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Rho-Dependent Transcription Termination in the Tryptophanase Operon Leader Region of *Escherichia coli* K-12

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Recent studies have suggested that expression of the tryptophanase (*tna*) operon of *Escherichia coli* is subject to transcription termination-antitermination control (V. Stewart and C. Yanofsky, J. Bacteriol. 164:731–740, 1985). In vivo studies have indicated that the transcribed leader region, *tnaL*, contains a site or sites of ρ-dependent transcription termination (ρ is the polypeptide product of the gene *rho*). We now report direct in vitro evidence that *tnaL* contains ρ-dependent termination sites. In vivo termination appeared to occur at the ρ-dependent termination sites identified in vitro. Transcription pausing analyses correlated sites of pausing in *tnaL* with sties of ρ-dependent termination.

Tryptophanase is a catabolic enzyme distinct from those of the tryptophan biosynthetic pathway. It is found exclusively in bacteria and catalyzes a variety of reactions involving tryptophan, indole, and related compounds (27). In *Escherichia coli*, tryptophanase synthesis is induced by tryptophan and is subject to catabolite repression (3, 28). Tryptophanase induction requires 3′,5′-cyclic AMP (cAMP) plus the cAMP receptor protein (CRP), a positive-acting factor required for transcription initiation at catabolite-sensitive promoters (reviewed in reference 8). However, other factors may also influence the catabolite repression of tryptophanase synthesis (3).

The tryptophanase structural gene, *tnaA*, and flanking regions have been cloned and sequenced (9). In vitro transcription experiments located the presumptive *tna* promoter over 300 nucleotides proximal to the *tnaA* initiation codon. This promoter requires cAMP-CRP for activity (10). The long transcribed leader region between the promoter and *tnaA* is termed *tnaL*. Within *tnaL* is a coding region, *tnaC*, for a 24-residue peptide (37). Recent studies suggest that translation of *tnaC* is required for full induction of the *tna* operon, as well as for setting the basal uninduced level of *tna* expression (V. Stewart and C. Yanofsky, submitted for publication).

Three major lines of evidence suggest that *tnaL* contains a site or sites of ρ-dependent transcription termination (ρ is the polypeptide product of the gene *rho*) and that induction by tryptophan suppresses termination, allowing readthrough into *tnaA* (37). First, measurements of in vivo RNA synthesis, under catabolite-derepressing growth conditions, indicate that expression from the *tna* promoter is constitutive, but that *tnaA* mRNA synthesis is induced by tryptophan. This establishes the leader region as the site of regulation by tryptophan and suggests that transcription termination-antitermination control regulates *tna* expression. Second, *rho* mutants are partially constitutive for tryptophanase production. This suggests that the regulatory region contains a site or sites of ρ-dependent transcription termination. Third, *cis*-acting tryptophan-independent constitutive mutants have single-base-pair changes in *tnaC*. These alterations are clustered some 90 nucleotides distal to the transcription initiation site. Most of these lesions define a sequence homologous to *boxA*, which is important for antitermination control in bacteriophage λ. *boxA*-like sequences may be involved in the function of the transcription fidelity factor NusA (13, 18, 29, 30; see also reference 38).

These observations led to a model for transcription termination/antitermination control of *tna* expression (37). According to this model, transcription initiation from the *tna* promoter is constitutive, regulated only by cAMP-CRP-mediated catabolite repression. In the absence of tryptophan, ρ-dependent transcription termination in *tnaL* prevents expression of the structural genes of the *tna* operon. In the presence of tryptophan, this termination is relieved, allowing *tna* expression. It was hypothesized that a tryptophan-responsive protein mediates transcription antitermination in *tnaL* to allow *tnaA* transcription (37).

We report here in vitro and in vivo analyses of transcription termination in *tna*L. Termination in vitro required ρ factor and occurred at several discrete sites. NusA enhanced the specificity of transcription termination in vitro. Transcription pausing also occurred at discrete sites, many of which were also ρ-dependent termination sites. Transcription in vivo appeared to terminate at the same sites as those found in vitro. These results provide direct evidence for ρ-dependent transcription termination within *tnaL*.

