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Ribonucleic Acid Release Activity of Transcription Termination Protein ρ Is Dependent on the Hydrolysis of Nucleoside Triphosphates†

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ABSTRACT: An assay is devised to measure ρ-dependent release of RNA chains from transcription complexes. It is shown that ρ stimulates the release of RNA from isolated ternary transcription complexes consisting of nascent RNA molecules, *Escherichia coli* RNA polymerase, and T7 DNA and that this stimulation is dependent on the presence of a nucleotide substrate for ρ–NTPase. Several experiments lead to the conclusion that release is a direct consequence of a NTP hydrolysis dependent ρ action on the RNA. First, the NTP requirement is satisfied by any of the nucleotides which are substrates for ρ–NTPase but is not satisfied by nucleotides that are not hydrolyzed by ρ action. Furthermore, the fact that some of the nucleotides that do activate release are poor substrates for RNA polymerase suggests that release is not dependent upon further nucleotide addition to the nascent chain. Second, the reaction conditions for release and RNA-dependent ATPase show similar requirements for optimum activity and similar sensitivity to inhibitors. The action of ρ in release shows no selectivity with nascent T7 RNA molecules that are longer than 300 nucleotides. When the transcription complexes contain T7 RNA molecules with a length distribution from 1000 to 2500 nucleotides, all the RNA chains are released. However, when complexes are used that have T7 RNA from 50 to 500 nucleotides long, there is a significant reduction in the release of molecules shorter than 300 nucleotides. It is also shown that the released RNA molecules appear to be the same size as the corresponding RNA molecules in the complex. Since a difference as large as 12 nucleotides out of 300 could be detected in the gel system used, this result rules out any mechanism that involves release by cleavage of the nascent RNA at some point more than 12 nucleotides from the 3′ end of the RNA in the complex.

RNA polymerase from *Escherichia coli* catalyzes the polymerization of RNA from a double-helical DNA template by a processive mechanism (Krakow et al., 1976). After initiation, an RNA chain is not released from its complex with the enzyme and the DNA until its synthesis is fully terminated. Since the rate of addition of nucleotides to a nascent RNA is not uniform at all sequences in the DNA (Darlix & Fromageot, 1972; Maizels, 1973; Rosenberg et al., 1978), there will be regions where RNA polymerase will pause temporarily—even for as long as several minutes—without being released. Although the synthesis of an RNA molecule may appear to be terminated temporarily, it is not *fully* terminated until the RNA molecule is released from the transcription complex.

Release of RNA molecules, and hence full termination of RNA synthesis, can occur spontaneously at certain sites on many DNA templates in reaction mixtures containing RNA polymerase as the only enzymatic transcriptional component (Richardson, 1969; Roberts, 1976; Adhya & Gottesman, 1978). However, at other sites, termination and release depend on the presence of a protein known as ρ (Roberts, 1969, 1976; Adhya & Gottesman, 1978). From studies of the transcription of λ DNA, T7 DNA, and DNA containing the Trp attenuator gene, it has been concluded that the sites where ρ acts correspond to those regions of the DNA template where RNA polymerase pauses (Darlix & Horaist, 1975; Rosenberg et al., 1978; Fuller & Platt, 1978). This conclusion suggests the possibility that the primary function of ρ in termination could be merely to release RNA from RNA polymerase molecules that have paused during transcription (Adhya & Gottesman, 1978; Richardson, 1978; Fuller & Platt, 1978).

ρ is an RNA-dependent NTPase[1] (Lowery-Goldhammer & Richardson, 1974), and its function in termination is dependent on the presence of substrates for the ρ–NTPase (Howard & de Crombrugghe, 1976; Galluppi et al., 1976). Thus, the release of RNA in termination could be a direct consequence of the NTP hydrolysis reaction.

In this paper a membrane filter binding assay is used to measure the release of RNA from isolated transcription complexes made from the action of *E. coli* RNA polymerase on T7 DNA. The results show that ρ stimulates the release of RNA when substrates for ρ–NTPase are present. An

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[1] Abbreviations used: NTPase, nucleoside triphosphate phosphohydrolase; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); App[CH_2]p, adenyl-5′-yl methylenediphosphate; App[NH]p, adenyl-5′-yl imidodiphosphate; Ap[CH_2]pp, adenosine 5′-α,β-methylenetriphosphate; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate, also known as cordycepin 5′-triphosphate; araATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; CpA, cytidylyl(3′–5′)adenosine; EDTA, (ethylenedinitrilo)tetracetic acid; NTP, nucleoside triphosphate.

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analysis is also made of the size distribution of the released RNA to show that ρ acts with very little specificity in release of T7 RNA molecules from the isolated complexes. Virtually all RNA molecules larger than 300 nucleotides are released directly without any evidence for cleavage of a part of the RNA during release.

