

Transcription termination at intrinsic terminators: The role of the RNA hairpin

(*Escherichia coli*/RNA polymerase/ ρ -independent termination)

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ABSTRACT Intrinsic termination of transcription in *Escherichia coli* involves the formation of an RNA hairpin in the nascent RNA. This hairpin plays a central role in the release of the transcript and polymerase at intrinsic termination sites on the DNA template. We have created variants of the λ tR2 terminator hairpin and examined the relationship between the structure and stability of this hairpin and the template positions and efficiencies of termination. The results were used to test the simple nucleic acid destabilization model of Yager and von Hippel and showed that this model must be modified to provide a distinct role for the rU-rich sequence in the nascent RNA, since a perfect palindromic sequence that is sufficiently long to form an RNA hairpin that could destabilize the entire putative 12-bp RNA-DNA hybrid does not trigger termination at the expected positions. Rather, our results show that both a stable terminator hairpin and the run of 6–8 rU residues that immediately follows are required for effective intrinsic termination and that termination occurs at specific and invariant template positions relative to these two components. Possible structural or kinetic modifications of the simple model are proposed in the light of these findings and of recent results implicating “inchworming” and possible conformational heterogeneity of transcription complexes in intrinsic termination. Thus, these findings argue that the structure and dimensions of the hairpin are important determinants of the termination–elongation decision and suggest that a complete mechanism is likely to involve specific interactions of the polymerase, the RNA terminator hairpin, and, perhaps, the dT-rich template sequence that codes for the run of rU residues at the 3' end of the nascent transcript.

Transcription in *Escherichia coli* is terminated by two distinct mechanisms. One depends on ρ , a hexameric protein that binds to the nascent RNA of the transcription complex and releases it at defined ρ -dependent terminators along the template. The other, called intrinsic (or ρ -independent) termination, also occurs at specific sites but depends only on signals encoded in the template. Intrinsic terminators all share a dyadic sequence that codes for a stable RNA hairpin followed by a string of uridine residues at the 3' end of the terminated RNA.

Transcript elongation and transcript termination (followed by RNA release) represent alternative and kinetically competitive pathways for transcription at each position along the DNA template. This view of the elongation–termination decision has been formulated quantitatively in terms of competitive free energy of activation barriers (1) and has been experimentally verified by examining the dependence of the termination efficiency and the rate of RNA release on the concentration of the next required nucleotide triphosphate (ref. 2; W. A. Rees, S. Weitzel, A. Das, and P.H.v.H., unpublished data). It has also been extended to deal with phage λ N

protein-dependent antitermination (Rees *et al.*, unpublished data) and is likely to be quite general. In this view transcript termination is considered to be possible, in principle, at every template position. In practice, however, termination does not occur at most template positions because the stability of the elongation complex results in characteristic half-times for complex dissociation and RNA release of hours or days, while the average dwell-time for elongation at a given template position at saturating NTP concentrations is 10–50 msec (3).

It has been shown for the λ tR2 terminator (2) and confirmed for the λ tR' terminator (Rees *et al.*, unpublished data) that the termination pathway becomes accessible at intrinsic terminators because the transcription complex is massively destabilized in the vicinity of these sites. This provides a “zone of opportunity” for termination along the template, within which the rates of transcript elongation and termination are comparable. The efficiency of termination at each template position within this zone is controlled by specific interactions of the complex with termination and antitermination factors and by kinetic and thermodynamic effects that depend on the sequence of the DNA template or on its manifestations in the nascent RNA (1, 4, 5).

The involvement of RNA hairpin formation in intrinsic termination is supported by a variety of evidence (for review, see refs. 6 and 7). However, the actual function of the hairpin in termination has remained unclear. Models have proposed that hairpin formation may partially disrupt the RNA-DNA hybrid within the ternary transcription complex and thereby facilitate the release of the RNA by partially destabilizing the complex (8), cause elongation complexes to pause at positions of termination and thus increase the probability of termination (9), or destabilize specific binding interactions between the polymerase and the nascent RNA (10).

