be due to the loss of Trp binding.

MATERIALS AND METHODS

In vitro translation assay and isolation of biotinylated mRNA-ribosome complexes. The *Escherichia coli* K-12 strains and plasmids used in this study are described in Table 1. To prepare S-30 cell extracts, we used *E. coli* strains derived from strain SR-14 (5), in which plasmid *prnCsacB* was replaced with one of the following plasmids: pNK (*rnmB* operon) (19), pNH153 (*rnmB* operon with an insertion of an adenine at position 751 in the 23S rRNA gene) (19), pKKU2609C (*rnmB* operon with a uridine-to-cytosine change at position 2609 in the 23S rRNA gene) (9), pNKA752C (*rnmB* operon with an adenine-to-cytosine change at position 752 in the 23S rRNA gene), or pNKA752T (*rnmB* operon with an adenine-to-thymidine change at position 752 in the 23S rRNA gene). S-30 cell extracts were also prepared from wild-type strain W3110 transformed with plasmid pL22K90W (containing an *rplV* mutant gene that specifies an L22 protein with a lysine-to-Trp change at position 90) (5). The S-30 extracts were prepared from 3-liter cultures (optical density at 600 nm of 0.7) grown in Luria broth plus 100 μ g/ml ampicillin and 2% glucose. The harvested cells were resuspended in 20 ml of a solution containing 50 mM Tris-acetate (pH 8.0), 10 mM magnesium acetate, 175 mM potassium acetate, 10 mM ammonium acetate, 2 mM dithiothreitol (DTT), and 100 μ g/ml phenylmethylsulfonyl fluoride and were disrupted using a French press. The debris was separated by centrifugation at 30,000 $\times$ g for 30 min, and the supernatant was distributed as small samples within each tube. The aliquots were frozen using dry ice and stored at -70°C . Biotinylated mRNAs were prepared by using DNA fragments obtained by PCR using plasmid pGF25-00 (*tnaC* mRNA) or pGF25-14 [*tnaC*(*Trp12Arg*) mRNA] (13), as described previously by Cruz-Vera et al. (4). The in vitro translation reactions were performed essentially as described previously (13). The mixture (100 μ l) contained 40 mM Tris-acetate (pH 8.0), 2 mM DTT, 0.5 mM GTP, 30 mM phosphoenolpyruvate, 0.3 U/ml pyruvate kinase, 2 mM ATP, 3.5% (wt/vol) polyethylene glycol 8000, 20 μ g/ml folinic acid, 10 mM ammonium acetate, 175 mM potassium glutamate, 1 mM spermidine, 7.5 mM magnesium acetate, 20 μ g/ml

tRNA (*E. coli* mixture), 40 μ g/ml mRNA, 0.37 mM of each amino acid except Trp, and either 30 μ l of the original S-30 extract or an S-30 preparation previously treated with the antibody anti-release factor 2 (RF2), as described previously (10). When [³⁵S]methionine (20 μ Ci; 3,000 Ci/mmol) was used to label TnaC, the corresponding nonlabeled amino acid was not added.

Isolation of biotinylated mRNA-ribosome complexes was performed according to the protocol provided by the Promega Co. To purify the biotinylated mRNA complexes using streptavidin paramagnetic beads, 1.5 ml of a suspension of streptavidin beads was washed three times with 1.0 ml of a wash buffer containing 35 mM Tris-acetate (pH 8.0), 1 mM DTT, 10 mM ammonium acetate, 175 mM potassium glutamate, and 10 mM magnesium acetate, and the beads were resuspended in 1.25 ml of this buffer with 2 mM Trp. After performing the in vitro translation reaction, 250 μ l of the total reaction mixture was removed, streptavidin beads were added, the suspension was mixed, and the final mixture was incubated for 10 min at room temperature to allow biotin-streptavidin interactions. The streptavidin beads with bound biotinylated mRNA-ribosome complexes were then separated by using an electromagnetic field and were washed once with washing buffer containing 2 mM Trp and twice with only washing buffer. The final bead preparations were resuspended in 100 μ l of washing buffer.

