Analysis of Tryptophanase Operon Expression in Vitro

ACCUMULATION OF TnaC-PEPTIDYL-tRNA IN A RELEASE FACTOR 2-DEPLETED S-30 EXTRACT PREVENTS Rho FACTOR ACTION, SIMULATING INDUCTION*

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Expression of the tryptophanase (tna) operon in Escherichia coli is regulated by catabolite repression and tryptophan-induced transcription antitermination. The key feature of this antitermination mechanism has been shown to be the retention of uncleaved TnaC-peptidyl-tRNA in the translating ribosome. This ribosome remains stalled at the tna stop codon and blocks the access of Rho factor to the tna transcript, thereby preventing transcription termination. In normal S-30 preparations, synthesis of a TnaC peptide containing arginine instead of tryptophan at position 12 (Arg12-TnaC) was shown to be insensitive to added tryptophan, i.e. Arg12-TnaC-peptidyl-tRNA was cleaved, and there was normal Rho-dependent transcription termination. When the S-30 extract used was depleted of release factor 2, Arg12-TnaC-tRNAPro was accumulated in the absence or presence of added tryptophan. Under these conditions the accumulation of Arg12-TnaC-tRNAPro prevented Rho-dependent transcription termination, mimicking normal induction. Using a minimal in vitro transcription system consisting of a tna template, RNA polymerase, and Rho, it was shown that RNA sequences immediately adjacent to the tnaC stop codon, the presumed boxA and rut sites, contributed most significantly to Rho-dependent termination. The tna boxA-like sequence appeared to serve as a segment of the Rho “entry” site, despite its likeness to the boxA element.

Escherichia coli and many other Gram-negative bacteria use the enzyme tryptophanase to degrade L-tryptophan to indole, pyruvate, and ammonia (1), allowing these microorganisms to utilize tryptophan as a source of carbon, nitrogen, and energy (2). The tryptophanase (tna) operon of E. coli has two major structural genes, tnaA, encoding tryptophanase, and tnaB, encoding a low affinity tryptophan permease (3, 4). Initiation of transcription of the tna operon is regulated by catabolite repression (5–7). Once initiated, continuation of transcription into the tnaA-tnaB structural gene region depends on tryptophan-induced transcription antitermination. Antitermination is achieved by some feature of induction that prevents Rho factor from terminating transcription in the leader region of the operon (5–7). The tna operon contains a 319-base pair transcribed leader region upstream of the tnaA initiation codon. The transcript of this leader region bears a coding segment, tnaC, specifying a 24-residue leader peptide, TnaC, that contains a single tryptophan residue. Synthesis of TnaC is essential for induction (3, 8, 9). Replacing the tnaC start codon by a stop codon (9, 10) or replacing Trp codon 12 by a codon for some other amino acid (10) prevents induction.

Evidence supporting the essential role of Rho factor in mediating transcription termination in the tna operon leader region was provided by analyses of Rho mutants (8), examination of Rho-inhibiting drugs (11), and deletion of a leader region sequence-rich in C residues (10). Mutations in rho that reduce Rho factor activity as well as addition of bicyclomycin, an inhibitor of Rho action, increase basal expression of the tna operon significantly (7, 8, 11). Similarly, deletion of a 23-nucleotide C-rich tna RNA sequence immediately following the tnaC stop codon (Fig. 1A) reduces transcription termination (10). Comparable sequences, called Rho utilization (rut) sites, have been identified in Rho transcription termination studies with other systems (12–14). Little is known about the features of the rut site required for Rho binding other than it generally has relatively little secondary structure (15) and is rich in cytosine residues (12, 16). Interaction between Rho and an RNA rut sequence is essential for Rho-dependent termination (17, 18).

