Characterization of Ribonuclease H Activity Associated with Yeast RNA Polymerase A

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The ribonuclease H activity associated with yeast RNA polymerase A degrades a variety of RNA-DNA hybrids with apparently no base specificity. The nuclease activity was characterized using (rA),- (dT), and (rG),- (dC), hybrids. It shows an absolute requirement for a divalent cation, Mg²⁺ or Mn²⁺. The pH curve is bimodal, with optima at pH 6.5 and 8. The optimal temperature depends strongly on the nature of the divalent cation. The activity is inhibited by low salt concentrations, EDTA, N-ethylmaleimide, and (rI). The nuclease activity is also drastically reduced under conditions in which polymerization can proceed, even with limiting concentrations of ribonucleoside triphosphates.

RNA polymerase A executes an exonucleolytic attack on the hybridized RNA, producing a mixture of a mononucleotide 5'-phosphate and of a dinucleotide with a 5'-phosphate end. The pattern of degradation products varies strongly with the incubation conditions. Depending on pH and divalent cation used, the dinucleotide/mononucleotide ratio can vary by a factor of 20 or more. During the course of these experiments it was found that Mn²⁺ ions, in absence of enzyme, catalyze the hydrolysis of (rA), to a mixture of acid-soluble oligonucleotides of decreasing length, terminated with a 3'-phosphate group.

After mild dissociation of RNA polymerase A with urea, the RNase H activity was recovered associated with the fractions containing both dissociated polypeptides, A₈₀ and A₅₄₅ subunits, and with the RNA polymerase A* which is lacking these subunits. The RNase H activity again co-migrated with A* enzyme upon polyacrylamide gel electrophoresis. Enzyme A* produced a mixture of dinucleotides and mononucleotides like Enzyme A, although in a significantly different ratio; and this ratio varied with the incubation conditions. The possibility of two distinct RNase H activities associated with yeast RNA polymerase A is discussed.

EXPERIMENTAL PROCEDURES

Materials

Enzymes - Yeast RNA polymerase A was prepared as previously described (7). The most purified fraction (phosphocellulose fraction) was used throughout this study. Homogeneous yeast RNA polymerase B (8) and Escherichia coli RNA polymerase (9) were prepared as previously described.

Nucleic Acids and Nucleotides - Synthetic polymers (rI), (dT)ₙ, and (dC)ₙ were obtained from GBICO (New York). [³H]rAₙ was purchased from Miles Laboratories. Phage T₇ [³H]DNA was kindly provided by A. Ruet and M. T. Sarroche (Saclay). [³H]rAₙ· (dT)ₙ hybrid was made by mixing equal amounts of [³H]rAₙ and (dT)ₙ strands in 0.1 M NaCl and 0.03 M Tris/HCl buffer, pH 7.8, and incubating at 37°C for 30 min as indicated by Riley et al. (10). ¹⁴C- or ³²P-labeled (rA)ₙ· (dT)ₙ and [³²P]rGₙ· (dC)ₙ were obtained from Amersham; the ³H- and ¹⁴C-labeled nucleoside triphosphates were obtained from P-L Biochemicals except for pAp, which was kindly synthesized for us by M. Blandin (Saclay). The ³²P-labeled nucleoside triphosphates were purchased from Amersham; the ³H- and ¹⁴C-labeled nucleoside triphosphates were obtained from C. E. N. Saclay. The melting curve of (rA)ₙ· (dT)ₙ hybrid was carried out in a Cary 15 spectrophotometer equipped with a thermostatically controlled cell-housing.

Methods

RNase H and RNA Polymerase Assays - The standard RNase H assay mixture contained, in 0.1 ml, [³H]rAₙ· (dT)ₙ (150 pmol of
Some Properties of RNA Polymerase-associated RNase H Activity

The properties reported below are those of RNase H activity associated with RNA polymerase A, purified as previously described (Ref. 7; phosphocellulose fraction, Peak 2, corresponding to the complete enzyme).

