JOURNAL OF BACTERIOLOGY, Mar. 1999, p. 1530–1536 Vol. 181, No. 5
0021-9193/99/$04.00+0

Role of Ribosome Release in Regulation of *tna* Operon Expression in *Escherichia coli*

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Received 3 June 1998/Accepted 17 December 1998

Expression of the degradative tryptophanase (*tna*) operon of *Escherichia coli* is regulated by catabolite repression and tryptophan-induced transcription antitermination. In cultures growing in the absence of added tryptophan, transcription of the structural genes of the *tna* operon is limited by Rho-dependent transcription termination in the leader region of the operon. Tryptophan induction prevents this Rho-dependent termination, and requires in-frame translation of a 24-residue leader peptide coding region, *tnaC*, that contains a single, crucial, Trp codon. Studies with a *lacZ* reporter construct lacking the spacer region between *tnaC* and the first major structural gene, *tnaA*, suggested that tryptophan induction might involve *cis* action by the TnaC leader peptide on the ribosome translating the *tnaC* coding region. The leader peptide was hypothesized to inhibit ribosome release at the *tnaC* stop codon, thereby blocking Rho's access to the transcript. Regulatory studies with deletion constructs of the *tna* operon of *Proteus vulgaris* supported this interpretation. In the present study the putative role of the *tnaC* stop codon in *tna* operon regulation in *E. coli* was examined further by replacing the natural *tnaC* stop codon, UGA, with UAG or UAA in a *tnaC*-stop codon-*tnaA′*-′*lacZ* reporter construct. Basal level expression was reduced to 20 and 50% when the UGA stop codon was replaced by UAG or UAA, respectively, consistent with the finding that in *E. coli* translation terminates more efficiently at UAG and UAA than at UGA. Tryptophan induction was observed in strains with any of the stop codons. However, when UAG or UAA replaced UGA, the induced level of expression was also reduced to 15 and 50% of that obtained with UGA as the *tnaC* stop codon, respectively. Introduction of a mutant allele encoding a temperature-sensitive release factor 1, *prfA1*, increased basal level expression 60-fold when the *tnaC* stop codon was UAG and 3-fold when this stop codon was UAA; basal level expression was reduced by 50% in the construct with the natural stop codon, UGA. In strains with any of the three stop codons and the *prfA1* mutation, the induced levels of *tna* operon expression were virtually identical. The effects of *tnaC* stop codon identity on expression were also examined in the absence of Rho action, using *tnaC*-stop codon-′*lacZ* constructs that lack the *tnaC-tnaA* spacer region. Expression was low in the absence of *tnaC* stop codon suppression. In most cases, tryptophan addition resulted in about 50% inhibition of expression when UGA was replaced by UAG or UAA and the appropriate suppressor was present. Introduction of the *prfA1* mutant allele increased expression of the suppressed construct with the UAG stop codon; tryptophan addition also resulted in ca. 50% inhibition. These findings provide additional evidence implicating the behavior of the ribosome translating *tnaC* in the regulation of *tna* operon expression.

Tryptophanase is a multifunctional enzyme that degrades L-tryptophan to indole, pyruvate, and ammonia (27) by a β-elimination reaction (34). Bacteria with tryptophanase activity can utilize tryptophan as a source of carbon, nitrogen, and energy (23). In addition, since the β-elimination reaction is reversible, tryptophanase can synthesize L-tryptophan from indole and L-serine, L-cysteine, or pyruvate and ammonia (34, 53).

Transcription of the *tna* operon of *E. coli* has been shown to be regulated by catabolite repression of transcription initiation (1, 39, 40) and by tryptophan-induced transcription antitermination (46). The tryptophanase (*tna*) operon has been cloned and sequenced from *Escherichia coli* (10), *Proteus vulgaris* (24), *Enterobacter aerogenes* (26), *Symbiobacterium thermophilum* (22), and the pathogen *Haemophilus influenzae* type b (30). The *tna* operon of *E. coli* contains two major structural genes, the promoter proximal gene, *tnaA*, encoding tryptophanase, and a distal gene, *tnaB*, encoding a low-affinity, high-capacity, tryptophan permease (10, 12). *tnaA* is preceded by a transcribed regulatory leader region containing a short open reading frame, *tnaC*, specifying a 24-residue leader peptide. Between *tnaC* and *tnaA* there is a ca. 200-bp spacer region containing several transcription pause sites. When cells grow in the absence of inducer, tryptophan, transcription is subject to Rho-dependent transcription termination (45) at these pause sites. In the presence of inducer, termination at these sites is prevented and expression is elevated 15- to 100-fold (46). Induction requires translation of *tnaC*, which encodes a peptide with a single Trp residue at position 12. Tryptophan induction is not observed when the *tnaC* start codon is replaced by a stop codon, or when the Trp codon at position 12 is replaced by codons specifying other amino acids (13, 17, 47). Additional residues in the TnaC peptide are also essential for induction, particularly several residues near the Trp residue (15).