MATERIALS AND METHODS

DNA templates. Restriction fragment *Alu*I-*Rsa*I619 contains the *tna* promoter and leader and the first 20 codons of *tnaA* (9). This fragment was cloned with *Bam*HI molecular linkers (5′-CCGGATCCGG-3′; Bethesda Research Laboratories, Bethesda, Md.) into the *Bam*HI site of pUC13 (21) to form plasmid pSVS14. Analogous restriction fragments carrying the *tnaC255* and *tnaC259* alleles (37) were used to generate plasmids pSVS14-255 and pSVS14-259. Control experiments established that both of the cloned templates retained their *tnaC* alterations, as defined by their in vivo phenotype (data not shown). Plasmid DNA was purified by CsCl-ethidium bromide equilibrium density gradient

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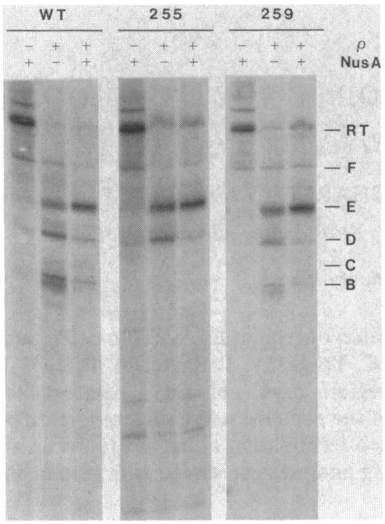

FIG. 1. ρ-Dependent transcription termination in vitro in *tnaL*. Transcription reactions contained 20 μg of ρ or NusA per ml, as indicated at the top of the figure. Lanes WT, Termination with the wild-type template; lanes 255, results with the *tnaC255* template; lanes 259, results with the *tnaC259* template. The positions of transcripts B through F and RT, the runoff transcript, are indicated on the right.

ultracentrifugation (7, 37). After digestion with the appropriate restriction enzyme (usually *Bam*HI), the insert DNA was isolated from 5% acrylamide gels (10). DNA concentrations were estimated by the wrap and ring method of Davis et al. (7).

In vitro transcription. Transcription reactions followed previously described protocols (12, 40), except that they contained 100 mM KCl, 2 mM cAMP, and 35 μg of CRP per ml. ATP, CTP, and UTP were present at 150 μM, and GTP was present at 20 μM; transcripts were labeled with [α-^{32}P]GTP. All transcription reactions were conducted at 37°C. Single-round transcription reactions were initiated by adding CTP, UTP, and rifampin (12, 17). Reactions were terminated by the addition of an equal volume of stop mix (31), heated at 95°C for 2 min, and loaded on 5% acrylamide–7 M urea gels (31). Bands were visualized after autoradiography for 12 to 96 h at −80°C. CRP preparations were gifts of Michael Deeley and Lori Stoltzfus (10, 22). ρ preparations were gifts of John Richardson and Malcolm Winkler (11, 40). RNA polymerase was a gift of Robert Fisher (12). NusA was purified in our laboratory by the method of Schmidt and Chamberlin (35).

Identification of RNA 3′ termini. We used the 3′-*O*-methyl nucleoside chain termination method to generate an RNA sequence ladder corresponding to the *tnaL* transcript (24, 32; E. A. Meighen, personal communication). Standard single-round transcription reactions containing NusA were prepared as described above, except that they contained a limiting concentration (20 μM) of CTP, GTP, or UTP. At 45 s after initiation, 3′-*O*-methyl CTP, GTP, or UTP, respectively, was added at a final concentration of 150 μM, and incubation was continued for 10 min. A complete single-round transcription reaction containing NusA was also conducted, and samples were taken at 20, 40, and 60 s after initiation. Samples (1.5 μl) were electrophoresed on a 5% acrylamide–7 M urea sequencing gel (37). The gel was dried, and bands were visualized after autoradiography with an intensifying screen and storage at −80°C for 1 week.

