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Termination of transcription by *Escherichia coli* RNA polymerase: Influence of secondary structure of RNA transcripts on ρ-independent and ρ-dependent termination

(rNTP base analog/rho ts15/ATP hydrolysis)

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ABSTRACT **The effect of RNA secondary structure on ρ-independent and ρ-dependent termination of transcription of T3 DNA by *Escherichia coli* RNA polymerase has been studied by incorporating, into nascent transcripts, base analogs that lead to altered base-pairing properties. A guanine → hypoxanthine substitution, with attendant weakening of secondary structure, abolished the ρ-independent termination at 20% of the genome; in contrast, replacement of cytosine with 5-bromocytosine, which forms stronger pairs with guanine, enhanced termination at this site. ρ-Independent termination was not altered by replacing uracil with 5-bromouracil. There are two major ρ-dependent termination sites on the T3 DNA—at 8 and 15%. The termination activity of ρ in this system also depended on RNA secondary structure. The incorporation of 5-bromouracil instead of uracil into RNA did not alter the site specificity of ρ action but ρ was rendered inactive when cytosine was replaced by 5-bromocytosine. In contrast, replacement of GTP with ITP in the reaction increased ρ-dependent inhibition of RNA synthesis, caused production of heterogeneous-sized transcripts, and stimulated ρ-mediated ATP hydrolysis. The ρ-associated ATPase activity, in the presence of isolated T3 RNA, was also stimulated by inosine substitution. Furthermore, the temperature-sensitive ρ isolated from *rho* 15 mutant of *E. coli*, which does not terminate transcription in the presence of the common rNTPs, was active when GTP was replaced with ITP. These results suggest that strongly paired G·C-rich regions in RNA stem-loop structures or RNA·DNA hybrids are essential for ρ-independent termination, whereas ρ-dependent termination requires weakly paired cytosine residues for its action.**

Termination of *Escherichia coli* RNA polymerase transcription, both *in vivo* and *in vitro*, is a highly specific process which occurs at a number of well-defined sites (terminators) on the DNA template (for a review, see ref. 1). Two different types of termination sites have been distinguished in *in vitro* transcription processes. Certain terminators, with varying efficiency, halt transcription in the presence of RNA polymerase alone whereas others require the *E. coli* protein ρ. At present, both termination mechanisms are poorly understood. Recent structural and biochemical studies indicate that, in ρ-independent as well as ρ-dependent termination, the secondary structure of the nascent transcript near its 3′ terminus or the stability of transient DNA·RNA hybrid formed during transcription, or both, plays an important role (1–6). We have examined these effects in an *in vitro* transcription system using bacteriophage T3 DNA template and *E. coli* RNA polymerase. This template has well-defined ρ-dependent and ρ-independent termination sites. Using such a system, we have found that incorporation, into RNA chains, of rNTP base analogs that alter the stability of the secondary structure of G·C-rich regions of the RNA formed during transcription substantially affects both the ρ-mediated and the ρ-independent termination reactions in opposite fashions.

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MATERIALS AND METHODS

E. coli RNA polymerase holoenzyme was purified by a modification of the procedure of Maitra and Hurwitz (7). ρ protein was purified from *E. coli* MRE-600 cells by the procedure of Roberts (8). Both preparations were free of detectable RNase (including RNase III), DNase, and nucleoside triphosphatase activities by criteria described previously. The temperature-sensitive ρ, isolated by a modification (9) of the procedure of Roberts (8), from *E. coli rho* 15 (10) was a kind gift of A. Das and S. Adhya (National Institutes of Health). The sources of all other reagents including bacteriophage T3 DNA were as described (11, 12).

Transcription of T3 DNA by *E. coli* RNA Polymerase. Reaction mixtures (0.1 ml) contained 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 50–200 mM KCl (as indicated), 4 mM dithiothreitol, the common ribonucleoside triphosphates or their analogs (as indicated) each at 0.4 mM and one labeled with ^{32}P in the α-position, 8.5 nmol of T3 DNA (expressed as deoxynucleotide residues), 2.4 μg of RNA polymerase, and ρ protein (as indicated); the mixtures were incubated for 20 min at 37°C. For measurement of the incorporation of labeled nucleotide into RNA, reactions were stopped by adding 0.5 ml each of 0.1 M $Na_4P_2O_7$ and 10% CCl_3COOH. The precipitated RNA was collected on Millipore filters (7) and assayed for radioactivity in a toluene-based scintillation fluid.