Several classes of *tna* operon constitutive mutants have been isolated; these show elevated expression of the operon in the absence of added tryptophan (46). Many constitutive mutants have single base pair changes in a 9-bp sequence at the distal end of *tnaC* (46) which has homology to *boxA* of bacteriophage λ (14). *boxA* is critical for proper antitermination, not termination, in phage λ (9) and other systems (21, 50). Deletion of a putative Rho utilization (*rut*) site located immediately following the *tnaC* UGA stop codon also results in constitutive

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expression of the operon (17). Because the *boxA*-like sequence in the *tna* operon does not behave like a typical *boxA*, it is conceivable that recognition of both the *boxA*-like sequence and the presumed *rut* site are required for Rho-dependent transcription termination in this operon. A third class of constitutive mutants contains +1 or −1 frameshift mutations in *tnaC* that allow translation to proceed well beyond the presumed *rut* site that follows the *tnaC* stop codon (17). Continued translation presumably interferes with Rho action. However, it is unlikely that tryptophan induction involves frameshifting, because stop codons introduced in the +1 and −1 reading frames between Trp codon 12 and the *tnaC* stop codon do not affect either basal level expression or tryptophan induction (15). Similarly, introducing stop codons in the +1 or −1 reading frame immediately following *tnaC* does not interfere with the effects of induction (25).

The precise mechanism by which added tryptophan induces *tna* operon expression is not known. Previous studies indicated that no unique regulatory factor other than TnaC is needed for induction (15, 16). Specifically, a plasmid containing a foreign promoter, the *E. coli tna* leader regulatory region, and a *lacZ* translational fusion to the first 20 codons of *tnaA* responded to tryptophan induction when introduced into two bacterial species that do not produce tryptophanase (15). Thus, if additional factors are required for induction, they are likely to be components common to many bacterial species, including those that lack a *tna* operon (15). All tests of TnaC *trans*-activation have given negative results (46).

A hypothesis consistent with all the observations made to date is that in the presence of inducer, the nascent TnaC peptide acts in *cis* on the ribosome translating *tnaC* and inhibits its release at the *tnaC* stop codon. The inhibited ribosome would interfere with Rho's binding to the *tna* transcript, thereby preventing transcription termination. If ribosome release or stalling were essential in the regulation of *tna* operon expression, then peptide chain release factors and the ribosome release factor could be influential in setting the basal or induced level of expression of the operon. In *E. coli*, two codon-specific peptide chain release factors (RF1 and RF2) direct termination of protein synthesis; RF1 recognizes UAG and UAA stop codons (54), whereas RF2 acts at the UGA and UAA stop codons (6). Unlike other prokaryotic RF2s, *E. coli* RF2 terminates translation weakly at UGA and UAA stop codons (33, 48). The third release factor, RF3, enhances the activity of RF1 and RF2 and lacks nonsense codon specificity (18, 31).

In previous studies performed to examine models invoking ribosome release or stalling, we designed and tested a construct that would not be subject to Rho regulation. This construct, *tnaC*-UGA-′*lacZ*, lacks the *tnaC-tnaA* spacer region within which Rho-dependent termination occurs (28). Its expression is dependent on translation of the UGA codon and synthesis of a TnaC-′LacZ fusion protein. Using this construct and various nonsense suppressors, we showed that addition of inducer inhibited β-galactosidase (β-Gal) formation and that this inhibition was dependent upon the presence of Trp residue 12 in the TnaC portion of the fusion protein (28). We found that inactivation of the structural gene for RF3 (55), increased β-Gal production 30-fold, and this increase was also reduced by the presence of tryptophan (28). In a parallel study with deletion derivatives of the *tna* operon of *P. vulgaris*, it was observed that a deletion that places the Shine-Dalgarno region for *tnaA* (in *tnaA′-′lacZ* fusion) near the *tnaC* stop codon results in tryptophan inhibition of *tnaA′-′lacZ* expression (25).

In this report, we extend our examination of the role of the *tnaC* stop codon by replacing the natural UGA stop codon with UAG and UAA. We show that the presence of UAG or UAA leads to a reduction of both basal and induced expression with *tnaC*-stop codon-*tnaA′-′lacZ* constructs containing the intact *tnaC-tnaA* spacer region. We also show that a temperature-sensitive mutation altering RF1 increases basal level expression in strains with UAG or UAA as the *tnaC* stop codon, but not in a strain with UGA. The induced levels of expression in these same strains are indistinguishable. We also examine the role of the three stop codons in a *tnaC*-stop codon-′*lacZ* fusion construct and show that tryptophan addition inhibits reading past the stop codon; however, this inhibition is not as pronounced when UAG or UAA replaces UGA. An RF1 mutation increased expression of the UAG construct in the presence and absence of tryptophan. These findings support the hypothesis that in the presence of tryptophan, TnaC peptide inhibition of ribosome release at the *tnaC* stop codon may be the crucial event that regulates Rho-dependent termination in the *tna* operon leader region.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
Strains		
CY15076	W3110 *tnaA2* Δ*lacU169*	This study
PDG1158	SVS1100 (λ*tna*$_p$ *tnaC*-UAA-*tnaA′-′lacZ*)	17
PDG1184	SVS1100 (λ*tna*$_p$ *tnaC*-UUA-*tnaA′-′lacZ*)	17
SVS1100	W3110 *bglR55*Δ (*lac-argF*)*U169*	46
SVS1144	SVS1100 (λ*tna*$_p$ *tnaA′-′lacZ*)	46
VK100	SVS1100 (λ*tna*$_p$ *tnaC*-UGA-′*lacZ*)	28
VK100/SerU^{UGA}	VK100 *serU* [Ser UG(A/G)] suppressor	28
VK700	CY15076 (λ*tna*$_p$ *tnaC*-UAG-′*lacZ*)	This study
VK700/SerU^{UAG}	VK700 *serU* (Ser UAG suppressor)	This study
VK701	VK700 *prfA1*(Ts)	This study
VK800	CY15076 (λ*tna*$_p$ *tnaC*-UAG-*tnaA′-′lacZ*)	This study
VK801	VK800 *prfA1*(Ts)	This study
VK805	VK801 *trpA9761*	This study
VK900	CY15076 (λ*tna*$_p$ *tnaC*-UAA-′*lacZ*)	This study
VK1100	CY15076 (λ*tna*$_p$ *tnaC*-UAG UUUGAC-*tnaA′-′lacZ*)	This study
VK1101	VK1100 *prfA1*(Ts)	This study
VK1145	SVS1144 *prfA1*(Ts)	This study
VK1159	PDG1158 *prfA1*(Ts)	This study
VK1185	PDG1184 *prfA1*(Ts)	This study
VK1200	CY15076 (λ*tna*$_p$ *tnaC*-UAG UUGACC-*tnaA′-′lacZ*)	This study
VK1201	VK1200 *prfA1*(Ts)	This study
Plasmids		
pGFIBI	*hisT su* (UAG suppressor)	35
pMY228	*trpT su7* (UAG suppressor)	36
pRS552	pBR322 derivative, *lac*-based vector	44
pSWC115	*trpT su7* (UAA suppressor)	36