Materials and Methods

Materials. ATP, GTP, CTP, UTP, and ATP-γ-S were purchased from Boehringer-Mannheim Corp. [^{3}H]UTP was obtained from ICN Pharmaceuticals Inc. Heparin was from Eli Lilly. Poly(C), App[CH_2]p, App[NH]p, and Ap[CH_2]pp were purchased from Miles Laboratories. dATP, ddATP, 3′-dATP and CpA were from Sigma Chemical Co. Bio-Gel A-15m, acrylamide, and bis(acrylamide) were obtained from Bio-Rad Laboratories. Urea, ultrapure grade, was a product of Schwarz/Mann. All other chemicals were of reagent grade. *E. coli* MRE 600 cells grown to late log phase in an enriched medium were purchased from Grain Processing Corp., Muscatine, IA.

Enzymes and DNA. ρ protein was isolated from *E. coli* MRE 600 by a procedure that will be published elsewhere (L. Finger and J. P. Richardson, unpublished experiments). RNA polymerase purified from *E. coli* MRE 600 by the method of Burgess & Jendrisak (1975) was purified further on DEAE-cellulose (Chamberlin & Berg, 1962). T7 DNA was prepared by phenol extraction of purified bacteriophage T7 (Thomas & Abelson, 1966).

Preparation and Isolation of Ternary Complexes. RNA synthesis reaction mixtures used for the preparation of ternary complexes contained, in a final volume of 0.5 mL, 0.04 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 25 mM KCl, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, 0.2 mM each of ATP, GTP, and CTP, 0.02 mM [^{3}H]UTP (0.4 μCi/nmol), 18 μg of T7 DNA, and 9 μg of RNA polymerase. In order to synchronize the initiation, RNA polymerase was first preincubated with the DNA for 5 min at 37 °C in the salt solution before adding the four nucleoside triphosphates. RNA synthesis was initiated by adding the four nucleoside triphosphates and arrested 3 min later by adding 20 μL of 0.5 M EDTA (to give 2 mol of EDTA/mol of $MgCl_2$). The ternary complex was isolated by chromatography on a 1.5 × 30 cm column of Bio-Gel A-15m in a solution containing 0.04 M Tris-HCl (pH 8), 25 mM KCl, 10^{-4} M $MgCl_2$, and 10^{-4} M dithiothreitol. The concentration of the RNA after isolation was 45% its concentration in the reaction mixture. Isolated complexes, stored at 0 °C, were used within 3 h of isolation.

In order to study the release of shorter RNAs, complexes were prepared in an RNA synthesis reaction mixture altered to contain 0.1 mM CpA and 8 μM each of ATP, GTP, CTP, and [^{3}H]UTP (6 μCi/nmol).

Release Assay. The standard reaction mixture for release contained, in a final volume of 50 μL, 0.04 M Tris-HCl (pH 8), 25 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 10^{-4} M dithiothreitol, 10^{-4} M EDTA, 25 μL of isolated complex [containing ~0.4 μg of T7 DNA (0.016 pmol), 0.2 μg of RNA polymerase (0.44 pmol), and 45 ng of RNA (0.08 pmol)], and 0.25 μg of ρ (0.9 pmol of hexameric ρ). Incubations were at 37 °C for the times indicated for individual experiments. ρ-Mediated release was terminated by the addition of 0.5 mL of filtration buffer [0.5 M KCl containing poly(C) and heparin (10 μg/mL each)]. After sitting 5 min at 0 °C, the diluted solution was filtered through a 1.3-cm Millipore HA nitrocellulose membrane filter, and the filter was washed with 0.2 mL of filtration buffer. After being dried, the filter was placed in 2 mL of a toluene-based scintillation fluid and counted in a Beckman LS 230 scintillation counter. Although the method measures the fraction of RNA remaining on the filter, the data are expressed as the fraction of RNA released, which is equal to 1 minus the fraction remaining on the filter.

These assay conditions were designed to avoid interference by the binding of free RNA to ρ or RNA polymerase. It is known that in 0.5 M KCl, binary complexes between RNA and these proteins dissociate while the ternary transcription complexes are stable (Richardson, 1966). Furthermore, the poly(C) and heparin will compete with the labeled RNA in the formation of binary complexes with ρ and RNA polymerase, respectively.

The validity of this assay method has been verified by using it to measure the effect of ρ on RNA release in complete transcription mixtures containing T7 DNA, RNA polymerase, and all four ribonucleoside triphosphates. On the basis of the known properties of the RNA polymerase reaction with T7 DNA (Richardson, 1966) and the known effects of ρ on transcription (Roberts, 1969), virtually all of the RNA made after 10 min in the absence of ρ should be in ternary complexes while most of the RNA made in that same time in the presence of ρ should be released. The results obtained with this release assay were as follows: 92% of the 10-min T7 RNA was retained on filters in the absence of ρ and 10% was retained in the presence of ρ (experiment not shown).

Polyacrylamide Gel Electrophoresis of RNA. Released RNA was purified from filtrates by the following procedure. After the addition of 20 μg of tRNA, the filtrate was extracted successively with equal volumes of H_2O-saturated phenol and of a chloroform–isoamyl alcohol mixture (24:1). Two volumes of ethanol was added to the aqueous layer, and the mixture was left to sit 15 h at −20 °C to precipitate the RNA.

