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J. Bacteriol. **1995, 177(24):7245.**

JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 7245–7254 Vol. 177, No. 24
0021-9193/95/$04.00+0

Evidence Suggesting *cis* Action by the TnaC Leader Peptide in Regulating Transcription Attenuation in the Tryptophanase Operon of *Escherichia coli*

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Received 5 July 1995/Accepted 3 October 1995

Expression of the tryptophanase (*tna*) operon in *Escherichia coli* is regulated by catabolite repression and transcription attenuation. Elevated levels of tryptophan induce transcription antitermination at one or more Rho factor-dependent termination sites in the leader region of the operon. Induction requires translation of a 24-residue coding region, *tnaC*, located in the 319-nucleotide transcribed leader region preceding *tnaA*, the structural gene for tryptophanase. In the present paper, we show that two bacterial species that lack tryptophanase activity, *Enterobacter aerogenes* and *Salmonella typhimurium*, allow tryptophanase induction and *tna* operon regulation when they carry a plasmid containing the *E. coli tna* operon. The role of *tnaC* in induction was examined by introducing mutations in a 24-nucleotide segment of *tnaC* of *E. coli* surrounding and including the crucial Trp codon 12. Some mutations resulted in a noninducible phenotype; these mostly introduced nonconservative amino acid substitutions in TnaC. Other mutations had little or no effect; these generally were in third positions of codons or introduced conservative amino acid replacements. A tryptophan-inserting, UGA-reading glutamine suppressor tRNA was observed to restore partial regulation when Trp codon 12 of *tnaC* was changed to UGA. Stop codons introduced downstream of Trp codon 12 in all three reading frames established that induction requires translation in the natural *tnaC* reading frame. Our findings suggest that the TnaC leader peptide acts in *cis* to prevent Rho-dependent termination.

Tryptophanase is a catabolic enzyme produced by many bacterial species. This enzyme degrades tryptophan to indole, pyruvate, and ammonia and allows the organism to utilize tryptophan as a source of nitrogen or of carbon and energy (13, 24). Tryptophanase also catalyzes the reverse reaction (43). In *Escherichia coli*, the *tna* operon consists of two major structural genes, *tnaA*, encoding tryptophanase (9), and *tnaB*, encoding a tryptophan permease (5, 32, 45). The leader region of the *tna* operon contains a region, designated *tnaC*, encoding a 24-amino-acid residue peptide. Transcription of the structural gene region of the *tna* operon is responsive to the presence of tryptophan but not other amino acids; addition of tryptophan to a growth medium results in a 10- to 100-fold elevation of the tryptophanase level (39). Although the precise mechanism of induction is unknown, previous studies established that addition of tryptophan induces transcription antitermination at one or more Rho factor-dependent termination sites located in the transcribed leader region of the operon (39, 40). Mutations in *rho* can elevate operon expression (39); also, deletion of a putative Rho utilization (*rut*) site, located immediately after the *tnaC* stop codon, resulted in constitutive operon expression (12). The major sites of termination were determined in vitro and in vivo and correspond to positions +234 and +291 relative to the start of transcription (38).

Translation of *tnaC* appears to be required for tryptophan-induced expression of the operon. Thus, replacement of the *tnaC* methionine start codon or introduction of a stop codon prevents induction (12, 40). The *tna* operons of two other enteric bacterial species, *Proteus vulgaris* and *Enterobacter aerogenes*, have been sequenced. These operons are organized like the operon of *E. coli* and also contain short leader peptide-coding regions (16, 17). Relatively few of the codons of *tnaC* are conserved in all three species; however, the single Trp codon, shown to be crucial in *E. coli* (40), is conserved, as are several adjacent codons (Fig. 1). These features suggest that translation of the central portion of the leader peptide-coding region, or its polypeptide product, may play a role in induction.

Mutations in the distal part of *tnaC* have been shown to result in partial constitutive expression (39). These mutations are in a 9-nucleotide segment that resembles the distal part of *boxA* of phage λ (39); *boxA* is necessary for proper antitermination in this bacteriophage (8, 10, 20). A segment of *tnaC* of *P. vulgaris* also shows some homology to *boxA* (16); however, it is located at a different position relative to the putative *boxA* in *E. coli tnaC*. A similar segment in *E. aerogenes tnaC* shows only weak homology to *boxA*.

The cellular component that presumably recognizes tryptophan and directs antitermination during transcription of the *tna* operon is unknown. Since methyl analogs of tryptophan, such as 1-methyltryptophan and 5-methyltryptophan, induce expression of the *tna* operon at concentrations similar to those at which tryptophan does (45) yet are at best poorly aminoacylated onto $tRNA^{Trp}$ (25), it is unlikely that charging of $tRNA^{Trp}$ is principally responsible for *tna* operon induction. Changing Trp codon 12 of *E. coli* to codons specifying other amino acids prevents amino acid induction (12). However, when Trp codon 12 was replaced by stop codons, suppressing tRNAs derived from $tRNA^{Trp}$ were effective in restoring induction. Other suppressing tRNAs were ineffective (12). It has also been shown that translation of *tnaC* in *trans* can result in inhibition of expression of the chromosomal copy of the *tna* operon (11). This effect depends upon translation of Trp codon 12 and occurs only when multiple copies of the inhibitory *tnaC*

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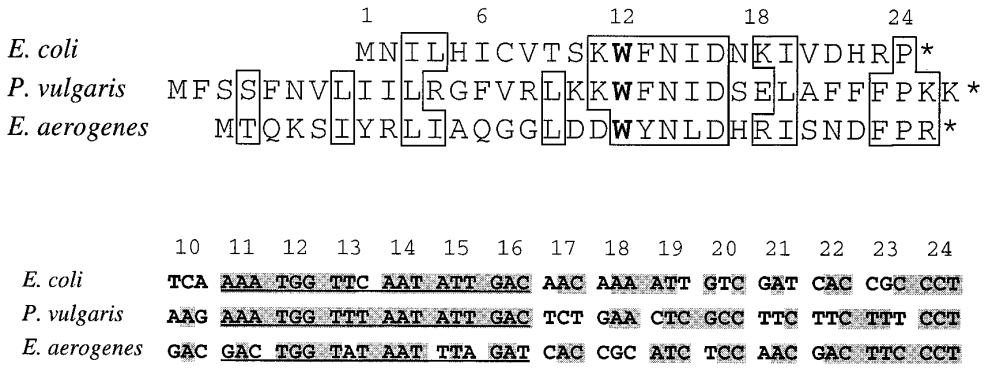

FIG. 1. TnaC leader peptides and a segment of the *tnaC* leader peptide-coding regions from three members of the family *Enterobacteriaceae*. The deduced amino acid sequences of the leader peptides from *E. coli*, *P. vulgaris*, and *E. aerogenes* are shown, with the nucleotides including and surrounding the conserved region presented underneath. Amino acid residues enclosed in boxes are those at a given position that are identical or similar. The *E. coli* residue numbers are indicated above the aligned sequences. The three species have a conserved Trp (W) residue near the center of the peptide. Nucleotides in *tnaC* that are conserved between at least two species are shaded. The central highly conserved nucleotide sequence, containing the TGG Trp codon, is underlined.

gene are present in the cell. Translation in *trans* of numerous copies of *tnaC* presumably titrates out or interferes with the function of a factor or event necessary for antitermination (11). Since this *trans*-acting inhibition of *tna* operon expression also substantially reduces the cell growth rate (10a), the putative factor may influence expression of other *E. coli* operons. These findings implicate Trp codon 12, its translation, or the presence of tryptophan in the TnaC leader peptide in the induction process.