ρ-ATPase Assay. For measurement of hydrolysis of ATP during transcription, reaction mixtures were the same as described above except that [γ-^{32}P]ATP (4×10^4 cpm/nmol) and [^3H]UTP (10×10^4 cpm/nmol) were used as the labeled nucleoside triphosphates. After incubation at 37°C for 20 min, the amount of [^3H]UMP incorporated into an acid-insoluble RNA product was determined by Millipore filtration (7); the release of $^{32}P_i$ by cleavage of [γ-^{32}P]ATP was determined by a modification of the method of Conway and Lipmann (13) as described (14). Control values for free $^{32}P_i$ present in reaction mixtures containing no ρ ranged between 0.4 and 0.6 nmol and were subtracted from all values reported here.

RESULTS

ρ-Independent Termination during Transcription of T3 DNA. Early in the infection of *E. coli* by phage T3, *E. coli* RNA polymerase holoenzyme initiates transcription at one or more promoters located at the left end of the T3 genome (within 1% on the standard genetic map) (15) to copy the early genes (0.3, 0.7 1, 1.1, and 1.3, respectively) and terminates at a site t_1 located near 20% of the genome (15). Although termination at this site occurs quite efficiently *in vivo* (16), *in vitro* the polymerase reads through it to copy a part of the late region (17, 18).

The presence of ρ factor in the *in vitro* transcription system restricts polymerase to transcribing the early genes only (17, 18).

The pattern of *in vitro* transcription of T3 DNA by *E. coli* RNA polymerase was followed by polyacrylamide gel electrophoresis of transcription products (Fig. 1). *In vitro*, the efficiency of termination at t_1 depended on the ionic conditions of the polymerase reaction. At low KCl concentration (50 mM), some RNA chains were terminated at the t_1 site to produce RNA of molecular weight 2.5×10^6. However, a significant portion of polymerase molecules read through t_1 to yield a larger RNA product, of apparent molecular weight 4.8×10^6 (Fig. 1, lane a). Presumably, a second terminator t_2 in the late region of the genome halts further progress of the polymerase. At higher KCl concentrations (200 mM and above), most of the transcripts terminate at t_1, giving rise to a single RNA species of molecular weight 2.5×10^6 (Fig. 1, lane b).

In order to study the influence of secondary structure of RNA on termination, GTP was replaced by ITP in the RNA polymerase reaction. Under such conditions, the polymerase incorporated IMP residues at those positions in RNA chains usually occupied by GMP, although with decreased efficiency (data shown in legend to Fig. 1). Because I·C base pairs are weaker than G·C pairs (20), RNA stem-loop structures and DNA·RNA hybrids containing such pairs are less stable. This base substitution resulted in the synthesis of a single RNA species of apparent molecular weight 4.5×10^6 (Fig. 1, lane c), indicating that no termination had occurred at t_1. Furthermore, this effect was independent of the salt concentration in the reaction (data not shown).