Degradation of Various Labeled RNA-DNA Hybrids—In a previous publication it was shown that RNA polymerase A contains a nuclease activity degrading the (rA)₆₀ moiety of the hybrid homopolymer duplex (rA₆₀ - (dT)₆₀). The kinetics of degradation of this hybrid is shown in Fig. 1 at pH 6.6 or 8. RNase H activity at pH 6.5 was about 10-fold higher than that at pH 8. The latter case, the labeled DNA strand was found to remain intact. The RNase H activity could be fulfilled either by Mg²⁺ (5 mM) or Mn²⁺ (1 mM). The concentration response curves were essentially the same at the two pH optima, 6.5 or 8 (Fig. 3). In contrast with these results, which were obtained with (rA)₆₀ - (dT)₆₀ as substrate, the hybrid (rG)₆₀ - (dC)₆₀ was degraded well only in the presence of Mn²⁺ ions, at a rather sharp optimal concentration around 1 mM. In the presence of Mg²⁺ (5 mM) the degradation rate of this synthetic hybrid was about 10-fold lower (result not shown).

Effect of pH—The RNase H activity was measured with (rA)₆₀ - (dT)₆₀ as substrate at different pH values, in Tris/maleate (pH 5 to 7) and Tris/HCl (above pH 7). The activity occurred within a very broad range of pH. Two pH optima were clearly found, near pH 6.5 and pH 8 (Fig. 2). As already shown in Fig. 1, the RNase H activity at pH 6.5 was about 50% lower than at pH 8. We checked that the single-stranded homopolymer (rA), resisted degradation under all these conditions (Fig. 2). Similar results were obtained with 30 mM MgCl₂ and 10 mM Tris buffer to minimize the variation in ionic strength due to the buffer.

Effect of Ions—The divalent cation requirement for the RNase H activity could be fulfilled either by Mg²⁺ (5 mM) or Mn²⁺ (1 mM). The concentration response curves were essentially the same at the two pH optima, 6.5 or 8 (Fig. 3). In contrast with these results, which were obtained with (rA)₆₀ - (dT)₆₀ as substrate, the hybrid (rG)₆₀ - (dC)₆₀ was degraded well only in the presence of Mn²⁺ ions, at a rather sharp optimal concentration around 1 mM. In the presence of Mg²⁺ (5 mM) the degradation rate of this synthetic hybrid was about 10-fold lower (result not shown).

The effect of a range of concentrations of monovalent cations, K⁺ or NH₄⁺ (in the presence of 5 mM Mg²⁺)
Inhibition - Concentrations of EDTA capable of binding all of the divergent cations present inhibited the activity completely. RNase H was also very sensitive to moderate ionic strength levels, as shown above (Fig. 3). Addition of a 4-fold excess of (rA)_n strands over (dT)_n reduced the activity 70%. A 4-fold excess of (dT)_n strand over (rA)_n inhibited the rate of reaction 85%. This inhibition was likely due to unproductive binding of the enzyme to the single-stranded polymer. Addition of a 10-fold excess of (rI), during the course of the reaction immediately stopped hydrolysis of the (rA)_n · (dT)_n hybrid. This supports a nonprocessive mechanism of degradation. Nuclease activity was also completely inhibited by the -SH reagent N-ethylmaleimide at 10^{-3} M. The exact sensitivity of the enzyme to the inhibitor was not determined, because the storage buffer for the enzyme contained an -SH compound. The inhibitory effect of ribonucleoside triphosphates, which is related to the tight association of the nuclease activity with RNA polymerase, is described separately below.

Inhibitory Effect of Ribonucleoside Triphosphates - We previously reported that addition of ATP prevented the degradation of the (rA)_n moiety of the (rA)_n · (dT)_n hybrid, whereas CTP, UTP, or GTP were ineffective (6). This observation was investigated in more detail, using two different hybrids, (rA)_n · (dT)_n or (rG)_n · (dC)_n, and the corresponding complementary ribonucleoside triphosphates, ATP or GTP, at varying concentrations. In the experiment reported in Fig. 5, both the polymerization and the degradation reaction caused by RNA polymerase occurred simultaneously. As shown in Fig. 5 the presence of relatively low concentrations of ATP, in the range of 5\cdot 10^{-5} to 10^{-3} M, drastically reduced the rate of hydrolysis of hybridized (rA)_n strands. In this range of ATP concentration, the rate of the (dT)_n-directed polymerization reaction remained very low. The rate of polymerization increased steeply at high concentrations of ATP, up to 10^{-3} M, but this was not followed by a further reduction in the degradation reaction which occurred at a significant background level. Basically, the same observations were made with the (rG)_n · (dC)_n polymer (Fig. 5), showing again that the polymerization and the degradation reaction were independent of each other.