How elevated tryptophan levels decrease Rho-dependent termination is also unknown. One hypothesis is that tryptophan induction alters the behavior of the ribosome translating the *tnaC* coding region so that it physically blocks Rho's access to the *tna* operon transcript. A second hypothesis is that the presence of elevated tryptophan levels favors formation of an RNA secondary structure (as in Rho-independent attenuation in the *E. coli trp* operon [18]) that prevents Rho's access to the transcript. This possibility is in agreement with the fact that Rho is known to bind poorly to highly structured segments of RNA (26). A third hypothesis is that the leader transcript contains binding sites for RNA-binding proteins other than Rho that either interfere with the entry of Rho, alter its termination activity, or render RNA polymerase immune to the action of Rho, as is believed to occur when N protein mediates antitermination in phage λ (8). The specific tRNAs required for *tnaC* translation, particularly $tRNA^{Trp}$, may influence the position of the ribosome, its rate of movement, and/or its choice of reading frame, thereby mediating attenuation by one or more of the above-described mechanisms. Last, the TnaC leader peptide itself, although apparently incapable of promoting induction in *trans*, may possess biological activity in *cis*, perhaps by interacting with the translating ribosome and mitigating Rho's action by one of the above-described mechanisms. In this paper we describe experiments that bear on each of the postulated mechanisms for preventing or enhancing Rho action in the *tna* operon. On the basis of our results, we propose that the TnaC leader peptide mediates induction by acting in *cis* on the translating ribosome to prevent Rho-dependent transcription termination.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Plasmid pKG1 contains the entire *E. coli tna* operon on a 5.3-kb *Bgl*II fragment inserted into the *Bam*HI site of the low-copy-number plasmid pACYC184 (6). Plasmids pPDG15, pPDG13, pPDG16, and pPDG19 (all provided by P. Gollnick) contain a *tnaA′-′lacZ* translational fusion inserted in the medium-copy-number plasmid pRLK15 (46). pPDG15 has the wild-type *tna* regulatory region, pPDG13 has *tnaC* Trp codon 12 replaced by TAG, pPDG16 has a frameshift mutation in *tnaC* resulting in constitutive expression of *tnaA* (12), and pPDG19 has the *tna* promoter replaced by the *tet* promoter from pBR322 (3, 41). Plasmid pKG800 also contains the *tna* operon leader regulatory region followed by a *tnaA′-′lacZ* fusion; transcription of the regulatory-fusion region is initiated at a *tet* promoter that replaces the *tna* promoter (Fig. 2). The introduced *tet* promoter initiates transcription 29 nucleotides upstream of the natural *tna* transcription start site. Plasmid pKG800 was constructed by inserting a 430-bp *Xho*I-*Bam*HI fragment into *Xho*I-*Bam*HI-digested pKG9. Plasmids pKG100 through pKG175 are identical to pKG800 except that each carries one or more unique mutations in *tnaC*. Site-directed mutagenesis was performed by the "megaprimer" PCR method described below. Plasmid pKG9 was made by inserting the *tet* promoter region from pBR322 (3), corresponding to nucleotides 1 to 76 (41), into *Eco*RI-*Bam*HI-digested pPDG55 (provided by P. Gollnick). Plasmid pPDG55 was made by inserting the 3.3-kb *Eco*RI-*Bal*I fragment from pMLB1034 (35), containing the *lacZ* gene with a 5′ deletion of 9 codons, into plasmid pSE400. Plasmid pSE400 (provided by S. Elledge) is a low-copy-number plasmid conferring resistance to spectinomycin.

Media and enzyme assays. Media, growth conditions, and enzyme assays were as described previously (11). The medium consisted of MOPS (morpholinepropanesulfonic acid)-based minimal medium (2, 23) supplemented with 1% acid-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
Strains		
E. coli K-12		
SVS1100 *tnaA2*	W3110 Δ(*lac-argF*)*U169 tnaA*	40
SVS1100 *tnaA*::Tn*10*	W3110 Δ(*lac-argF*)*U169 tnaA*	This study
SVS1144	SVS1100 (λ*tna*$_p$ *tnaC tnaA′-′lacZ*)	40
PDG1170	SVS1144 (λ*tna*$_p$ *tnaC* Trp-12→TGA *tnaA′-′lacZ*)	12
SE5000	MC4100 *recA56* Δ(*lac-argF*)*U169 thi*	36
E. aerogenes T-17R1	Prototrophic, *tna lac*	F. Gibson
S. typhimurium LT2	Prototrophic, *tna lac*	R. Bauerle
Plasmids		
pKG1	*tna*$_p$ *tnaC*$^+$ *tnaAB*$^+$	This study
pKG800	*tet*$_p$ *tnaC*$^+$ *tnaA′-′lacZ*	This study
pKG100-175	*tet*$_p$ *tnaC tnaA′-′lacZ*	This study
pPDG15	*tna*$_p$ *tnaC*$^+$ *tnaA′-′lacZ*	P. Gollnick
pPDG13	*tna*$_p$ *tnaC* Trp-12→TGA *tnaA′-′lacZ*	P. Gollnick
pPDG16	*tna*$_p$ *tnaC261 tnaA′-′lacZ*	P. Gollnick
pPDG19	*tet*$_p$ *tnaC*$^+$ *tnaA′-′lacZ*	P. Gollnick

