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# Effects of DNA base analogs on transcription termination at the tryptophan operon attenuator of *Escherichia coli*

(*in vitro* transcription/ρ-independent termination/deoxyribonucleotide analogs/primed synthesis/bacteriophage M13)

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ABSTRACT **We have devised a method to specifically incorporate deoxyribonucleotide base analogs *in vitro* into either strand of the tryptophan (*trp*) operon attenuator region, using primed synthesis on bacteriophage M13 derivatives carrying cloned *trp* attenuator DNA. We have employed these techniques to extend previous studies implicating both RNA–RNA and RNA–DNA interactions in transcription termination in an attempt to determine the nature of the contribution from the template DNA molecule in termination regions. In general, we find that the dramatic effects upon transcription termination seen with base analog incorporation into mRNA do not occur when similar analogs are incorporated into the DNA. Only the analog 2,6-diaminopurine deoxyribonucleotide triphosphate (dDapTP), which strengthens A·T or A·U base pairing, elicits a significant response: in the template DNA strand, the presence of this analog increases readthrough at the *trp* attenuator. The analog 5-bromouracil deoxyribonucleoside triphosphate (BrdUTP), which also strengthens pairing with its complementary base, has no detectable effect on termination when it is placed in either strand of the *trp* attenuator or the mutant attenuator *trp a1419*. Surprisingly, though the analog 5-iodocytosine deoxyribonucleoside triphosphate (IdCTP) does not affect termination, it has a great effect on initiation of transcription, depressing *trp* promoter activity as well as stimulating transcription from other regions. These results support the postulated interaction between terminal uridines in mRNA and the template DNA strand in enhancing termination and suggest that there are no significant additional contributions from the DNA. In addition, the novel use of M13 derivatives for incorporating analogs into the DNA on a preparative scale provides a technique for introducing mutations in a general but controlled fashion as a new means for studying other regulatory regions.**

Utilizing the filamentous bacteriophage M13 (whose life cycle includes both double-stranded and single-stranded stages) we can introduce changes in cloned DNA regions by substituting deoxyribonucleotide analogs for the normal nucleotides in a primed synthesis reaction on a single-strand template. Because the orientation in which the desired DNA fragment inserts into the double-stranded M13 vector (the replicative form) determines which strand will be found in the single-stranded phage DNA, both the template and the nontemplate strand can be isolated. This specific incorporation of DNA analogs into either strand can be carried out easily on a preparative scale and permits a controlled perturbation of the primary structure of the cloned region.

We describe below the application of this method to study termination of transcription at the *trp* attenuator *in vitro*. Like other ρ-independent termination sites in *E. coli*, this site contains a G+C-rich region of dyad symmetry followed by a series of uridines in the RNA (1). Models for transcription termination have postulated major roles for an mRNA hairpin (resulting from the dyad symmetry) and the stability of the rU–dA interaction between transcript and template (2–4). The construction of synthetic termination sites and analysis of several related terminators are consistent with the involvement of both RNA–RNA and RNA–DNA interactions (5). However, the precise contribution of the DNA itself has not been determined. To circumvent the ambiguities inherent in our own previous work on termination with ribonucleotide analogs, we have incorporated similar base analogs into the DNA of the template and nontemplate strands and then transcribed from these templates using normal ribonucleoside triphosphates. Our results suggests that DNA–DNA interactions do not play a key role in termination.

## METHODS

**Cloning of *trp a* and *trp a1419*.** We used the cloning vehicle bacteriophage M13mp2, in which part of the *lac* operon from *E. coli* has been inserted into M13 RF DNA and an *Eco*RI site created in a region coding for the $NH_2$ terminus of β-galactosidase (6). Primer fragment was isolated from an *Eco*RI digest of the plasmid pMH232 (7) which produces a 90-base-pair fragment adjoining the *Eco*RI cloning site in M13mp2. This fragment can be used as a primer for all clones. The double-stranded replicative form of M13 was isolated after infection of *E. coli* K-12 71-18 cells (6).