Inhibition of the enzyme for the hybrid structure (6), we investigated the effect of temperature on synthesis of hybridic having very different thermal stability. Hydrolysis of (rG)_n · (dC)_n, in the presence of Mn^{2+} ions, was optimal at 50°C (Fig. 4). With (rA)_n · (dT)_n, the optimum temperature was dependent upon the nature of the divergent cation. With Mg^{2+}, the rate of degradation was optimal at 37°C, whereas with Mn^{2+} ions the rate of reaction increased up to 50°C and was still quite high at 60°C. This was surprising, since under these precise ionic conditions (with 1 mM Mn^{2+}) the melting temperature of (rA)_n · (dT)_n was 53°C (see Fig. 4); (with 5 mM Mg^{2+} the melting temperature of this polymer was 63°C which is considerably higher). The explanation for this unexpected finding is that, above 50°C, Mn^{2+} ions catalyze an extensive degradation of (rA)_n polymer to acid-soluble products (Fig. 4). No such degradation of (rG)_n was observed up to 60°C nor was any degradation of (rA)_n seen with Mg^{2+} ions. Similar observations had been previously reported (13).

Inhibitory Effect of Ribonucleoside Triphosphates - We previously reported that addition of ATP prevented the degradation of the (rA)_n moiety of the (rA)_n · (dT)_n hybrid, whereas CTP, UTP, or GTP were ineffective (6). This observation was investigated in more detail, using two different hybrids, (rA)_n · (dT)_n or (rG)_n · (dC)_n, and the corresponding complementary ribonucleoside triphosphates, ATP or GTP, at varying concentrations. In the experiment reported in Fig. 5, both the polymerization and the degradation reaction caused by RNA polymerase occurred simultaneously. As shown in Fig. 5 the presence of relatively low concentrations of ATP, in the range of 5\cdot 10^{-5} to 10^{-3} M, drastically reduced the rate of hydrolysis of hybridized (rA)_n strands. In this range of ATP concentration, the rate of the (dT)_n-directed polymerization reaction remained very low. The rate of polymerization increased steeply at high concentrations of ATP, up to 10^{-3} M, but this was not followed by a further reduction in the degradation reaction which occurred at a significant background level. Basically, the same observations were made with the (rG)_n · (dC)_n polymer (Fig. 5), showing again that the polymerization and degradation reaction were independent of each other.
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FIG. 5. Inhibition of RNase H activity by complementary ribonucleoside triphosphates. Upper panel, the mixtures used for the assay of the polymerase and RNase H reactions contained (in 0.1 ml) 0.07 M Tris-HCl, pH 8.1, 1 mM dithiothreitol, 5 mM MgCl₂, [3²P](rA), -(dT), (60 pmol of polymerized [³²P]AMP, 400 cpm/pmol, and 125 pmol of dTMP), 3 µg of RNA polymerase A, and increasing concentrations of ATP, as indicated. Duplicate assays were run, one with unlabelled ATP to monitor the ³²P-labeled acid-soluble material released by RNase H (O--O), the other with [³H]ATP to measure (rA), -(dT), synthesis by a conventional acid precipitation technique (■-■). Lower panel, basically the same experiment was carried out, except that [³²P](rG), -(dC), was used as template-substrate (60 pmol of polymerized [³²P]GMP, 805 cpm/pmol and 325 pmol of dCMP), Mg²⁺ ions were replaced by 2.5 mM MnCl₂ and increasing concentration of GTP were added instead of ATP. In all cases, incubation was for 40 min at 37°C. Degradation of [³²P](rG), -(dC), in the presence of unlabeled GTP (△-△). Synthesis of [³H](rG), in the presence of [³H]GTP (■-■).