Detection of in vivo terminated transcripts. A growing culture of *E. coli* was pulse-labeled with [^{3}H]uridine, and RNA was isolated as described (37). RNA was hybridized to *Hin*dIII-digested pSVS14 DNA that had been denatured and immobilized on nitrocellulose filters (37). The filters were not treated with RNase. RNA was eluted from the filters (2), ethanol precipitated, and electrophoresed on a 5% acrylamide–7 M urea gel. The gel was fixed and treated with sodium salicylate (5). Bands were visualized by autoradiography at −80°C for 2 weeks.

Prediction of RNA secondary structure. Potential RNA secondary structures were generated by the program of Zuker and Stiegler (41). This program uses a dynamic algorithm to search for the potential secondary structures with the lowest theoretical free energy of formation. Free energies were assigned according to the values compiled by Salser (34).

RESULTS

In vitro transcription termination. The DNA template *Alu*I-*Rsa*I619 contains the *tna* promoter and leader and the first 20 codons of *tnaA* (9). Transcription initiation at the previously defined *tna* promoter is expected to generate a 385-nucleotide runoff transcript (10). (This includes nucleotides from the *Bam*HI linker; see Materials and Methods.) Indeed, the major transcript produced in a minimal transcription system containing RNA polymerase, nucleoside triphosphates, [α-^{32}P]GTP, and cAMP-CRP was of this size (band RT, runoff transcript, Fig. 1). In some gels, this transcript appeared as a closely spaced doublet, consistent with the two initiation points previously defined (10). In addition, no runoff transcript was detected when CRP was omitted from the transcription mixture (data not shown).

The *Alu*I-*Rsa*I619 templates were usually excised with *Bam*HI from pSVS14 (see Materials and Methods). Excision of this fragment with *Eco*RI and *Hin*dIII extended the *tnaA* end of the template by 23 nucleotides, derived from the pUC13 multiple cloning site (21). Transcription on this template generated an appropriately larger runoff transcript (data not shown). We conclude that the apparent 385-nucleotide transcript resulted from transcription initiation at the previously defined *tna* promoter (10). Addition of ρ factor eliminated production of the runoff transcript, and a series of new transcripts appeared (bands B through E, Fig. 1). Synthesis of all of these transcripts was dependent upon CRP. Further, transcripts of identical mobilities were generated from the *Eco*RI + *Hin*dIII-excised templates (data not shown). We conclude that these transcripts resulted from ρ-dependent termination of transcription initiating from the *tna* promoter.

Effects of NusA on transcription termination. NusA had no detectable effect on transcription in the absence of ρ (data not shown). However, NusA had a reproducible effect on the terminated transcripts. NusA suppressed production of smaller ρ-dependent transcripts, especially bands B and D, and enhanced termination at band E (Fig. 1).

Transcription termination in *tnaC* mutants. Previous work defined a *boxA*-like sequence in the *tna* leader region. Genetic alterations leading to constitutive tryptophanase production were found to be single nucleotide changes within this sequence. It was concluded that the *boxA*-like sequence is required for proper transcription termination in vivo (37).

We chose two *tnaC* alleles for in vitro analysis. *tnaC255* changes the *boxA*-like sequence from CGCCCTTGA to CGCCCTTGG and results in the weakest constitutive phe-

notype observed in vivo. *tnaC259* changes the sequence to AGCCCTTGA and results in a very strong constitutive phenotype in vivo (37). Both mutant templates produced normal runoff transcripts in both the presence and absence of NusA (Fig. 1, and data not shown). ρ-Dependent termination on the *tnaC255* template was clearly different from that on the wild-type template. Bands B and C were not visible, and an increased amount of runoff transcript was apparent (Fig. 1). This pattern was consistently observed in four independent experiments. In contrast, the *tnaC259* template produced a banding pattern that differed little from that produced by the wild type (Fig. 1). In some experiments, *tnaC259* seemed to produce a slightly greater amount of runoff transcript (Fig. 1), but it was not as striking nor as consistent as that produced by *tnaC255*. More significantly, the *tnaC259* template produced wild-type amounts of transcripts B through D (Fig. 1). NusA had a similar effect on transcription termination on all three templates: it suppressed proximal termination sites and enhanced distal sites. Thus, the altered termination pattern produced by *tnaC255* was not due to a defective interaction with NusA (see below).