A nucleic acid destabilization model for intrinsic termination was developed by Yager and von Hippel (8). They proposed that the net stability of the elongation complex at any template position reflects the algebraic sum of three thermodynamic components: (i) the destabilizing free energy change associated with the opening of the DNA transcription bubble; (ii) the stabilizing free energy change associated with the formation of the RNA-DNA hybrid within the transcription bubble; and (iii) the stabilizing free energy change associated with the interactions of the nucleic acid (DNA and RNA) framework of the complex with core RNA polymerase (8). Components i and ii can be calculated for known sequences by using base-pair stability data, and component iii can be estimated by difference, since at intrinsic terminators the net heights of the free energy of activation barriers to elongation and termination must be approximately equal (1, 8).

To use this approach to calculate changes in transcription complex stability at intrinsic terminators, a specific nucleic acid framework model for the elongation complex must be

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Abbreviation: TE, termination efficiency.

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defined. Based on the experimental information then available (7, 8), a model for this complex was postulated that consisted of an ≈ 17 -bp DNA bubble and an ≈ 12 -bp RNA-DNA hybrid located at the downstream end of the bubble. Both the structure of this framework and its interactions with polymerase were assumed to remain approximately invariant as the transcription complex moved along the template. In this model the major change in the overall stability of the elongation complex at intrinsic terminators was attributed to a destabilization of the RNA-DNA hybrid, resulting from the combined effects of RNA hairpin formation in competition with the upstream portion of the hybrid and the relative instability of the rU-dA sequence in the portion that remained.

On this basis, the net stability of the transcription complex was calculated as a function of position along the DNA template for intrinsic terminators with known termination positions and termination efficiencies (TEs), and it was shown that the model could accurately predict the positions of termination for most of these terminators (2, 8). The nucleic acid destabilization model was subjected to further experimental test by measuring the stability of the elongation complex as a function of template position at the λ tR2 terminator (2). The results showed that the position along the template of the zone of opportunity for termination for the tR2 terminator was also consistent with the simple nucleic acid destabilization model.

On the other hand, such calculations also showed that this purely thermodynamic model could not predict the effects of small changes in terminator sequence and structure on TE (1, 5). This should not have been surprising, since it was already known that changes in transcription rates could significantly alter TEs, suggesting the need to incorporate kinetic considerations into a complete termination model. However, this conclusion could also be reached on the basis of the calculations themselves, since the decrease in the stability of the transcription complex at terminators that was calculated by using the nucleic acid destabilization model was on the order of +18 kcal/mol (1 cal = 4.184 J), with an estimated standard error of approximately ± 3 kcal/mol (8), while the activation energy formalism showed that stability changes that altered the relative heights of the free energy of activation barriers to elongation and termination by ± 2 kcal/mol sufficed to cover the entire range of TEs (1). As a consequence it was clear that any of a variety of minor changes in transcription rate or complex stability could significantly perturb this parameter, while the prediction of termination positions should be relatively robust (1, 8).

To subject the nucleic acid-destabilization model to more rigorous test and to attempt to elucidate the actual mechanistic role(s) of the terminator hairpin in intrinsic termination, we undertook a systematic study of the effects on termination of designed variants of the tR2 hairpin. The results presented in this paper show that the simple version of the nucleic acid destabilization model is not fully consistent with our findings. In *Discussion*, we describe the changes in the simple model that are required to accommodate our measurements and consider other termination models in the light of these results.

MATERIALS AND METHODS

RNA Polymerase and DNA Templates. The holoenzyme form of *E. coli* RNA polymerase was purified as described (11). Variants of the tR2 terminator were constructed from ptR2-4, a plasmid that contains the tR2 sequence just downstream of the strong T7A1 promoter (2). To make plasmid ptR2-5, ptR2-4 was digested with *Stu* I, which cuts at two sites within the tR2 hairpin-encoding sequence, followed by ligation of the ends of the plasmid DNA. The other variants of tR2 were constructed by inserting various double-stranded oligonucleotides at the unique *Stu* I site of ptR2-5. Plasmids ptR2-16 and

ptR2-17, featuring changes in the sequence immediately upstream of the tR2 hairpin, were both constructed by site-directed mutagenesis of ptR2-4. All plasmids were purified from transformed JM105 *E. coli* cells and digested with *Rsa* I, which cuts these plasmids into four pieces. One of these pieces contains the T7A1 promoter and the tR2 terminator sequence or one of its variants. After extraction with phenol/chloroform [1:1 (vol/vol)], and precipitation with ethanol, the *Rsa* I-digested plasmids were used as templates in transcription reactions.