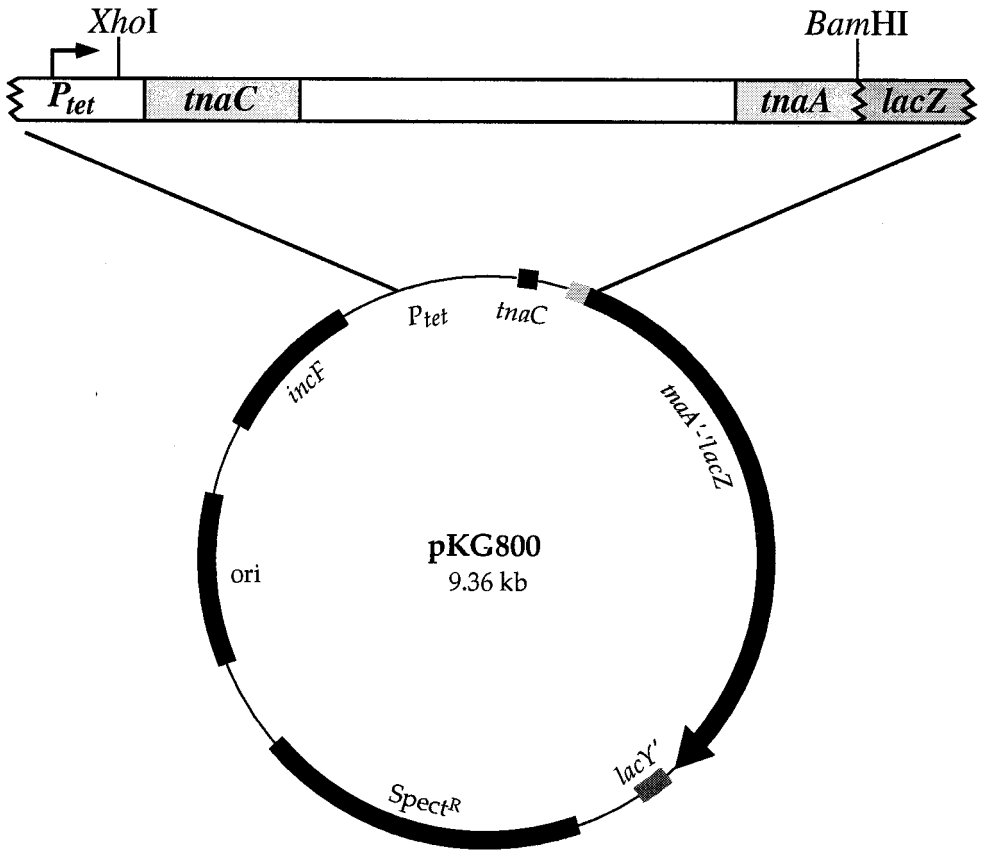

FIG. 2. Schematic diagram of plasmid pKG800. A 430-bp fragment containing the *tna* leader region up to *tnaA* codon 21 was inserted between the *tet* promoter and the *lacZ* gene on a low-copy-number plasmid. The product has a functional *tnaA'-'lacZ* fusion under the control of the *tna* regulatory region, with constitutive transcription initiation from the *tet* promoter. The site of transcription initiation is 29 nucleotides upstream of the natural *tna* start site; thus, the natural *tnaC* ribosome binding site and start codon are preserved.

hydrolyzed casein, 1 μg of thiamine per ml, and 100 μg of spectinomycin per ml. When indicated, Vogel-Bonner minimal medium (42), supplemented with 0.2% glycerol, 0.05% acid-hydrolyzed casein, and 20 μg of chloramphenicol per ml, was used. When appropriate, media were supplemented with tryptophan or 1-methyltryptophan. β-Galactosidase assays were performed by a slight modification of the method of Miller (21); sonicated cell extracts were used instead of permeabilized whole cells. All extracts were assayed in duplicate. Enzyme activity was found to be linear with respect to the amount of extract added. Deviation among duplicate samples was less than 10%.

Megaprimer PCR mutagenesis. Megaprimer PCR (31) is a fast, simple, and efficient method for introducing site-directed mutations. The desired mutations are specified during synthesis of an oligonucleotide (primer) complementary to the region of mutagenesis. Primer A has a stretch of nucleotides identical to the region upstream of *tnaC* and contains nucleotides for an *Xho*I restriction site at its 5′ end. The mutagenic primer (primer M) was used in a standard PCR with Deep Vent polymerase (New England Biolabs). The PCR product obtained (approximately 120 bp) was purified after electrophoresis on a 2% SeaPlaque low-melting-point agarose (FMC) gel. This purified DNA was then used as a megaprimer for a second round of PCR with primer B, which anneals to the downstream *lacZ* gene, just past a unique *Bam*HI site. The 496-bp PCR product was then digested with *Xho*I and *Bam*HI, and the resulting 430-bp fragment was ligated into *Xho*I-*Bam*HI-cut pKG9. With this procedure, each site-directed mutation was readily introduced in the *tnaC* leader peptide-coding region. Neither the *tet* promoter nor the *lacZ* reporter gene was mutagenized, since these regions reside in the vector, on either side of the *Xho*I and *Bam*HI sites. Mutations were confirmed by double-stranded dideoxy sequencing (30) with a Sequenase 2.0 sequencing kit (U.S. Biochemicals).

Degenerate oligonucleotides were also used for mutagenesis. These oligonucleotide primers were doped with incorrect phosphoramidites during chemical synthesis, such that each nucleotide from *tnaC* codon 13 to codon 24 had a 95% probability of matching the $tnaC^+$ sequence and a 5% chance of being one of the three incorrect bases. Because of the doping procedure followed, each oligonucleotide mixture had a 16% probability of introducing no base substitutions, a 30% probability of introducing a single base change, and a 54% probability of introducing two or more base changes. Since synthesis of the degenerate positions in the oligonucleotide primers was completely random, the only bias affecting the final distribution of incorrect base pair incorporation occurred during the annealing steps in the megaprimer PCR and was found to be minor.

RNA secondary structure analysis. Stable RNA secondary structures were predicted by using the MFOLD program (Genetics Computer Group) on a SPARCServer1000 computer (Sun Microsystems) and by using the DNASIS program (Hitachi Software Engineering America, Ltd.) on a Macintosh Quadra 800 computer (Apple Computer). Each *tnaC* allele was modeled for effects of the mutation on enhancing or decreasing the stabilities of putative RNA structures in *tnaC* leader mRNA. In constructs in which the *tet* promoter replaced the *tna* promoter, an additional 29 nucleotides are present at the 5′ end of the transcript; these extra nucleotides did not alter the predicted RNA secondary structures for any of the various *tnaC* alleles.

RESULTS

Expression of the *E. coli tna* operon in *Salmonella typhimurium* and *E. aerogenes* species that lack tryptophanase. Attempts to detect a gene encoding a presumed *trans*-acting factor that would be responsible for tryptophan-induced expression of the *tna* operon of *E. coli* have been unsuccessful. Mutagenesis of *E. coli* was performed by using nitrosoguanidine, UV irradiation, or Tn*10* transposon insertion; no *trans*-acting, *tna*-specific regulatory factor was detected (11a). Additionally, transformation of *E. coli* with a plasmid-based genomic library of *E. coli* did not result in any transformants with increased tryptophanase expression (9a). An alternative explanation of induction is that all of the information necessary for tryptophan-induced expression is located in the leader region of the operon; i.e., there is no unique *trans*-acting *tna* antitermination factor. According to the latter view, only nor-

TABLE 2. Tryptophanase levels in *tna* plasmid-containing strains

Strain/plasmid[a]	TNase act (ratio)[b]		
	Basal	With 1-MT	With Trp
SVS1100 *tnaA2*/pKG1	0.94	32 (34)	30 (32)
E. aerogenes/pKG1	0.04	7.9 (200)	6.4 (170)
S. typhimurium/pKG1	0.16	15 (93)	11 (69)

