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Synthetic sites for transcription termination and a functional comparison with tryptophan operon termination sites *in vitro*

(*in vitro* transcription/ρ-independent termination/mutant RNA polymerase/ribonucleotide analogs/mutant attenuator)

GAIL E. CHRISTIE*, PEGGY J. FARNHAM, AND TERRY PLATT†

Department of Molecular Biophysics and Biochemistry, Yale University, 333 Cedar Street, New Haven, Connecticut 06510

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ABSTRACT **Termination of transcription by *Escherichia coli* RNA polymerase *in vitro* appears to depend primarily on two structural features of the termination site—a G+C-rich region of dyad symmetry and a series of terminal uridine residues in the transcript. To determine whether these two features are sufficient to specify ρ-independent termination *in vitro*, we have introduced new sequences within a tryptophan (*trp*) operon structural gene to create two sites with these characteristics. Transcription with wild-type RNA polymerase *in vitro* demonstrates that discrete termination occurs at one of these new sites, although at a low level. Use of the mutant RNA polymerase *rpo203*, which is more sensitive to certain weak terminators than is the wild-type enzyme, increases termination at both sites. We have compared the activity of our synthetic terminators with those of several termination sites in the *E. coli trp* operon. Under normal conditions of transcription *in vitro*, termination becomes more efficient with an increase in the length of the stem in the RNA hairpin or an increase in the number of consecutive uridine residues. Transcription with the *rpo203* polymerase and with ribonucleotide analogs gives changes consistent with these general trends. These results support a model for termination involving separate but essential roles for the RNA hairpin and the stretch of uridines in the transcript.**

Termination of transcription by *Escherichia coli* RNA polymerase generally occurs at specific sites on a DNA template. Although termination is modulated by the transcription termination factor ρ *in vivo* (1), many terminators function *in vitro* in the absence of ρ. Sequence comparison of ρ-independent termination sites reveals common features (2, 3). These include a G+C-rich region of dyad symmetry, which can result in an RNA hairpin, and a series of terminal uridine residues in the transcript immediately 3′ to the region of dyad symmetry. Our studies have suggested that these two features reflect the involvement of both RNA–RNA and RNA–DNA interactions in termination at ρ-independent sites (4). The presumed role of the RNA hairpin in inducing a pause by RNA polymerase is supported by experimental evidence (2, 5, 6). The extreme instability of rU·dA base-pairing (7) probably enhances the release of the nascent transcript at the run of uridines. According to our model, the balance between elongation and dissociation of RNA polymerase at a termination site depends on the potential stability of the RNA hairpin and the weakness of the interaction between the terminal region of the transcript and the template.

Results obtained by varying conditions of transcription *in vitro* are consistent with this model. Termination can be affected not only by nucleotide sequence, but also by the nature of the incorporated ribonucleotides and the RNA polymerase (4, 8–10). Our previous studies have focused on the *trp* attenuator termination site (*trp a*) as a model system. Removal of four of the eight terminal uridine residues from this site by the deletion *trp a1419* eliminates termination of transcription *in vitro* (4, 11). Incorporation into the nascent transcript of the nucleotide analog 5-iodocytidine 5′-triphosphate (ICTP), which strengthens G·C base pairing, partially restores termination at this altered site. Termination can also be restored by the RNA polymerase mutation *rpo203* (12). Comparison of this mutant polymerase with the wild-type enzyme (*rpo*$^+$) under several conditions suggests that the *rpo203* enzyme is more sensitive to the instability of a rU·dA hybrid and therefore requires fewer uridine residues for termination to occur (4). The importance of the rU·dA base-pairing in termination is supported by the observation that incorporation of the analog 5-bromouridine 5′-triphosphate (BrUTP) into the RNA, which would strengthen the transcript–template interaction, increases readthrough levels at *trp a* (4).