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and the plasmids used in this study are listed in Table 1. Strains VK700, VK800, and VK900 and their derivatives are all single lysogens carrying λRS45 (44) with various inserts. To prepare these strains, the *tnaC*-UA(G/A)-′*lacZ* and the *tnaC*-UAG-*tnaA′-′lacZ* fusions from pRS552 (Table 1) were independently crossed into phage λRS45 (44) and the recombinant phage genome was inserted into the chromosome of CY15076 (Table 1). *prfA1*(Ts) (37, 38) or the *serU* suppressors [su UAG or su UG(A/G)] (2, 28) were introduced into VK100 (28), VK700, or VK800 by P1 transduction. Mutant *trpA9761* is an amber mutant of *trpA* in *E. coli*. The *trpA9761* allele was introduced into appropriate strains by transduction. Plasmids were introduced into various strains by transformation (41), selecting for the appropriate antibiotic resistance marker.

Media and enzyme assay. Vogel and Bonner minimal medium (49) was used throughout. For β-Gal assays (32), cultures were generally grown with shaking at 30 or 37°C in minimal medium plus 0.2% glycerol–0.05% acid-hydrolyzed casein, with or without L-tryptophan (100 μg/ml). When appropriate, media were supplemented with kanamycin (30 μg/ml), tetracycline (15 μg/ml), or ampicillin (100 μg/ml). β-Gal assays were performed as described by Miller (32); β-Gal activity

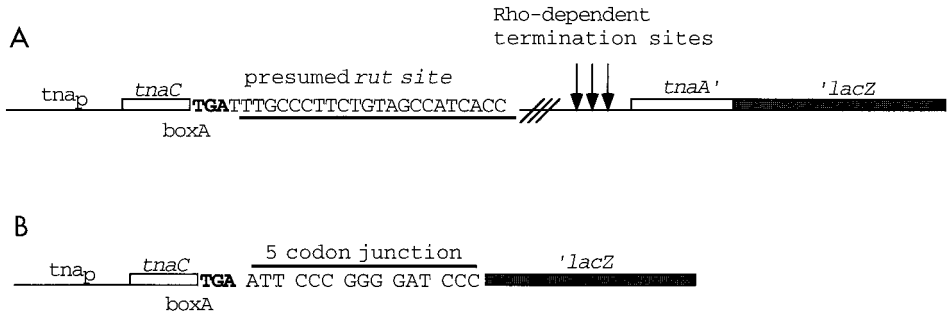

FIG. 1. Schematic representations of the basic *tna* operon-*'lacZ* fusions employed in this study. (A) *tnaC*-UGA-*tnaA'-'lacZ* construct in strain SVS1144. The underlined *rut* site is part of the natural *tnaC-tnaA* spacer region, which also contains Rho-dependent termination sites (45). (B) *tnaC*-UGA-*'lacZ* construct in strain VK100. *tnaC*, its UGA stop codon, and a five-codon junction are in phase with *lacZ* lacking its first eight codons. The UGA stop codon (shown in boldface type) in both constructs was also changed to UAG or UAA.

is reported in Miller units (32). For tryptophan synthetase (TSase) α and β_2 assays, cultures were grown overnight at 37°C in the same minimal medium supplemented with 0.2% glycerol, 0.05% acid-hydrolyzed casein, and indole (4 μg/ml). Cells were collected by centrifugation, washed, and frozen. Each frozen pellet was resuspended in 0.1 M Tris buffer, pH 7.8, and disrupted by sonication. The sonication extracts were centrifuged and the cell supernatants were assayed for TSase activity in the indole plus serine to tryptophan reaction (8) in the presence and absence of excess wild-type TSase α or β_2. For a definition of the unit of specific activity, and for other assay conditions, see the work of Creighton and Yanofsky (8).