The precipitated phenol-purified RNA was collected by centrifugation, washed with 0.5 mL of 95% ethanol, and dissolved in 20 μL of electrophoresis buffer (90 mM Tris, 90 mM boric acid, 4 mM Na_3EDTA, and 1% sodium dodecyl sulfate) containing 20% sucrose, 0.01% bromophenol blue, 0.01% xylene cyanol, and 0.01% methyl orange. Individual samples were applied to slots at the top of a 14 cm wide, 0.15 cm thick, 18 cm high verticle slab gel made with a 7.5–3% linear gradient of polymerized acrylamide [ratio of acrylamide to bis(acrylamide) was 30:0.8] in electrophoresis buffer containing 7 M urea. Electrophoresis was for 4 h at 100 V. For the location of the ^{3}H-labeled RNA after electrophoresis, the gel was impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974), dried, and exposed to X-ray film.

Results

ρ Facilitates the Release of RNA from Isolated Transcription Complexes. Ternary transcription complexes containing *E. coli* RNA polymerase, T7 DNA, and nascent T7 RNA molecules can be isolated from complete transcription mixtures by chromatography on Bio-Gel A-15m. When the complexes have been prepared by incubation of the transcription mixture for 3 min, the nascent T7 RNA molecules range in size from 1000 to 2500 nucleotides with an average of 1800 nucleotides. About 90% of the RNA isolated by that procedure is retained on the filters, when filtered by the standard procedure. When these complexes are incubated at 37 °C with 0.25 μg of ρ and ATP, the RNA is rapidly and quantitatively released from the transcription complexes (Figure 1). Although a small fraction (from 10 to 20%, depending on the preparation of complexes used) of the RNA is released rapidly in the absence of ρ, the majority of the RNA molecules remain stably bound to the complexes. The effect of ρ is also dependent upon the incubation temperature; when

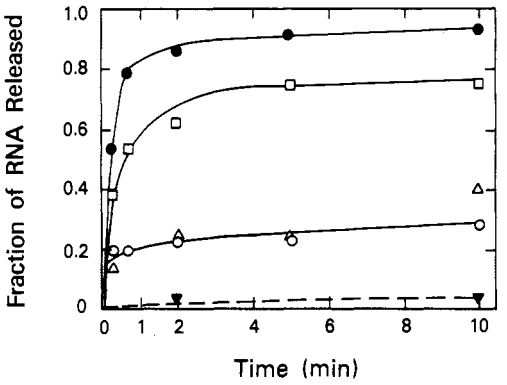

FIGURE 1: Kinetics of RNA release. The fraction of RNA released was measured at the indicated times in standard assay solutions containing the following: (●) ATP and 0.25 μg of ρ; (□) ATP and 0.12 μg of ρ; (○) 0.25 μg of ρ and *no* ATP; (△) no ρ; (▼) ATP and 0.25 μg of ρ at 0 °C. Initially, the complexes contained 5400 cpm of [^{3}H]T7 RNA.

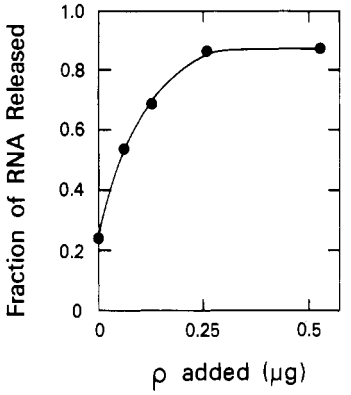

FIGURE 2: Dependence of release of the concentration of ρ protein. The fractions of RNA release was measured in standard assays and incubated for 2 min with the indicated concentrations of ρ protein. Initially, the complexes contained 7400 cpm of [^{3}H]T7 RNA.

left at 0 °C less than 3% of the RNA is released by 10 min. This result suggests that the mere presence of ρ in the release mixture is not sufficient to cause release.

Measurements of the extent of release after 2 min as a function of ρ (Figure 2) indicate that the minimum amount of ρ needed to release all the RNA in this assay is 0.25 μg, which is equivalent to 10 ρ hexamers/nascent RNA or 2/RNA polymerase molecule. The kinetics of release measured with half that amount of ρ (Figure 1) suggest that ρ is functioning stoichiometrically and not catalytically; with 0.12 μg of ρ/assay, the initial rate of release is rapid but the reaction stops with only 80% release. Since the saturation of the ρ effect occurs when the number of ρ molecules is about equivalent to the number of RNA polymerase molecules but is in excess of RNA molecules (only 20% of the RNA polymerase molecules were active in RNA synthesis in the preparation used), this result raises the possibility that ρ functions in release as a one-to-one complex with RNA polymerase.

ATP Requirement. Figure 1 shows that the effect of ρ on release depends on the presence of ATP; the release in the mixture containing ρ and no ATP is the same as in the mixture without ρ. This requirement for ATP suggests that the release reaction is related to the RNA-dependent NTPase activity of ρ. The requirement for NTP is satisfied by low concentrations of ATP (Figure 3); half maximum release is achieved with an ATP concentration of 20 μM. Since the K_m for ATP in the ρ–NTPase reaction is 9 μM (Lowery & Richardson, 1977), the two reactions show a similar dependence on ATP concentration.