A major prediction of this hypothesis (4) is that termination of transcription *in vitro* should occur at any site where dyad symmetry sufficient for hairpin formation is followed by a series of thymidines in the DNA sequence. We report here the construction and analysis of two such synthetic terminators. In addition, studies of both synthetic and natural termination sites using base analogs and the *rpo203* polymerase begin to distinguish between the relative contributions to termination of the RNA–RNA and RNA–DNA interactions.

MATERIALS AND METHODS

Preparation of Plasmids and Templates. Isolation of the *trp a* and *trp a1419* template fragments has been described previously (4). A plasmid carrying the *trp a135* mutation was the generous gift of C. Yanofsky. This mutant terminator is identical to *trp a* except for a T to G base change within the run of uridines (13). The plasmid pWU11, containing the *trp* terminator (*trp t*) next to the *trp* promoter, is described in Wu *et al.* (14). The plasmid pLD102, which was used in construction of synthetic terminators, is described in Christie and Platt (15). After digestion with the appropriate restriction endonucleases, template fragments were isolated by electrophoresis on polyacrylamide gels in 50 mM Tris–borate (pH 8.3)/1 mM EDTA and eluted electrophoretically in 40 mM Tris–acetate (pH 7.9). *Eco*RI and *Bam*HI linkers (Collaborative Research, Waltham, MA) were phosphorylated by incubation with ATP and phage T4 polynucleotide kinase (Boehringer Mannheim). Blunt-end ligation reactions were carried out in 10 μl containing 0.5 μg of linker, 0.8 pmol of pLD102, 70 mM Tris·HCl (pH 7.6), 20 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM ATP, and 1 (Weiss) unit of T4

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Abbreviations: ICTP, 5-iodocytidine 5′-triphosphate; BrUTP, 5-bromouridine 5′-triphosphate.

* Present address: Dept. of Molecular Biology, Univ. of California, Berkeley, CA 94720.

† To whom reprint requests should be addressed.

DNA ligase (P-L Biochemicals) and were incubated 16–18 hr at 4°C. Ligation mixtures were subsequently heated to 65°C for 10 min to inactivate the enzyme, diluted into the appropriate digestion buffer, and digested with restriction endonuclease *Eco*RI or *Bam*HI. The resulting linear plasmid molecules were purified by electrophoresis in horizontal 1.4% agarose slab gels in 40 mM Tris–borate (pH 8.3)/1 mM EDTA. The DNA was eluted from the agarose by electrophoresis into hydroxylapatite, recovered in 1.0 M sodium phosphate (pH 6.5), and chromatographed on Bio-Gel P60 (Bio-Rad). The plasmid DNA was then religated in 50 μl with 0.02–0.05 unit of T4 DNA ligase for 16–18 hr at 4°C and used to transform W3110 *trpΔAE1*. DNA was isolated from ampicillin-resistant colonies and screened for the presence of the new *Eco*RI or *Bam*HI restriction site.

***In Vitro* Transcription.** Wild-type *E. coli* RNA polymerase and the mutant *rpo203* polymerase were purified by the method of Burgess and Jendrisak (16). The *rpo203* polymerase was also a gift from W. McClure. [α-^{32}P]GTP (10–30 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. The base analogs ICTP and BrUTP were a gift from A. Waldrop and D. Ward. Standard 10-μl transcription reaction mixtures included 0.2–1.0 pmol of template, 0.05–0.2 μg of wild-type polymerase or 0.2–1.0 μg of *rpo203* polymerase, 20 mM Tris–acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 4 mM $Mg(OAc)_2$, 150 mM KCl, 200 μM each ATP, CTP, and UTP, and 3–4 μCi (about 15 μM) [α-^{32}P]GTP. We have previously shown that this GTP concentration is high enough to avoid transcription artifacts (4, 6). The base analogs ICTP and BrUTP were used in place of CTP and UTP, respectively, at final concentrations of 200 μM. Incubations were carried out for 10–20 min at 37°C. Reactions were terminated by adding 100 μl of 0.3 M sodium acetate/1 mM EDTA/0.5 mg of tRNA per ml. After ethanol precipitation, the samples were analyzed by electrophoresis on 7% or 10% acrylamide/7 M urea gels. RNA bands were located by autoradiography and were quantitated by excising the transcripts from the gel and measuring their radioactivities directly in the ^{3}H channel of a liquid scintillation counter (Čerenkov radiation) or by scanning the autoradiographs with a densitometer.