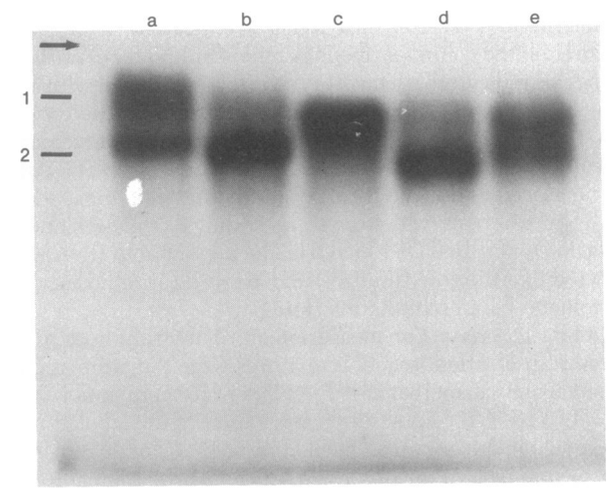

FIG. 1. Autoradiograms of polyacrylamide-agarose gels of T3 RNAs synthesized by *E. coli* RNA polymerase *in vitro* in the presence of rNTP base analogs. T3 DNA was transcribed under standard reaction conditions using [α-^{32}P]ATP (3×10^5 cpm/nmol) and various rNTP base analogs substituted for corresponding normal substrates as indicated: lanes a and b, normal rNTPs; lane c, ITP replaced GTP; lane d, BrCTP replaced CTP; lane e, BrUTP replaced UTP. The RNA in lane b was synthesized in a reaction mixture containing 200 mM KCl; all other reaction mixtures contained 50 mM KCl. The amount of [α-^{32}P]AMP incorporated in reaction mixtures containing the normal rNTP at 50 mM KCl was 1.2 nmol; it was 0.4–0.5 nmol in reaction mixtures containing the base analogs. ^{32}P-Labeled RNA transcripts were separated electrophoretically on vertical slab gels that contained 2% acrylamide and 0.5% agarose (12). Electrophoresis, with Peacock's buffer (19) containing 0.1% sodium dodecyl sulfate, was at 70 V for about 4 hr at room temperature until the bromophenol blue dye marker reached the bottom of the gel. After electrophoresis, gels were analyzed by autoradiography. Molecular weights of RNA species are represented by numbers at the left: 1, 4.8×10^6; 2, 2.5×10^6. Arrow, origin of electrophoresis.

Two other ribonucleotide base analogs, 5-bromo CMP and 5-bromo UMP, when substituted for CMP and UMP, respectively, in a RNA chain, stabilize RNA·DNA hybrids and RNA·RNA double-stranded structures greatly by forming the stronger BrC·G and BrU·A base pairs (21, 22). When 5-bromocytidine triphosphate (BrCTP) replaced CTP, the read-through beyond the t_1 site was prevented, giving rise to a single transcript; substitution of BrUTP for UTP in the polymerase reaction caused no apparent change in the efficiency of termination at t_1 (Fig. 1).

ρ-Dependent Termination of Transcription on T3 DNA. The progress of transcription of T3 DNA by *E. coli* RNA polymerase in the presence of ρ was also followed by gel electrophoresis (Fig. 2). At 50 mM KCl and in the presence of ρ factor, the 2.5×10^6 and 4.8×10^6 molecular weight RNA species were not observed. Instead, two new major RNA species of molecular weights 1.0 and 1.4×10^6 and several minor species (molecular weight range of $0.5–0.7 \times 10^6$) were formed (Fig. 2, lane b). The major species corresponded to termination sites at 8 and 15% on the T3 genome, respectively. Apparently, these sites lose their susceptibility to ρ at increased salt concentrations (200 mM KCl) (Fig. 2, lane c) because the only major RNA product formed under these conditions was molecular weight 2.5×10^6 (corresponding to termination at the ρ-independent site t_1).

The effect of the secondary structure of RNA on ρ-mediated termination was examined by substituting the nucleoside triphosphate analogs ITP, BrCTP, and BrUTP for the corre-