An additional sequence element shown to be essential for maximum termination is located near the distal end of tnaC (8, 19). This sequence is homologous to the boxA elements found in studies of the phage λ nut antitermination system (20, 21) and with bacterial rrr operons (22, 23) (Fig. 1B). boxA of these operons is required for prevention of Rho-dependent termination, not for facilitation of Rho-dependent termination. The N protein of phage λ relieves both Rho-dependent termination and intrinsic transcription termination (24). Several host factors called Nus factors are involved in antitermination at sites of Rho-dependent termination (21, 25). For example, rrr boxA is the loading site for the E. coli S10 (or NusE) and NusB proteins (26). boxA of rrr operons appears to be sufficient to prevent Rho-dependent termination in vivo (27), while the phage λ nut antitermination system, boxA and boxB sequences are both required for specific N binding and subsequent formation of the RNA polymerase-N-NusA-NusB-NusG-S10-nut antitermination complex (21, 25, 28). In contrast, the boxA sequence in the tna operon does not behave like a typical boxA sequence, mutations in this presumed boxA sequence decrease rather than increase transcription termination in the tna operon leader region (8).

Attempts to detect a gene encoding a presumed trans-acting factor that is responsible for tryptophan-induced expression of the tna operon of E. coli have been unsuccessful (29). The findings obtained in vivo and in vitro studies of tna operon regulation suggest that all the genetic information necessary for tryptophan-induced expression of the tna operon is located in its leader region. The in vivo regulatory features of tna operon expression have been confirmed in vitro using an S-30

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Regulation of the E. coli tna Operon in Vitro

In this paper we show that depleting RF-21 from an S-30 extract results in the accumulation of uncleaved Arg12-TnaC-trRNAgly in the translating ribosome in the absence of added tryptophan. This accumulation of uncleaved Arg12-TnaC-trRNAgly prevents Rho factor action establishing that ribosome stalling at the tnaC stop codon is sufficient to eliminate Rho-mediated translation termination. We provide additional in vitro evidence demonstrating that the RNA sequence marked by the ribosome stalled at the tnaC stop codon serves as the Rho “entry” site. Our findings suggest that the tna boxA sequence, despite its sequence similarity to boxA elements of bacteriophage λ and rrr operons, contributes to Rho-dependent termination by serving as a segment of the Rho entry site.

Materials and Methods

Enzymes and Reagents—Restriction endonucleases were purchased from New England Biolabs. E. coli 100% e-saturated (α32P) RNA polymerase (RNAP) was purchased from Epicentre Technologies Corp. (Madison, WI). E. coli RF protein was kindly supplied by Dr. Peter von Hippel (University of Oregon), and E. coli cAMP receptor protein (CRP protein) was kindly provided by Dr. Ronald Somerville (Purdue University). Purified E. coli RF-1, RF-3, S. typhimurium RF-2, and anti-RF-2 polyclonal antiserum were generous gifts from Dr. Koichi Ito and Dr. Yoshikazu Nakamura (The University of Tokyo, Japan). Protein A-Sepharose 4B Fast Flow was purchased from Sigma (catalog no. P-9424). [α-32P]UTP (−1000 Ci/mmol) and [35S]methionine (−1000 Ci/mmol) were obtained from PerkinElmer Life Sciences.

Cell-free Transcription-Translation—S-30 extracts were prepared as described by Zabary (31) using cells of E. coli strain A19 RNase1-containing trpR lacZ trpA2 tnaAbgl::Tn10. Reaction conditions and procedures for preparation of the self-ligated DNA templates used to direct in vitro S-30 reactions have been detailed (7). Single round analyses of transcription coupled to translation, using cell-free extracts, have been described (7).