Transcription. Reactions were run essentially as described (2). Open-promoter initiation complexes were formed with 75 nM RNA polymerase and 30 nM *Rsa* I-digested plasmid DNA in 14 μ l of transcription buffer (200 mM potassium glutamate/10 mM magnesium glutamate/1 mM dithiothreitol/30 mM HEPES, pH 7.9) by incubating at 37°C for 10 min. Radiolabeled elongation complexes stalled at template position +24 (termed +24 complexes) were then formed by adding 100 μ M ApU, 50 μ M ATP, 50 μ M CTP, 1.5 μ M GTP, and 1.5 μ M [α -³²P]GTP (800 Ci/mmol; 1 Ci = 37 GBq), bringing the final volume to 19 μ l, and the reaction was incubated at 30°C for 2 min. The resultant reactions, containing +24 complexes, were transferred to a water bath at 37°C for 1 min, elongated with all four NTPs, and quenched with 2 vol of formamide buffer [95% (vol/vol) formamide/50 mM Tris-HCl, pH 8.0/0.025% xylene cyanol/0.025% bromphenol blue]. The RNA products of the transcription reaction were separated by gel electrophoresis and quantitated as described (2).

RESULTS

TE of tR2 RNA Hairpin Variants. In previous work (2), we had characterized the properties of the wild-type tR2 terminator. The exact site of termination was mapped to two consecutive template positions (U7 and U8) that correspond to the seventh and eighth rU residues within the rU-rich sequence on the 3' side of the RNA hairpin (Fig. 1). The total TE of the tR2 terminator is only about 0.40 under approximately physiological conditions [all four NTPs (each 1 mM) at 37°C] and is easily changed by manipulating reaction conditions (2). In particular, the TE of the tR2 terminator can be increased by reducing the concentration of UTP, which is the next nucleotide required to extend the nascent RNA at both termination positions. The observed hyperbolic dependence of the TE on UTP concentration is also consistent with the control of this parameter by the rate of the next RNA elongation step at each template position (1, 2).

In the current study, we wished to construct variants of tR2 that might be expected to increase TE. We focused on the structure of the tR2 hairpin, since the RNA sequence downstream of the hairpin was already very uridine-rich (a canonical feature of many efficient intrinsic terminators; ref. 14). To confirm that the tR2 hairpin is necessary for termination [as observed *in vivo* (15)], we deleted all or parts of the hairpin-forming sequence (variants tR2-5 and tR2-7). No termination was observed with these variants *in vitro* (Fig. 2), even when the concentration of UTP was reduced to prolong significantly the dwell-time of complexes at U7 and U8. To determine whether TE is correlated with the stability or the specific sequence of the RNA hairpin, we tested the effect of reversing the top 5 bp of the hairpin stem (variant tR2-6). This change alters the sequence of the hairpin while leaving its stability and structure relatively unchanged. Fig. 2 shows that this variant is a functional terminator, and Table 1 indicates that its TE is slightly increased over that of wild-type tR2. Thus this specific change in sequence of the hairpin stem does not have a significant effect on TE as measured *in vitro*, though we note that the measured TE of tR2 *in vivo* was decreased markedly when the two G-C pairs at the base of the stem were reversed (15).