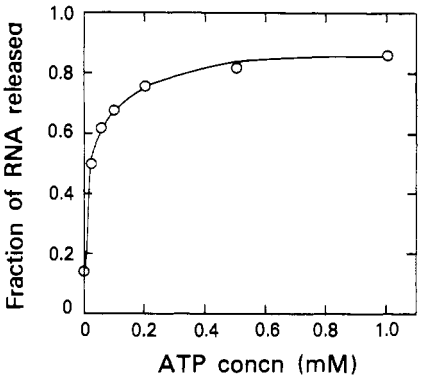

FIGURE 3: Dependence of release on the concentration of ATP. Standard assay solutions containing 0.25 μg of ρ protein and the indicated amounts of ATP were incubated for 2 min. Initially, the complexes contained 6900 cpm of [^{3}H]T7 RNA.

Table I: Effect of Nucleotides on Release of RNA[a]

nucleotide	fraction of RNA released	nucleotide	fraction of RNA released
none	0.14	araATP	0.89
ATP	0.86	ddATP	0.14
GTP	0.82	Ap[CH_2]pp	0.13
UTP	0.86	App[CH_2]p	0.17
CTP	0.84	App[NH]p	0.17
2′-dATP	0.84	ATP-γ-S	0.17
3′-dATP	0.72	ADP	0.15

[a] Standard assay solutions containing the indicated nucleotides at 1 mM and 0.25 μg of ρ were incubated for 2 min. Initially, the complexes contained 6900 cpm of [^{3}H]T7 RNA.

The two reactions also have identical nucleotide substrate specificities. RNA is released readily when ATP is replaced by any one of the other three ribonucleoside triphosphates or by 2′-dATP, 3′-dATP, or araATP (Table I). All of these nucleotides are good substrates for ρ–NTPase. However, no ρ-promoted release is found when ATP is replaced by ddATP, Ap[CH_2]pp, App[CH_2]p, App[NH]p, ATP-γ-S, or ADP. None of these ATP analogues has any appreciable activity in the ρ–NTPase reaction. The lack of activity with the β,γ-methylene and imido analogues has already been reported (Galluppi et al., 1976). Tests of NTPase activity with ddATP, with Ap[CH_2]pp using a phosphate release assay, or with ATP-γ-S by assaying for the formed ADP indicate that the rates of cleavage of these analogues by ρ action are less than 0.01 times the rate of cleavage of ATP. Since some of these nonhydrolyzed analogues of ATP can often replace ATP in reactions that require merely the binding of the nucleotide (Yount, 1975), the finding that ρ-dependent release occurs only when a nucleotide is present that can be hydrolyzed by ρ leads us to conclude that release is dependent on the hydrolysis of nucleoside triphosphates. Although many of the nucleotides that satisfy the requirement for the release activity could be used as substrates for RNA polymerase, some, such as 2′-dATP, are very poor substrates for RNA polymerase [in the absence of $MnCl_2$; see Hurwitz et al. (1972)]; thus, it is unlikely that the release reaction is dependent upon further addition of nucleotides to the nascent RNA chain.

Influence of Mg^{2+} Ions on Release. The ability of ρ plus ATP to stimulate release of RNA is dependent on the presence of Mg^{2+} ions at low levels. Although the intrinsic rate of release of RNA in the absence of ATP (or in the absence of ρ) is highest with no $MgCl_2$, the ρ–ATP combination does not stimulate release unless $MgCl_2$ is present (Figure 4). The addition of low levels of $MgCl_2$ increases dramatically the extent of release by the ρ–ATP combination while it decreases

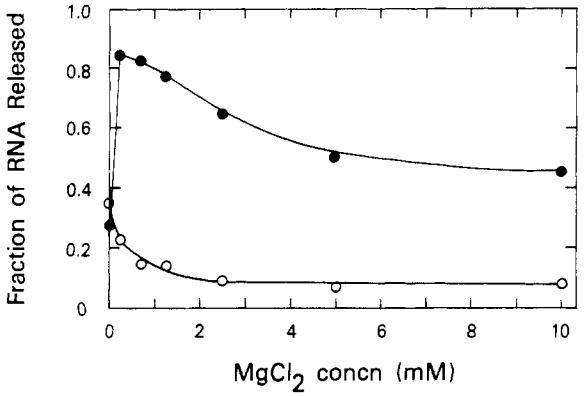

FIGURE 4: Effect of $MgCl_2$ concentration on RNA release. Standard assay solutions containing 0.25 μg of ρ and ATP (●) or 0.25 μg of ρ and *no* ATP (○) and the indicated amounts of $MgCl_2$ were incubated for 2 min. The fraction of RNA released in the absence of ρ in a solution containing no $MgCl_2$ was 0.3. Initially, the complexes contained 7300 cpm of [^{3}H]T7 RNA.