FIG. 6. Analysis of (rG), -(dC), degradation by thin layer chromatography. Top, incubation mixture (0.5 ml) contained [³²P](rG), -(dC), (60 pmol of CMP, 230 cpm/pmol), 0.06 M Tris/HCl, pH 8, and 1 µg of RNA polymerase A. Incubation was at 37°C. At various times, 0, 2, 5, 10, 20, 30, and 40 min, two 20-µl aliquots were withdrawn, one was used to measure acid-soluble radioactivity, the other was supplemented with 5 µl of 0.1 M EDTA and directly applied to a polyethyleneimine cellulose plate, which was developed with 0.6 M phosphate buffer as described under "Methods" and then subjected to radioautography. The control (called C) on the left, corresponds to a sample of [³²P](rG), -(dC), incubated for 60 min at 37°C in absence of enzyme. Bottom, the three spots, revealed by autoradiography, were scraped off, eluted with 0.1 M HCl, and their radioactivity determined by liquid scintillation. Results are given as percent of total radioactivity recovered at the level of the different spots; percent of total input radioactivity solubilized in acid (△-△); per cent radioactivity present in Spot 1 (□-□) and in spot 2 (■-■).

Mode of Action of Ribonuclease H

Partial Digest of (rG), -(dC), - In the above experiments, the degradation reaction was followed by monitoring the release of acid-soluble radioactive products. In the following, breakdown products were isolated by chromatography on polyethyleneimine cellulose sheets. Aliquots of the reaction mixture containing [³²P]-labeled (rG), -(dC), were withdrawn at various times and subjected to chromatography with phosphate buffer (Fig. 6). Three spots were found on the autoradiogram. The material remaining at the origin, corresponding to the undegraded polymer, progressively disappeared, and gave rise to two new components (Spots 1 and 2). From their Rₚ values, these spots could correspond, respectively, to a mononucleotide and dinucleotide. There was no evidence of trailing of the radioactive material at the origin. To quantify these observations the compounds were eluted from the chromatogram with 0.1 M HCl and their radioactivity determined. The percentage of total radioactivity recovered in Spots 1 and 2 as a function of time is shown in Fig. 6. These breakdown products accounted for more than 80% of the hybrid degrada-

tion, as measured in parallel by acid precipitation. The ratio of radioactivity present in Spot 1 versus that in Spot 2 was 1:6. (A minor spot, faster than Spot 1, possibly free phosphate, was not taken into account.)

Nature of Degradation Products - The degradation products from [³²P](rG), -(dC), were identified by two-dimensional chromatography on thin layer cellulose plates (Fig. 7). The technique allows the resolution of a complex mixture of ribonucleotides and related compounds (11). Co-chromatography with several unlabeled markers, pG, Gp, ppG, ppGp, allowed the identification of Spot 1 as 5'-GMP. The nature of
Spot 2 was inferred after alkaline hydrolysis. The compound was eluted with 0.3 M KOH and treated with alkalai for 16 h at 37°C. Upon alkaline hydrolysis Spot 2 disappeared and gave rise to two new spots which co-migrated with the 2'(3')-guanosine diphosphate isomers of pGp. This indicated that Spot 2 corresponded to the dinucleotide pGpG, hydrolyzed to pGp with alkalai.

Similarly, hydrolysis of [3P]rA(rA) by RNA polymerase A released two compounds corresponding to 5'AMP (pA) and to the dinucleotide pApA (Fig. 7). A minor spot with the migration behavior of adenosine was also found with 14C-labeled substrate (result not shown). Further proof for the identification of the products was provided after alkaline hydrolysis by chromatography of the digest on polyethyleneimine cellulose with 0.8 M borate buffer. The compounds co-chromatographed perfectly with authentic pA and pAp. The rate of release of the two products was compared by chromatography. The compounds were eluted and their radioactivity determined. The molar ratio of pA versus pApA was found in the range of 1:3, which corresponded well to the molar ratio of pG versus pGpG found previously (once the appropriate corrections were made for their phosphate content) (see Fig. 6).