Single Round in Vitro Transcription Analyses Using a Purified System—DNA templates bearing mutations were prepared by megaprimer PCR methods (32) and were gel-purified. Buffering conditions used in the coupled transcription-translation system (7) were adopted for use with the purified transcription system. The standard transcription reaction mixture (in a total volume of 25 μl) contained 2 units of RNAP (Epicentre), 35 mM Tris acetate, pH 8.0, 10 mM magnesium acetate, 200 mM potassium glutamate, 30 mM ammonium acetate, 2 mM dithiothreitol, 2 mM CAMP, 1 mM ATP, and 35 μg/ml CRP protein, 10 μg/ml bovine serum albumin, and 10 mM linear DNA template. TTP and GTP were present at 1 μM. Single round transcription reactions were initiated by adding 20 μM UTP, 20 μC of [α-32P]UTP, and 50 μg/ml rifampicin after 10 min of preincubation at 37 °C (33). Reactions were terminated by phenol extraction after another 10-min incubation and analyzed on a 6% polyacrylamide, 7M urea gel. When specified, a high level of Rho factor (1 μg hexamer) was included in the preincubation reaction mixture. Gels were dried and exposed to a phosphorimaging screen or x-ray film. Levels of 32P-labeled readthrough (RT) bands were quantified by using a phosphorimaging device (molecular Image System G3 363, BioRad).

Immunoprecipitation of RF-2—To remove RF-2 from the S-30 extract directly, a Protein A prebinding step was performed to enrich for antibodies. 150 μl of Protein A-Sepharose 4B slurry (−100 μl of packed beads) was prewashed with TMNK S-30 buffer (35 mM Tris acetate, pH 7.8, 10 mM magnesium acetate, 30 mM ammonium acetate, 60 mM potassium glutamate) three times. After each wash, the beads were recovered by centrifugation at 2500 × g for 1 min. 100 μl of anti-RF-2 antiserum was added, and the mixture was placed on a rotating wheel for 1 h at room temperature. The beads were recovered, washed with TMNK buffer containing 5 μg/ml leupeptin three times, and distributed evenly into three siliconized tubes. 100 μl of S-30 was added to one tube and mixed with the beads for 2 h at 4 °C. After centrifugation at 10,000 × g for 2 min, the supernatant was recovered. This immunoprecipitation step was repeated twice with the same S-30 extract. The S-30 extract depleted of RF-2 was stored at −80 °C. An S-30 extract treated identically with a pre-bled serum was used as a control.

Results

Preparation of an RF-2-depleted S-30—In previous studies using an in vitro S-30 system it was shown that tryptophan induction of tna operon expression is a consequence of tryptophan inhibition of cleavage of newly synthesized TnaC-pepti-
dyl-tRNA\textsuperscript{Pro} (7). This peptidyl-tRNA remained in a complex with the stalled translating ribosome and its associated \textit{tna} transcript. It was demonstrated that both the presence of tryptophan and synthesis of TnaC-tRNA\textsuperscript{Pro} were necessary for stalling of the translating ribosome at the \textit{tnaC} stop codon. The stalled ribosome appears to prevent Rho factor from binding to the \textit{tna} transcript, thereby preventing transcription termination. In the absence of added tryptophan the synthesized TnaC-tRNA\textsuperscript{Pro} was cleaved, the translating ribosome dissociated from the \textit{tna} transcript, Rho bound to the \textit{tna} transcript, and transcription was terminated (30). On the basis of these findings we presumed that if the S-30 extract were depleted of RF-2 the translating ribosome would stall at the \textit{tnaC} stop codon in the absence of tryptophan and that Rho action would be blocked. To examine this possibility, an anti-RF-2 antiserum was used to deplete the S-30 of RF-2, as detailed under “Materials and Methods.” Although we did not experimentally determine that all of the RF-2 in the extract had been removed, the \textit{in vitro} translation results presented below demonstrate that there is insufficient RF-2 in the depleted S-30 to carry out normal translation termination.