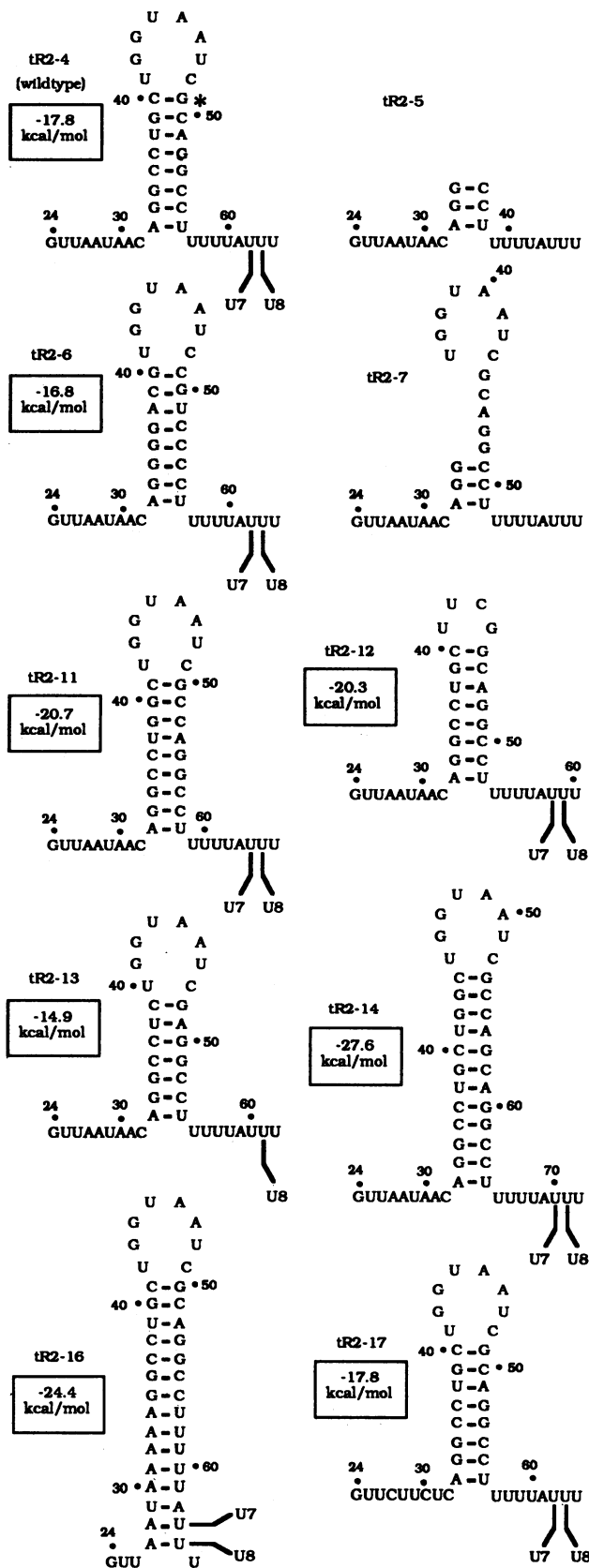


FIG. 1. Variants of the tR2 terminator shown as RNA sequences folded into the calculated most stable secondary structure. The predicted stabilities of the RNA hairpin structures, determined by using the data sets of refs. 8 and 12, are also shown, and the termination positions for all functional tR2 variants are indicated. The predicted stabilities of the tR2-4 and tR2-12 RNA hairpins were confirmed experimentally by thermal melting (13).

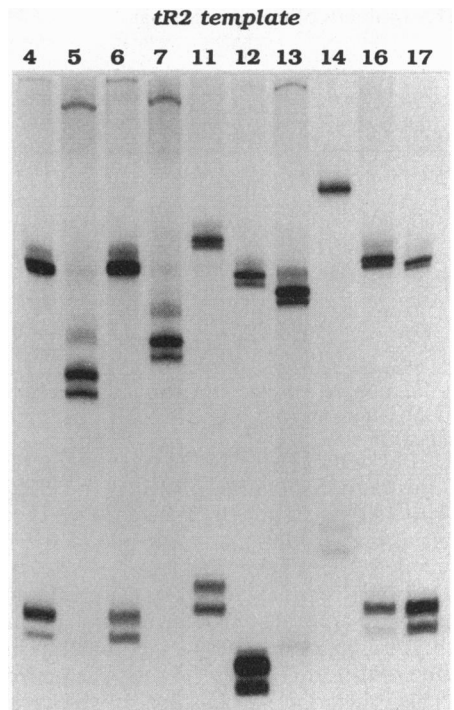


FIG. 2. Transcription products using templates containing the tR2 terminator variants shown in Fig. 1. For variants tR2-4, tR2-6, tR2-11, tR2-12, tR2-14, tR2-16, and tR2-17, termination occurs at two consecutive template positions (U7 and U8) that correspond to the faster migrating doublet bands. Termination occurs with low efficiency at a single template position for variant tR2-13 and is not detectable for tR2-5 and tR2-7. The full-length products appear as doublet bands, corresponding to the run-off transcript with and without an extra 3'-terminal nucleotide.