Puromycin protection assays. Puromycin competition assays were carried out with 10 μ l of bead complexes obtained from in vitro translation reactions performed with [³⁵S]methionine after washing and resuspension. Solutions were mixed with the same volume of water or a 20 mM Trp solution. In some instances, complexes were incubated in the presence of puromycin, which was added to induce the hydrolysis of the peptidyl-tRNA. After the reaction was stopped by adding 10 μ l of a solution containing 10 mM Tris (pH 6.8), 10% sodium dodecyl sulfate, and 2 mM DTT to the mixture, the components of the final reaction mixtures were resolved by electrophoresis on 10% Tricine-sodium dodecyl sulfate protein gels. The dried gels were exposed to X-ray films, and the sections of the dried gels that contained TnaC-tRNA^{Pro} and TnaC were excised. The radioactivity (counts per minute [cpm]) in these bands was measured by using a scintillation counter. The amount of [³⁵S]methionine-TnaC-tRNA^{Pro} remaining after the reaction with puromycin was determined by dividing the cpm in the band corresponding to TnaC-tRNA^{Pro} by the combined cpm of the bands corresponding to TnaC-tRNA^{Pro} and the TnaC peptide.

Footprinting assays. Footprinting assays were performed as described previously (16). The isolated complexes were resuspended in 100 μ l of the appropriate buffer and were modified by exposure to 4 μ l of dimethyl sulfate (DMS) (1:6 dilution in ethanol) for 10 min at room temperature. DMS reactions were stopped by adding 50 μ l of stop buffer (1 M Tris-HCl [pH 8.0], 1 M 2-mercaptoethanol, and 1 mM EDTA) to the reaction mixture. The final reaction mixtures were diluted with a 10 mM EDTA solution to reach a total volume of 500 μ l, and the RNA was then extracted by treatment with phenol.

Modification by methylation was monitored by primer extension analyses using avian myeloblastosis virus reverse transcriptase (GIBCO) and 5'-³²P-labeled deoxyoligonucleotide primers (27). Primers 5'-TCCGGTCTCTCGTACT-3' and 5'-CTATCCTCACTCAAGGCTC-3', complementary to nucleotides 2654 to 2674 and 2102 to 2122 of 23S rRNA, were used to detect modified nucleotides in 23S rRNA. The positions of the modified nucleotides were identified by reference to dideoxy sequencing ladders obtained using the fmol@ DNA cycle sequence system kit (Promega). DNA from plasmid pNK that contained the 23S

FIG. 1. Model displaying the presumed region of the *E. coli* 50S ribosomal subunit of the stalled ribosome complex that contains TnaC-tRNA^{Pro} and free Trp. Shown at the left is the trinucleotide CCA of TnaC-tRNA^{Pro}, attached to TnaC, in the P site of the peptidyl transferase center. Also displayed is the segment of the TnaC peptide believed to be located in the ribosomal exit tunnel. The TnaC peptide was drawn as an α -helix since polypeptide chains are known to adopt a helical structure in the ribosome exit tunnel (31). The presumed locations of selected ribosomal residues, free Trp, sparsomycin, and puromycin in the 50S ribosome subunit are also indicated. The model is based on the ribosome structure of *E. coli* determined previously by Schuwirth et al. (26). Shown are the nucleotides (cyan) and amino acid residues (brown) analyzed in this study as well as some of the nucleotides involved in the peptidyl transferase reaction (violet) and the nucleotides affected by sparsomycin interaction (A2058 and A2059). The positions of the antibiotics sparsomycin (yellow) (based on the ribosome structure of *H. marismortui*) (24) and puromycin (black) (based on the ribosome structure of *H. marismortui*) (15) as well as the putative location of the bound free Trp (blue) (the exact location has not been demonstrated) are shown in relation to the positions of the P site occupied by the trinucleotide CCA (lavender-blue) and A site (occupied by a puromycin molecule) of the ribosome. Residues in green, Pro24, Asp16, and Trp12, are in the TnaC peptide; they are conserved and have been shown to be essential for *tna* operon induction (11, 28). The model was prepared using PyMOL software (7).

rRNA gene (19) and the same primers were used for primer extension of rRNA genes.