Transcription pausing. Transcription pausing was detected in single-round transcription experiments (12, 17, 40). Open promoter complexes were allowed to form, and then transcription was initiated by the addition of CTP and UTP. Simultaneous addition of rifampin prevented reinitiation. Samples were removed into stop mix at defined times after transcription initiation. Subsequent visualization of the transcript bands showed the time course of transcription. Transcribing RNA polymerase paused for significant periods at defined positions on the template (Fig. 2 and 3). Others have observed that ρ-dependent termination sites correlate with transcription pause sites (20, 25, 33), and indeed, the paused *tnaL* transcripts B through E comigrated with major ρ-terminated transcripts (data not shown; compare Fig. 1, 2, and 3). Transcript F, visible in both the absence and presence of ρ (Fig. 1), appeared to be a very long-lived pause transcript (Fig. 2 and 3).

We compared transcription pausing on the wild-type, *tnaC255*, and *tnaC259* templates. In the absence of NusA,

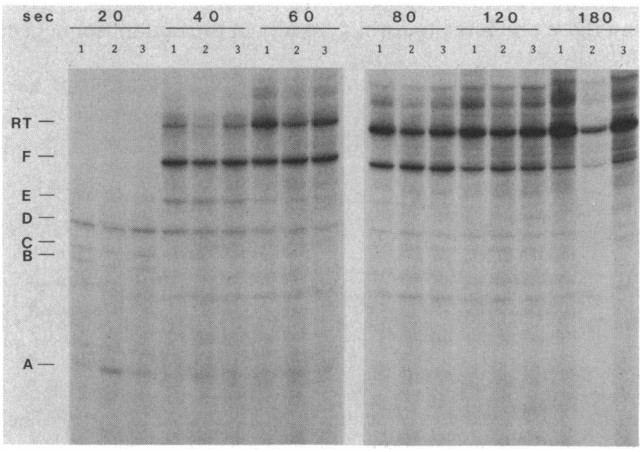

FIG. 2. Transcription pausing in the absence of NusA. Samples were taken at 20, 40, 60, 80, 120, and 180 s after initiation, as indicated at the top of the figure. Lanes 1, Transcription mixtures with the wild-type template; lanes 2, *tnaC255* template; lanes 3, *tnaC259* template. The position of transcripts A through F and RT, the runoff transcript, are indicated on the left.

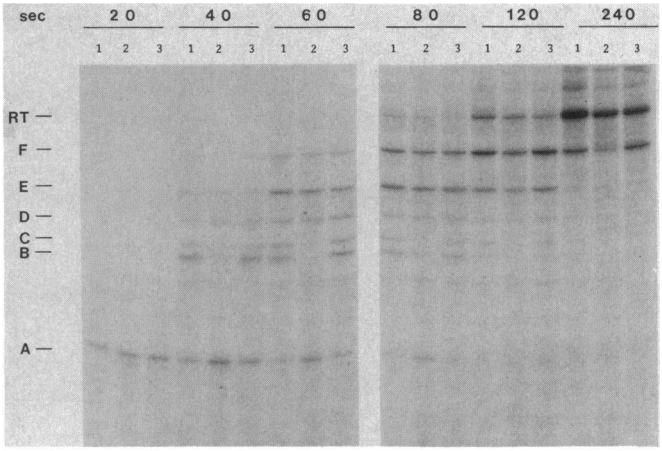

FIG. 3. Transcription pausing in the presence of NusA. Samples were taken at 20, 40, 60, 80, 120, and 240 s after initiation, as indicated at the top of the figure. Lanes 1, Transcriptions with the wild-type template; lanes 2, *tnaC255*; lanes 3, *tnaC259*. The positions of transcripts A through F and RT, the runoff transcript, are indicated on the left.