We next explored the relationship between hairpin stability and TE by lengthening and shortening the hairpin stem and by altering the hairpin loop. When one G-C pair was removed from the top of the hairpin stem (variant tR2-13), we observed a significant decrease in TE relative to wild-type tR2 (Table 1). Conversely, insertion of an additional G-C pair at the same position in the stem (variant tR2-11) slightly increased TE. When the hairpin was stabilized by replacing the tR2 loop sequence with the stabilizing (12) UUCG "tetra-loop" (variant tR2-12), TE also increased slightly (we confirmed the expected increase in hairpin stability due to this loop substitution experimentally by thermal melting measurements).

Based on these results one might predict that additional stabilization of the tR2 RNA hairpin by further lengthening of the stem should continue to increase TE. The results were opposite. Rather we found that the insertion of an additional 4 bp into the top of the stem (variant tR2-14) decreased the total TE to 0.20, one-half of the wild-type tR2 level (Table 1). Similar results had been obtained in *in vivo* experiments by Cheng *et al.* (15). These findings suggested that the relationship between hairpin structure and TE must be more subtle. Examination of two additional tR2 variants strengthened this view. The RNA hairpin of wild-type tR2 can, in principle, be extended (and thus stabilized) significantly further by pairing the adenine-rich sequence upstream of the hairpin with the string of downstream uridine residues. However, this extended hairpin involves mismatches and so might not be sufficiently stable to form during transcription elongation (or it might form too slowly). Two additional variants were constructed to investigate this issue. In one the RNA sequence just upstream of the hairpin was made perfectly complementary to the 3'-terminal uridine sequence (variant tR2-16), while in the other the upstream segment was made completely noncomple-

Table 1. TE of variants of the tR2 terminator

Terminator	TE					
	Position U7		Position U8		Total	
	- NusA	+ NusA	- NusA	+ NusA	- NusA	+ NusA
tR2 (wild type)	0.11 ± 0.01	0.56 ± 0.03	0.32 ± 0.02	0.68 ± 0.11	0.40 ± 0.01	0.86 ± 0.06
tR2-6	0.24 ± 0.02	0.32 ± 0.04	0.41 ± 0.02	0.58 ± 0.04	0.56 ± 0.02	0.71 ± 0.01
tR2-11	0.28 ± 0.01	0.65 ± 0.01	0.35 ± 0.01	0.49 ± 0.04	0.54 ± 0.01	0.82 ± 0.02
tR2-12	0.28 ± 0.02	0.38 ± 0.01	0.56 ± 0.03	0.66 ± 0.05	0.69 ± 0.02	0.79 ± 0.03
tR2-13	0*	0.10 ± 0.02	0.11 ± 0.02	0.55 ± 0.05	0.11 ± 0.02	0.61 ± 0.04
tR2-14	0.12 ± 0.01	0.40 ± 0.03	0.09 ± 0.01	0.12 ± 0.01	0.20 ± 0.02	0.47 ± 0.03
tR2-16	0.09 ± 0.01	0.51 ± 0.02	0.30 ± 0.03	0.67 ± 0.03	0.36 ± 0.03	0.84 ± 0.02
tR2-17	0.22 ± 0.01	0.56 ± 0.02	0.58 ± 0.03	0.76 ± 0.02	0.67 ± 0.01	0.89 ± 0.01

TE values shown represent the average of three measurements, and the errors represent the 95% confidence interval ($\pm ts/\sqrt{N}$, where $t = 4.3$, $s =$ standard deviation, and $N = 3$).