Site-directed mutagenesis. Two PCR-based methods (28) were used to introduce point mutations in the *tnaC* region. In the standard PCR approach, the VK1 primer, 5′-CGG AAT TCA GCT TCT GTA TTG GTA AG-3′, has a sequence of nucleotides identical to the region upstream of *tnaC* and contains an *Eco*RI site at its 5′ end. The mutagenic TNAC-UAG primer, 5′-GGG ATC CCC GGG AAT CTA AGG GCG GTG ATC-3′, and TNAC-UAA primer, 5′-TCC CCC GGG AAT TTA AGG GCG GTG-3′, contain a *Bam*HI site and a *Sma*I site at their respective 5′ ends. Primer pairs VK1 and TNAC-UAG or VK1 and TNAC-UAA were used with *Taq* polymerase (Boehringer Mannheim Co., Indianapolis, Ind.) to amplify the 353- and 347-bp PCR products, respectively. These products were individually cloned into the pCRII vector (Invitrogen Co., San Diego, Calif.), and sequences were confirmed by dideoxy sequencing (42). The 353-bp insert in the pCRII vector was digested with *Eco*RI and *Bam*HI, purified by using the GENECLEAN II kit (BIO 101 Inc., La Jolla, Calif.), and subcloned into the *Eco*RI-, *Bam*HI- cleaved pRS552 (44) to give the *tnaC*-UAG-*'lacZ* construct. To prepare the *tnaC*-UAA-*'lacZ* construct, the 347-bp insert in the pCRII vector was first cloned into the *Eco*RI-, and *Sma*I- cleaved sites of pBluescript (41). The resulting recombinant was digested with *Eco*RI and *Bam*HI, and the insert was subcloned into the *Eco*RI-, *Bam*HI-cut sites of pRS552 (44).

The megaprimer PCR method (43) was used to make the *tnaC*-UAG-*tnaA'-'lacZ* construct. First, the LACZ-RT primer, 5′-GCG ATT AAG TTG GGT AAC GCC AGG-3′, and the mutagenic TNAC-UAG-TNA primer, 5′-CAC CGC CCT TAG TTT GCC CTT CTG-3′, were used with *Taq* polymerase to amplify a 305-bp product. This product was then purified by using a PCR purification kit (QIAGEN Inc., Chatsworth, Calif.) and combined with the VK1 primer, 5′-CGG AAT TCA GCT TCT GTA TTG GTA AG-3′, to amplify the final 675-bp PCR product. This product was also cloned into the pCRII vector and sequenced (42). The resulting 675-bp insert was cleaved with *Eco*RI and *Bam*HI, purified by using the GENECLEAN II kit, and subcloned into the *Eco*RI-, *Bam*HI-cleaved pRS552 vector (44). This approach was also used to introduce the primer sequences 5′-CGC CCT TAG TTT GAC CTT CTG TAG CCA TCA-3′ and 5′-CAC CGC CCT TAG TTG ACC CTT CTG TAG CCA TCA-3′ (with mutational changes underlined) into the *tnaC*-UAG-*tnaA'-'lacZ* construct.

RESULTS

Basal and induced expression from *tnaC*-stop codon-*tnaA'-'lacZ* constructs in which UAG or UAA replaces UGA as the *tnaC* stop codon. To determine whether the identity of the *tnaC* stop codon influences basal or induced expression of the *tna* operon, constructs containing each of the three stop codons were prepared and integrated into the *E. coli* chromosome, and the β-Gal activities of the resulting strains were determined. The natural *tnaC* stop codon of *E. coli* is UGA (Fig. 1) (10, 46). With constructs containing the UAG and UAA stop codons, basal expression of the operon was 20 and 50%, respectively, of that observed with the UGA stop codon (Table 2). These findings are consistent with evidence demonstrating that in *E. coli*, RF1 terminates translation more efficiently than RF2 (7, 48). Following growth with tryptophan as inducer, the same pattern was observed; induced expression was only 15 or 60%, respectively, of that obtained with the *tnaC*-UGA stop codon construct (Table 2). These findings establish that both basal and induced expression of the *tna* operon are influenced by the identity of the *tnaC* stop codon.

In strain PDG1184, the *tnaC* stop codon was replaced by a leucine codon, UUA (17). This change allows translation to proceed five codons beyond the last sense codon of *tnaC*, to a UAG termination codon (17). Such a construct exhibits expression levels much like those of the UAG construct VK800; however, basal level expression is two-fold higher (Table 2).

Effects of the presence of a mutant RF1 on *tna* operon expression. Mutant allele *prfA1* encodes a temperature-sensitive RF1. This allele was introduced into appropriate strains, and basal and induced expression were measured (Table 3). Since strains with *prfA1* are temperature sensitive for growth, they were grown at the permissive temperature, 30°C (38). The *prfA1* allele increased basal expression of the constructs with the UAG and UAA stop codons 60- and 3-fold, respectively; it reduced both basal and induced expression of the construct with the wild-type *tnaC* UGA stop codon by 50%. Induced expression levels of strains with the *prfA1* allele and any of the

TABLE 2. Basal and induced expression in strains with constructs containing one of three stop codons (at 37°C)

Strain	Relevant features	Avg β-Gal activity (Miller units) ± SD[b]		
		−Trp	+Trp	+Trp/−Trp[c]
SVS1144	*tnaC*-UGA-*tnaA'-'lacZ*	518 ± 20	14,019 ± 1,159	27
VK800	*tnaC*-UAG-*tnaA'-'lacZ*	99 ± 12	1,919 ± 252	19
VK800/pMY228[a]	VK800 with su UAG (Trp)	367 ± 33	1,199 ± 82	3
PDG1158	*tnaC*-UAA-*tnaA'-'lacZ*	223 ± 17	8,344 ± 940	37
PDG1184	*tnaC* stop to UUA[d]	239 ± 9	2,076 ± 97	9

[a] pMY228, a plasmid carrying a Trp-inserting $tRNA^{Trp}$ UAG suppressor

[b] Cultures were grown at 37°C in minimal medium (49) plus 0.2% glycerol and 0.05% acid-hydrolyzed casein, with (+Trp) or without (−Trp) 100 μg of L-Trp per ml. For β-Gal assay conditions, see Materials and Methods. Values are for at least five cultures of each type.