RESULTS

Construction and Analysis of Synthetic Terminators. To determine whether the two features common to ρ-independent termination sites are sufficient to specify termination, we constructed synthetic sites with these characteristics. DNA sequence analysis of *trpC* (17) revealed a region of dyad symmetry at nucleotides 73–80 that included a *Bal* I cleavage site and was

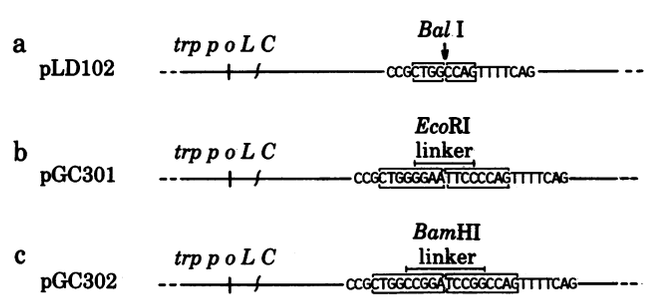

FIG. 1. Construction of synthetic termination sites. DNA sequence of the *trpC* region surrounding the *Bal* I site in the parental plasmid, pLD102, and the new sequences after insertion of the *Eco*RI and *Bam*HI linkers are shown. Insertion of a single intact linker was confirmed by DNA and RNA sequence analysis. Boxes indicate the regions of dyad symmetry; the nucleotides contributed by the linkers are overlined.

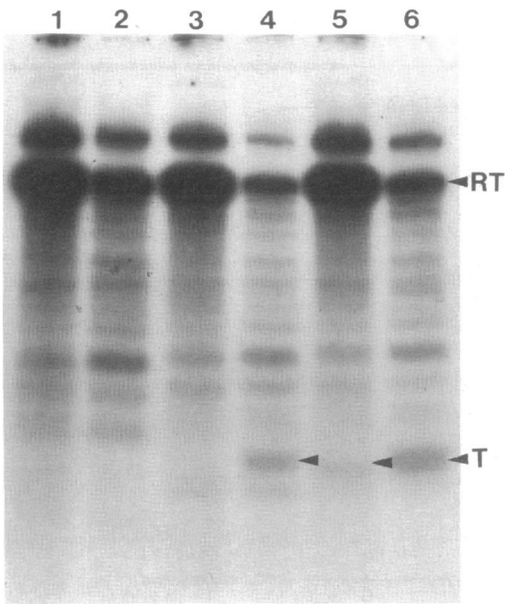

FIG. 2. Termination at the synthetic sites. *Bst*NI fragments (see Fig. 3) were isolated from the plasmids and used in standard transcription reactions with either wild-type (*rpo*$^{+}$) or *rpo203* polymerase. This autoradiograph of the *in vitro* transcription products demonstrates that termination occurs on templates carrying the synthetic terminators. The bands arising from termination at the new sites (Fig. 1) are designated by a T; RT indicates the *trp*-promoted readthrough transcript. Lane 1, pLD102, *rpo*$^{+}$; lane 2, pLD102, *rpo203;* lane 3, pGC301, *rpo*$^{+}$; lane 4, pGC301, *rpo203;* lane 5, pGC302, *rpo*$^{+}$; lane 6, pGC302, *rpo203*. The prominent band of intermediate molecular weight is *trp*-promoted, and terminates in the *trpC* coding sequence at what may be a naturally occurring polar site.