*No detectable termination.

mentary to this sequence (variant tR2-17). The TEs observed with these variants were again unexpected. The TE of tR2-16 was the same as that of wild-type tR2, while the TE of tR2-17 was significantly higher (Table 1).

Relation Between Template Positions of Termination and of RNA Hairpin Nucleation. The first termination position of the wild-type tR2 terminator is located 14 residues downstream of the nucleotide residue that closes the hairpin loop (residue G* in Fig. 1). This is also the first position at which a tR2 RNA hairpin could be stably nucleated if the 3' end of the nascent RNA is involved in a 12-bp RNA-DNA hybrid within the ternary elongation complex, as proposed in the simple nucleic acid-destabilization model (8). On the same basis this model predicts that the first termination positions for variants tR2-11 and tR2-14 should fall upstream of the wild-type tR2 termination positions. For tR2-14, in particular, it predicts that the termination positions should move upstream by four positions relative to those of wild-type tR2. The termination positions expected for each tR2 variant were calculated as described (2, 8). To test these predictions the termination positions for each variant were mapped by RNA sequencing, using a 3'-dNTP chain-terminating method as described (2). The results show that the termination positions for all the tR2 variants studied occur at the same positions, U7 and U8 (variant tR2-13 is a slight exception, in that it displays only a single termination site at U8). Thus, contrary to earlier findings based on a variety of terminators from the literature (2, 8), these results suggest that intrinsic termination positions may not be determined solely by the template position at which the RNA terminator hairpin can first be stably nucleated.

An alternate interpretation of these results might be that these termination positions are kinetically (rather than thermodynamically) determined, if the rates of RNA elongation are unusually slow at U7 and U8. To address this, we slowed elongation upstream of positions U7 and U8 on templates containing tR2-13 and tR2-14 by lowering the UTP concentration to 5 μ M. We found that although transcription complexes could be induced to pause briefly at positions A6, U7, and U8 by this procedure, the positions of termination on these templates remained unchanged (data not shown), arguing against the proposal that these termination positions are uniquely kinetically defined.

Effects of Saturating NusA Concentrations on TE of the tR2 Variants. Many intrinsic terminators, including tR2, become more efficient in the presence of NusA, an elongation factor that interacts with the core polymerase of the elongation complex after the σ subunit has been released (16). NusA would also have been present in the earlier *in vivo* studies of tR2 terminator variants (15). As a consequence, we have looked for specific hairpin-dependent effects of NusA on the measured *in vitro* TEs of our tR2 variants. Table 1 shows that saturating concentrations of NusA increase the total TE of all

the tR2 variants, though the magnitude of the effect varies. Precedents from other work (ref. 7; also Rees *et al.*, unpublished data) suggest that the observed NusA effect may largely reflect a general NusA-induced decrease in the overall rate of elongation.

DISCUSSION

Effects of Terminator Hairpin Structure and Dimensions on TE. This study of the tR2 terminator was designed to provide a critical test of the nucleic acid destabilization model for intrinsic termination described by Yager and von Hippel (8). Our results and the largely congruent *in vivo* study of Cheng *et al.* (15) provide some insights on the role of the RNA hairpin in termination. It appears that the optimal hairpin for maximizing TE contains a stem of 8 or 9 mostly G-C base pairs and a loop of 4–8 (or more?) residues and that within these structural limits, TE is moderately increased by increasing the stability of the hairpin. Lengthening or shortening the stem beyond these limits decreases (or abolishes) termination activity.

In addition, these results clearly show that the run of 6–8 rU residues located just downstream of the hairpin stem (comparison with other intrinsic terminators shows that the rA residue located in this part of the tR2 terminator is not required) plays an important role of its own and that the possibility of incorporating this run of rUs into the stem of a lengthened hairpin has little effect on the observed TE (or may even be slightly inhibitory). Most critically, our results show that no matter how we alter the hairpin (and, within limits, perturb the observed TE), termination invariably occurs at positions U7 and U8 within the run of rU residues. This suggests that these 6–8 rU residues must be transcribed before termination can take place.