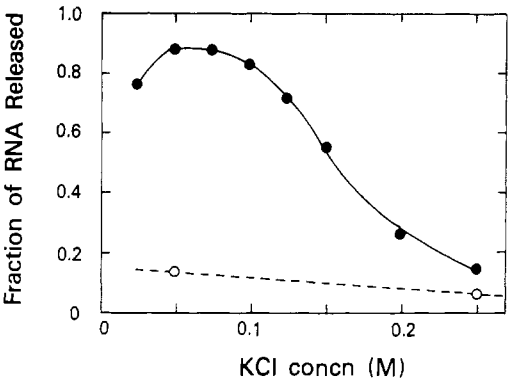

FIGURE 5: Effect of KCl concentration on RNA release. Standard assay solutions containing 0.25 μg of ρ and ATP (●) or 0.25 μg of ρ and no ATP (○) and the indicated amount of KCl were incubated for 2 min. Initially, the complexes contained 6200 cpm of [^{3}H]T7 RNA.

the dissociation of RNA with ρ in the absence of ATP or in the absence of ρ (not shown). The optimum $MgCl_2$ concentration for ρ-mediated release is in the range of 1 mM, and higher concentrations inhibit the ρ-mediated release reaction.

These effects appear to reflect the requirement and influence of $MgCl_2$ on the ATP hydrolysis reaction. With isolated T7 RNA, that reaction is optimum in 0.6 mM $MgCl_2$ (with 1 mM ATP) and is inhibited by higher concentrations of $MgCl_2$ (J. P. Richardson and M. R. Macy, unpublished experiments). However, the two reactions do differ in the degree of the inhibition. When measured in 10 mM $MgCl_2$, the extent of release after 2 min is 50% the extent measured in 0.3 mM $MgCl_2$, while the rate of ATP hydrolysis with isolated RNA is 2% the rate measured in 0.6 mM $MgCl_2$. This lack of a perfect quantitative correlation between release and ρ–NTPase activity does not necessarily imply that release is independent of ATP hydrolysis because the release assay measures the consequence of a single event, whereas the assay for ATP hydrolysis measures a steady-state reaction. The amount of ATP hydrolysis required to release a single RNA may be quite small yet hydrolysis will continue with free RNA long after release has occurred.

Inhibitors of Release. If the ρ-mediated release reaction is coupled to the RNA-dependent NTPase activity of ρ, the release activity should be inhibited by compounds or by conditions that prevent ρ from binding to the RNA.

A specific condition that is known to affect greatly the binding of ρ to RNA is the ionic strength; the binding of ρ to T7 RNA is inhibited in 0.25 M KCl (unpublished observation), and this loss of binding is reflected in the NTPase and termination activities of ρ (Galluppi et al., 1976). The results presented in Figure 5 show that this sensitivity to salt concentration affects the release activity as well; the extent of release is maximum from 0.05 to 0.075 M KCl and decreases to near zero as the KCl concentration is increased to 0.25 M.

The binding of ρ to the RNA to be released can also be inhibited by the presence of a competing RNA or a polyanion such as heparin. ρ binds very tightly to poly(C) (Galluppi & Richardson, 1980) and the interaction is strong enough for poly(C) to act as a potent inhibitor of the ρ function in transcription termination (Bektesh, 1979). The polyanion, heparin, which is a potent competitive inhibitor of ρ–NTPase with isolated T7 RNA [although not with poly(C)], also inhibits the ρ function in termination (Küpper et al., 1978). When present at 10 μg/mL, both of these substances prevent the stimulation of RNA release by ρ (data not shown). This action is one reason for their inclusion in the filtration buffer for the release assay.

The good correlation between the effect of salt and competing polyanions on the interaction between ρ and RNA on the release activity of ρ is thus further evidence that the release is a consequence of a direct interaction between ρ and the nascent RNA. Since poly(C) is a very effective activator of ρ–NTPase, the fact that nascent RNA molecules are not released in its presence indicates further that ρ-catalyzed hydrolysis in the same reaction mixture is not a sufficient condition for release. Rather, the requirement for NTP hydrolysis is that it be coupled to an action of ρ on the RNA to be released.

Selectivity of the Release Reaction and the Nature of the RNA Product. The results of Figure 1 showed that ρ can facilitate the release of all RNA molecules in isolated transcription complexes containing nascent RNA molecules with an average size of 1800 nucleotides. Although this suggests that there may be little or no selectivity in the release reaction, the following experiments indicate that the RNA has to be larger than a certain size to be released. When complexes are prepared by short incubation with low concentration of nucleoside triphosphates, the RNAs in the complexes are not released as readily as the RNAs from the complexes made under the standard conditions. By 10 min only 25, 34, and 57% of the RNA are released from complexes prepared by 1.5, 3, and 4.5 min of synthesis, respectively (Figure 6). Under the conditions of synthesis used, the average chain growth rate is ~100 nucleotides/min. Thus, as the average size of the RNA molecule increases, the fraction of molecules that can be released by ρ action increases.