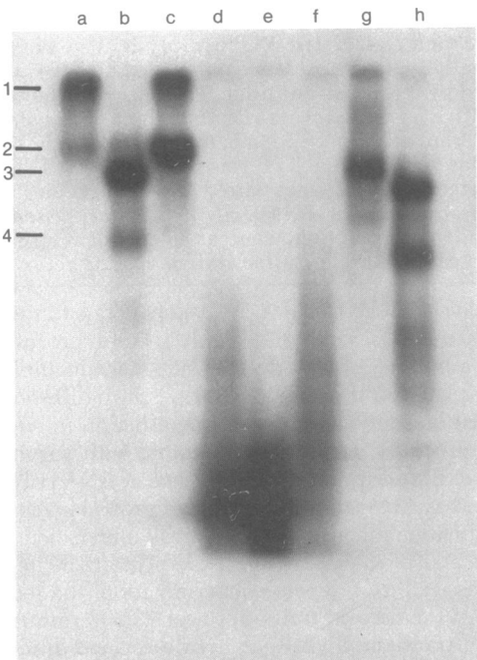

FIG. 2. Autoradiograms of polyacrylamide-agarose gels of *E. coli* RNA polymerase transcripts of T3 DNA synthesized in the presence of ρ (0.75 μg), rNTP base analogs, and [α-^{32}P]ATP (3×10^5 cpm/nmol). The amount of ρ used was above saturating levels for the polymerase reactions under the conditions of the assay. Substrates and KCl additions were as follows: lane a, normal rNTPs, 50 mM KCl, no ρ; lane b, normal rNTPs, 50 mM KCl, ρ; lane c, normal rNTPs, 200 mM KCl, ρ; lane d, ITP replaced GTP, 50 mM KCl, ρ; lane e, ITP replaced GTP, 200 mM KCl, ρ; lane f, ITP replaced GTP, 50 mM KCl, 2 μg of *rho* 15 (incubated at 32°C); lane g, BrCTP replaced CTP, 50 mM KCl, ρ; lane h, BrUTP replaced UTP, 50 mM KCl, ρ. RNA products were analyzed electrophoretically as described in Fig. 1. Molecular weights of RNA species are represented by numbers at the left: 1, 4.8×10^6; 2, 2.5×10^6; 3, 1.4×10^6; 4, 1.0×10^6.

sponding normal substrates in the transcription reaction. When GTP was replaced by ITP, the specificity of ρ-dependent termination was largely lost; under these conditions, the transcripts synthesized were heterogeneous in size and were detected as a smear in the gel (Fig. 2, lane d). Analysis of the products by determining the radioactivity in 1-mm slices of cylindrical gels after polyacrylamide urea electrophoresis (12) also showed size heterogeneity with molecular weights ranging from 1×10^5 to 4×10^5 (data not shown). It appears that incorporation of IMP into the nascent transcripts potentiates the action of ρ, causing it to terminate chains at several as yet undefined sites on the template. In contrast to the GTP reaction, ρ was equally active at low and at high salt concentrations (Fig. 2, lane e). When BrCTP was substituted for CTP in the transcription reaction, however, ρ was inactive in terminating RNA chains at the 8 and 15% sites. The RNA products were the same size as those obtained in the absence of ρ (compare Fig. 1 lane d and Fig. 2 lane g). Substitution of BrUTP for UTP in the transcription reaction caused little apparent change in ρ-mediated termination on T3 DNA (Fig. 2, lane h).

Effect of Substitution of Base Analogs on ρ-Dependent Inhibition of RNA Synthesis. Termination activity of ρ also could be measured as inhibition of *in vitro* transcription of T3 DNA by *E. coli* RNA polymerase (Fig. 3). Replacement of GTP with ITP in the reaction caused RNA synthesis to be abruptly inhibited; maximal inhibition occurred at much lower ρ concentrations and the extent of inhibition was greater. In contrast, replacement of CTP with BrCTP in the transcription reaction inhibited the action of ρ: the amount of RNA synthesis under these conditions was similar to that obtained in the absence of ρ. Replacement of UTP with BrUTP had no effect in ρ-mediated inhibition of RNA synthesis. The inclusion of low concentrations of ITP or BrCTP in reactions containing four normal rNTPs had no effect on ρ-mediated inhibition of RNA synthesis, indicating that the base analogs do not act as effectors on ρ action (data not shown).