A Strict Requirement for the rU Sequence Does Not Fit the Simple Nucleic Acid Destabilization Model. The simple nucleic acid destabilization model (8) would predict that an RNA hairpin sufficiently long to compete with the entire putative 12-bp hybrid (variant tR2-14) should be able to trigger termination without the participation of the rU sequence. As a result one would expect the positions of intrinsic termination to be shifted significantly upstream for this variant (to position U3). This is not observed. Instead the sites of termination remain invariant at positions U7 and U8. What then might be the role of the rU run? One possibility, which represents only a minor elaboration of the simple nucleic acid destabilization model, is that RNA hairpin invasion and competitive displacement are limited by structural features of the complex to approximately the 6 upstream bp of the putative 12-bp hybrid. The downstream 6 bp must then be rU-dA, since only this hybrid sequence is sufficiently less stable than its all-DNA homologue to provide the necessary level of RNA-DNA hybrid destabi-

lization. Other (nonthermodynamic) possibilities also exist and will be considered below. The observed maximum in TE at hairpin stem lengths of 7 or 8 bp is not relevant to the model, since the base pairs closest to the loop in longer stems would not contribute to the destabilization of a 12-bp RNA-DNA hybrid in any case.

Is the Structure of Transcription Complexes Invariant with Template Position? One of the postulates of the original nucleic acid destabilization model was that the nucleic acid framework of the transcription complex and the interactions of this framework with polymerase remain largely invariant with template position. Recent work by others has raised important questions about the general validity of this assumption. Thus footprinting studies of advancing transcription complexes have revealed that elongation can involve alternating stretches of monotonic and inchworm-like movement of the polymerase complex (17). Over extended sequences the polymerase seems to maintain the same conformation (footprint), moving uniformly along the template. However, this monotonic movement can be interrupted when the complex encounters DNA sequences that appear to halt the advance of the front-end domain of the polymerase while the nascent RNA continues to be elongated. It is particularly relevant that one DNA sequence that induces a cycle of inchworm-like movement is the dA-dT-rich stretch of the tR2 terminator that codes for the rU sequence of the nascent RNA.

Nudler *et al.* (17) have suggested that this inchworming process may lead to the progressive accumulation of internal strain within the polymerase, until the front-end is released and leaps several positions downstream to restore the relaxed monotonic conformation. This connection implies that intrinsic termination might involve an inchworming cycle, triggered either by movement of the polymerase into or across the dT-dA-rich sequence of the template or by hairpin formation, and that this event might participate in destabilizing the ternary complex and triggering RNA release.

We still know nothing about the effects (if any) of inchworming on the structure of the nucleic acid framework of the transcription complex. It is possible that inchworming may be required to permit the RNA hairpin to form in competition with the putative hybrid. In this view, a kinetic block to hairpin formation within the hybrid region during monotonic movement of the elongation complex along the template could be responsible for preventing the formation of long potential RNA hairpins (such as that of variant tR2-14) in the absence of the required adjacent downstream run of rU residues in the nascent RNA and thus prevent such thermodynamically adequate sequences from triggering termination.

What Is the Role of the RNA Hairpin in Intrinsic Termination? Despite the fact that our results can be accommodated within a modified nucleic acid destabilization model, the results presented here and by others suggest that the dimensions and structure of the hairpin, and not just its stability, are important determinants of termination. Several observations imply that the hairpin interacts with the polymerase and that this interaction helps to regulate termination. (i) This idea, which was suggested previously in the context of an *in vivo*

study of the TE of tR2 variants (15), could explain the lower termination efficiency of tR2-14 compared to wild-type tR2 and the lower TE of tR2-16 relative to tR2-17. (ii) The invariant location of the termination positions of the tR2 variants with respect to the hairpin structure, and the observation that the distance from the base of the hairpin to the first termination position is conserved for terminators for which termination positions have been mapped precisely (2, 18, 19), could also be explained by a specific interaction between the RNA hairpin and the polymerase. (iii) The importance of geometric details of the hairpin structure in termination observed herein is also supported by the common features of other known intrinsic terminators, which are characterized by short stable G-C-rich hairpin structures with surprisingly constant dimensions. Thus 85% of the 148 compiled terminators have stem lengths of 7 (± 3) bp and 85% display loops of 4 (± 1) nt (14).

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