\textbf{Cleavage of TnaC-tRNA\textsuperscript{Pro} in an RF-2-depleted S-30 Preparation Is Dependent on Added Functional RF-2; Tryptophan Addition Inhibits the Action of Added RF-2}—We showed previously, and confirmed in this study, that TnaC-tRNA\textsuperscript{Pro} accumulates in the presence of inducing levels of tryptophan in the control S-30 system. In this system, in the absence of added tryptophan, only free TnaC was detected (Fig. 2A, compare lanes 1 and 2) (7). When an RF-2-depleted S-30 was used in this transcription-translation coupled system, TnaC-tRNA\textsuperscript{Pro} was also detected in the absence of added tryptophan (lane 3). Addition of tryptophan increased the intensity of the TnaC-tRNA\textsuperscript{Pro} band slightly (compare lanes 3 and 4). When purified RF-2 was added to the RF-2-depleted S-30, in the absence of added tryptophan, no TnaC-tRNA\textsuperscript{Pro} was detected, only free TnaC was observed (lane 5). As expected, addition of tryptophan restored the accumulation of TnaC-tRNA\textsuperscript{Pro} in the presence of purified RF-2 (lane 6). These results show that RF-2 must be added to the RF-2-depleted S-30 to achieve TnaC-tRNA\textsuperscript{Pro} cleavage.

The anti-RF-2 antiserum employed cross-reacts very weakly with RF-1\textsuperscript{2}; thus, depletion of RF-1 as well as RF-2 could be contributing to the results obtained above. To rule out this possibility, purified RF-1 was added to the RF-2-depleted S-30 to determine whether it could mediate TnaC-tRNA\textsuperscript{Pro} cleavage (Fig. 2B). TnaC-tRNA\textsuperscript{Pro} was accumulated in the RF-2-depleted S-30 preparation in the absence of added tryptophan (lane 1). The accumulation of TnaC-tRNA\textsuperscript{Pro} was unchanged in the presence of added purified RF-1 (lane 2). Consistent with the results shown in Fig. 2A, addition of purified RF-2 led to TnaC-tRNA\textsuperscript{Pro} cleavage, and this cleavage was inhibited by added tryptophan (lanes 3 and 4). These findings indicate that strict stop codon recognition is maintained in the S-30 system. Recall that it was previously observed that when centrifugation-purified TnaC-tRNA\textsuperscript{Pro}-ribosome complexes were subjected to RF-1 treatment, RF-1 addition did lead to cleavage of the peptidyl-tRNA despite the fact that the \textit{tnaC} stop codon is UGA (30).

It was shown previously that replacing Trp\textsuperscript{12} of TnaC by Arg eliminates tryptophan induction \textit{in vivo} (10). As expected, when an Arg\textsuperscript{12} template was used in control S-30 reactions, no accumulation of Arg\textsuperscript{12}-TnaC-tRNA\textsuperscript{Pro} was detected either in the absence or presence of added tryptophan; only free Arg\textsuperscript{12}-TnaC peptide was observed (Fig. 2C, lanes 1 and 2). By contrast, Arg\textsuperscript{12}-TnaC-tRNA\textsuperscript{Pro} was accumulated in the RF-2-depleted S-30 preparation in the absence of tryptophan (lane 3). Arg\textsuperscript{12}-TnaC-tRNA\textsuperscript{Pro} was mostly cleaved and barely detected when purified RF-2 was added to the reaction mixture (lane 4). As expected, there was no effect of added tryptophan on Arg\textsuperscript{12}-TnaC-tRNA\textsuperscript{Pro} cleavage (lane 5).

\textbf{Mimicry of Tryptophan Induction: Constitutive Expression of the tna Operon in the RF-2-depleted S-30}—When the S-30 extract is depleted of RF-2, the ribosome translating tnaC presumably stalls at the \textit{tnaC} UGA stop codon. As occurs upon induction, the stalled ribosome should prevent Rho factor from binding to the transcript, thereby allowing transcription of the \textit{tna} operon to continue. To test this possibility, single round transcription analyses (coupled with translation) were monitored in S-30 extracts directed by either the wild type Trp\textsuperscript{12} template (Fig. 3) or the mutant Arg\textsuperscript{12} template (Fig. 4). Fig. 3A shows the \textsuperscript{33}P-labeled mRNA bands observed in a control S-30 reaction with or without added tryptophan. In the absence of added tryptophan, due to the action of Rho factor followed by RNA degradation (7), multiple pause transcript species are visible in early time samples, and these bands subsequently