[c] Ratio of β-Gal activities with and without Trp.

[d] Adds five sense codons to *tnaC* to a UAG.

TABLE 3. Effects of a *prfA1* mutation on basal and induced expression in strains with one of three stop codons (at 30°C)

Strain	Relevant features	Avg β-Gal activity (Miller units) ± SD[a]		
		−Trp	+Trp	+Trp/−Trp[b]
SVS1144	*tnaC*-UGA-*tnaA′-′lacZ*	729 ± 24	16,244 ± 729	22
VK1145	SVS1144 *prfA1*	421 ± 52	6,833 ± 875	16
VK800	*tnaC*-UAG-*tnaA′-′lacZ*	49 ± 3	1,102 ± 104	22
VK801	VK800 *prfA1*	3,093 ± 295	7,096 ± 548	2.3
PDG1158	*tnaC*-UAA-*tnaA′-′lacZ*	163 ± 3	6,554 ± 357	40
VK1159	PDG1158 *prfA1*	459 ± 21	7,634 ± 861	17
PDG1184	*tnaC* stop to UUA[c]	72 ± 4	626 ± 80	9
VK1185	PDG1184 *prfA1*	513 ± 23	881 ± 45	1.7

[a] Cultures were grown at 30°C in minimal medium (49) plus 0.2% glycerol and 0.05% acid-hydrolyzed casein, with (+Trp) or without (−Trp) 100 μg of L-Trp per ml. For β-Gal assay conditions, see Materials and Methods. Values are for at least five cultures of each type.
[b] Ratio of β-Gal activities with and without Trp.
[c] Adds five codons to *tnaC* to a UAG.

TABLE 5. Introducing the UGA stop codon in the −1 or +1 reading frame immediately following *tnaC*-UAG does not appreciably alter the response to *prfA1*

Strain	Relevant features[a]	Avg β-Gal activity (Miller units) ± SD[b]		+Trp/−Trp[c]
		−Trp	+Trp	
VK800	*tnaC*-UAG-*tnaA′-′lacZ*	52 ± 4	1,218 ± 164	23
VK801	VK800 *prfA1*	953 ± 83	3,090 ± 434	3.2
VK1100	*tnaC*-UAG UUUGCC to *tnaC*-UAG UU**UGA**C	182 ± 15	1,270 ± 101	7
VK1101	VK1100 *prfA1*	953 ± 118	2,074 ± 300	2.2
VK1200	*tnaC*-UAG UUUGCC to *tnaC*-UAG U**UGA**CC	27 ± 5	857 ± 94	32
VK1201	VK1200 *prfA1*	261 ± 18	841 ± 81	3.2

[a] Underlined changes introduce stop codons in the −1 or +1 reading frame following *tnaC*-UAG. The out-of-frame UGA stop codon is in boldface type.
[b] Cultures were grown at 30°C in minimal medium (49) plus 0.2% glycerol and 0.05% acid-hydrolyzed casein, with (+Trp) or without (−Trp) 100 μg of L-Trp per ml. For β-Gal assay conditions, see Materials and Methods. Values are for at least five cultures of each type.
[c] Ratio of activities with and without Trp.

three *tnaC* stop codons were virtually identical (Table 3). The *prfA1* allele increased basal level expression of the construct in strain PDG1184 sevenfold, but there was little induced expression (Table 3).

Test for suppression of the *trpA9761* amber mutation in VK801 derivatives. The high basal level expression observed in strain VK801 (*tnaC*-UAG-*tnaA′-′lacZ*/*prfA1*) (Table 3) could be due to specific suppression of the *tnaC* UAG stop codon by a resident UAG suppressor. Suppression would allow translation to continue four codons downstream from the *tnaC* stop codon. Previous findings argue against this possibility. First, suppression of the *tnaC* stop codon should at best approach expression in strain PDG1184, in which a sense codon replaces the *tnaC* stop codon. As shown in Table 2, strain PDG1184 does not exhibit high basal expression in the absence of tryptophan. Second, suppression of the *tnaC* UAG stop codon in strain VK800 (*tnaC*-UAG-*tnaA′-′lacZ*) gives basal and induced expression levels comparable to those of strain PDG1184 (Table 2).

Two tests were performed to rule out significant amber suppression in strain VK801 containing *prfA1*. An amber mutation that is known to respond to all tested amber and ochre suppressors, *trpA9761*, was introduced into VK801, yielding strain VK805 (Tables 1 and 4). Isolates of VK805 were observed to be tryptophan-dependent, indicating that the *prfA1* alteration does not allow natural suppression of the *trpA9761* amber codon by insertion of an amino acid that restores TSase α activity. Although *trpA9761* is known to be suppressed to prototrophy by known amber and ochre suppressors, it is conceivable that the *prfA1* mutation allows insertion of an amino acid that does not restore TSase α activity. The *trpA* system provides an excellent test of this possibility since virtually all *trpA* missense mutants produce an enzymatically inactive protein that nevertheless can complex with TSase β_2 and activate this subunit 30-fold in the indole plus serine to tryptophan reaction. Two isolates of strain VK805 were examined, and no significant TSase α protein was observed, demonstrating the absence of significant amber suppression (Table 4). Other data also indicate that the increased *tna* operon expression associated with the *prfA1* allele is not due to significant amber suppression (see Table 7).