[a] SVS1100 *tnaA2* is an *E. coli* strain with a mutation in the chromosomal *tnaA* gene (5, 40). pKG1 contains the intact *tna* operon of *E. coli.*

[b] Strains were grown in Vogel-Bonner minimal medium (42) plus 0.2% glycerol, 0.05% acid-hydrolyzed casein, and 20 μg of chloramphenicol per ml with or without 20 μg of DL-1-methyltryptophan (1-MT) per ml or 100 μg of L-tryptophan (Trp) per ml. Tryptophanase (TNase)-specific activity is expressed as micromoles of *S-o*-nitrophenyl-L-cysteine converted to *o*-nitrothiophenolate per minute per milligram of protein. The ratio of the specific activity with the addition of 1-MT or Trp to the basal specific activity is given in parentheses.

mal cell constituents and events would be needed for tryptophan induction. To examine this possibility, we introduced plasmids bearing different versions of the *tna* operon of *E. coli* into two bacterial species that lack tryptophanase, and we measured inducibility. We examined plasmids containing the intact *tna* operon (pKG1), a translational fusion of *tnaA* to *lacZ* driven by the *tna* promoter (pPDG15) or the *tet* promoter (pPDG19), the translational fusion operon with a leader region constitutive mutation (pPDG16) (12), and this translational fusion operon with a *tnaC* nonsense mutation that results in noninducibility (pPDG13) (12). The resulting plasmid-containing strains were grown in a variety of media, with and without the inducer DL-1-methyltryptophan or L-tryptophan, and assayed for tryptophanase or TnaA–β-galactosidase. Representative data from these experiments are presented in Tables 2 and 3. In tests with the three species with a plasmid containing the intact *tna* operon, there was appreciable induction by DL-1-methyltryptophan and L-tryptophan, although basal-level expression and induced expression of the operon were lower in *E. aerogenes* and *S. typhimurium* than in *E. coli* (Table 2). The induction ratios of tryptophanase specific activity were higher in *E. aerogenes* and *S. typhimurium* than in *E. coli*. In tests with all three species with *tnaA'-'lacZ* fusion plasmids, a plasmid with the wild-type *tna* regulatory region showed normal induction; however, the maximum induced levels of β-galactosidase were 4- to 12-fold lower in the heterologous species (Table 3). The fold induction was also lower in *S. typhimurium* than in *E. coli*. Analyses with a homologous plasmid that expresses the *tnaA'-'lacZ* fusion constitutively in *E. coli*, pPDG16, showed that expression was comparable in *S. typhimurium* and half of this level in *E. aerogenes*. Thus, the reduced expression in the other two species is not due to less effective use of the *E. coli tna* promoter or the *tnaA* ribosome binding site. Analyses with a plasmid carrying the *tnaA'-'lacZ* fusion in which the *tet* promoter replaced the *tna* promoter indicated that there was significant induction in all three species, although again, the fold induction was lower in *S. typhimurium*. A noninducible plasmid, pPDG13, bearing a replacement of *tnaC* Trp codon 12 by the stop codon, TAG, was noninducible in each species. In other studies it was shown that introducing a plasmid containing *mtr*, the gene encoding the tryptophan-specific permease, did not affect induction in the other two species (data not shown), arguing against the possibility that tryptophan transport differences limited induction (45). In other studies (data not shown) induction was also observed with constructs in each of the three species in MOPS minimal medium supplemented with 1% acid-hydrolyzed casein (12), and in Vogel-Bonner minimal medium containing glycerol and five amino acids but no aromatic amino acids (45), or 1% lactate as carbon source. *tna* operon expression in the three species was also measured in media containing glucose. In each of the species, expression of the *tna* operon was subject to catabolite repression, but the addition of tryptophan led to a normal fold induction (data not shown). These findings establish that in two species that lack tryptophanase, plasmids bearing the *tna* regulatory region are subject to both basal-level control and tryptophan induction.

Noninducible mutants. The above-described results with heterologous species suggest that the information contained in the *E. coli tnaC* leader region is sufficient for *tna* operon regulation. However, previous studies did not establish whether the nucleotide sequence of *tnaC* or the amino acid sequence of TnaC mediates transcription antitermination. Most of the amino acid residues that are conserved in the TnaC leader peptides of *E. coli*, *P. vulgaris*, and *E. aerogenes* lie adjacent to the crucial Trp residue (Fig. 1); this region also is highly conserved at the nucleotide level (Fig. 1). Therefore, this central, semiconserved segment, corresponding to *E. coli tnaC* codons 11 to 19, was targeted for random mutagenesis by the megaprimer PCR method (31) with degenerate oligonucleotide primers.

Approximately 200 random mutants were isolated and assayed for effects on tryptophan regulation of a tet_p *tnaA'-'lacZ* reporter construct in a low-copy-number plasmid. Some mutants continued to show regulation by tryptophan, but most mutants were noninducible. These noninducible mutants generally had single nucleotide changes in the central region of *tnaC*, the region that is conserved in *E. coli*, *P. vulgaris*, and *E. aerogenes* (Fig. 1). The changes in, and the characteristics of, the noninducible mutants are presented in Table 4. Each of these mutations, except *tnaC279* (a slight induction was observed for *tnaC279*), eliminated tryptophan induction, and each changed the predicted TnaC amino acid sequence. Several of the predicted amino acid changes were conservative,

TABLE 3. β-Galactosidase activities of various plasmid-containing bacterial strains[a]

Strain	Plasmid	β-Galactosidase activity[b]		
		Basal	With 1-MT	Ratio[c]
SVS1100 *tnaA*::Tn*10*	pPDG15	58	1500	26
	pPDG16	2,400	2,400	1.0
	pPDG19	53	1,100	20
	pPDG13	30	32	1.1
E. aerogenes	pPDG15	9	400	44
	pPDG16	1,000	1,100	1.1
	pPDG19	18	450	25
	pPDG13	7	7	1.0
S. typhimurium	pPDG15	19	125	6.6
	pPDG16	2,200	2,200	1.0
	pPDG19	47	210	4.5
	pPDG13	10	10	1.0

[a] Cultures were grown in Vogel-Bonner minimal medium (42) supplemented with 0.2% glycerol and 0.05% acid-hydrolyzed casein, with or without 20 μg of DL-1-methyltryptophan (1-MT) per ml. All plasmids contained the same *tnaA'-'lacZ* translational fusion. pPDG15, wild-type *tna* regulatory region; pPDG16, *tna* constitutive mutant (12); pPDG19, *tet* promoter replaces the *tna* promoter; pPDG13, homologous to pPDG15 with Trp codon 12 replaced by TAG. Neither the *E. aerogenes* parental strain nor the *S. typhimurium* recipient strain has β-galactosidase activity. SVS1100 *tnaA*::Tn*10* is an *E. coli* strain with a Tn*10* insertion in *tnaA*.

[b] *o*-nitrophenol liberated; OD_{400} per 30 min per 1 OD of cells at 600 nm.