\footnote{K. Ito, personal communication.}
The regulation of the E. coli tna operon in vitro involves the use of a Tryptophan (Trp) template. In control S-30 reactions with this template, Rho-dependent termination was observed both in the absence and presence of added tryptophan (Fig. 4A). Multiple paused transcripts visible in early time samples, and these bands subsequently disappeared. These results confirm that the Trp residue at TnaC position 12 is crucial for tryptophan-induced inhibition of Rho action (10, 30). When we examined the Arg\textsuperscript{12} template in an RF-2-depleted S-30 extract, in the absence of added tryptophan, Rho-dependent termination was prevented (Fig. 4B, −RF2 lanes). Intermediate length bands appeared, then they continued to elongate, and consequently the RT band became more prominent. Also evident was the RNA doublet band. The addition of purified RF-2 restored Rho-dependent termination; paused transcripts appeared in early time samples and subsequently were mostly degraded. A small amount of RT species was observed (Fig. 4B, +RF2 lanes). These findings establish that a translating ribosome stalled at the tnaC stop codon prevents Rho factor action.

**Contributions of the boxA Sequence and the rut Site Adjacent to the tnaC Stop Codon to Rho-dependent Termination in Vitro**—A sequence resembling a boxA sequence, and a presumed rut site (Rho utilization), are located in the vicinity of the tnaC stop codon (Fig. 1). Their identification was based on sequence similarities and in vivo mutational studies (8, 10). Deletion of the rut site results in semiconservative expression of the tna operon in vivo (10), as does deletion or genetic alteration of the boxA sequence. These findings suggest that both sequences play a role in mediating Rho-dependent transcription termination in the tna operon leader region (8, 34).

It has been well established that boxA sequences of phage λ (20) and of rRNA operons (22) are required for transcription antermination, not termination. Thus the boxA-like sequence at the distal end of tnaC appears to behave differently. When templates with or without the boxA and rut sequences were tested in vitro in single round transcription experiments with purified RNA polymerase and Rho, both sites were observed to contribute significantly to Rho-dependent transcription termination (Fig. 5). Under the conditions used, Rho addition to the wild type template resulted in almost complete elimination of the RT species (Fig. 5A, WT + Rho lane). The RT (%) levels with boxA-1 (Δ6 bp), boxA-2 (Δ13 bp), and rut (Δ23 bp) templates (Fig. 5A) were 13, 35, and 34%, respectively (Fig. 5, A and B). Most importantly, when the template lacked both the boxA and rut sites, boxA-rut (Δ56 bp), the RT level was 96% (Fig. 5, A and B). With a template bearing a 36-bp deletion of a sequence near the 5′ end of the tna operon leader region (Δ + 7 to +42, Fig. 5A) there was little reduction in the efficiency of Rho-dependent termination, compared with the WT template (Fig. 5B). This finding suggests that the differences found in the levels of RT transcripts when templates were used that bear specific tna leader deletions cannot simply be attributed to the changes in length of the corresponding transcripts.

Because the interaction of Rho with RNA is known to be very sensitive to RNA secondary structure (15), we used the MFOLD program of Zuker (35) to predict the secondary structures of the boxA and rut deletion transcripts. Computer modeling revealed that no new secondary structural elements were formed in the remaining sequences for any of the deletion constructs. In addition, the deletions introduced did not further stabilize any existing tnaC secondary structure; rather, most of the deletions (ΔboxA-1, ΔboxA-2, and ΔboxA-rut) destabilized a wild type structure (Fig. 1C). Taken together, our results suggest that the changes in Rho-dependent termination activity observed with our deletion templates must be due primarily to the loss of the specific deleted RNA segments and not to changes in length of the deletion transcript or to changes in an