TABLE 4. TSase α and β_2 activities of *trpA prfA1*(Ts) strains[a]

Strain	Characteristics	Sp act of[b]:		TSase α/TSase β_2[c]
		TSase α	TSase β	
VK800	*tnaC*-UAG-*tnaA′-′lacZ*	2.8	3.3	0.85
VK801	VK800 *prfA1*	3.4	4.9	0.69
VK805[d]	VK801 *trpA9761*	0.17[e]	15	0.01
VK805[d]	VK801 *trpA9761*	0.07[e]	13	0.01

[a] Cultures were grown overnight in minimal medium (49) supplemented with 0.2% glycerol, 0.05% acid-hydrolyzed casein, and 4 μg indole per ml. For TSase assay conditions, see Materials and Methods.
[b] Indole plus serine to tryptophan reaction.
[c] Ratio of specific activities of TSase α and TSase β_2.
[d] Two different VK805 isolates were used.
[e] Corrected for the inherent activity of TSase β_2 in the indole to tryptophan reaction.

Effects of introducing UGA stop codons in the −1 or +1 potential reading frames immediately following the *tnaC* UAG stop codon. Point mutations were introduced in the spacer region of the *tnaC*-UAG-*tnaA′-′lacZ* construct to test whether there is ribosomal frameshifting beyond the *tnaC* UAG stop codon in the *prfA1* mutant background (Table 5). The resulting constructs *tnaC*-UAG UU**UGA**C and *tnaC*-UAG U**UGA**CC, introduce out-of-frame UGA stop codons (shown in boldface type). In a *prfA1* mutant background, any −1 or +1 frameshift would allow translation to terminate at a UGA codon immediately following the normal UAG stop codon and would presumably result in β-Gal production similar to that obtained in strain PDG1184 (Table 3). In Table 5, the presence of the *prfA1* allele resulted in a 5- to 10-fold increase in the basal level of β-Gal activity in strains with the constructs *tnaC*-UAG UUUGAC (strain VK1101) and *tnaC*-UAG UUGACC (strain VK1201), respectively; a similar increase (18-fold) in the basal level of β-Gal expression was observed with the control strain VK801 (Table 5). These findings indicate that ribosomal frameshifting beyond the *tnaC* UAG stop codon is not responsible for the increase in the basal level expression in a *prfA1* mutant background. Note that the changes introduced following the *tnaC* stop codon do influence the absolute levels of basal and induced expression.

Tryptophan inhibition of translational readthrough beyond the *tnaC* stop codon in constructs lacking the *tnaC-tnaA* spacer region. In a previous study (28) we designed and tested a construct that we believed would permit analysis of the effects of inducer in the absence of Rho-dependent termination. This construct, *tnaC*-UGA-*′lacZ*, lacks the *tnaC-tnaA* spacer region.

TABLE 6. Tryptophan inhibition of translation beyond the *tnaC* stop codon in constructs lacking the *tnaC-tnaA* spacer region

Strain	Relevant features	Avg β-Gal activity (Miller units) ± SD[f]		+Trp/ −Trp[g]
		−Trp	+Trp	
SVS1144	*tnaC*-UGA-*tnaA'-'lacZ*	465 ± 42	15,728 ± 1,353	34
VK100	*tnaC*-UGA-*'lacZ*	3	0.4	0.1
VK100/SerU[a]	VK100 with su UGA (Ser)	85 ± 6	18 ± 2	0.2
VK700	*tnaC*-UAG-*'lacZ*	0.1	0.03	0.3
VK700/pMY228[b]	VK700 with su UAG (Trp)	430 ± 42	490 ± 39	1
VK700/SerU[c]	VK700 with su UAG (Ser)	190 ± 21	130 ± 9	0.7
VK700/pGFIBI[d]	VK700 with su UAG (His)	709 ± 63	475 ± 48	0.6
VK900	*tnaC*-UAA-*'lacZ*	5 ± 0.5	2 ± 0.4	0.4
VK900/pSWC115[e]	VK900 with su UAA (Trp)	9 ± 1	4 ± 0.4	0.4

[a] SerU, Ser-inserting $tRNA^{Ser}$ UGA suppressor.
[b] pMY228, plasmid carrying a Trp-inserting $tRNA^{Trp}$ UAG suppressor.
[c] SerU, Ser-inserting $tRNA^{Ser}$ UAG suppressor.
[d] pGFIBI, plasmid carrying a His-inserting $tRNA^{His}$ UAG suppressor.
[e] pSWC115, plasmid carrying a Trp-inserting $tRNA^{Trp}$ UAA suppressor.
[f] Cultures were grown at 37°C, in minimal medium (49) plus 0.2% glycerol and 0.05% acid-hydrolyzed casein, with (+Trp) or without (−Trp) 100 μg of L-Trp per ml. For β-Gal assay conditions, see Materials and Methods.
[g] Ratio of activities with and without Trp.

It contains the *tna* promoter through the *tnaC* UGA stop codon, has an added in-phase five-codon junction, and is followed by *lacZ* minus its first eight codons (5) (Fig. 1). Since this construct lacks the *tnaC-tnaA* spacer region, Rho action should be eliminated. Our previous findings supported this expectation (28).