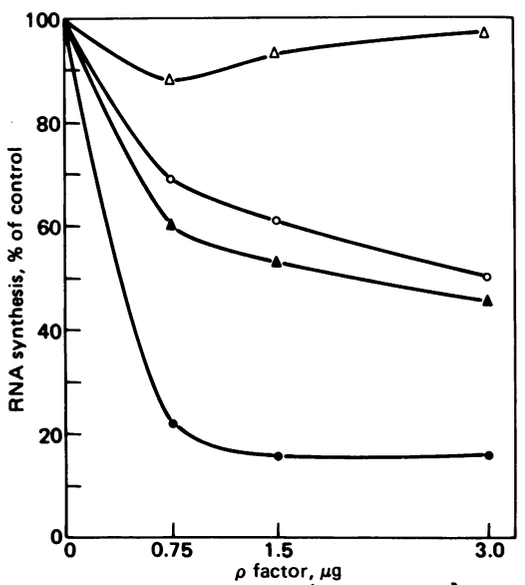

FIG. 3. Effect of rNTP base analogs on ρ-mediated inhibition of RNA synthesis. Polymerase reactions were carried out with ρ additions as indicated. Substrates for the reactions were as follows: ○, normal rNTPs; ●, ITP in place of GTP; △, BrCTP in place of CTP; ▲, BrUTP in place of UTP. Control values refer to [α-^{32}P]UMP incorporated in the absence of ρ under conditions specified (e.g., analog or normal rNTPs). These values were 1.2 nmol in reaction mixtures containing the normal rNTPs and 0.4–0.5 nmol in reactions containing base analogs.

ATPase Activity of ρ. Transcription in the presence of ρ is accompanied by ρ-dependent hydrolysis of ATP to ADP + P_i (23–26). During transcription of T3 DNA in the presence of ρ, there was a small but measurable amount of ATP hydrolysis to ADP in P_i (Fig. 4). This hydrolysis was dependent on ρ as well as on T3 DNA-directed RNA synthesis (data not shown). Replacement of GTP with ITP in the polymerase reaction resulted in a marked stimulation of ATPase activity. No detectable ATPase was observed in reaction mixtures that contained BrCTP in place of CTP. The use of BrUTP in place of UTP led to a decrease in the amount of ATP hydrolyzed. It is unlikely that BrCTP itself inhibits the ATPase activity because neither this analog nor ITP has any effect on poly(C)-dependent ATPase activity of ρ within a wide range of analog concentrations tested (data not shown).

In addition to exhibiting a transcription-dependent ATPase activity, ρ factor also catalyzes a RNA-dependent hydrolysis of ATP in the absence of other components of the transcription system (23–26). In order to study more directly the interaction of ρ with RNA products formed in the presence of GTP and ITP in T3 DNA-directed RNA polymerase reactions, the respective transcripts were isolated and used to direct ρ-dependent ATPase activity. T3 RNA transcripts that contained IMP in place of GMP residues supported ρ-catalyzed hydrolysis of ATP at a much faster rate and to a greater extent than did normal GMP-containing RNA (Fig. 5). In either case, the ATPase activity was not significantly affected by KCl concentrations in the range 0–300 mM (data not shown).

Properties of *rho* 15. The observations described above were confirmed and extended by carrying out similar experiments with a mutant rho protein, *rho* 15 (10). This protein, isolated from one of the polarity suppressor ts mutants of *E. coli*, does not show transcriptional termination activity *in vivo* (29) and *in vitro* at any temperature tested (10); however, it catalyzes a poly(C)-dependent ATPase activity at low temperatures and is temperature sensitive for this activity (10).

At the permissive temperature, 32°C, a small but measurable amount of ATP hydrolysis was detected during transcription

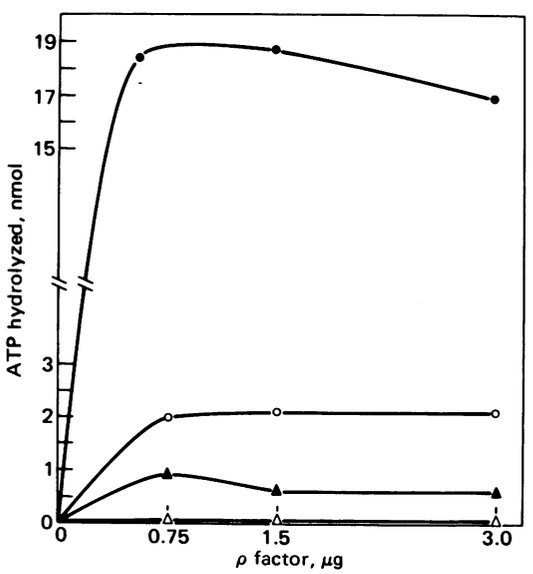

FIG. 4. Effect of rNTP base analogs on ATPase activity of ρ during transcription of T3 DNA by *E. coli* RNA polymerase with indicated amounts of ρ. Substrate additions were as follows: ○, normal rNTPs; ●, ITP replaced GTP; △, BrCTP replaced CTP; ▲, BrUTP replaced UTP.