followed by four T residues (Fig. 1*a*). The plasmid pLD102, in which *trpC* was fused to the *trp* promoter by the internal deletion *trpΔLD102* (15) provided an ideal starting point, because no termination occurs *in vitro* at this site (Fig. 2, lanes 1 and 2). We increased the dyad symmetry by inserting an 8-base pair *Eco*RI linker (G-G-A-A T-T-C-C) or a 10-base pair *Bam*HI linker (C-C-G-G-A-T-C-C-G-G) into the *Bal* I site, creating the plasmids pGC301 and pGC302, respectively (Fig. 1). Because only four T residues follow the dyad symmetry, we did not expect these sites to function well with wild-type RNA polymerase. With the more sensitive *rpo203* polymerase, however, termination should be detectable even at a very inefficient site. The *rpo203* polymerase can indeed terminate transcription at the *trpC301* site, though wild-type polymerase cannot (Fig. 2, lanes 3 and 4). The *trpC302* site can cause termination of transcription with both *rpo203* and wild-type polymerase molecules to a detectable extent (Fig. 2, lanes 5 and 6). The addition of ρ to the standard transcription reaction mixtures has no detectable effect on termination. These results confirm that dyad symmetry followed by a run of uridines in the transcript can cause ρ-independent termination.

Transcription with *rpo203* Polymerase and Ribonucleotide Analogs. To assess the relative contributions of the strength of the hairpin and the polyuridine stretch in regulating the amount of termination, we compared transcription termination at the synthetic sites and four other terminator structures derived from sites in the *trp* operon. The DNA templates used for *in vitro* transcription are summarized in Fig. 3.

Fig. 4 illustrates the sequence and presumed secondary structure of the transcript at each termination site. We transcribed each template with both wild-type and *rpo203* polymerases, and separated the transcription products by gel elec-

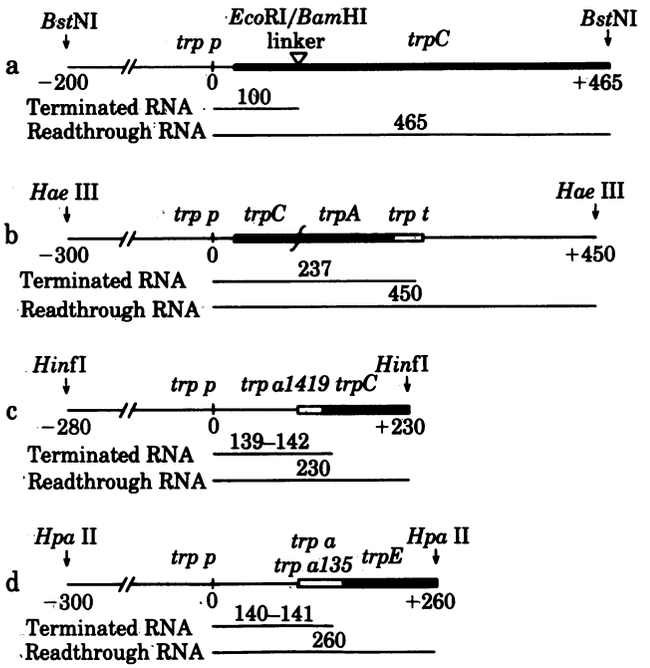

FIG. 3. Templates used in comparative studies of termination sites. The heavy lines indicate coding sequence, and the genes are named above. Distances are measured in nucleotides from the beginning of *trp*-promoted transcription. The open boxes correspond to regions of terminator sequence. (*a*) The *trpC301* and *trpC302* synthetic terminators. The site of insertion of the linkers is shown by the triangle. (*b*) The *trp t* template isolated from pWU11 (11). (*c*) Template containing the *trp a1419* deletion, which is fused to *trpC*. (*d*) Template for the wild-type *trp* attenuator and the point mutant *trp a135*.