In strain VK100 containing this construct (*tnaC*-UGA-*'lacZ*) with or without UGA suppressors, the presence of tryptophan led to an 80% decrease in β-Gal activity (28). To measure expression in strains with derivatives of this construct in which the UGA stop codon was replaced by UAG or UAA, strains VK700 (*tnaC*-UAG-*'lacZ*) and VK900 (*tnaC*-UAA-*'lacZ*) were prepared (Table 6). When grown in minimal medium with or without tryptophan, strains VK100 (*tnaC*-UGA-*'lacZ*), VK700 (*tnaC*-UAG-*'lacZ*), and VK900 (*tnaC*-UAA-*'lacZ*) had very low β-Gal levels, indicating that these strains lack active nonsense suppressors (Table 6). Introducing appropriate suppressors into these strains did lead to an increase in expression, in the presence or absence of tryptophan. In contrast to our results with the *tnaC*-UGA-*'lacZ* construct (Table 6), the presence of the UAG or UAA stop codon coupled with the appropriate suppressor allowed only modest tryptophan inhibition of expression (30 to 60% inhibition with UAG or UAA versus 80% inhibition with UGA).

To examine the effects of the temperature-sensitive RF1 on translation beyond the *tnaC* UAG stop codon, *prfA1* was introduced into strain VK700 (*tnaC*-UAG-*'lacZ*), yielding strain VK701 (*tnaC*-UAG-'lacZ/*prfA1*) (Table 7). Note that all the strains in Table 7 were grown at 30°C. VK701 showed very low levels of expression, with or without inducer. This result supports the finding that strain VK801 (Table 3) lacks an effective UAG suppressor. Addition of a plasmid containing a UAG-reading $tRNA^{His}$ suppressor (35) into control strain VK700 increased basal expression appreciably and allowed 40% inhibition by the inducer (Table 7). Introduction of this suppressor into strain VK701 (containing *prfA1*) increased expression almost threefold compared to VK700 bearing a suppressor and allowed 50% inhibition of expression by tryptophan.

DISCUSSION

Studies with the *tna* operon of *E. coli* have identified features of the *tna* leader region that are necessary for this operon's regulation by transcriptional attenuation. In the absence of an inducer, transcription is terminated by the action of Rho factor, at one of several transcription pause sites located in the leader region preceding *tnaA*, the first major structural gene of the operon (45). Mutational changes that allow high-level constitutive expression of the operon in the absence of inducer have identified cell components and sequences that are necessary for transcription termination. These mutations alter Rho factor, change bases in critical regulatory sequences in the *tna* leader region (*boxA*, the *rut* site), or allow translation from the *tnaC* coding sequence to proceed in other reading frames, beyond the presumed *rut* site in the *tna* transcript. These observations and other experimental findings suggest that when cells grow without inducer, Rho factor generally binds to the leader segment of the *tna* transcript, moves 3′ on the transcript until it interacts with a paused polymerase molecule in the leader region, and then directs the polymerase to terminate transcription.

In the presence of added tryptophan, Rho-dependent termination in the leader region of the *tna* operon is prevented. Induction requires translation of a 24-residue peptide coding region, *tnaC*, located near the 5′ end of the transcript (17). Several residues of TnaC are essential for induction, suggesting that the peptide as such participates in the induction process. Some tryptophan analogs which do not appear to be incorporated into protein also function as inducers; thus, an inducer may act without being incorporated into the TnaC leader peptide (15, 16). Stop codons introduced in the +1 or −1 reading frames within *tnaC*, or following *tnaC*, do not affect either basal or induced expression, establishing that tryptophan induction does not involve frameshifting (15, 16, 25). Introducing an in-phase stop codon within the distal segment of *tnaC* does prevent induction (15).

How added tryptophan is used as the signal that leads to inhibition of Rho-dependent termination is a basic unanswered question. One possibility is that the TnaC peptide is modified in some manner when produced in cells growing with excess tryptophan and that the altered peptide blocks Rho action. Alternatively, in the presence of added tryptophan some cell component could interact with the TnaC peptide, altering its properties so that it blocks Rho action. Two previous studies (28, 55) and this report have focused on how the

TABLE 7. Effects of a *prfA1* mutation on tryptophan inhibition of translation of the *tnaC*-UAG-*'lacZ* construct (at 30°C)

Strain	Relevant features	Avg β-Gal activity (Miller units) ± SD[b]		+Trp/ −Trp[c]
		−Trp	+Trp	
VK700	*tnaC*-UAG-*'lacZ*	0.05	<0.01	
VK700/pGFIBI[a]	VK700 with su UAG (His)	274 ± 18	173 ± 22	0.6
VK701	VK700 *prfA1*	4 ± 0.8	1.5 ± 0.4	0.4
VK701/pGFIBI[a]	VK701 with su UAG (His)	715 ± 42	355 ± 59	0.5

[a] pGFIBI, plasmid carrying a His-inserting $tRNA^{His}$ UAG suppressor.
[b] Cultures were grown at 30°C in minimal medium (49) plus 0.2% glycerol and 0.05% acid-hydrolyzed casein, with (+Trp) or without (−Trp) 100 μg of L-Trp per ml. For β-Gal assay conditions, see Materials and Methods.
[c] Ratio of activities with and without Trp.