RESULTS

The narrow region of the ribosome exit tunnel is important for Trp-mediated induction of *tna* operon expression. Previously, we described studies with mutants bearing alterations in 23S rRNA (9, 19) or changes in ribosomal protein L22 that prevent the Trp-mediated induction of *tna* operon expression (5, 28). Our results suggested that interactions between these residues in the ribosome exit tunnel and Trp12 of TnaC are essential for some event required for induction (5). The location of Trp12 of TnaC-tRNA^{Pro} in the narrow region of the ribosome exit tunnel (Fig. 1) is based largely on the experimental findings that K11 of TnaC-tRNA^{Pro} can be cross-linked to 23S rRNA nucleotide A750 and that Trp12 of TnaC-tRNA^{Pro} protects 23S nucleotide A788 from methylation (5). These results imply that these residues are very near one another in the 50S subunit (5). The mutants examined have single nucleotide changes in 23S rRNA, U2609C (9), or an A insertion at position 751 (+A751) (19) or the single amino acid

change K90W in ribosomal protein L22 (5). The locations of these residues in the 50S ribosome subunit of *E. coli* are shown in Fig. 1. These residues are in the narrow region of the ribosome exit tunnel (26), a region that we believe is adjacent to Trp12 of TnaC-tRNA^{Pro} (5). Also shown in Fig. 1 is nucleotide A752, which is located between nucleotides U2609 and A751. To determine if this nucleotide is also required for Trp-mediated induction, we replaced it with a C or a T. We introduced these changes into a expressible 23S rRNA gene present on a plasmid and transformed this plasmid into a strain with all the chromosomal *rm* genes deleted that had an integrated λ *tna_p tnaC tnaA'-lacZ* expression construct (5) (Table 1). Expression of *tnaA'-lacZ* was determined by measuring β -galactosidase levels during growth in the presence or absence of Trp, as described previously by Cruz-Vera et al. (5). We observed that the ratio of *tnaA'-lacZ* expression with Trp to that without Trp, when the wild-type 23S rRNA gene was expressed, was 26 (Table 2). When expression was measured in strains producing 23S rRNA with the change A752C or A752T, the induction ratio was 1.2 (Table 2). These results indicate that the nucleotide at position 752 can also influence *tna* operon induction.

The narrow region of the ribosome exit tunnel is involved in the Trp inhibition of puromycin hydrolysis of TnaC-tRNA^{Pro}. To obtain a better understanding of how residue changes in the ribosomal exit tunnel affect Trp-mediated induction, mutationally altered 23S rRNA or the L22 protein was incorporated into ribosomes, and the behavior of these ribosomes was examined in vitro in *tna* operon induction experiments. Strains in which all the chromosomal *rm* genes had been deleted, strains containing a plasmid overproducing a mutant 23S rRNA, were used to produce ribosomes containing only mutated 23S rRNAs (1) (Table 1). To obtain mutant ribosomes with the L22 K90W mutant protein, a plasmid overproducing L22 K90W was introduced into a wild-type strain containing the *rm* operon genes and the L22 gene (W3110) (Table 1). Cell extracts (S-30) prepared from these strains were employed in in vitro translation reactions with separately prepared *tnaC* mRNAs. As shown previously, with wild-type ribosome complexes, the presence of an excess of Trp inhibits RF2-mediated translation termination, resulting in the accumulation of the TnaC-peptidyl-tRNA, TnaC-tRNA^{Pro} (13). As shown in Fig. 2A, S-30s prepared with ribosomes with the 23S rRNA change A752C, +A751, or U2609C or the L22 change K90W, unlike the S-30 prepared with wild-type ribosomes, were unable to

TABLE 2. Nucleotide changes in 23S RNA that affect *tnaA'-lacZ* expression in strain $\Delta 7rm \Delta lac$ (λ *tna_p-tnaC tnaA'-lacZ*)

<i>rm</i> Plasmid	β -Gal activity (Miller units) ^a $\pm$ SD		Induction ratio (+Trp/−Trp) ^b
	−Trp	+Trp	
23S wild type	150 $\pm$ 14	3,900 $\pm$ 33	26
23S A752C	110 $\pm$ 10	135 $\pm$ 14	1.2
23S A752T	120 $\pm$ 13	143 $\pm$ 14	1.2

^a Cultures were grown in minimal medium plus 0.2% glycerol and 0.05% acid-hydrolyzed casein. β -Galactosidase (β -Gal) assays were performed in three independent experiments.