the pattern of pausing on the wild-type and *tnaC259* templates was indistinguishable (Fig. 2). The *tnaC255* template yielded a slightly different pattern: pause transcript A was more prominent, and B and C were less prominent than those of the other templates (Fig. 2). Distal transcripts from all three templates were indistinguishable. In the presence of NusA, the overall rate of transcription was lower (35), so the pausing time course appeared to be expanded (12, 17, 20). Again, the wild-type and *tnaC259* templates generated indistinguishable pause patterns (Fig. 3). The pattern of *tnaC255* was clearly different. Paused transcript A was noticably longer lived, while transcripts B and C were barely visible. Again, the distal transcripts appeared identical on all three templates. Thus, *tnaC255* appeared to reduce the levels of both paused and terminated transcripts B and C and also enhanced the lifetime of paused transcript A; these effects were independent of the presence of NusA. In contrast, *tnaC259* had little effect on pausing or termination, irrespective of NusA.

3′ Ends of paused transcripts. We used 3′-*O*-methyl nucleoside chain termination (24, 32; see Materials and Methods) to locate the 3′ ends of paused transcripts A through F with an apparent accuracy of plus or minus 1 nucleotide (data not shown). The lengths of transcripts A through F were approximately 103, 165, 176, 199, 234, and 291 nucleotides, respectively.

Transcription termination in vivo. We used denatured *Alu*I-*Rsa*I619 DNA as a hybridization probe to search for *tnaL*-derived transcripts in vivo (2). [^{3}H]uridine-labeled RNA isolated from growing *E. coli* was hybridized to pSVS14, eluted, and visualized after electrophoresis and autoradiography. Apparent terminated transcripts corresponded closely in size to ρ-terminated transcripts produced in vitro (Fig. 4). In a second experiment we electrophoresed ^{32}P-labeled in vitro transcripts adjacent to ^{3}H-labeled in vivo transcripts on the same gel, and nearly all of the bands comigrated (data not shown). This suggests that the ρ-dependent termination sites characterized in vitro are also active in vivo.

RNA was isolated from a culture that was only partially induced for *tna* expression; we estimate that the differential rate of *tnaA* mRNA synthesis was only about one-third of

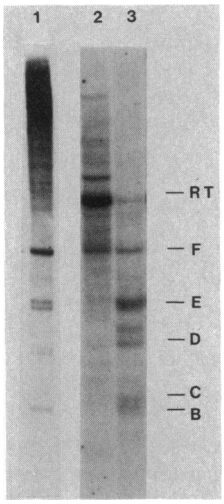

FIG. 4. Transcription termination in vivo in *tnaL*. Lane 1, [^{3}H]uridine-labeled RNA isolated from a partially induced culture by hybridization to plasmid DNA containing *tnaL*. Lanes 2 and 3, In vitro transcription reactions conducted in the absence and presence of ρ, respectively (see Fig. 1).

the theoretical maximum (V. Stewart and C. Yanofsky, unpublished data). Thus, we detected significant amounts of terminated transcripts in *tnaL*. We believe that the heterogeneous, high-molecular-weight RNA detected in vivo represents antiterminated transcripts that extended into the *tna* operon, well past the hybridization probe DNA present in pSVS14 (Fig. 4).

The relative amounts of each of the terminated transcripts detected in vivo were apparently different from the amounts produced in vitro; in particular, band F is much more prominent in the in vivo RNA preparation (Fig. 4). We attribute at least one reason for this difference to the translation of *tnaC* in vivo (37). ρ-Dependent termination is more effective on untranslated RNA (reviewed in references 23 and 39; see also reference 18). Indeed, we have found that elimination of *tnaC* translation caused a large decrease in *tna* operon expression (Stewart and Yanofsky, submitted for publication). Thus, we believe that in vivo, *tnaC* translation may interfere with ρ-dependent termination at the early sites; this inhibition would not be observed, of course, in the minimal in vitro transcription reactions (see reference 18).