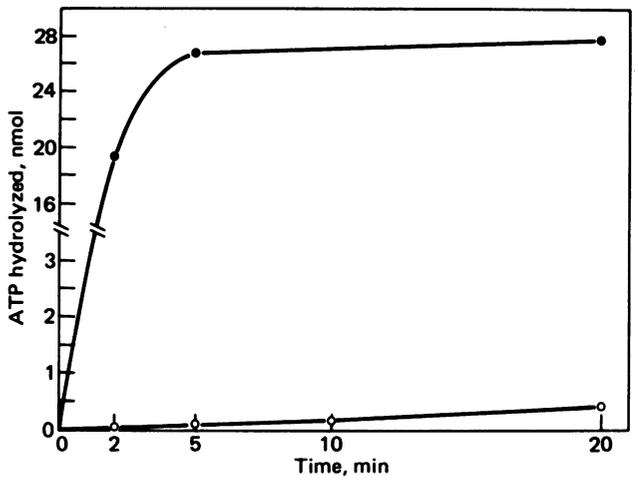

FIG. 5. ATPase activity of ρ in the presence of isolated T3 RNA formed with ITP (●) or GTP (○). Reaction mixtures (0.1 ml) contained 50 mM Tris·HCl (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 0.5 mM [γ-^{32}P]ATP (20 cpm/pmol), 0.75 μg of ρ, and 0.66 nmol (as nucleotide residues) of isolated ^{3}H-labeled T3 RNA formed *in vitro* with *E. coli* RNA polymerase. After incubation at 37°C as indicated, reactions were terminated and the amount of $^{32}P_i$ released was measured.

of T3 DNA by *E. coli* RNA polymerase in the presence of *rho* 15. Under these conditions, no inhibition of RNA synthesis was observed (Table 1). When GTP was replaced by ITP, however, *rho* 15 catalyzed a more extensive hydrolysis of ATP at 32°C. Furthermore, RNA synthesis was now inhibited approximately 70%. Analysis of RNA products made under these conditions revealed random termination of transcription producing heterogeneous RNA transcripts (Fig. 2, lane f). Both activities of *rho* 15 in the ITP-containing system were temperature sensitive (Table 1).

Table 1. Action of *rho* 15 in the presence of rNTP base analogs

System measured	Reaction measured at: 32°C	52°C
RNA synthesis, % of control:		
GTP system	100	100
ITP system	29	100
ATP hydrolyzed (nmol) during transcription in the presence of rNTPs		
GTP system	1.1	0.5
ITP system	18.6	1.2
ATP hydrolyzed (nmol) in the presence of isolated RNA containing		
GMP residues	1.4	0.8
IMP residues	20.9	4.1
Poly(C)	26.0	3.7

In each experiment, two sets of reaction mixtures were prepared. One set was incubated at 32°C and the other was incubated at 52°C. The assay systems in the first two experiments were similar to those described in the legends to Figs. 3 and 4, respectively, except, where indicated, ITP replaced GTP in the RNA polymerase reaction mixtures. *rho* 15 (2 μg) was added where appropriate. In the first experiment (RNA synthesis), control values represented [α-^{32}P]UMP incorporated in the absence of ρ (0.24 and 0.12 nmol at 32 and 52°C, respectively). The assay system used in the third experiment was similar to that described in the legend to Fig. 5 except that 23 nmol of poly(C) or 0.37 nmol of GMP-containing or IMP-containing T3 RNAs formed *in vitro* by *E. coli* RNA polymerase was added as indicated.

At 32°C, the mutant ρ protein and the wild-type protein behaved similarly with respect to their RNA-dependent ATPase activities. In the presence of the mutant ρ, ATP was hydrolyzed 14-fold more in the presence of IMP-containing T3 RNA than with the corresponding GMP-containing RNA (Table 1). The ATPase activity, as expected, was reduced considerably at 52°C.