[c] Ratio of activity with DL-methyltryptophan to basal activity.

TABLE 4. Mutations in *tnaC* codons 12 through 19 that do or do not prevent tryptophan-induced antitermination

Allele	*tnaC* codon[a]:								Amino acid substitution	Relative β-galactosidase activity[b]		
	12	13	14	15	16	17	18	19		Basal	With Trp[c]	Ratio[d]
tnaC$^+$	TGG	TTC	AAT	ATT	GAC	AAC	AAA	ATT		6.8	100[e]	15
tnaC265	CGG								W→R	5.4	4.7	0.9
tnaC266	CGG	TGG							WF→RW	21	18	0.7
tnaC279		ATC							F→I	6.3	13	2.1
tnaC280		TGC							F→C	6.5	6.6	1.0
tnaC281		TTG							F→L	5.6	5.7	1.0
tnaC283			ATT						N→I	6.1	7.3	1.2
tnaC284			AGT						N→S	5.2	6.6	1.3
tnaC286			AAA						N→K	5.8	5.7	1.0
tnaC287				TTT					I→F	4.8	4.5	0.9
tnaC288				AAT					I→N	12	12	1.0
tnaC299					GAA				D→E	12	12	1.0
tnaC305								ACT	I→T	12	12	1.0
tnaC306								AAT	I→N	12	12	1.0
tnaC267	TGG								F→W	60	180	3
tnaC282		TTT							F	7.7	97	13
tnaC285			AAC						N	8.2	121	15
tnaC300					GAT				D	16	86	5.4
tnaC301						AAT			N	8.0	83	10
tnaC302							CAA		K→Q	5.4	39	7.2
tnaC307								ATA	I	14	86	6.1
tnaC308								ATC	I	24	80	3.3

[a] Each nucleotide changed by mutation is underlined.
[b] The induced specific activity for strain SE5000 containing plasmid pKG800 was set at 100%.
[c] L-Tryptophan (Trp) (100 μg/ml) was added to the growth medium.
[d] Ratio of induced to uninduced specific activities.
[e] 6.6 μmol of *o*-nitrophenol formed per min per mg of protein.

e.g., *tnaC279*, Phe-13 to Ile; *tnaC281*, Phe-13 to Leu; *tnaC287*, Ile-15 to Phe; and *tnaC299*, Asp-16 to Glu (Table 4). Several mutations that resulted in the noninducible phenotype had nonconservative substitutions in TnaC, e.g., *tnaC265*, Trp-12 to Arg; *tnaC280*, Phe-13 to Cys; *tnaC283*, Asn-14 to Ile; *tnaC284*, Asn-14 to Ser; *tnaC286*, Asn-14 to Lys; *tnaC288*, Ile-15 to Asn; *tnaC305*, Ile-19 to Thr; and *tnaC306*, Ile-19 to Asn. Replacing Trp-12–Phe-13 by Arg-12–Trp-13 (*tnaC266*) eliminated induction, as previously reported (12). Base-level expression was increased in some of the mutants two- to threefold (Table 4). These mutants were not induced by amino acids present in the 1% acid-hydrolyzed casein. These data suggest that the nucleotide sequence of the transcript and/or the amino acid sequence of TnaC is crucial for induction by tryptophan.

Tryptophan-regulated mutants. A second class of randomized *tnaC* mutants showed either partial or full induction by tryptophan (Table 4). All but two of these had a change in the third position of a codon that did not alter the TnaC amino acid residue specified. Six mutants were phenotypically similar to the wild type and showed an appreciable induction ratio upon addition of tryptophan (alleles *tnaC282*, *tnaC285*, *tnaC300*, *tnaC301*, *tnaC302*, and *tnaC307*). However, several mutants showed higher basal levels than the wild type (*tnaC 267*, *tnaC300*, and *tnaC308* mutants), one had a higher induced level than the wild type (*tnaC267* mutant), and one had a reduced induced level (*tnaC302* mutant). The *tnaC267* change was previously characterized as one leading to reduced induction by tryptophan (12); it carries a conservative Phe-13-to-Trp substitution. This tandem Trp codon configuration at codons 12 and 13 caused the induced level to rise nearly twofold and elevated base-level expression nearly eightfold, thus providing only a threefold response to tryptophan. A mutant with a nonconservative change, Lys to Gln (*tnaC302*), was induced to only 40% of the normal induced level; however, its base-level expression was lower than that of the wild type, resulting in a sevenfold overall increase in the response to tryptophan. Both (i) this good correlation between no amino acid change or a conservative amino acid substitution in TnaC and retention of tryptophan inducibility and (ii) the noninducible, amino acid-substituted mutants presented in Table 4, suggest that the amino acid sequence of the TnaC peptide may be crucial to tryptophan induction. In addition to the tryptophan-regulated and the noninducible mutants described above, we also isolated constitutive mutants with changes in *tnaC*. These are currently under investigation.

A tryptophan-inserting glutamine opal suppressor tRNA restores weak induction. It was shown previously that replacing *tnaC* Trp codon 12 by the TAG amber or TGA opal nonsense codon and suppressing these stop codons with a variety of nonsense suppressors that insert amino acids other than tryptophan did not lead to constitutive expression of the operon or allow induction of the operon by tryptophan or other amino acids (12). However, suppression of amber or opal codon 12 by tryptophan-inserting $tRNA^{Trp}$ amber or opal suppressors did restore induction in response to tryptophan (12). In more-recent mutational studies, codons for 16 amino acids have been introduced at *tnaC* position 12 (9b). Induction was observed only when codon 12 was TGG, the Trp codon. The fact that only Trp-inserting $tRNA^{Trp}$ suppressor tRNAs allowed induction raised the possibility that reading of codon 12 by $tRNA^{Trp}$ or insertion of Trp during translation of codon 12, or both, was necessary for induction. To distinguish between these possibilities, we examined the ability of an altered $tRNA^{Gln}$ opal suppressor tRNA that inserts tryptophan, described by Rogers et al. (29), to allow tryptophan induction. The data in Table 5 indicate that replacing Trp codon 12 by TGA (strain PDG 1170) eliminates induction and appreciably reduces base-level expression of the operon, as expected. The reduction of the

TABLE 5. A tryptophan-inserting $tRNA^{Gln}$ UGA suppressor restores weak induction

Strain	*tnaC* change	Suppressor	β-Galactosidase activity[a]		
			Basal	With Trp	Ratio
SVS1144	None	None	1,800	19,000	11
PDG1170	TGG-12→TGA	None	62	43	0.7
		$tRNA^{Gln}$ (UGA)[b] (inserts Trp)	93	190	2.1
		$tRNA^{Trp}$ (UGA)[b] (inserts Trp)	290	1,100	3.7