While in the course of these experiments we were interested to analyze the products of the hydrolysis of [3P]rA(rA) by RNA polymerase A, it was noted that some activity was also present at the level of RNA polymerase A* (6). However, RNA polymerase A* had a specific activity, which was lower than that of RNA polymerase A in terms of RNase H. This suggested that one or both polypeptides dissociated from RNA polymerase A, it was noted that some activity was recovered as a major peak of protein together with some residual Enzyme A. The salt concentration required for their catalyzed by Mn2+ ions at 60°C (see Fig. 4). Chromatography of the breakdown products on polyethyleneimine cellulose sheets with borate buffer revealed the formation of a mixture of oligonucleotides of increasing length, along with some 3'-AMP. Upon alkaline hydrolysis of the partial digest, only trace amounts of mononucleotide were found in this respect. With Mg2+ ions, at pH 8, the major degradation product of (rA)2(dT)6 was the dinucleotide pApA, whereas, with Mn2+ ions, the mononucleotide pA predominated slightly (Table I). A strong variation in the ratio of these two products was also found as a function of pH. At pH 6.56 and with Mg2+ ions, only trace amounts of mononucleotide was found, and the dinucleotide:mononucleotide ratio increased by a factor of 5 compared to pH 8.1 (Table I). With Mn2+ ions an increase of incubation temperature from 37°C to 50°C enhanced the dinucleotide spot and lowered the mononucleotide one.

**Factors Affecting Pattern of Degradation Products**—The pattern of degradation products was not invariant. The mononucleotide/dinucleotide ratio depended upon the incubation conditions. The influence of the nature of the activator cation was most striking in this respect. With Mg2+ ions, at pH 8, the major degradation product of (rA)2(dT)6 was the dinucleotide pApA, whereas, with Mn2+ ions, the mononucleotide pA predominated slightly (Table I). A strong variation in the ratio of these two products was also found as a function of pH. At pH 6.56 and with Mg2+ ions, only trace amounts of mononucleotide was found, and the dinucleotide:mononucleotide ratio increased by a factor of 5 compared to pH 8.1 (Table I). With Mn2+ ions an increase of incubation temperature from 37°C to 50°C enhanced the dinucleotide spot and lowered the mononucleotide one.

**Attempt to Isolate Polypeptide with RNase H Activity**

In the initial report on the association of RNase H activity with RNA polymerase A, it was noted that some activity was also present at the level of RNA polymerase A*. However, RNA polymerase A* had a specific activity, which was lower than that of RNA polymerase A in terms of RNase H. This suggested that one or both polypeptides dissociated from RNA polymerase A (Subunits A3 and A4, of 48,000 and 34,500 daltons) were somehow involved in the nucleic activity. To investigate this point further, RNA polymerase A was subjected to a mild dissociation treatment with 1.5 mM urea in order to favor the formation of A* enzyme and accumulate the two polypeptides. After the urea treatment the enzyme was applied to a phosphocellulose column; the proteins were eluted by a gradient of ammonium sulfate in absence of urea, and the fractions were assayed for RNA polymerase and RNase H activity (Fig. 8). Addition of urea strongly increased the dissociation of the enzyme; RNA polymerase A* was recovered as a major peak of protein together with some residual Enzyme A. The salt concentration required for their
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Fig. 8. Chromatography on phosphocellulose of RNA polymerase A after urea treatment. RNA polymerase A (2 mg of phosphocellulose step, Peak II (7)) was diluted to 0.17 mg/ml with a buffer containing 20 mM Tris/HCl, pH 8, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA, 0.05 M ammonium sulfate, 10% glycerol, and 1.5 mM urea, at 0°C. The sample was loaded onto a phosphocellulose column (10 cm x 1.25 cm) equilibrated with the above buffer. The column was washed with 30 ml of buffer without urea and the proteins \( \text{A}_{280} \) were eluted with 100 ml of a linear gradient from 0.05 to 0.4 M ammonium sulfate in the same buffer without urea. Fractions of 1.2 ml were collected and 20 \( \mu \text{l} \) was assayed for RNA polymerase (\( \text{A}_{280} \)) and RNase H activity using the standard assays described under "Methods." Fractions 90 to 100 were first concentrated about 3-fold by dialysis at -20°C against the same buffer containing 70% glycerol then assayed for RNase H activity (---). Conductivity measurements indicated that Enzyme A* was eluted at 0.19 M ammonium sulfate, Enzyme A at 0.25 M, and the Subunits A and A at 0.35 M ammonium sulfate. The fractions were pooled as indicated in the figure. Aliquots of the proteins pooled were precipitated with 15% trichloroacetic acid and analyzed by dodecyl sulfate electrophoresis on slab gel (\( \text{A}^{*} \)); the numbers on the gel correspond to the number of the fractions pooled.

elution was characteristic of these two forms of enzyme which were also identified by their subunit content (Fig. 8, inset). The dissociated subunits were eluted tandemly at a higher salt concentration, as evidenced by sodium dodecyl sulfate gel electrophoresis. RNase H activity co-chromatographed with Enzyme A, but also, despite the dissociation treatment, with Enzyme A*. Interestingly, the fractions containing the dissociated polypeptides were also found, after concentration, to have RNase H activity (Fig. 8).