An analysis of the size of the nascent T7 RNA molecules in the complexes isolated after 4.5 min of synthesis shows a broad distribution of chain lengths from 200 to 550 nucleotides (Figure 7). The fact that several distinct bands are resolved is consistent with other published evidence (Darlix & Fromageot, 1972) that the rate of addition of nucleotides on the nascent RNA during its polymerization is not uniform for all sequences of T7 DNA. When this RNA is compared with the RNA that has been released by ρ action, it is evident that the efficiency of release changes significantly at a chain length of ~300 nucleotides. Estimations of the fractions released from integration of densitometer scans of the radiofluorographs indicate that for chains shorter than 300 nucleotides 35% is released while for the RNA molecules longer than 300 nucleotides 70% is released. The results in Figure 7 also confirm that the effect of ρ on release depends on ATP and that there

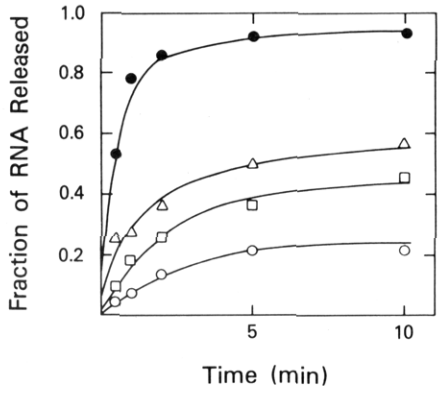

FIGURE 6: Kinetics of RNA release from complexes containing RNA molecules shorter than ρ-terminated T7 RNA. Release was measured as a function of time in standard reaction solutions containing 0.25 μg of ρ and complexes prepared with low concentrations (8 μM) of nucleoside triphosphates and incubated for 1.5 min [(○) initially 650 cpm of [^{3}H]T7 RNA], 3.0 min [(□) initially 1600 cpm of [^{3}H]T7 RNA], and 4.5 min [(△) initially 2670 cpm of [^{3}H]T7 RNA]. The kinetics of release from complexes prepared by synthesis for 3 min with the standard transcription mixture are also shown [(●) initially 5400 cpm of [^{3}H]T7 RNA]. In all cases, the extent of release from the complexes with the shorter RNA was less than 10% when incubated for 10 min in solution containing 0.25 μg of ρ and no ATP.

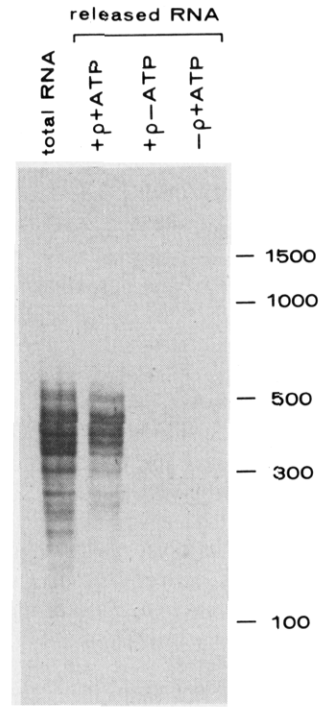

FIGURE 7: Radiofluorograph of total and released [^{3}H]T7 RNA after electrophoresis on a polyacrylamide gel. Complexes containing short nascent T7 RNA chains were prepared by incubating reaction mixtures containing CpA and low concentrations (8 μM) of the nucleoside triphosphates for 4.5 min. Complete release mixtures contained 30000 cpm of [^{3}H]T7 RNA in 125 μL of complex, 1.25 μg of ρ, and 1 mM ATP in a total volume of 250 μL. After 5 min at 37 °C, 1 mL of a solution containing 0.5 M KCl and 10 μg of poly(C)/mL was added, and the mixture was left at 0 °C for 5 min before filtration through a Millipore filter. The released RNA in the filtrate was isolated by phenol extraction as described under Materials and Methods. The gel shows samples of RNA released in a complete reaction mixture (63% released), in a reaction mixture without ρ (6% released), and in a reaction mixture without ATP (9% released). Total RNA was isolated from 125 μL of unfiltered complexes by treatment with 0.4% sodium dodecyl sulfate and extraction with phenol. The gel was exposed for 1 day. The numbers on the right-hand side indicate the chain lengths of the RNA molecules.

is no significant release of RNA when ATP is added without ρ.

A comparison of the bands from the released RNA with those from the RNA molecules in the complex that are larger than 300 nucleotides (Figure 7) shows an excellent correspondence with respect to the sizes and relative amounts of individual species of RNA. Thus, there does not appear to be any discrimination made in the release of RNA chains larger than 300 nucleotides, and the RNA chains appear to be released intact. With this gel system, a difference in size of 12 nucleotides for the RNA molecules ~300 nucleotides long would cause a detectable difference in the mobility of the RNA. Thus, if release occurs by a cleavage of the nascent RNA near the site of attachment, the cleavage would have to be within 12 nucleotides of the 3′ end, which would be in the domain of the RNA bound to the RNA product site of RNA polymerase as defined by pancreatic ribonuclease digestion experiments (Kumar & Krakow, 1975; Rohrer & Zillig, 1977). Although ρ could cleave within that region, we favor the interpretation that ρ causes release through complete dissociation of the intact RNA.