Isolation of the 560-base-pair *trp a* and 510-base-pair *trp a1419* fragments carrying the *trp* promoter has been described (3, 8). These fragments were mixed with *Eco*RI-cut M13mp2 and the overhanging ends filled by using Pol I Klenow fragment and 1 μM of each of the deoxyribonucleoside triphosphates in 20 mM Tris·HCl/10 mM $MgCl_2$/1 mM dithiothreitol/1 mM EDTA/50 μM rATP. After 30 min on ice, 1 unit of T4 DNA ligase was added. The reaction was incubated at 15°C for 12–15 hr and used to transfect *E. coli* 71-18 cells. The single-stranded phage were screened for orientation of the inserts by plaque hybridization of $^{32}P$-labeled *trp* leader RNA from an *in vitro* transcription reaction and nick-translated leader DNA to duplicate filters carrying the candidates. The RNA hybridizes only to cloned inserts in one orientation, the DNA to both.

After these phage were grown-up and single-stranded DNA isolated, they were checked for the presence of the cloned inserts by using a short primed synthesis reaction similar to that used in dideoxy sequencing (9, 10). An analytical restriction digest of this synthesized DNA can be visualized by autoradiography of acrylamide gels, and each orientation in which the

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Abbreviations: dDapTP, 2,6-diaminopurine deoxyribonucleoside triphosphate; IdCTP, 5-iodocytosine deoxyribonucleoside triphosphate; BrdUTP, 5-bromouracil deoxyribonucleoside triphosphate; ICTP, 5-iodocytosine-5′-triphosphate; BrUTP, 5′-bromouridine-5′-triphosphate.

fragment can insert into the M13 vector will give a different pattern of restriction bands in the area of interest. If the radioactive triphosphate is diluted with unlabeled triphosphate after a short time, only the bands immediately adjacent to the primer will be labeled, resulting in an easily interpretable restriction pattern. Thus, rescreening insures correct identification of the clone. This method is especially useful if RNA complementary to the cloned fragment cannot be easily obtained.

**Construction of Templates.** For each clone, single-stranded DNA was isolated from 5-ml phage-infected overnight cultures. For preparative work, the complementary strand was synthesized on 5–10 pmol of single-stranded DNA from each clone by extension from a 90-base-pair *Eco*RI *lac* primer fragment (also 5–10 pmol), by using 1 unit of Pol I Klenow DNA polymerase in 5 mM Tris·HCl/6 mM $MgCl_2$/5 mM dithiothreitol/50 $\mu$M deoxyribonucleotide triphosphates (Fig. 1). The DNA analogs were used at 50 $\mu$M for 5-iodocytosine deoxyribonucleoside triphosphate (IdCTP), 10 $\mu$M for 2,6-diaminopurine deoxyribonucleoside triphosphate (dDapTP), and 50 $\mu$M for 5-bromouracil deoxyribonucleoside triphosphate (BrdUTP). The resultant double-stranded DNA was digested with *Bst*NI, and the attenuator-containing fragment was identified by ethidium bromide staining on 5% acrylamide gels and eluted for use in transcription reactions. The analogs IdCTP and BrdUTP (P-L Biochemicals) were 96% pure. The dDapTP was a gift from Dave Ward and had been purified on HPLC. To test whether template synthesis might be the result of contamination in our normal triphosphates, we tried the reaction with all possible combinations of three triphosphates and found no detectable synthesis.

***In Vitro* Transcription.** *E. coli* RNA polymerase was purified by the method of Burgess and Jendrisak (11). [$\alpha$-$^{32}$P]GTP (10–40 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels and [$\alpha$-$^{32}$P]UTP (450 Ci/mmol) were obtained from New England Nuclear. Standard 10-$\mu$l transcription reactions contained about 0.1 $\mu$g of template and 0.2 $\mu$g of polymerase in 20 mM Tris acetate, pH 7.9/0.1 mM dithiothreitol/4 mM $MgAc_2$/150 mM KCl and 200 $\mu$M each of the unlabeled triphosphates. Incubations were carried out at 37°C for 20 min and were terminated by addition of 100 $\mu$l of 0.3 M sodium acetate/1 mM EDTA/tRNA (0.5 mg/ml). After ethanol precipitation, the samples were analyzed by electrophresis on 10% polyacrylamide/7 M urea gels. RNA bands were located by autoradiography, eluted from the gel, and subjected to T1 RNase digestion and two-dimensional analysis as described (2).