Assuming that the RNase H corresponded to one of the dissociated polypeptides, it was intriguing to find a residual RNase H activity in RNA polymerase A*. The RNase H activity of the A* enzyme was about 4-fold lower than that of Enzyme A. Based on the results of the gel electrophoresis with dodecyl sulfate, it was unlikely that A* retained more than 10% of the amount of the A and A subunits. Nevertheless another technique was used to confirm the above observation. RNA polymerase was subjected to electrophoresis in a slab gel under non denaturing conditions, which resolves A* and A forms of enzymes (7). One part of the gel was stained to locate the protein bands, and the other was sliced and used to assay RNase H activity (Fig. 9). The main band of protein migrated as RNA polymerase A*, ahead of a minor band of Enzyme A. After slicing the gel, RNase H activity was again clearly found at the level of A*. The dissociated polypeptides being basic proteins (14) do not enter the gel under these conditions.

RNA polymerase A* also produced a mixture of mononucleotides and dinucleotides, like Enzyme A, although in a significantly different ratio (see Table I). Less mononucleotide was produced compared to Enzyme A. Sometimes the mononucleotide spot was barely detectable. It can be seen in Table I that the pattern of degradation products varied with the incubation conditions, as with A enzyme.
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The persistence of an RNase H activity in homogeneous preparations of yeast RNA polymerase A is most interesting. This nuclease activity behaves as an exonuclease which produces a mixture of two different products, a 5'-phosphate mononucleotide and a dinucleotide with a 5'-phosphate end. The exonucleolytic mechanism of degradation is inferred from the fact that these products accounted for all of the acid-soluble radioactivity recovered. Oligonucleotides of increasing length were not observed. If the RNase H activity was that of an endonuclease, short oligonucleotides which cannot form stable hybrids at 50°C or even at 37°C would accumulate and never yield mononucleotides as final degradation products (15). The drastic and immediate inhibitory effect of a competitor nucleic acid, such as (ri)n, when added during the course of degradation, was suggestive of a nonprocessive mechanism of degradation. The nuclease appears to have a broad band specificity because it degraded the (rG)n strand of (rG)n-(dC)n hybrids as well as the (rA)n strand of (rA)n-(dT)n polymers, provided that the appropriate activator cation was added. The RNA-DNA hybrid made with denatured T1 DNA as template by yeast RNA polymerase B was degraded as well. Therefore the presence of a 5'-triphosphate end does not prevent the degradation reaction. However we have no conclusive evidence, up to now, concerning the direction of degradation.

Several unusual properties of the nuclease activity suggested the possibility that we may be dealing with more than one form of ribonuclease H. There was first the bimodal pH curve with optima at pH 6.5 and pH 8; also the fact that the temperature response curve depended strongly upon the divalent cation used; the release of two different breakdown products, as well as the large variation in the mononucleotide/dinucleotide ratio were particularly striking. These particularities are not in themselves decisive and do not eliminate the possibility that a single polypeptide is responsible for the activity. There are examples of enzymes having a bimodal curve of pH (16). A mixture of products is also produced by phage SP1 RNase which hydrolyzes denatured DNA to give 5'-phosphodinucleotides with some 5'-phosphotrinucleotides (17). Finally the mode of action of various nucleases was shown to depend on the incubation conditions (18, 19). However, the most striking evidence in favor of different RNase H was the separation of nuclease activity into two distinct protein fractions after dissociation of RNA polymerase A with urea. The activity was recovered with RNA polymerase A* and also the two dissociated polypeptides (Subunits A48 and A34). The presence of an RNase H activity associated with A* was confirmed by gel electrophoresis. The nuclease activity appears, therefore, to be associated with different polypeptides. Preliminary experiments indicated that the mixture of the dissociated subunits produced mainly the mononucleotide, but the low amount of protein available and the instability of the activity prevented a thorough study of their mode of degradation. It was puzzling that RNA polymerase A* produced, like Enzyme A, a mixture of mono- and dinucleotides, although in a different ratio; therefore a decisive proof of the existence of two distinct RNase H in RNA polymerase A will have to rest on the identification of the polypeptides responsible for the activity.