DISCUSSION

In a previous study, we proposed a model for transcription termination/antitermination control of tryptophanase operon expression (37). One prediction of this model is that the transcribed leader region, *tnaL*, contains a site or sites of ρ-dependent transcription termination. This paper presents both in vitro and in vivo evidence that supports that prediction. We observed that in vitro transcription termination in *tnaL* was dependent upon added ρ factor. Further, we isolated in vivo transcripts that appeared to result from termination at the same positions that we observed in vitro. We also found that most transcription pause sites identified in vitro corresponded to sites of ρ-dependent transcription termination. The defined endpoints of the paused, terminated, and runoff transcripts detected in *tnaL* with the *Alu*I-*Rsa*I619 template are schematically represented in Fig. 5.

ρ-Dependent termination. Models for ρ-dependent termination postulate at least two distinct events: ρ binding to untranslated nascent RNA, and RNA polymerase pausing at specific sites on DNA (1, 4, 18, 20, 25, 26). Transcription antitermination, mediated by the bacteriophage λ *N* gene product, results from an altered RNA polymerase that is insensitive to termination signals (14). A common feature of ρ-dependent terminators is a relatively long (100 to 300 nucleotides) stretch of untranslated RNA that is poor in G residues (reviewed in references 23 and 39). Indeed, the *tna* leader contains a 220-nucleotide stretch of untranslated RNA; the composition of this region is roughly 17% G.

In λtR1, ρ-dependent termination occurs at multiple, defined positions within the region of untranslated RNA (18, 20, 24; reviewed in references 23 and 39). Further, termination sites detected in vitro correspond to those used in vivo (6, 19, 20, 24, 33). Not all transcription pause sites are also ρ-mediated release sites for termination, the apparent strengths of the pauses do not necessarily correlate with the rates of transcript release (20, 26). In the qualitative analysis presented in this paper, termination in *tnaL* followed these generalizations. Pause transcript A (Fig. 5) did not seem to be terminated by ρ, and the apparent half-lives of paused transcripts B through F did not correlate with their apparent frequencies of ρ-dependent termination (compare Fig. 1, 2, and 3).

Potential role of RNA secondary structure in transcription pausing. We experimentally determined the 3′ endpoints for each of the major pause transcripts in *tnaL*. We used the RNAFLD computer program of Zuker and Stiegler (41) to search for potential stable secondary structures within the entire *tnaL* region. Indeed, potentially stable structures were found just proximal to sites A, B, E, and F (Fig. 6). These were among the most stable potential structures predicted in the entire *tnaL* region. A relatively weak structure was found associated with site D, and no stable structure could be located immediately proximal to site C (Fig. 6). We also searched for potential secondary structures in the *tnaC* mutant sequences. The predicted structures in *tnaC259* were essentially identical to those in the wild-type sequence (not shown). In contrast, the single-base change in *tnaC255* had striking effects on the predicted secondary structures. Structure a′ is theoretically more stable than the wild-type structure a. In addition, a new structure, x, that is theoretically more stable than b, is predicted to prevent the formation of structure b in this mutant (Fig. 6). The distal structures, d, e, and f, are predicted to form normally in the mutant sequence (not shown).

Pausing at the 1:2 hairpin in the *trp* leader transcript has

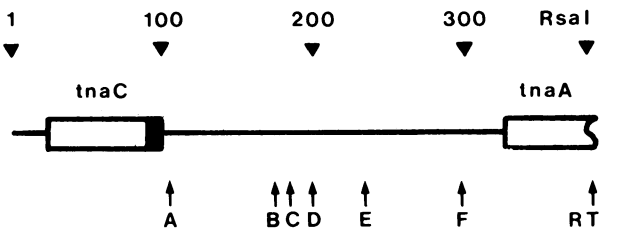

FIG. 5. Schematic representation of transcribed RNA from the *Alu*I-*Rsa*I619 template, drawn to scale; length in nucleotides is indicated by the triangles. Transcription initiates at the left (1) and proceeds to the right. The *tnaC* and *tnaA* coding regions are shown as open boxes. The *boxA*-like sequence ("black-box") in *tnaC* is indicated by the filled-in portion of the *tnaC* region. The positions of paused or terminated transcripts A though F and the runoff transcript (RT) are indicated.