## RESULTS

The previously described DNA fragments spanning the *trp* operon promoter (*trp p*) and the nearby attenuator (*trp a*) region (3, 8) were cloned into a unique site in the double-stranded replicative form of the vector M13mp2 (see Fig. 1). The nucleotide sequences of the wild-type and mutant attenuator regions are shown in Fig. 2. The orientation in which each fragment inserts into the M13 cloning site determines which strand will be found in the single-stranded phage DNA. We obtained clones for each in both orientations (Fig. 3) and thus could specifically incorporate analogs into either the template or nontemplate strand. Preparative amounts of double-stranded DNA were prepared from each of the single-stranded circular DNAs in primed synthesis reactions by using either the four normal nucleotide triphosphates or completely substituting for one with an appropriate base analog. The analogs IdCTP, BrdUTP, and dDapTP were used in the primed synthesis reaction (substituted for dCTP, dTTP, and dATP, respectively) to produce a total of 16 templates. All of these analogs strengthen base pairing with

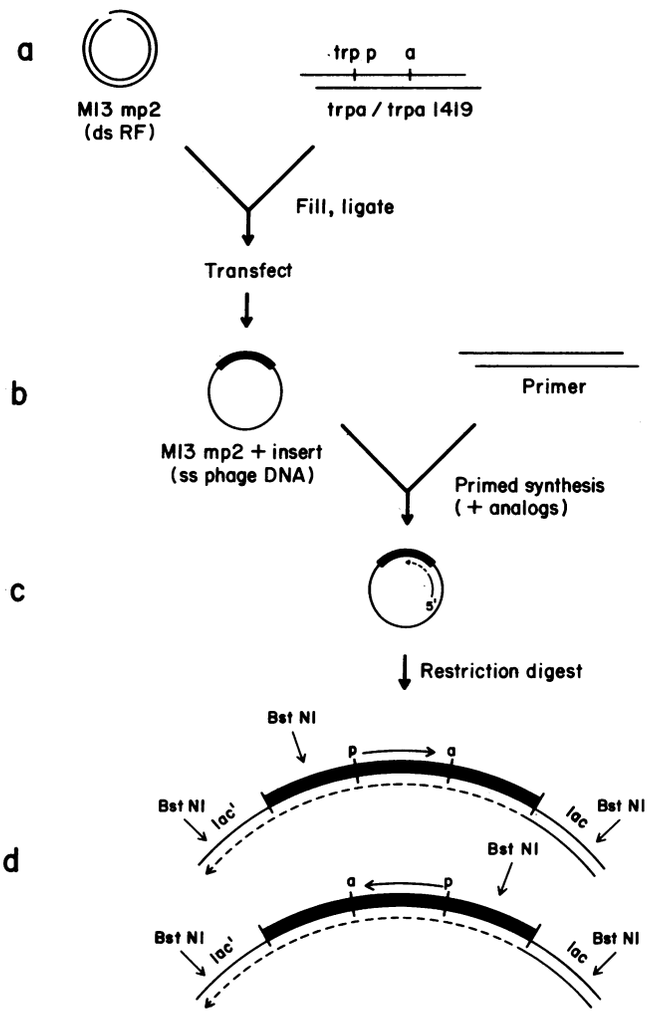


FIG. 1. Schematic of template preparation. Shown here is the method by which the deoxyribonucleotide base analogs were incorporated into the normal and mutant *trp* attenuator DNA. The double-stranded vector M13mp2, which has been cut at the single *Eco*RI site, is mixed with either the *Hpa*II 560-base-pair *trp a* fragment or the *Hinf* 510-base-pair *trp a1419* fragment (*a*). Each of these fragments contains the *trp* promoter and attenuator regions. The overhanging ends of the vector and fragment are filled and then ligated. 71-18 cells are transfected with the mix and phage containing inserts are identified by using plaque hybridization to RNA and DNA probes. The single-stranded DNA isolated from these phage is used in a primed synthesis reaction with a 90-base-pair *lac* primer (*b*). A specific analog is substituted for its homologous normal triphosphate and incorporated into the strand which is being synthesized (*c*). A *Bst*NI digest of the resulting double-stranded DNA then produces the template for transcription reactions. As there are two possible orientations for each cloned insert, two different *Bst*NI restriction fragments containing the *trp* promoter and attenuator will be obtained for both *trp a* and *trp a1419* (*d*).