Observations available at the moment indicate that one of the two polypeptides which can dissociate from Enzyme A (7), called Subunit A48, is in fact identical with yeast RNase H1. RNase H1 is one of two ribonucleases H which have been recently identified in yeast cells (1, 2). The experiments demonstrating the identity of RNase H1 with Subunit A48 will be described elsewhere. The polypeptide(s) responsible for the nuclease activity present in Enzyme A* have not yet been identified.

Whatever the outcome of further attempts to resolve the nuclease from RNA polymerase A, it is clear that ribonuclease H activity is not required for the transcription process. Thus, under conditions in which polymerization can proceed, the nuclease activity is drastically reduced, even with limiting concentrations of ribonucleoside triphosphates. This probably reflects the processivity of the polymerization reaction and the fact that the RNase H remains tightly bound to and concealed within the transcribing RNA polymerase molecule.2 A number of preparations of RNA polymerase B were tested and found to have no RNase H activity. Therefore, the role of this ribonuclease H activity is still obscure. However, the

1 J. Huet and F. Iborra, unpublished experiments.
2 An alternative explanation could be imagined, assuming that RNase H digests the hybridized RNA in a 3' → 5' direction. Elongation of the pre-existing hybridized [32P]pA chains by incorporation of unlabeled AMP residues would then prevent the release of 32P-labeled material. To interpret in this way the results of Fig. 5, one has to assume also that chain elongation occurs preferentially at low nucleotide triphosphate concentration, whereas initiation of new chains predominates at high substrate concentration. This hypothesis is not unlikely. However, using [α-32P]ATP, no formation of [32P]pA or [32P]pApA could be detected by chromatography during the course of RNA synthesis on (rA)n-(dT)n template.

FIG. 9. RNase H activity at the level of A* enzyme upon polyacrylamide gel electrophoresis. RNA polymerase A (15 μg; glycerol gradient step 7) was subjected to electrophoresis on 5% polyacrylamide slab gel prepared essentially according to Laemmli (12) excepted that sodium dodecyl sulfate was omitted (nondenaturing conditions). Electrophoresis was run at 4°C, under constant current (50 mA/gel; 1 mm thick) for 3.5 h. One strip of gel was stained and identified. The Position of the protein bands is indicated in the figure. One adjacent strip was cut into 1-mm slices which were incubated into 0.15 ml of RNase H assay mixture containing [3H](rA)n-(dT)n (350 pmol of AMP residues; 36 cpmpmol) for 2 h at 37°C. Acid-solubilized radioactivity was determined as usual.

Discussion

The persistence of an RNase H activity in homogeneous preparations of yeast RNA polymerase A is most interesting. This nuclease activity behaves as an exonuclease which produces a mixture of two different products, a 5'-phosphate mononucleotide and a dinucleotide with a 5'-phosphate end. The exonucleolytic mechanism of degradation is inferred from the fact that these products accounted for all of the acid-soluble radioactivity recovered. Oligonucleotides of increasing length were not observed. If the RNase H activity was that of an endonuclease, short oligonucleotides which cannot form stable hybrids at 50°C or even at 37°C would accumulate and never yield mononucleotides as final degradation products (15). The drastic and immediate inhibitory effect of a competitor nucleic acid, such as (rI)n, when added during the course of degradation, was suggestive of a nonprocessive mechanism of degradation. The nuclease appears to have a broad band specificity because it degraded the (rG)n strand of (rG)n-(dC)n hybrids as well as the (rA)n strand of (rA)n-(dT)n polymers, provided that the appropriate activator cation was added. The RNA-DNA hybrid made with denatured T1 DNA as template by yeast RNA polymerase B was degraded as well. Therefore the presence of a 5'-triphosphate end does not prevent the degradation reaction. However we have no conclusive evidence, up to now, concerning the direction of degradation.

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presence of such nuclease activity supports the hypothesis that eukaryotic RNA polymerases are part of a large multienzyme system containing a number of enzymatic or non enzymatic proteins that could be involved in RNA or DNA metabolism or even chromatin structure.

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