^b Ratio of values for cultures grown with Trp (+Trp) and those grown without Trp (−Trp).

FIG. 2. Examination of the products generated from the translation of *tnaC* mRNA variants using different cell extracts in the presence of Trp. (A) In vitro translation reactions performed with *tnaC* mRNA and cell extracts containing wild-type (wt) or mutant ribosomes. (B) In vitro translation reactions performed with cell extracts containing wild-type ribosomes and *tnaC* mRNA or noninducible *tnaC*(Trp12Arg) mRNA. The cell extracts were prepared from the $\Delta 7rm$ strain (Table 1) containing the 23S rRNA gene indicated in the figures or were obtained from a wild-type strain containing all the wild-type *rm* genes, a copy of the *rplV* wild-type gene, and a plasmid with a mutant *rplV* gene that overproduces the L22 protein with the K90W change (Table 1). The protein products formed in the absence (–) or presence (+) of Trp were labeled by adding 35 S-labeled methionine and resolved by electrophoresis on Tris-Tricine polyacrylamide gels. Northern blot assays were performed as described previously by Cruz-Vera et al. (4), and degradation assays using RNase A were performed to verify the identity of TnaC-tRNA^{Pro}, as described previously by Gong and Yanofsky (13). This experiment was performed four times, with essentially the same results. An asterisk marks the principal background-labeled band that is produced using cell extracts without *tnaC* mRNA (see lanes 5 and 6 in B).

accumulate TnaC-tRNA^{Pro} in the presence of Trp (Fig. 2, compare lane 2 with lanes 4, 6, 8, and 10). Furthermore, the accumulation of detectable amounts of the TnaC peptide was poor in reactions performed with mutant ribosomes, with or without Trp, and the intensity of the TnaC band was similar to the intensity of the band obtained with wild-type ribosomes in the absence of added Trp (Fig. 2A). A noninducible mutant *tnaC*(Trp12Arg) mRNA was also used in these in vitro translation reactions (Fig. 2B). Interestingly, expression of this mutant *tnaC*, in the presence or the absence of Trp, was also poor compared with the expression observed with wild-type *tnaC* mRNA in the presence of Trp (Fig. 2B, compare lanes 1 and 2 with 4). However, when we performed in vitro translation assays with the corresponding wild-type or mutant RF2-depleted S-30 extracts, we observed higher levels of TnaC-tRNA^{Pro} and the TnaC peptide in the presence or absence of Trp with either *tnaC* mRNA or *tnaC*(Trp12Arg) mRNA (data not shown). These results establish that the mutant ribosomes that we prepared and examined can still translate wild-type

tnaC mRNA and the noninducible mutant *tnaC*(Trp12Arg) mRNA. In addition, we observed similar levels of the background protein produced in all our in vitro translation assays (Fig. 2). Thus, the ability of our mutant ribosomes to translate this other mRNA appears to have been unaffected. These results can be interpreted as follows: mutant ribosomes translate *tnaC*, translation is terminated, and the TnaC peptide produced, the translating ribosome, and the *tnaC* mRNA are released. The TnaC peptide and *tnaC* mRNA are then degraded, as observed with wild-type ribosomes in the absence of added Trp (data not shown). However, with wild-type ribosomes, when Trp is present, some fraction of the TnaC-tRNA^{Pro} resists hydrolysis, unlike the result observed when Trp is present with mutant ribosomes. Thus, in Trp-induced stalled wild-type ribosomes, both the TnaC peptide and the *tnaC* mRNA are protected from degradation (data not shown).