[a] Strains were grown in MOPS minimal medium (2, 23) containing 1% acid-hydrolyzed casein as a carbon source, with or without 100 μg of L-tryptophan (Trp) per ml. Activity is expressed in Miller units (21). Ratio, ratio of activity with Trp to basal activity.
[b] $tRNA^{Gln}$ (UGA) and $tRNA^{Trp}$ (UGA) are present on plasmids psu^+2op (29) and pSWC101 (27), respectively, which were introduced into strain PDG1170 by transformation.

basal level is typical of constructs in which translation to the end of *tnaC* is prevented. Introduction of the tryptophan-inserting glutamine suppressor tRNA into strain PDG1170 consistently resulted in a twofold increase in expression levels in response to tryptophan. Note that the basal level increased only slightly in the presence of this suppressor, suggesting that this suppressor tRNA is inefficient in reading this opal codon. As a control, we measured suppression of the same opal codon by a tryptophan-inserting $tRNA^{Trp}$ suppressor. This suppressor increased base-level expression nearly fivefold and allowed almost fourfold induction by tryptophan. In other studies it was shown that replacing Trp codon 12 by a glutamine codon did not allow induction (9b). These findings suggest that insertion of a Trp residue at amino acid position 12, rather than translation of codon 12 by $tRNA^{Trp}$, is essential for induction.

Introduction of stop codons in all three *tnaC* reading frames. The results described above suggest that proper translation of *tnaC* is essential for tryptophan induction of *tna* operon expression. A previously reported frameshift mutation in codon 18 of *tnaC* that presumably allows translation to proceed beyond the *tnaC* stop codon results in constitutive *tna* operon expression (12). To examine the possibility that induction requires translation of *tnaC* in an altered reading frame, stop codons were introduced in all three reading frames, at seven different locations in *tnaC* (Table 6). Changing Trp-12 or Phe-13 to TAG or changing Lys-18 to TAA abolished induction by tryptophan. Stop codons introduced in the −1 reading frame (in *tnaC282*, *tnaC300*, and *tnaC301*; codon 13, 16, or 17) had little effect upon induction, as did insertion of a stop codon in the +1 reading frame (*tnaC307*; codon 19). These results suggest that frameshifting in *tnaC* is not an essential event in tryptophan-mediated induction, whereas translation past codon 18 in the natural reading frame is necessary.

Lack of correlation of phenotype and predicted stability of RNA secondary structures. While most of the data presented here suggest a role for the TnaC peptide, an RNA secondary structure in leader RNA could also play a role in attenuation control of *tna* operon expression. The *tnaC* mutants described in Table 4 fell into two phenotypic categories: inducible and noninducible by tryptophan. To determine if these phenotypes correlated with changes in the predicted stabilities of RNA secondary structures, the leader RNA segment containing each of the *tnaC* alleles was modeled for predicted RNA secondary structures. The wild-type *tnaC* transcript could form either of two overlapping structures (Fig. 3) located approximately 218 and 189 nucleotides, respectively, upstream of the major site of Rho-dependent termination in vivo (38). There was no correlation between the phenotype caused by a mutation and its effect on the stability of either of these RNA secondary structures (Table 7). For example, allele *tnaC280*, which rendered the *tna* operon noninducible (Table 4), had no effect on structure 1 but nearly doubled the predicted stability of structure 2, to −13.0 kcal (ca. −54.4 kJ)/mol (Table 7). However, alleles *tnaC284*, *tnaC283*, and *tnaC286*, which also are noninducible (Table 4), had little or no effect on structure 2 and partially destabilized structure 1 (Table 7). Additionally, structure 2 is destabilized in alleles *tnaC293* and *tnaC305*, yet these changes also produced a noninducible phenotype (Table 4). The *tna* leader RNA segments of *P. vulgaris* and *E. aerogenes* were also examined for RNA secondary structures, and they were predicted to form only weakly stable structures (data not shown). There were no homologous predicted structures for

TABLE 6. Effects of introducing stop codons in the three reading frames of *tnaC*

Allele	Stop codon[a]	*tnaC* codon[b]:									Relative β-galactosidase activity[c]		
		12	13	14	15	16	17	18	19	20	Basal	With Trp[d]	Ratio[e]
tnaC$^+$		TGG	TTC	AAT	ATT	GAC	AAC	AAA	ATT	GTC	11	100[c]	9.1
tnaC263	0	T**A**G									14	7.4	0.5
tnaC323	0		T**A**G								7.9	7.6	1.0
tnaC282	−1		TT**T**								11	120	11
tnaC300	−1					GA**T**					24	110	4.6
tnaC301	−1						AA**T**				11	82	7.5
tnaC314	0						AA**T**	T**A**A			8.7	7.1	0.9
tnaC307	+1								AT**A**		17	110	6.5

[a] Reading frame in which the introduced stop codon is present.
[b] Each nucleotide changed by mutation is underlined. None of the mutations shown alter the natural TnaC amino acid sequence, except when stop codons TAG or TAA are introduced.
[c] The induced specific activity was set at 100%.
[d] L-Tryptophan (Trp) (100 μg/ml) was added to the growth medium.
[e] Ratio of induced to uninduced specific activities.
[f] 3.8 μmol of *o*-nitrophenol formed per min per mg of protein.

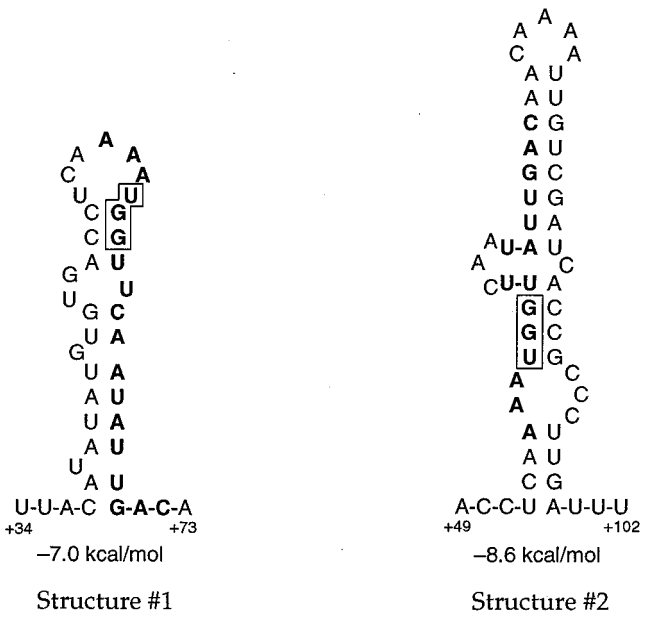

FIG. 3. Predicted RNA secondary structures present in *tnaC* leader RNA. Structures were predicted by using the MFOLD program (Genetics Computer Group). Structure 2 corresponds to structure a described by Stewart et al. (38). Each *tnaC* change was modeled for its effect on the stabilities of these and other putative structures. No strong correlation was found between secondary structure stability and *tnaA'-'lacZ* expression (Table 7). The central conserved segment (*tnaC* codons 11 to 16) is in boldface type. Trp codon 12 (UGG) is boxed. Nucleotide positions are numbered relative to the normal *tna* start of transcription. Actual transcription is initiated 29 nucleotides upstream, from the *tet* promoter. The major site of in vivo Rho-dependent termination occurs at position +291 (38). One kilocalorie = 4.184 kJ.

transcripts from any pair of the three species. Combined, these data support the conclusion that the predicted RNA secondary structures are not responsible for tryptophan induction.