their complementary DNA or RNA base (12), and allow us to examine the consequences of altering the DNA in both the G+C-rich region of dyad symmetry and the terminal AT stretch of $\rho$-independent terminators.

**Transcription of M13pf16.** Because this phage (Fig. 3*a*) contains the nontemplate strand of the *trp* attenuator, primed synthesis will incorporate analogs into the template strand. Fig. 4*a* displays the products resulting from transcription of this DNA template. Lane 1 is the control reaction with the normal template; the prominent band (L) is *trp* leader and the slower moving species is readthrough (RT) transcript. Lane 2 shows

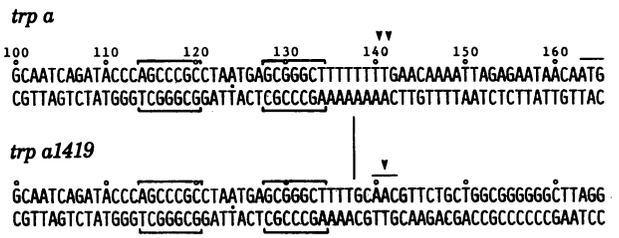


FIG. 2. Sequence of the *trp* attenuator regions. The wild-type *trp a* leader region is compared to the *trp a1419* sequence. The boxes indicate the dyad symmetry which can result in a stable hairpin formation in the RNA. The arrows indicate the known termination points in the leader sequence. Primed synthesis can result in 100% analog substitution in one strand or the other.

that incorporation of BrdUTP into the template strand had little effect on the amount of termination versus readthrough (<2% change in the amount of termination). The incorporation of IdCTP (lane 3) shows only faint bands corresponding to L and RT, with the former being nearly obscured by a new prominent band. This new species just above the leader band was not derived from the leader region, as shown by fingerprint analysis.

The difficulty in obtaining interpretable data when IdCTP is incorporated into the template is evidently due to a strong inhibition of correct initiation by this analog. To circumvent this problem here, we performed a kinetic analysis of the primed synthesis reaction. At a time (≈5 min under our conditions) when the *trp a* region was synthesized but the promoter region had not yet been reached, the IdCTP was diluted with a 100-fold excess of dCTP. On this template, in which the attenuator region but not the promoter region contained the analog, initiation was normal and termination was unaffected (data not shown). With dDapTP (lane 4) the ratio of readthrough to leader