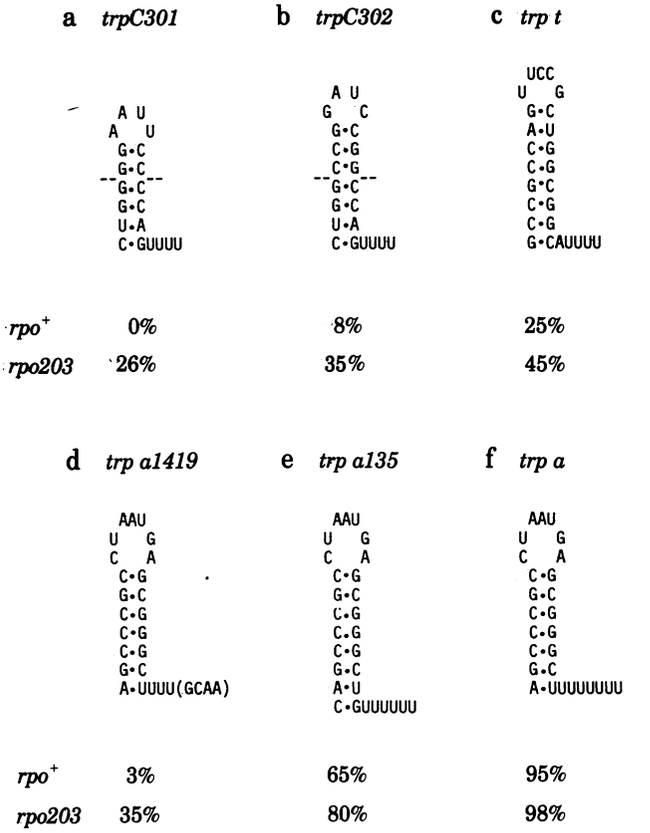

FIG. 4. Sequence and presumed secondary structure of transcripts at their termination sites. The dashed lines delineate linker sequences inserted during terminator construction. The extent of termination with wild-type (*rpo*$^+$) and *rpo203* polymerases is expressed as mol %. The exact location of the 3′ end has been determined previously for the *trp a*, *trp a1419*, *trp a135*, and *trp t* transcripts. For the two synthetic terminators, two-dimensional analysis of oligonucleotides narrowed down the endpoint to within the predicted region (data not shown). Because our intent in these studies was to determine the extent of termination we did not further pursue the identity of the 3′ ends.

trophoresis. The terminated species transcribed from each template were analyzed by standard two-dimensional electrophoresis/homochromatography of T1 ribonuclease digestion products to verify that they corresponded to the transcripts indicated in Fig. 3. From the relative yield of the terminated and readthrough transcription products, we calculated the amount of termination obtained with each polymerase. Termination is greater in all cases with the more sensitive *rpo203* enzyme, the most dramatic effects occurring at those sites having the shortest stretch of uridines. This supports the previous suggestion (4) that this mutant polymerase is more sensitive to the transcript–template instability.

The two ribonucleotide analogs used in these studies increase the stability of base-pairing. Incorporation of ICTP should affect primarily the strength of the RNA hairpin, whereas BrUTP should stabilize the terminal rU–dA interaction. Templates containing each of the six terminators shown in Fig. 4 were transcribed using each of the analogs. In addition, the *rpo203* polymerase was used in combination with these base analogs; this allows us to alter the response to the hairpin and transcript–template interactions separately or simultaneously. The products of these transcriptions are displayed in Fig. 5. The results are summarized in Table 1 and discussed below.

DISCUSSION

Sites for ρ-independent termination of transcription *in vitro* are characterized by the potential to form a hairpin followed by a terminal stretch of uridines in the transcript. We have demonstrated that these two features are sufficient to define a termination site, by constructing two synthetic terminators within the *trpC* gene of *E. coli*. In addition, we have compared these synthetic sites with several other terminators. The transcripts differ from one another in the length and composition of the hairpins and in the number of uridine residues, and their structures are presented in Fig. 4. The three attenuator derivatives have essentially the same hairpin (the *trp a135* stem is longer by one G•C base pair), but differ from each other in the number of terminal uridines. Conversely, four of the terminators have the same number of terminal uridine residues, allowing us to compare the effect of variations in the hairpin on termination efficiency.