DISCUSSION

The mechanism of tryptophan-induced expression of the *tna* operon is not known. Induction appears to depend on the cell sensing extracellular tryptophan or increased intracellular tryptophan levels and responding by reducing Rho-dependent transcription termination in the *tna* leader region. Previous studies demonstrated that translation of the *tnaC* leader peptide-coding region is essential for induction (40). The central segment of the TnaC peptide is conserved between *E. coli* (residues 11 to 16) and *P. vulgaris* (residues 19 to 24) (16). Residues 17 to 22 of TnaC of *E. aerogenes* contain amino acids identical or very similar to those of *E. coli* and *P. vulgaris* (17) (Fig. 1). The nucleotide sequences of these transcript segments also are nearly identical (Fig. 1). These conserved segments contain a single Trp codon which has been shown to be essential for induction of the *E. coli tna* operon (12). These are the only enteric bacterial species from which the *tna* operon has been cloned and sequenced.

The short, conserved segment of the *tnaC* leader peptide-coding region could have regulatory importance for one or more of the following reasons: (i) the nucleotides in this segment of the transcript may be critical for forming, or preventing the formation of, one or more important RNA secondary structures, as is observed in *trp* operon leader RNA (18); (ii) this RNA segment may constitute all or part of a binding site for an RNA-binding protein, like the TRAP protein of *Bacillus subtilis* (1, 34), the BglG protein of *E. coli* (14), or ribosomal protein L4 (33), each of which regulates transcription attenuation; (iii) the conserved *tnaC* codons may be translated at a particular rate, and that rate establishes a precise spacing between the transcribing RNA polymerase and the translating ribosome (spacing of this nature has been shown to be critical for transcription attenuation in many operons) (44); (iv) the conserved segment of the *tnaC* transcript may promote or retard ribosomal frameshifting, hopping, or stop-codon readthrough, as observed, respectively, during translation of transcripts of the *prfB* gene (7), phage T4 gene *60* (15), and the *fdhF* gene (47); or (v) the TnaC leader peptide may act in *cis* and to alter the behavior of the translating ribosome, as has been observed for a 5-amino-acid residue segment of the *cat-86* leader peptide (19, 22, 28). We consider our findings in relation to each of these possibilities and propose a working hypothesis that explains many of the features of *tna* operon regulation.

RNA secondary structures. RNA secondary structures have been predicted to be capable of forming in the leader segment of the *tna* transcript of *E. coli* (38) (Fig. 3). Although the putative structures correspond to sites of transcription pausing in vitro and Rho-dependent termination in vivo and in vitro (40), there are two structures that include the central segment of the *tnaC* transcript that has been studied here (Fig. 3). The phenotypes of mutants altered in this transcript segment do not correlate with the stabilities of the predicted RNA secondary structures. Thus, cells with alleles *tnaC280* and *tnaC283* have identical phenotypes in that neither responds to tryptophan induction (Table 4), yet these alleles have different effects on the stabilities of the predicted RNA structures (Table 7). Also, the changes in alleles *tnaC282* and *tnaC286* destabilize predicted structure 1 to similar extents, yet these alleles have different phenotypes (Table 4). Furthermore, no homologous RNA structures can be predicted for the corresponding segments of the *P. vulgaris* and *E. aerogenes tna* transcripts. Transcripts from these species are predicted to form different, although less stable, secondary structures (data not shown). We conclude that although one or more of these RNA structures may form in vivo, other features of the *tna* leader transcript must be predominantly responsible for the behavior of our mutants and for proper transcription termination control.

TABLE 7. Mutations in *tnaC* and their effect on RNA secondary structure stability

Allele	Nucleotide change	Phenotype	Predicted change (ΔG) in structure stability[a]	
			Structure 1	Structure 2
tnaC280	U-62→G	Noninducible	0.0	−6.0
tnaC284	A-65→G	Noninducible	+2.4	−1.7
tnaC283	A-65→U	Noninducible	+2.7	0.0
tnaC286	U-66→A	Noninducible	+3.2	0.0
tnaC293	G-70→A	Noninducible	−0.3	+3.1
tnaC303	A-76→G	Noninducible	0.0	0.0
tnaC305	U-80→C	Noninducible	0.0	+0.9
tnaC282	C-63→U	Regulated	+3.1	0.0
tnaC285	U-66→C	Regulated	−1.2	0.0
tnaC307	U-81→A	Regulated	0.0	+1.2
tnaC308	U-81→C	Regulated	0.0	+1.2
tnaC300	C-72→U	Regulated	0.0	+1.2
tnaC301	C-75→U	Regulated	0.0	−0.7

[a] A positive change indicates decreased stability; values are expressed in kilocalories per mole (1 kcal = 4.184 kJ). The free energies of formation for structures 1 and 2 are −7.0 and −8.6 kcal/mol, respectively (Fig. 3).

RNA binding site. As mentioned above, RNA-binding proteins have been shown to modulate transcription termination. Thus, the NusB-S10 heterodimer appears to bind to phage λ *boxA* sequences in vitro (20) and, along with other viral and cellular factors, to promote antitermination past the Rho-dependent terminator λ tR1 (8, 10, 20). A segment at the end of the *tnaC* transcript of *E. coli*, codons 23 to 25, shows some homology to λ *boxA*. This segment, when mutated, can result in partial constitutive expression of the *tna* operon (39), which is the opposite of the phenotype observed in *boxA* mutants of phage λ (10).

Conserved codons 11 to 16 of *tnaC* of *E. coli* could constitute all or part of a recognition site for a unique RNA-binding antitermination protein. Three lines of evidence argue against this possibility. First, introducing the *E. coli tna* operon into two species that lack tryptophanase allows tryptophan-induced expression of the operon in these species (Tables 2 and 3). It seems unlikely that two species that lack tryptophanase would both possess an RNA-binding protein specific for antitermination in the *tna* operon. Second, attempts to detect a gene in *E. coli* encoding such a presumed *trans*-acting factor have failed. Mutations that would render the factor nonfunctional, temperature sensitive, or reduced in function have been sought but have not been found; overexpression of randomly cloned wild-type *E. coli* genes from an *E. coli* genomic library also failed to detect a genetic fragment that would specify a *trans*-acting, *tna*-specific regulatory protein in *E. coli*. Third, some amino acids encoded in the conserved segment of *tnaC* appear to be crucial for proper induction. Thus, single amino acid changes resulting from alleles *tnaC281*, *tnaC286*, and *tnaC299* prevent tryptophan induction, whereas near-normal induction is observed in cells containing alleles *tnaC282*, *tnaC285*, and *tnaC 300* (Table 4), yet each pair of alleles (*tnaC281* and *tnaC282*, *tnaC286* and *tnaC285*, and *tnaC299* and *tnaC300*) contains a mutation at an identical nucleotide position. These results argue against a *trans*-acting *tna*-specific RNA-binding protein being responsible for antitermination.