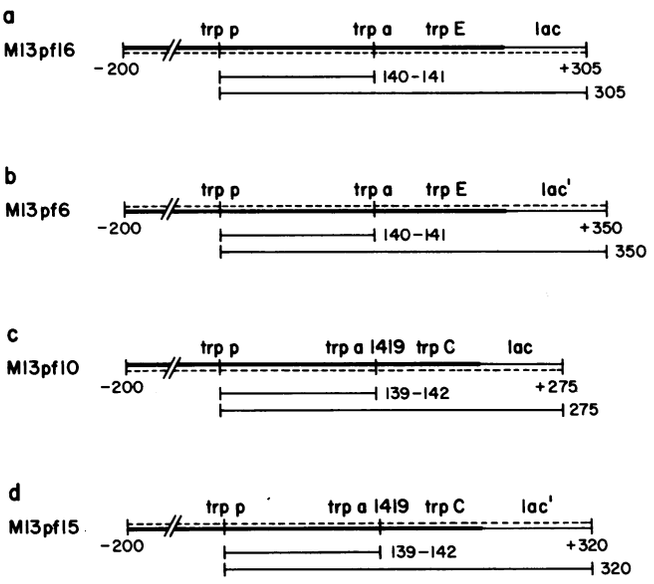


FIG. 3. Comparison of the *trp* DNA templates. The DNA fragments from the four clones were isolated and used as templates for transcription *in vitro*. Numbers indicate distance from the start point of transcription. The location of the *Bst*NI site at −200 is approximate. The products of transcription from the *trp* promoter are the leader RNAs (terminating at either *trp a* or *trp a1419*) and readthrough RNAs. In the wild-type DNA, *trp a* precedes the *trpE* structural gene by about 20 nucleotides. In the template carrying the *trp a1419* deletion, the attenuator is fused to the *trpC* sequence about 150 nucleotides from the distal end of that gene. The dashed lines indicate the strands in which the analogs are incorporated.

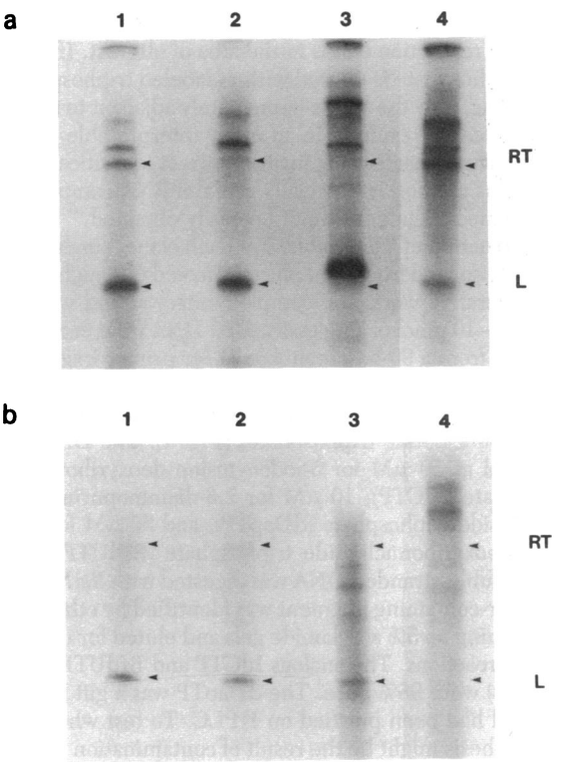


FIG. 4. Autoradiographs of *in vitro* transcripts from the *trp a* templates. *a* shows transcription from M13pf16 (analogs in template strand) and *b* is from M13pf6 (analogs in nontemplate strand). In each case lane 1 represents transcription from DNA that has been synthesized with only normal triphosphates. The remaining lanes represent transcription from DNA containing BrdUTP (lane 2), IdCTP (lane 3), or dDapTP (lane 4). RT indicates *trp*-promoted readthrough; L is the leader RNA terminated at the *trp* attenuator. The intense band just above leader in lane 3 of *a* is not *trp*-promoted, nor are the bands just below RT in lane 3 of *b*. These are all a result of anomalous initiation of transcription (see *Discussion*).

increased, approximately 2- to 4-fold. This is the only case in which the presence of an analog had a significant effect on termination (see *Discussion*).

**Transcription of M13pf6.** This attenuator fragment (Fig. 3*b*) is in the opposite orientation to M13pf16, thus allowing analogs to be introduced into the nontemplate strand. Fig. 4*b* presents transcription results from this clone. As before, lane 1 shows leader and readthrough RNA produced from DNA synthesized with normal triphosphates. Lane 2 shows that there was no difference in the results when BrdUTP is incorporated. Lane 3, which is transcription from DNA containing IdCTP, displays not only leader but also other bands. In neither of these cases was there a significant deviation from the usual ratio of L to RT. For the experiment of lane 4 we encountered a serious technical problem as yet not solved: we were unable to make preparative amounts of DNA (containing the dDapTP) from this clone. Thus the transcription levels are very low. This problem with analog incorporation into specific strands of DNA has been seen by others (D. Ward and J. Bodnar, personal communication). Because dDapTP can be incorporated into this strand of the *trp a1419* DNA (see below), we surmise that the replacement of the normal leader sequence (containing 14 adenines in the equivalent region) is responsible for alleviating the synthesis problem.