Discussion

We have shown that ρ protein facilitates the dissociation of T7 RNA molecules from isolated complexes containing nascent RNA bound to RNA polymerase and T7 DNA and that this activity depends on the ATP hydrolysis activity of ρ. A similar effect of ρ and ATP on the release of RNA has also been demonstrated recently by using complexes with λ DNA and a gel electrophoresis separation technique to distinguish the released RNA (Galluppi & Conaway, 1979). We have also used the gel electrophoresis assay procedure to measure release of the RNA from complexes with T7 DNA, and the results obtained were in good qualitative agreement with those obtained with the filter binding assay (unpublished observations).

Several of the characteristics of the release reaction indicate that the activity being measured with isolated complexes is representative of a functioning of ρ in transcription termination. First, the conditions that allow release correlate well with conditions that allow termination. Both reactions are favored at low salt concentrations (~25–50 mM KCl) and are fully inhibited when the KCl concentration is above 0.25 M. Second, neither reaction occurs when the nucleoside triphosphates are replaced by nonhydrolyzable analogues. Third, both reactions are completely inhibited by low (10 μg/mL) concentrations of heparin or poly(C). Fourth, the release reaction appears to be adequately rapid; with a saturating amount of ρ, most RNA molecules are released within a minute, which is a time scale that could be sufficient for a termination function. Fifth, the amounts of ρ required for release correspond roughly to the amounts of ρ required for termination.

When assayed for termination activity with the amounts of DNA and RNA polymerase that are used in the release assays, termination activity is saturated with 0.05 μg of ρ (results not shown). The data in Figures 1 and 2 indicate that it takes 5 times as much ρ to saturate the release assay. It is not clear why the correspondence is not better than this. The fact that it takes about as many ρ molecules as RNA polymerase to saturate the release reaction while there is a 10-fold excess of ρ over RNA molecules suggests that the function of ρ could be controlled by an interaction with RNA polymerase and that this interaction can occur regardless of whether the RNA polymerase has been active in making an RNA molecule. However, in the termination assay, the reaction is saturated when there is roughly one ρ molecule/RNA molecule. The

significance of the difference in sensitivity of the two assays will require further analysis. It may reflect a fundamental difference in mechanism or an abnormality of the release assay. Perhaps higher amounts of ρ are needed to release RNA from RNA polymerase molecules that are not at normal ρ termination sites. Alternatively, the structure of the complexes could have been altered sufficiently during the isolation to make them less sensitive to ρ action than the complexes that have not been isolated.

The conclusion that the release activity of ρ depends on the NTP hydrolysis reaction is based on the finding that the NTP requirement for release cannot be satisfied by ATP analogues that are not substrates for ρ–NTPase whereas the requirement is satisfied by other nucleoside triphosphates that are substrates for ρ–NTPase. The conclusion is also supported by the correlation in the requirements and influence of $MgCl_2$ on the release and the ATP hydrolysis reactions. Furthermore, the fact that poly(C), which is a very potent activator of ρ–NTPase, inhibits ρ action in RNA release suggests that release is a consequence of NTP hydrolysis activated by the RNA molecule being released. Since chemical or mechanical energy can be derived from the hydrolysis of ATP to ADP and P_i, perhaps this reaction is being used to drive the dissociation of the RNA. A nascent RNA molecule is bound very tightly to RNA polymerase and DNA during transcription. This is indicated from the fact that the isolated complex remains intact over a period of several hours in the absence of ρ (Richardson, 1966; Naito & Ishihama, 1975) and from the evidence that there is no exchange of nascent RNA molecules between DNA molecules during polymerization (Bremer & Konrad, 1964). Hence, the expenditure of chemical energy may be needed to overcome the strong binding energy that holds the RNA to RNA polymerase and DNA.

Mechanistically, the function of NTP hydrolysis could be to allow ρ to pry the nascent RNA away from the transcription complex. ρ is able to bind firmly to RNA (Richardson, 1970), and studies of the effects of ATP on the properties of the RNA in ρ–RNA complexes suggest that ATP hydrolysis may cause the RNA to become wrapped around ρ (Galluppi & Richardson, 1980). If ρ could bind to the nascent T7 RNA at a specific site (Bektesh & Richardson, 1980) and use the ATP hydrolysis reaction to activate the other interactions that would lead to the wrapping of the RNA, these interactions would bring ρ into contact with the RNA polymerase. Such a contact could act as the point of leverage for ρ to pry the nascent RNA away through a continued action of the forces causing the wrapping. Another possible mechanism is that the contact between ρ and RNA polymerase caused by a wrapping of the RNA around ρ could trigger a conformational change in the RNA polymerase that would result in the release of the RNA. This variation of the model would fit well with the genetic evidence that a specific ρ–RNA polymerase interaction is crucial to the termination process (Das et al., 1978; Guarente, 1979).

Whatever the actual mechanism, ρ appears to act indiscriminately to release T7 RNA molecules so long as the RNA is a certain size. The size requirement could reflect the necessity for ρ to bind firmly to some site on the RNA before it can effect release of the RNA. Either the T7 RNA has to be at least 300 nucleotides long for ρ to make this contact or a specific binding site is not present in the T7 RNA chains until they are that long. Since ρ binds to a specific site on λ cro RNA (Bektesh & Richardson, 1980), we favor the interpretation that the T7 RNAs shorter than 300 nucleotides do not contain a site for the initial interaction with ρ.