DISCUSSION

In the present communication, we describe the *in vitro* termination of RNA chains formed in the presence of rNTP base analogs, T3 DNA, and *E. coli* RNA polymerase. The effects of salt concentration and various base analog substitutions on ρ-independent termination could be attributed to the requirement for a strongly base-paired RNA stem-loop, or RNA·DNA hybrid structure involving G·C-rich sequences for this type of termination. Secondary structures involving A·U (or A·dT) pairs are probably not important for ρ-independent termination because the replacement of UMP with BrUMP in RNA was without effect on the efficiency of termination of RNA chains at the 20% site on the T3 DNA. Similar results have been obtained for termination at the *trp* attenuator (3) as well as ρ-independent termination of RNA formed *in vitro* with DNA from phages T7 and T3 (4). It is noteworthy that sequence studies of RNA chains that terminate in the absence of ρ reveal the presence of a relatively stable G·C-rich sequence immediately preceding the U-rich 3′ end of the transcripts (for reviews, see refs. 1 and 2). It must be emphasized, however, that our studies do not distinguish between the importance of intramolecular RNA·RNA hydrogen bonding and of intermolecular DNA·RNA interactions in termination.

Our studies on the influence of base-analog substitutions during RNA synthesis on termination with ρ underline the importance of ρ–RNA interactions. Changes in the RNA secondary structure lead to changes in the effectiveness of ρ. Replacement of GMP residues in RNA with IMP stimulates ρ activity greatly, causing ρ to terminate RNA chains more or less randomly; the presence of BrCMP in RNA or of high concentrations of salt in polymerase reaction mixtures virtually abolished the action of ρ. These results suggest that ρ requires weakly structured RNA or RNA·DNA hybrid regions containing exposed CMP residues for which ρ has high affinity (25) in order to interact with the polymerase at specific sites on the template. Secondary structures involving A·U (or A·dT) interactions are apparently not involved in determining this site specificity because BrUMP substitution in RNA (in place of UMP) does not alter the action of ρ.

It is probable that ρ possesses duplex RNA or DNA·RNA hybrid melting activity that is essential for termination. The inability of *rho* 15 to terminate RNA chains at any temperature under conditions in which the normal four rNTPs were present suggests that *rho* 15 is deficient in this melting activity. The weakening of RNA secondary structure by replacing GMP residues on nascent transcripts with IMP permits the mutant protein to bypass this requirement and terminate RNA chains at 32°C. This melting activity may require ATP hydrolysis. ρ would thus possess two types of ATPase activities—one dependent on RNA and uncoupled from termination, and the other dependent on RNA polymerase and the nascent transcript at the termination site as recently suggested by Das *et al.* (9). In our studies the correlation between ATPase and termination activities of ρ are not immediately apparent because the termination-defective *rho* 15 catalyzed ATP hydrolysis during

transcription at 32°C in the presence of the normal rNTPs. It is likely that the uncoupled ATPase activity is potent enough to mask any deficiency in the termination-dependent ATPase.

The alteration of the secondary structure of RNA, therefore, has opposite effects on ρ-dependent and ρ-independent reactions: strengthening the secondary structure of RNA interactions hinders ρ activity but stimulates ρ-independent termination. In contrast, weakening such interactions stimulates ρ activity and inhibits ρ-independent termination of RNA chains. This apparent anomaly can be reconciled if we postulate that the two types of termination signals represent a spectrum of RNA polymerase pause sites on the DNA. Thus, the ρ-independent termination sites induce a relatively longer pause whereas the ρ-dependent terminators induce only a small pause of RNA polymerase movement. A hydrogen-bonded RNA·RNA duplex or DNA·RNA hybrid region would be necessary for such pauses. Strong base pairing would increase the chances for a longer pause leading to ρ-independent termination. At other sites, an interaction of ρ with pausing RNA polymerase involving weakly paired CMP residues in RNA would be necessary for termination but not sufficient; ρ then would have to melt the duplex region before the completed transcript and polymerase could be released.

Note Added in Proof. We have recently observed that ρ is as active in hydrolyzing ATP with poly(5-BrC) as with poly(C). This suggests that the observed inhibition of ρ-dependent termination in the presence of BrCTP (see Figs. 2 and 3) is due not to altered interaction of ρ with the substituted cytosine residues in nascent transcripts but to stronger base pairing between BrC and G residues.

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