**Fig. 3.** Single round in vitro transcription (coupled with translation) analyses examining the effects of RF-2 depletion on RNA synthesis, using a Trp\textsuperscript{12} template. A, single round transcription pattern in a control S-30 preparation. B, single round transcription pattern in the RF-2-depleted S-30 extract. S-30 reactions (50 µl) were incubated at 37 °C for 10 min, then 20 µCi of [\textsuperscript{3}H]UTP, 200 µM CTP, and 100 µg/ml rifampicin were added together to the reaction mixture. Samples (10 µl) were taken at the indicated time points, the reaction was stopped by phenol extraction, and the samples were loaded on an RNA gel. 20 µg/ml chloramphenicol (Cm) was added when required. The RT transcript (430 nucleotides nt), pause site RNAs, and the RNA doublet (D) locations are indicated. Arrows mark the locations of the 165- and 176-nucleotide transcripts.

**Fig. 4.** Single round in vitro transcription (translation-coupled) analyses using an Arg\textsuperscript{12} template. A, analyses using the control S-30. B, analyses using the RF-2-depleted S-30. Reactions were performed as described in the legend to Fig. 3 except that an Arg\textsuperscript{12} template was used. The RT transcript (430 nucleotides nt), pause site RNAs, and the RNA doublet (D) locations are indicated. Arrows mark the locations of the 165- and 176-nucleotide transcripts.
region completed synthesis of TnaC and then dissociated from the tnaC stop codon, rendering the transcript accessible to Rho. In the presence of the inducer features of the leader peptide lead to the formation of a tryptophan binding site in the ribosome, and, when tryptophan is bound, RF-2 cannot activate peptidyltransferase cleavage of the TnaC-peptidyl-tRNAPro (30).

Our data show that depletion of RF-2 from the S-30 extract alters the requirements for prevention of Rho factor action. In a normal S-30 extract, inducing levels of tryptophan and the nascent wild-type TnaC-peptidyl-tRNA are required to prevent Rho-dependent termination (Fig. 3). In the RF-2-depleted S-30 extract, neither inducing levels of tryptophan nor synthesis of the nascent WT TnaC is required. Thus, when the Arg12-TnaC template was tested in the RF-2-depleted S-30 extract, accumulation of 32P-labeled Arg12-TnaC-tRNAPro was observed (Fig. 2), and Arg12-TnaC-tRNAPro was shown to be associated with its translating ribosome. This accumulation was correlated with prevention of Rho-dependent termination (Fig. 4). When translation of tnaC in the RF-2-depleted S-30 extract was inhibited by chloramphenicol addition, there was no inhibition of Rho-dependent termination (Fig. 3). Thus, translation of the Arg12-TnaC mutant template is required in the RF-2-depleted S-30 extract for prevention of Rho action (Fig. 4).

We also show using the RF-2-depleted S-30 extract that elevated levels of tryptophan induce tna operon expression by inhibiting the action of RF-2, not RF-1. Our study was further investigated using fully purified structures of the tna operon, and we observed that RF-1 and RF-2 were equally effective in mediating TnaC-tRNAPro cleavage at the tnaC UGA stop codon and that tryptophan inhibited the action of both release factors (30). Either some factor is removed during ribosome isolation that discriminates between the different release factors or stop codon recognition is no longer necessary for release factor participation in peptidyltransferase activation in our isolated ribosome complexes.

A striking characteristic of Rho-dependent transcription ter-

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**Fig. 5.** Single round *in vitro* transcription analyses using highly purified components, RNA polymerase and Rho, to examine the contributions of the boxA and rut sites to Rho-dependent termination. A and B, the effect of various deletions on Rho-dependent transcription termination. C, the effect of single and double point mutations in boxA on the termination activity of Rho. RT species are marked by arrows. For the WT template, the length of the RT transcript is about 430 nucleotides. The locations of the 165- and 176-nucleotide transcripts are shown. Levels of 32P-labeled RT bands were quantified by using a phosphorimaging device (Bio-Rad). RT(%): amount of RT species obtained with Rho/amount obtained without Rho, with the same DNA template.