**Transcription of M13pf10.** Because this phage carries the nontemplate strand of the mutant *trp a1419* (Fig. 3*c*), primed synthesis incorporates analogs into the template strand. This attenuator normally gives nearly 100% readthrough (Fig. 5*a*,

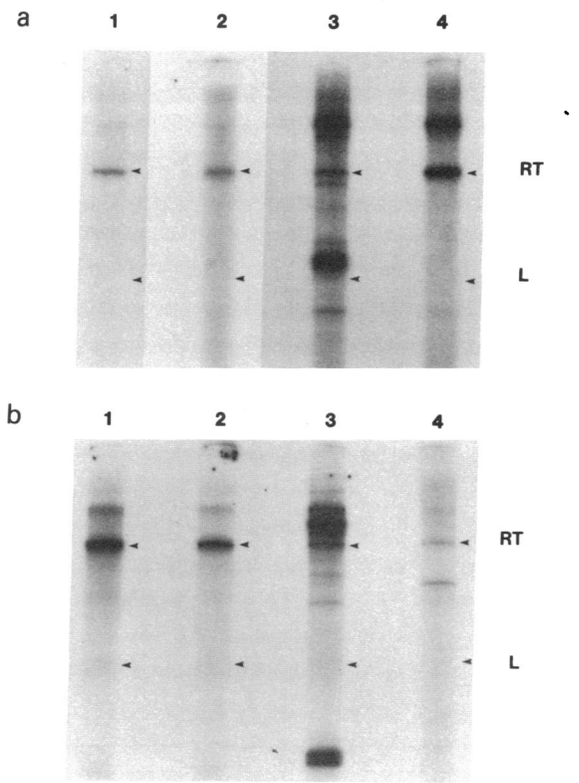


FIG. 5. Autoradiographs of *in vitro* transcription from the *trp a1419* templates. *a* shows transcription from M13pf10 (analogs in template strand) and *b* is from M13pf15 (analogs in nontemplate strand). In each set, lane 1 represents transcription from DNA that has been synthesized with only normal triphosphates. The remaining lanes represent transcription from DNA containing BrdUTP (lane 2), IdCTP (lane 3), or dDapTP (lane 4). RT indicates *trp*-promoted readthrough; L is the leader RNA terminated at the *trp* attenuator. The dark bands in lane 3 of *a* and lanes 3 and 4 of *b* do not correspond to *trp*-promoted RNA species (see *Results* and *Discussion*).

lane 1). Neither BrdUTP (lane 2) nor dDapTP (lane 4) in the DNA can elicit detectable levels of termination. However, lane 3 shows that when IdCTP was incorporated, though termination was not affected, extra RNA species were produced. Two-dimensional analysis of these species after T1 RNase digestion demonstrated that they did not contain any oligonucleotides specific to *trp* leader mRNA.

**Transcription of M13pf15.** The single-stranded DNA from this clone corresponds to the template strand of the *trp a1419* attenuator (Fig. 3*d*), and the analogs are therefore incorporated into the nontemplate strand. As seen before, transcription on this attenuator gave 100% readthrough with normal triphosphates (Fig. 5*b*, lane 1) and was unchanged with the incorporation of BrdUTP (lane 2). Once again, the presence of IdCTP in the DNA gave more RNA species (lane 3). The incorporation of dDapTP (lane 4), in this case, resulted in the appearance of an RNA species smaller than RT. Fingerprint analysis demonstrated that it was unrelated to *trp* leader mRNA.

## DISCUSSION

We have employed M13 as a means of incorporating analogs into specific DNA regions. By modifying the primed synthesis reaction on single-stranded M13 DNA, analogs can be placed into either strand of the cloned fragment. Because vector and primer are easily isolated, once the conditions for each clone have been optimized analytically, preparative amounts of double-stranded DNA containing chosen analogs may be obtained.* Substituted template DNA is suitable for *in vitro* transcription studies to probe aspects of gene control.