Trp is known to inhibit the hydrolysis of TnaC-tRNA^{Pro} by puromycin, an analog of an aminoacyl-tRNA, as well as hydrolysis initiated by RF2 (11). Conceivably, the ribosomal mutations that prevent induction do so by preventing Trp binding at the peptidyl transferase center. Consistent with this explanation, studies with *tnaC* have shown that puromycin action is inhibited by ribosome-bound Trp (5, 11). We therefore tested whether Trp would block puromycin action with mutant ribosome complexes. To perform these analyses, TnaC-tRNA^{Pro}-ribosome-mRNA complexes were isolated using streptavidin beads following pretreatment of ribosomal extracts with an anti-RF2 antiserum (see Materials and Methods). This treatment eliminates the RF2 protein, the protein responsible for the cleavage of TnaC-tRNA^{Pro} in ribosomes in the absence of added Trp (10). Accordingly, TnaC-tRNA^{Pro}-ribosome-mRNA complexes accumulate in the presence or absence of added Trp (10). Using these anti-RF2-treated wild-type and mutant ribosome complexes, the ability of Trp to protect TnaC-tRNA^{Pro} from puromycin cleavage was examined. As shown in Fig. 3, when puromycin was added to wild-type ribosome complexes, TnaC-tRNA^{Pro} was cleaved, and a prominent cleaved TnaC band was observed (compare lane 1 with lane 2 in the wild-type panel). This cleavage was blocked by added Trp (Fig. 3, compare lane 2 with lanes 3 and 4). With all the mutant ribosome complexes examined, however, Trp was incapable of protecting TnaC-tRNA^{Pro} from hydrolysis by puromycin; consequently, a prominent TnaC band was observed (Fig. 3, compare lane 2 with lanes 3 and 4 in the mutant panels). The TnaC peptide band is prominent in these isolated complexes because it is not degraded to the extent observed following translation termination in in vitro assays using crude, nonisolated complexes (Fig. 2). Accordingly, the levels of TnaC peptide observed with isolated complexes (Fig. 3) are higher than the levels observed in complete S-30 in vitro reactions (Fig. 2). The results in Fig. 3 cannot be due to greater susceptibility of mutant ribosomes to puromycin action since we observed the same TnaC-tRNA^{Pro} hydrolysis ratio using different concentrations of puromycin with wild-type or mutant ribosomes in the absence of Trp (not shown). Thus, Trp is unable to protect TnaC-tRNA^{Pro} from puromycin action with complexes formed with specific mutant ribosomes in the absence of RF2. Therefore, either these mutant ribosomes are incapable of binding Trp or, when they bind Trp, this amino acid cannot block puromycin action.

FIG. 3. Examination of TnaC-tRNA^{Pro} hydrolysis by puromycin using isolated mutant and wild-type (wt) ribosome complexes. Isolated ³⁵S-labeled TnaC-tRNA^{Pro}-ribosome-*tnaC* mRNA complexes containing the 23S rRNA genes or L22 mutant protein K90W, indicated in the figure, were incubated with different concentrations of Trp and then exposed to puromycin (Puro) action by adding (+) 0.02 mM puromycin to 10 nM complexes. The mixtures were then incubated for 10 min at 37°C, the reaction was stopped, and the labeled products were resolved by electrophoresis on Tris-Tricine polyacrylamide gels. The percentage of TnaC as TnaC-tRNA^{Pro} was calculated by dividing the cpm of the band corresponding to TnaC-tRNA^{Pro} by the combined cpm of the bands corresponding to TnaC-tRNA^{Pro} plus the band corresponding to the TnaC peptide. This experiment was performed two times, with essentially the same result.