**Fig. 6.** Model for tna operon regulation in *E. coli*. 1, in the absence of inducing levels of tryptophan the translating ribosome reaches the tnaC stop codon, RF-2-mediated TnaC-tRNAPro cleavage occurs, and the translating ribosome subsequently releases from the template. Rho factor then has access to its entry site on the transcript in the vicinity of the tnaC UGA stop codon, it contacts a paused polymerase, and causes transcription termination. 2, in the presence of inducing levels of tryptophan, the combined action of ribosome-associated uncleaved TnaC-tRNAPro and ribosome-bound tryptophan inhibits RF-2-mediated TnaC-tRNAPro cleavage. As a result, the translating ribosome stalls at the tnaC stop codon. The stalled ribosome blocks the access of Rho to its entry site on the transcript. Transcription termination is thereby prevented, and RNA polymerase continues transcription into the tnaC-tnaB coding region.

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3. F. Gong and C. Yanofsky, unpublished data.
mination is the requirement for a specific entry site for Rho on the synthesized transcript. Binding to this site is a prerequisite for the activation of the ATPase activity of Rho. This is required for its subsequent helicase and transcription termination functions (14, 36). Our data and previous findings suggest that the RNA sequence in the vicinity of tnaC stop codon constitutes the critical entry site for Rho. The previously identified boxA sequence, as well as the rut site, appear to be required for maximal Rho-dependent termination in our purified in vitro system, in the absence of Nus factors. Deletion of the 23-nucleotide rut site significantly reduced the action of Rho in vitro (Δrut, 35% RT, Fig. 5). Most importantly, deletion of both the boxA and rut sites (ΔboxA-rut, 96% RT, Fig. 5) virtually eliminated transcription termination by Rho. The tna boxA sequence, like the rut site, is rich in C residues (Fig. 1).

The boxA sequence recognized in phase λ Nnut antitermination (37) and in rrr operon regulation (22) is required for prevention of Rho-dependent termination. It has been shown that nucleotides 2–6 of both rrr and λ boxAs are specifically involved in the assembly of the antitermination complex (26, 28). For example, the boxA 4U → G mutation impairs the ability of rrr boxA to support transcription antitermination in vivo (26). The same mutation in λ boxA prevents the formation of the complete complex (RNA polymerase-NusA-NusE-NusG-S10-nut) (28). Note that tna boxA has a C instead of U at position 4 (Fig. 1B). Thus the resemblance of the tna boxA sequence to the classic boxA sequence may simply be coincidental. Indeed, previous in vivo studies with nusA1, nusA100, and nusE100 mutant strains (38, 39) failed to detect an effect of these alterations on termination in the tna operon (34). The key role played by NusA in the phase λ Nnut (boxA+boxB) antitermination system has been documented recently (24).

In view of the importance of ribosome stalling in tna operon induction, it is obvious that coupling of translation with transcription must play a significant role in tna operon regulation as it does in trp operon regulation (40). There are two presumed hairpin structures near the 5’ end of tna mRNA (Fig. 1C), one containing the tnaC Shine-Dalgarno sequence and start codon and one formed from the very end of the tnaC coding region. It was shown previously that this latter secondary structure serves as a transcription pause structure in vitro (6). We assume there is some mechanism of disrupting the 5’ structure, allowing ribosome binding and translation initiation, and that transcription pausing at the more 3’ structure may help to delay the time when transcription resumes.

These kinetic features essential to tna operon regulation have not yet been addressed.

A second basic feature of tna operon regulation that remains to be elucidated is how, under inducing conditions, synthesis of the nascent TnaC peptide-tRNA participates in the creation of a tryptophan binding site. Also unknown is how the presence of ribosome-bound TnaC peptide-tRNA and bound tryptophan prevent RF-2 activation of ribosomal peptidyltransferase activity. Answers to these questions may provide an understanding of how features of a nascent peptide are recognized by a translating ribosome (41–43).

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