If the role of the RNA hairpin is to induce pausing by polymerase and the role of the polyuridines is to promote dissociation of the transcript from the template, several specific predictions can be made as to the relative efficiencies of these terminators. An increase in the strength of the hairpin should enhance termination, whereas an increase in the stability of the interaction between the transcript and the template in the terminal U-rich region should increase readthrough. Our observations are generally consistent with these predictions (Table 1).

Consequences of Variations in the Hairpin. The initial step in the events leading to termination of transcription is believed to be a pause of the transcription complex on the template. RNA polymerase has been shown to hesitate at regions of dyad symmetry regardless of whether termination can occur, and this pause can be accentuated by factors that increase the strength of the hairpin (5, 6, 18). Previous studies on the *trp a1419* tem-

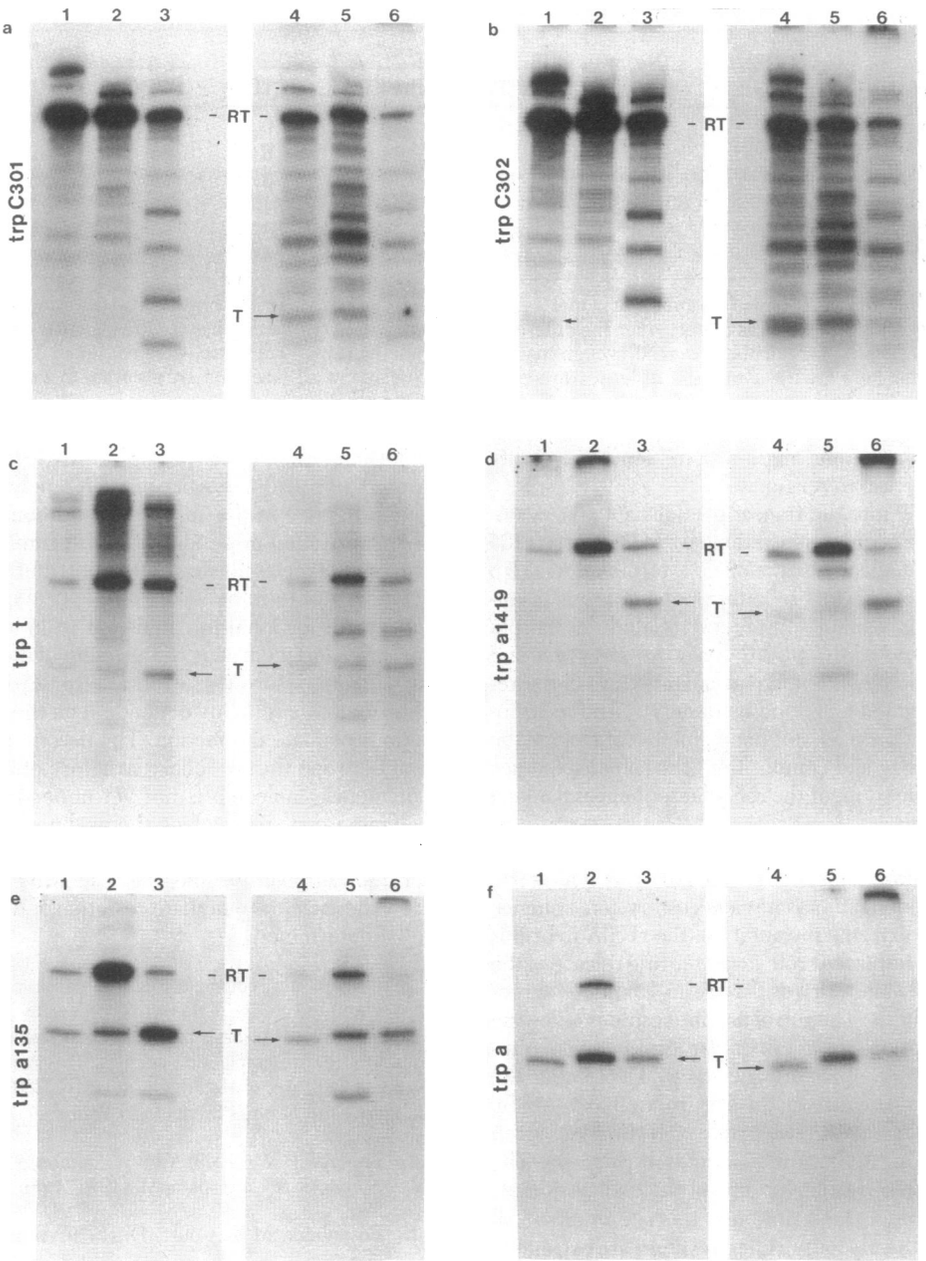

FIG. 5. Autoradiographs of *in vitro* transcripts from the various terminator templates. The first three lanes are transcription with *rpo*$^+$ and normal ribonucleotides (lane 1), BrUTP (lane 2), or ICTP (lane 3). The next three lanes are *rpo203* and normal ribonucleotides (lane 4), BrUTP (lane 5), or ICTP (lane 6). RT indicates the *trp*-promoted readthrough RNA, T is the terminated species. (*a*) *trpC301*, (*b*) *trpC302*, (*c*) *trp t*, (*d*) *trp a1419*, (*e*) *trp a135*. The band beneath the species designated T represents a strong pause in the *trp* leader region and has been discussed elsewhere (6). (*f*) *trp a*. For transcripts with incorporated analogs, the digestion products from T1 ribonuclease can vary considerably in their positions on a two-dimensional analysis relative to their normal counterparts. Thus, interpretation of oligonucleotide patterns of these species cannot always provide unambiguous identification. We verified for the *trp* leader transcript containing ICTP that the RNA was indeed *trp* promoted, and therefore we believe that the criterion of apparent size on acrylamide gels provides valid identification of the transcripts. In several cases (for example, lane 3 in *a* and *b*), new transcription products appear with the analogs. Two-dimensional analysis of T1 ribonuclease products demonstrated that these transcripts are not *trp* promoted, and we did not pursue their identity.

plate suggested that ICTP, which increases the strength of G·C base pairs, can increase the extent of termination (4). The studies presented here have allowed us to compare terminators with stems ranging from six to eight base pairs and to assess the relative effects of strengthening the stem on their termination potential.

In general, terminators with shorter stems in the hairpins are less effective than terminators with longer stems. Among the four terminators that have only four terminal uridines, the least efficient is *trpC301*, with a stem of only six base pairs (Fig. 4*a*). Increasing the stem by a single G·C base pair to yield the *trpC302* terminator increases termination at this site (Fig. 4*b*). The *trp a1419* site, which resembles *trpC302* in base composition and length of the stem in the hairpin, is also a very poor terminator (Fig. 4*d*). The *trp t* terminator, with the strongest hairpin, is the most efficient of these weak sites. The *rpo203* and wild-type polymerases respond in essentially the same way to these changes in stem length, as would be expected if the *rpo203* mutation primarily affects the response to the rU–dA interaction. This does not preclude the possibility that other

Table 1. Effect of ribonucleotide analogs and a mutant polymerase on termination of transcription

Terminator	No. of G-Cs/ stem length	No. of uridines	*rpo*$^+$			*rpo203*		
			NTPs	BrUTP	ICTP	NTPs	BrUTP	ICTP
(*a*) *trpC301*	5/6	4	0	0	0	26	21	36
(*b*) *trpC302*	6/7	4	8	4	6	35	30	24
(*c*) *trp t*	7/8	4	25	8	25	45	15	41
(*d*) *trp a1419*	6/7	4	3	6	64	35	17	79
(*e*) *trp a135*	7/8	6	65	21	90	80	61	94
(*f*) *trp a*	6/7	8	95	76	99	98	93	94