Changes in 23S rRNA and the L22 protein prevent Trp from inhibiting sparsomycin binding to the ribosome. In previous studies of induction of the wild-type *tna* operon, it was shown that Trp can compete with sparsomycin, an antibiotic that interacts with the peptidyl transferase center (15, 20). Trp reduces sparsomycin's ability to increase DMS methylation of nucleotides A2058 and A2059 of 23S rRNA of wild-type ribosomes (Fig. 4A, lanes 3 and 4; also see Fig. 1); the effect on A2059 methylation is more prominent (4). Competition assays were therefore also performed between sparsomycin and Trp using complexes containing mutant ribosomes. Unlike wild-type complexes, complexes with the A752C, +A751, U2609C, or L22 K90W changes were not protected by Trp from sparsomycin-induced RNA methylation (Fig. 4A, compare lane 4 with lanes 6, 8, and 10, and B, compare lane 2 with lane 4). These findings suggest that in complexes containing mutant ribosomes with changes that prevent Trp-mediated induction, Trp cannot compete with sparsomycin. Thus, Trp binding and inhibition of sparsomycin action appear to have been eliminated by these ribosomal mutations.

It was shown previously with wild-type complexes (4) that bound Trp reduced the methylation of A2572, a nucleotide located in the vicinity of the peptidyl transferase center (Fig. 1). Trp did not reduce the methylation of A2572 in complexes containing the ribosomal changes examined in the present study to the extent observed with wild-type ribosomes (Fig. 4C, compare lanes 1 and 2 with the remaining lanes). These findings also suggest that Trp is incapable of binding effectively to the mutant ribosome complexes that were examined.

DISCUSSION

The mechanism of Trp-mediated induction of *tna* operon expression is an example of RNA-based gene regulation in which residues of a nascent leader peptide interact with regions of the translating ribosome, directing the ribosome to bind a specific signal molecule, in this case, Trp (8, 17, 18, 21). When bound, Trp inhibits ribosomal peptidyl transferase activity, causing the stalling of the translating ribosome on the *tna* transcript. This stalling prevents Rho-factor-mediated transcription termination in the leader region of the operon, allowing the transcription of the structural gene region of the operon to proceed. In previous studies, cross-linking analyses were used to identify the approximate location of Trp12 of TnaC in the ribosome exit tunnel (5). Amino acid residue Lys11 of TnaC was shown to cross-link to nucleotide A750 of 23S rRNA in the narrow region of the ribosome exit tunnel (5). It was also shown that the change U2609C, altering a 23S rRNA nucleotide close to the presumed location of Trp12 of TnaC (Fig. 1), prevented Trp-mediated induction. In addition, the insertion of an additional A residue, at position 751 of the 23S rRNA gene, or replacing residue K90 of ribosomal protein L22 with Trp, prevented Trp-mediated induction (5). Both A751 of 23S rRNA and K90 of L22 are also presumably located close to Trp12 of TnaC (Fig. 1). These findings imply that a region of the exit tunnel recognizes features of Trp12 of TnaC and that this recognition is essential for free Trp binding and Trp-mediated induction. In the current study, we prepared ribosomes with these alterations and asked a specific question:

FIG. 4. Footprinting analysis of the effects of residue changes in the peptidyl transferase center upon adding Trp to isolated complexes containing wild-type or mutant ribosomes. Isolated TnaC-tRNA^{Pro}-ribosome-*tnaC* mRNA complexes were obtained from in vitro translation reactions performed using cell extracts obtained from $\Delta 7rm$ strains that express the 23S rRNA genes indicated or using cell extracts obtained from a wild-type (wt) strain producing the wild-type or mutant L22 protein. The isolated complexes were incubated without (–) or with (+) Trp for 10 min at 37°C. A sparsomycin (Sps) solution was (+) or was not (–) added to the reaction mixture, and the mixture was incubated for an additional 10 min. The mixtures were then exposed to DMS for 10 min at room temperature. Total RNA was extracted and used as a template in primer extension assays. (A and B) An oligonucleotide complementary to nucleotides 2102 to 2122 was used in primer extension assays. (C) Primer extension analyses with mutant and wild-type ribosomes were performed to examine Trp protection from DMS methylation of nucleotide A2572. No sparsomycin was added. An oligonucleotide primer complementary to nucleotides 2654 to 2674 was used.

do ribosomal changes that prevent Trp-mediated induction act by preventing effective binding of free Trp at the A site of the translating ribosome?