If ρ is functioning primarily as an RNA release factor, how can we reconcile the fact that ρ-mediated termination of transcription yields specific RNA molecules with the fact that it shows so little discrimination in the release reaction? We believe that much of the specificity in termination is a consequence of the nonuniform rate of transcription. Certainly some of the specificity depends on the ability of ρ to interact with the nascent RNA. ρ will not act on an RNA that does not have a binding site nor will it act before a site has been formed. Furthermore, in the cell, ρ would not be able to act if the site is covered or blocked by some other component such as a ribosome. However, once ρ can bind to a nascent RNA, that RNA could be released as soon as ρ catches the RNA polymerase, and that is more likely to occur at a given site where the rate of transcription is slow than at a site where the rate of transcription is fast. Thus, much of the specificity of ρ-mediated termination would be determined by the interactions among RNA polymerase, the nascent RNA, and the DNA template that govern the rate of nucleotide addition.

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Mechanism of Action of Polymeric Aurintricarboxylic Acid, a Potent Inhibitor of Protein–Nucleic Acid Interactions†

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ABSTRACT: The mechanism of inhibition of protein–nucleic acid complex formation by polymeric aurintricarboxylic acid (ATA) was investigated by proton magnetic resonance spectroscopy. The approach was the synthesis of totally deuterated ATA, followed by a 100-MHz proton magnetic resonance study of its interaction with bovine pancreatic ribonuclease A (RNase), a model nucleic acid binding protein. The binding of ATA to RNase elicited chemical shift changes and line broadening in the C(2)–H resonances of histidyl residues 12 and 119, both of which are located in the active site, whereas that of histidyl residue 105, which resides on the exterior of the protein structure, is unaffected. (Histidyl residue 48 is not observed under our conditions except at high pH.) The ϵ-methylene protons of the lysyl side chains were also broadened upon the binding of ATA. Polymeric ATA displaces cytidine 2′-monophosphate and cytidine 3′-monophosphate from the active site of the enzyme as revealed by nuclear magnetic resonance spectroscopy. These observations suggest that the mechanism of action of ATA involves competition between the nucleic acid and the polymeric ATA for binding in the active site of the protein. Electron spin resonance spectroscopy reveals that polymeric ATA is a stable free radical, thus accounting for the major line broadening effect upon binding to protein. This finding may provide a powerful means of probing the nucleic acid binding site of proteins by proton magnetic resonance spectroscopy.

Aurintricarboxylic acid (ATA)[1] has been extensively utilized by molecular biologists as a powerful inhibitor of proteins whose biological function depends on the formation of a complex with nucleic acid. Numerous studies have employed ATA in the investigation of both prokaryotic and eukaryotic systems [for reviews, see Apirion & Dohner (1975), Grollman & Huang (1976), and Schleich et al. (1978)]. Interpretation of the mechanism of inhibitory activity of ATA has been difficult because commercially available preparations of this reagent contain numerous components, not all of which possess inhibitory activity (Huang & Grollman, 1972; Givens & Manly, 1976; Steward et al., 1977; Blumenthal & Landers, 1973; Tsutsui et al., 1978). Recent work in our laboratory revealed that the active components present in ATA preparations are a collection of heterogeneous polymers of the phenol–formaldehyde type (Schleich et al., 1978; González et al., 1979).

The objective of the present study was the elucidation of the mechanism by which ATA prevents the formation of a protein–nucleic acid complex. Our approach was the synthesis of totally deuterated ATA, followed by a proton magnetic resonance study of its interaction with ribonuclease A (RNase A), a model nucleic acid binding protein (Jensen & von Hippel, 1976, and references cited therein). RNase A was chosen because of its well-known ability to bind both RNA and DNA, because of its ready availability, and because this protein has been extremely well characterized by a variety of physical techniques including ^{1}H nuclear magnetic resonance (NMR) spectroscopy [for reviews, see Roberts & Jardetzky (1970) and Benz & Roberts (1973)].

Materials and Methods

Bovine pancreatic RNase, type XII-A, the free acids of cytidine 2′-monophosphate (2′-CMP), and cytidine 3′-monophosphate (3′-CMP) were obtained from Sigma Chemical Co. "Aluminon" grade ATA was purchased from Aldrich (lot 061757). Nitrobenzene-d_5 (99 atom % ^{2}H), paraformaldehyde-d_2 (98 atom % ^{2}H), methanol-d_4 (99 atom % ^{2}H), and sulfuric acid-d_2 (99 atom % ^{2}H) were obtained from Merck Sharpe & Dohme Canada Ltd.

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[1] Abbreviations used: ATA, aurintricarboxylic acid; 2′-CMP, cytidine 2′-monophosphate; 3′-CMP, cytidine 3′-monophosphate; RNase, bovine pancreatic ribonuclease A (EC 3.1.4.22); NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; HMDS, hexamethyldisiloxane; ppm, parts per million.

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