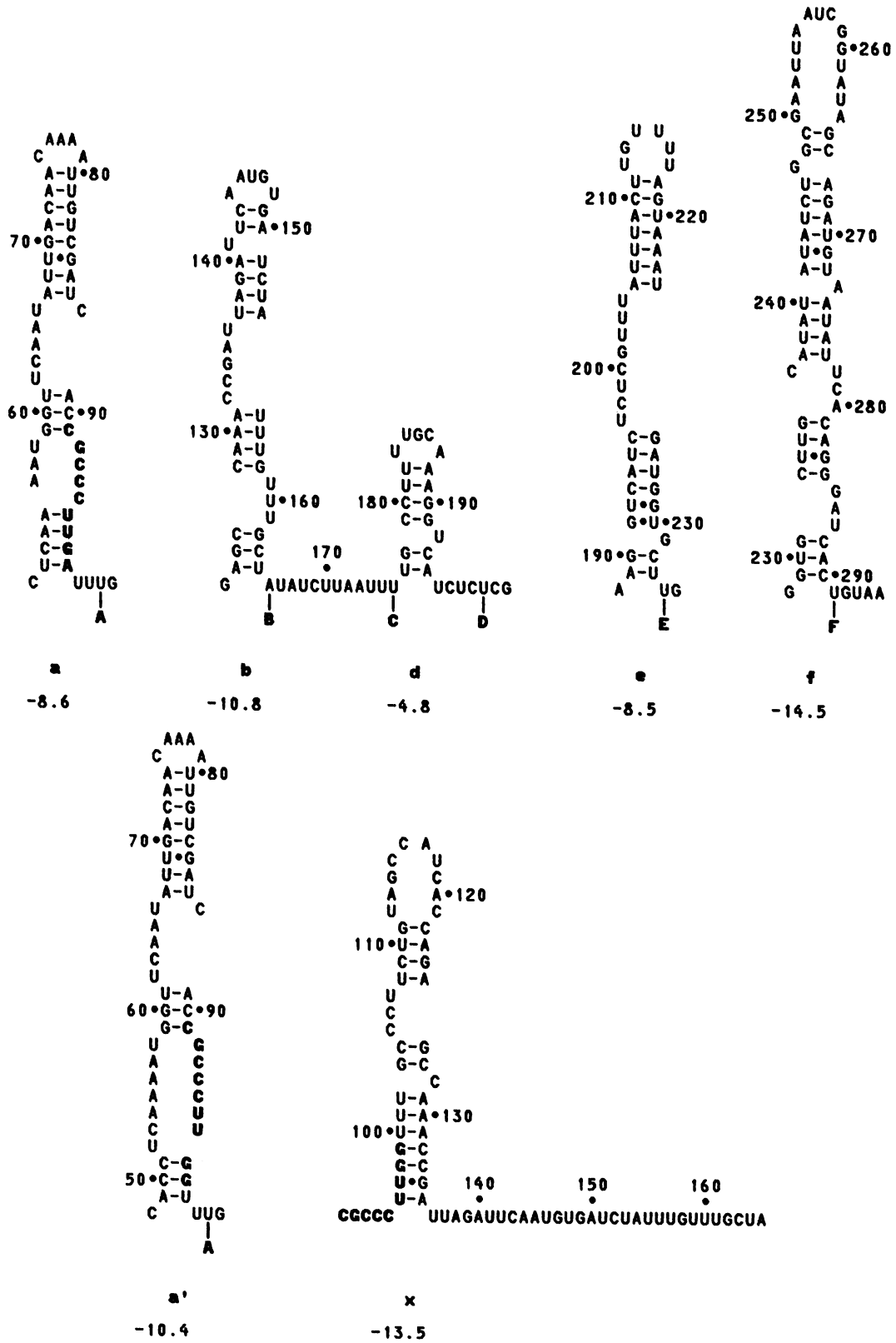

FIG. 6. Predicted RNA secondary structures in the transcript of *tnaL*. Potential RNA secondary structures were predicted by the RNAFLD program of Zuker and Steigler (41). The 3′ endpoints of paused transcripts A through F are marked with vertical bars. The *boxA*-like sequence from positions 91 to 99 is marked in boldface. Calculated free energies of formation are indicated below each structure. Potential structures a, b, d, e, and f are from the wild-type sequence. Structures a′ and x are from *tnaC255*. The single-base change of *tnaC255* is A to G at position 99.