Our findings suggest that residues in the ribosome exit tunnel region, nucleotide U2609, and the A-rich region, A749-A750-A751-A752-A753, as well as amino acid residue K90 of ribosomal protein L22 are involved in the recognition of Trp12 of TnaC-tRNA^{Pro} or of transmitting this information. The binding of free Trp in the ribosome is an essential event required for the induction of *tna* operon expression (Fig. 1). Changing any of these residues reduced or eliminated the ability of Trp to inhibit the termination of translation of *tnaC* or to induce the stalling of the ribosomes engaged in translating *tnaC* (Fig. 2). These changes were shown to have the following additional effects: (i) they eliminated Trp protection of TnaC-tRNA^{Pro} from puromycin hydrolysis (Fig. 3), (ii) they eliminated Trp protection from sparsomycin interaction with the ribosome (Fig. 4), and (iii) they eliminated the ability of Trp to protect nucleotide A2572 of 23S rRNA from methylation (Fig. 4).

Sparsomycin and puromycin are antibiotics that are known to interact with the ribosomal peptidyl transferase center (Fig. 1) (15, 20, 23, 25). They compete with one another for interaction with the ribosome (14); however, they do not interact identically with the peptidyl transferase center (Fig. 1) (24). It has been suggested that the changes in the peptidyl transferase center produced by bound sparsomycin affect the interactions of puromycin with the ribosome (24). Sparsomycin apparently

contacts nucleotide A2602 in structural models of the ribosome in translation (Fig. 1) (15, 20). This interaction appears to affect the conformation of both the A and the P sites of the ribosome of *Deinococcus radiodurans* (2). Nucleotides of the ribosome of *D. radiodurans* corresponding to nucleotides A2438, C2452, C2499, U2500, and U2584 of the ribosome of *E. coli*, nucleotides implicated in A-site binding, change their conformation upon an interaction of the ribosome with sparsomycin (2). It has also been shown that when puromycin interacts with the A site of the *Haloarcula marismortui* ribosome, it induces specific movements of the nucleotides corresponding to nucleotides 2583 to 2585 and 2506 of the 23S rRNA of *E. coli* (24). These changes presumably reorient the ester group of the peptidyl-tRNA, making it accessible for attack (24). Interestingly, both puromycin and sparsomycin affect the orientation of nucleotide 2584, located in the vicinity of nucleotide 2585. Nucleotide 2585, when changed, affects the extent of hydrolysis induced by a release factor during translation termination (30). In the TnaC example, Trp can inhibit the binding and action of both antibiotics (Fig. 3 and 4). The results presented in this paper suggest that bound Trp may alter the location of critical nucleotides, i.e., nucleotides 2602, 2584, and 2585, in the peptidyl transferase center when the wild-type ribosome is translating *tnaC* mRNA. These positional changes presumably prevent RF2 from inducing hydrolysis of TnaC-tRNA^{Pro}; they also appear to alter the interaction of the ribosome with sparsomycin, puromycin, or an aminoacyl-tRNA (4).

Ribosome recognition of features of the TnaC peptide results in the specific binding of free Trp in the ribosome, inhibiting peptidyl transferase activity. Interference assays examining UV-light-induced cross-linking between sparsomycin and ribosomal nucleotides indicate that sparsomycin binds at a site close to the location of critical residue A2602 of the peptidyl transferase center (20). The findings in this paper (Fig. 4) show that free Trp inhibits sparsomycin binding to ribosomes containing nonmutant TnaC-tRNA^{Pro}. Since nucleotide U2609 is near A2602 and is connected to it (Fig. 1) (20), it is conceivable that Trp12 of TnaC-tRNA^{Pro} acts by displacing nucleotide U2609. This may alter the location of A2602 in the peptidyl transferase center. If the center contains a bound free Trp molecule, altering the position of A2602 may make the ribosome susceptible to Trp inhibition. Alternatively, the displacement of U2609 by Trp12 of TnaC-tRNA^{Pro} may shift the position of A2602 and thereby create a free Trp binding site in the peptidyl transferase center. Experiments must be performed to distinguish between these possibilities.

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