Numbers indicate the extent of termination in mol %. The ratio of radioactivities incorporated into the terminated and readthrough species (see Fig. 5) was corrected for the difference in length, based on the number of G residues in each transcript. For the purposes of these calculations, readthrough species other than the run-off transcript were not taken into account. The values reported here are the average of at least two separate experiments in all cases and are accurate to within ±5%.

features of termination regions might also influence the ability of the mutant polymerase to terminate.

Incorporating ICTP into the transcripts does not give a consistent pattern. While termination at the *trp a1419* and *trp a135* sites is increased dramatically by the analog, termination at *trp t* and the synthetic terminators by either polymerase is insensitive to ICTP. This cannot simply be related to hairpin strength, because *trp t* and *trpC301* have the strongest and weakest stems, respectively. Some particular feature of the region around the attenuator may be especially sensitive to incorporation of ICTP. We have not been able to control for the contribution of loop size and composition, the actual sequence of base pairs within the stem, or the role that sequences flanking a terminator might play.

Consequences of Alterations in the Uridine Stretch. Although RNA polymerase pauses at regions of dyad symmetry, termination of chain elongation is not a necessary consequence of such a pause. The extreme instability of the rU·dA hybrid is postulated to play an important role in permitting release of the nascent transcript (7), and our results are in complete agreement with this hypothesis. The run of uridine residues following the hairpin at termination sites is critical for termination of transcription in the absence of factors such as ρ. Decreasing the number of uridines at the attenuator site from eight to six by the *trp a135* mutation severely depresses termination, even though the strength of the hairpin is increased (Fig. 4*e*). The *trp a1419* deletion, which removes four of the eight uridines, eliminates termination of transcription at this site *in vitro*. All of the terminators with only four uridine residues are extremely inefficient; two of them, *trp a1419* and *trpC301*, are incapable of allowing termination by wild-type polymerase, one terminates at a very low level (*trpC302*), and one, *trp t*, functions with an efficiency of 25–30%. Confirmation that a strong hairpin alone is insufficient for termination was provided by inversion of the *trp t* site. This effectively removes all of the uridines while preserving the hairpin, and eliminates termination (data not shown).

We can vary the effect of the rU–dA interaction in two ways—by transcription with the mutant polymerase *rpo203* or by incorporation of the nucleotide analog BrUTP. The *rpo203* enzyme appears to be more sensitive than *rpo*$^+$ to an rU·dA hybrid region, and it enhances termination at all of the sites we examined. BrUTP stabilizes the rU–dA interaction, making dissociation of the RNA from the DNA more difficult, and results in a decrease in termination at all of these sites. As expected, the *rpo203* polymerase can partially reverse the BrUTP effect (see Table 1).

These results all support a simple model for ρ-independent termination of transcription. Analysis of our synthetic termination sites confirms that a region of dyad symmetry capable of forming a stable hairpin in the RNA, followed by a series of consecutive uridines, can cause RNA polymerase to cease elongation *in vitro* in the absence of additional factors. Moreover, characterization of ρ-independent terminators with highly differing efficiencies illustrates the contributions of structure to the efficiency of termination. There appears to be a minimum requirement for a hairpin sufficient to induce RNA polymerase to pause, and increasing the strength of the hairpin can increase termination. However, even at strong pause sites, the extent of termination is highly dependent on the weakness of the transcript–template interaction. The precise nature of the interaction between the secondary structure of the transcript and the polymerase molecule is not yet understood. The behavior of polymerase at sites lacking the uridine residues, such as ρ-dependent terminators and sites of premature termination of transcription in polarity, must be responsive to additional factors. How these factors modulate cessation of RNA synthesis remains to be determined.

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