Rate of translation of *tnaC*. The spacing between a translating ribosome and a transcribing RNA polymerase can be crucial for regulation of transcription termination, as in the leader regions of many biosynthetic operons, in which the coding region for a leader peptide is immediately followed by a Rho-independent terminator (44). The rate of translation of the codons of *tnaC* and the subsequent spacing between the translating ribosome and RNA polymerase could be sensitive to the charged $tRNA^{Trp}$ concentration and could modulate the termination-antitermination decision by physically blocking Rho's access to the *rut* site on the transcript. However, mutations in *tnaC* that prevent tryptophan induction cannot be explained simply by altered translation rates. Thus, changing Phe codon 13 to Ile codon ATC (*tnaC279*) or Cys codon TGC (*tnaC280*) or changing Asn codon 14 to Ile codon ATT (*tnaC283*) renders *tna* operon expression mostly noninducible by tryptophan (Table 4), yet these new codons represent slowly (TGC) versus rapidly (ATT and ATC) translated codons (4, 37). Also, the substitution of 33 unique codons (encoding 16 different amino acids) for Trp codon 12 did not allow induction by any of the specified amino acids (9b), yet the rates of translation of these substituted codons would be quite varied. Thus, there is little correlation between the rate of translation of individual codons present at positions 12 to 14 of *tnaC* and the phenotype of cells carrying mutations in these codons. Therefore, some other feature of the leader transcript must be responsible for the *tna*-specific antitermination-termination decision. Nevertheless, translation of Trp codon 12, or the context of this codon, appears to play a crucial role in regulation.

Ribosomal frameshifting. Mutations that introduce frameshifts in the *tnaC* coding region can cause antitermination, presumably by allowing ribosomes to translate in the −1 reading frame for an additional 75 nucleotides beyond *tnaC* to the next in-phase stop codon (12). Presumably, continued translation physically blocks Rho's access to its binding site, *rut* (12). To test the possibility that tryptophan induction of *tna* operon expression involves frameshifting during translation of *tnaC*, stop codons were introduced at seven different positions in *tnaC*, in all three reading frames. In alleles *tnaC282*, *tnaC300*, and *tnaC301*, stop codons were introduced in the −1 frame (Table 6), yet none of these altered amino acids specified by the natural *tnaC* reading frame. These changes did not prevent induction. In addition, in allele *tnaC307* a stop codon was introduced in the +1 frame; this change altered the third position of a codon in the natural reading frame but did not prevent induction. However, the changes in alleles *tnaC263*, *tnaC323*, and *tnaC314* eliminated induction; in each a stop codon was introduced in the natural frame (Table 6). These results demonstrate that although translation of *tnaC* in its natural reading frame is essential for induction, translation of *tnaC* in other reading frames does not appear to be required for normal regulation of operon expression. Several of the mutant constructs examined in the work presented in Table 4 were analyzed for *trans* effects on the chromosomal *tna* operon. Noninducible mutants were not influenced by the presence or absence of a chromosomal *tna* operon, and chromosomal *tna* operon expression showed normal regulation in response to tryptophan (data not shown).

Role of the TnaC peptide. Since TnaC production in *trans* could not overcome *cis*-acting mutations in *tnaC*, it is possible that this peptide acts solely in *cis* to modulate the termination-antitermination decision. The five-residue peptide MVKTD, a portion of the *cat-86* leader peptide, and the eight-residue peptide MSTSKNAD, from the *cmlA* leader peptide, both act in *cis* to inhibit the peptidyl transferase activities of their associated translating ribosomes (19, 22, 28). The central regions of *E. coli* and *P. vulgaris* TnaC have the identical amino acid sequence KWFNID, whereas the same region of *E. aerogenes* TnaC has a similar sequence, DWYNLD (Fig. 1). Every mutation we examined that changed this conserved amino acid sequence resulted in the loss of tryptophan induction (Table 4). By contrast, mutations that altered third positions of codons allowed induction when the replacing codon did not specify an amino acid change (in alleles *tnaC282*, *tnaC285*, *tnaC300*, *tnaC301*, *tnaC307*, and *tnaC308*). However, mutational changes at some of these same positions did eliminate induction when a different amino acid was specified (in alleles *tnaC281*, *tnaC 286*, and *tnaC299*) (Table 4). Also, as shown previously (12), cells with a Trp-12-to-stop codon mutation were uninducible, but these cells were inducible when a tryptophan-inserting $tRNA^{Trp}$ suppressor was present (Table 5). Interestingly, we observed partial induction when a tryptophan-inserting glutamine tRNA suppressor was present (Table 5); non-tryptophan-inserting tRNA suppressors did not restore induction (12). Although the general conclusion that the TnaC peptide appears to act in *cis* during tryptophan induction can be drawn, inspection of the data in Table 4 reveals that there are significant changes in either the uninduced or induced levels of expression of the operon in different mutants. These facts suggest that these changes affect operon expression in other ways.

Our combined data show a strong correlation between the presence of the correct amino acid sequence in the tryptophan-containing segment of the TnaC peptide and inducibility of the *tna* operon. Our findings also argue against other possible roles

for the *tnaC* leader peptide-coding region. We conclude, therefore, that synthesis of a particular segment of the TnaC peptide is essential for tryptophan-induced expression of the *tna* operon and that the peptide probably acts in *cis* to facilitate induction. We propose that the TnaC peptide may act on its translating ribosome and, by affecting elongation, stalling, or release of the ribosome, modulate access of Rho factor to the nascent transcript. The role of tryptophan availability in the induction process remains unclear. Also unexplained is the *trans* inhibitory effect of translation of a second copy of *tnaC* when present in the same cell on a multicopy plasmid (11). We also do not understand the role of the *boxA*-like sequence in *tnaC* in mediating Rho termination. Despite these unexplained features, our findings suggest that the *tna* operon is regulated by a novel form of transcription attenuation in which a *cis*-acting leader peptide promotes antitermination past Rho-dependent transcription termination sites.

ACKNOWLEDGMENTS

We are grateful to Paul Gollnick for the gifts of plasmids pPDG13 to pPDG19, to Dieter Söll for plasmid psu$^+$2op, and to Steve Elledge for plasmid pSE400. We are also indebted to Virginia Horn and Tina Lai for assistance in strain construction and enzyme assays and to Mark Eshoo for providing data on Trp-12 mutants. We thank Ajith Kamath, Enrique Merino, and Joe Sarsero for critical readings of the manuscript.

This work was supported by Public Health Service grant GM 09738. K.G. was a predoctoral fellow supported in part by a National Science Foundation Predoctoral Fellowship and by the National Institutes of Health Graduate Training Program in Biotechnology.

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