These methods were applied to test current models of $\rho$-independent transcription termination. These models suggest that the extent of termination depends upon a strong hairpin structure in the RNA followed by a series of 3′-terminal uridines (3, 5). In particular, numerous studies that have utilized ribonucleotide base analogs for transcription have demonstrated a profound effect of these analogs on the behavior of RNA polymerase at termination sites. For example, we believe the increased termination upon incorporation of 5-iodocytidine-5′-triphosphate (ICTP) is largely due to an enhancement of RNA–RNA interactions in the hairpin stem, whereas the incorporation of 5′-bromouridine-5′-triphosphate (BrUTP) decreases termination because it increases the stability of the hybrid rU–dA interaction. Using the above approach, we have tested directly the involvement of the DNA in several aspects of this model.

We find that the dramatic effects on termination seen with the incorporation of analogs into the RNA (3–5, 15–17) do not occur when the analogs are present in the DNA. The most striking change in termination occurred when the analog dDapTP was used. The incorporation of this analog into the coding strand of the DNA in the *trp* attenuator should mimic the effect of using BrUTP in the RNA, because both strengthen the rU–dA interaction at the terminal uridines. As predicted, this analog did increase readthrough transcription at the *trp* attenuator. That this increase was not due to DNA–DNA interactions was shown by the incorporation of BrdUTP into the same region of DNA on the nontemplate strand. Although this substitution increases the dU–dA interaction, it should not affect base pairing with the RNA, and we have shown here that this incorporation had no effect on termination. The dDapTP had no effect on termination when incorporated into either strand of *trp a1419*.

The incorporation of BrdUTP into either strand of the *trp a* or the *trp a1419* templates had no effect on termination. Even substitution for the run of eight thymidines in the nontemplate strand at the end of the attenuator region (where maximal effect might be expected) did not elicit a detectable response. We conclude that the contribution of strengthened pairing between thymidine and its complementary DNA (or RNA) base is negligible.

The results with IdCTP are more complicated. We postulated that the enhanced termination seen on the *trp a1419* template when ICTP was used in the transcription mix was due to increased stability of the RNA stem. Accordingly, the presence of IdCTP in the DNA should not give this same result. In all cases, the IdCTP caused new transcription products to appear. Two-dimensional analysis of the major transcripts from each clone showed that these RNAs were not *trp*-promoted; thus the incorporation of IdCTP apparently changes the specificity of polymerase initiation on these DNAs. There may be another sequence in this region that can function—although at a low level—as a promoter (unpublished data), and with IdCTP in the DNA, it is possible that promotion from this region becomes more efficient than that from the *trp* promoter. When IdCTP was selectively incorporated into the termination region of M13pf16 but not in the promoter, the transcription products were identical to those that used normal triphosphates (data not

* A careful choice of the restriction enzyme used to obtain the template may be necessary, since the presence of an analog in the restriction site can alter the specificity and rate of cleavage of a restriction enzyme (13, 14). For instance, *Hin*cII, *Hae* III, *Hha* I, and *Hpa* II will not cleave if IdCTP is in the DNA (unpublished observation).

shown). Therefore, it seems that the presence of IdCTP in the DNA has a far greater effect on initiation than on termination.

This change in promoter function caused by both IdCTP and dDapTP could be due to an alteration of external groups on DNA bases that serve as recognition points for RNA polymerase. It has been postulated that external groups such as methyls are important contact sites for RNA polymerases. The effect of these analogs on initiation may be more related to their causing alterations in the major and minor grooves of the DNA helix than to their base-pairing properties.

In summary, the use of M13 for incorporating analogs into the DNA has allowed us to test the contributions of template and nontemplate DNA strands to transcription termination. The only major effect seems to be from the template strand in the A-rich region complementary to the 3′ end of the transcript. Since BrUTP incorporated into RNA has a similar effect (3) but BrdUTP in the DNA does not, we infer that the important contribution to termination is from RNA–DNA interactions at this site (4). In the G+C-rich region, which has been postulated to enhance termination by impeding the progress of RNA polymerase, we can detect no contribution due to IdCTP in either strand of DNA. Because ICTP in the RNA has a strong effect, these results are consistent with the postulate that RNA–RNA interaction in a hairpin structure is the other major factor contributing to $\rho$-independent termination (1, 3–5).

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