been correlated with the relative predicted free energies of the wild-type and mutant hairpins at this position (17). Indeed, transcription pausing at site A in the mutant *tnaC255* was enhanced relative to the wild type (Fig. 2 and 3); perhaps a more stable structure in the mutant template is responsible for this effect (Fig. 6). In addition, pausing and termination at sites B and C was nearly abolished by the *tnaC255* alteration (Fig. 1, 2, and 3), consistent with the prediction that structure b may not form in this mutant (Fig. 6). Termination and pausing at distal sites appeared to be unaffected. Obviously, these explanations are highly speculative and offer only one hypothesis to explain the in vitro effects of the *tnaC255* alteration.

Effects of NusA. The *E. coli nusA* gene product, NusA, appears to interact with RNA polymerase during RNA chain elongation (15). In vitro, NusA mediates the interaction of both termination and antitermination factors with RNA polymerase (16, 36). In addition, NusA modulates the RNA polymerase response to pause and termination sites (20). Lau et al. suggest that NusA can influence both the extent of transcription pausing and the initial kinetics of ρ-dependent transcript release, and therefore can potentially increase or decrease the termination efficiency at any given site (20). NusA had similar effects on in vitro pausing and termination in *tnaL*; it enhanced pausing and altered the pattern of ρ-dependent transcription termination.

Role of the *boxA*-like sequence in *tnaL*. A site within λtR1, *boxA*, is required for antitermination control of early rightward transcription. Friedman and coworkers have proposed that *boxA* acts as a recognition site for NusA, which is also required for antitermination at λtR1 (13, 29, 30; see also references 18 and 38). An analogous *boxA*-like site in *tnaL* was defined by genetic lesions that presumably reduce in vivo transcription termination in the *tna* leader region (37).

Lesions in the *boxA*-like sequence in *tnaC* cause constitutive *tnaA* expression in vivo, presumably by preventing efficient transcription termination in *tnaL*. If *boxA*-like sequences serve as recognition sites for NusA (13, 18, 29, 30, 38), then this implies that NusA acts as a transcription termination factor at *tnaL* (37). In its simplest form, this hypothesis predicts that templates carrying *tnaC* alterations should produce wild-type transcription pausing and termination patterns in the absence of NusA, but should show altered patterns in the presence of NusA.

We tested two *boxA*-like alleles, *tnaC255* and *tnaC259*, for their effects on transcription termination and pausing in vitro. *tnaC259*, which causes strong constitutive expression in vivo, had virtually no effect on either termination or pausing in vitro, irrespective of the presence of NusA. *tnaC255*, which has a relatively weak in vivo effect, showed a consistent effect in vitro: it increased readthrough, it enhanced the apparent lifetime of paused transcript A, and it reduced or eliminated paused and terminated transcripts B and C (Fig. 5). However, it had no detectable effect on more distal transcripts D and E, and its effects on the proximal transcripts were apparently not related to the presence of NusA. We do not know whether the in vivo effects of *tnaC255* are due solely to its *boxA* alteration or whether it also affects transcription termination similarly to the way it apparently does in vitro.

Therefore, the relationship between genetic alterations in *tnaC* and altered transcription termination could not be demonstrated in vitro. The *boxA*-like sequence in *tnaL* overlaps the coding region for the *tnaC* peptide. It is probable that translation in this region is crucial for the proper action of both transcription termination and transcription antitermination factors (Stewart and Yanofsky, submitted for publication).

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