peptide may act in preventing termination. In prior analyses with a suppressed *tnaC*-stop codon-′*lacZ* fusion lacking the *tnaC-tnaA* spacer region, the addition of tryptophan led to inhibition of translation beyond the *tnaC* stop codon (28). Tryptophan addition also reversed the increased expression resulting from inactivation of RF3 (28). In related studies with the *tna* operon of *P. vulgaris*, it was shown that added tryptophan inhibited expression of a *tnaA*′-′*lacZ* translational fusion in which the *tnaA* ribosome binding site was placed close to the *tnaC* stop codon (25). These effects of inducer were not observed when the critical tryptophan codon in the leader peptide coding region was replaced by some other codon (28). The most straightforward interpretation of these observations is that in the presence of tryptophan, the TnaC leader peptide acts in *cis* on the translating ribosome, preventing its release at the *tnaC* stop codon. The stalled ribosome would block Rho's access to the transcript, thereby inhibiting termination.

In the present study we examined the effect of changing the natural *tnaC* UGA stop codon of *E. coli* to the other stop codons, UAG and UAA. We also analyzed the effects of introducing a temperature-sensitive mutant allele, *prfA1*, that alters RF1. With a *lacZ* reporter construct that allows Rho-dependent termination, changing the natural UGA stop codon to UAG lowered basal and induced expression of the operon by ca. 80%. Changing the UAA stop codon to UGA reduced basal and induced expression by 50%. The reduced expression observed with the UAG stop codon relative to the UGA stop codon could be explained most simply if RF1 dissociates the translating ribosome from the *tnaC* UAG stop codon more rapidly than does RF2 at the UGA stop codon. More rapid release could allow more rapid binding of Rho factor. This interpretation is consistent with the conclusion that in *E. coli* RF1 terminates translation more efficiently than RF2 (33, 48). Support for this interpretation is also provided by the observation that a mutant allele specifying a temperature-sensitive RF1, when examined at the permissive temperature, led to a 60-fold increase in basal level expression when the *tnaC* stop codon was UAG (Table 3). In fact, expression was increased only twofold further by the addition of tryptophan.

It is also conceivable that in the presence of the mutant RF1, the nucleotide sequence including the UAG stop codon permits efficient translational frameshifting and reading into the presumed *rut* site. However, introduction of a UGA stop codon in the −1 or +1 reading frame immediately following the *tnaC* UAG stop codon did not eliminate the increased basal level expression of the *tnaC*-UAG construct in a *prfA1* mutant background. Furthermore, our findings with an in-phase *tnaC*-UAG-5 codon junction-′*lacZ* construct (Table 6) that requires suppression of the UAG stop codon for β-galactosidase production also argue against this possibility. Thus, it appears that the rate of ribosome release at the *tnaC* stop codon (and not ribosomal frameshifting beyond the *tnaC* UAG stop codon) may be the key event that determines whether Rho factor will terminate transcription in the leader region of the operon.

In an early study with the *tnaC*-UGA-5 codon junction-′*lacZ* construct, we observed that under a variety of conditions allowing in-phase reading of the *tnaC* UGA stop codon, addition of tryptophan to the culture medium led to ca. 80% inhibition of β-Gal production (28). In this study, the UGA stop codon was changed to UAG or UAA, and the resulting constructs were tested in the presence of appropriate suppressors. We found that in all but one strain (containing a Trp-inserting UAG suppressor) tryptophan addition had a 30 to 60% inhibitory effect on β-Gal production (Table 6). Introduction of the *prfA1* alteration resulted in a threefold increase in translation of the UAG stop codon (Table 7). In this case, the altered RF1 clearly allows increased in-phase translation. Tryptophan addition to this strain led to 50% inhibition of β-Gal production (Table 7). These findings demonstrate that stop codon identity does influence the ability of tryptophan to inhibit ribosome movement beyond the suppressed *tnaC* stop codon.

Inhibition of ribosome function by a leader peptide has been documented in other systems. In prokaryotes, the five-residue leader peptide, MVKTD, encoded by the *cat* leader sequence (19, 29), and the eight-residue peptide, MSTSKNAD, encoded by the *cmlA* leader sequence, act in *cis* to block movement of the translating ribosome (29) by inhibiting its peptidyltransferase activity (20, 29). In both cases, blocking the translating ribosome allows downstream translation of a structural gene conferring drug resistance. In eukaryotes, the 24-residue upstream open reading frame (uORF) encoded upstream of the *arg-2* gene of *Neurospora crassa* (51, 52) and the 22-residue uORF2 encoded by the gp48 gene of cytomegalovirus (3, 4, 11) have been reported to act in *cis* to block release of their translating ribosomes. In these cases, stalling of the translating ribosome at the respective uORF termination codon appears to inhibit translation of a downstream ORF. Additional analyses focused on the TnaC peptide of the *tna* operon should reveal how TnaC acts to increase this operon's expression.

ACKNOWLEDGMENTS

prfA1(Ts) was kindly supplied by S. M. Rydén. *serU* [su UG(A/G)] and *serU* (su UAG) were gifts from E. Murgola and J. R. Beckwith, respectively. We are indebted to Virginia Horn for her technical assistance with *E. coli* strain production. We are also grateful to Peter Margolis, Kristin Black, and M. C. Yee for their critical reading of this manuscript.

These studies were supported by grant GM09738 to C.Y. from the United States Public Health Service. K.V.K. is a Postdoctoral Fellow supported by grant GM09738.

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