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T7 gene 6 exonuclease has an RNase H activity

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ABSTRACT

T7 gene 6 exonuclease has been shown to have an RNase H activity as well as a double-strand specific DNase activity by the following experiments: The RNase H activity coelutes with the DNase activity from DEAE-cellulose, phosphocellulose, hydroxyapatite, and Sephadex G-200 columns. Gene 6 exonuclease specified by a T7 strain with a temperature sensitive mutation in gene 6 has an extremely heat-labile RNase H activity as well as a heat-labile DNase activity. T7 gene 6 exonuclease degrades the RNA region of a poly(A)·poly(dT) hybrid polymer exonucleolytically from the 5' terminus, releasing a ribonucleoside 5'-monophosphate product. When the RNA strand of a ϕX174 RNA·DNA hybrid molecule synthesized with E. coli RNA polymerase is degraded, a ribonucleoside triphosphate is produced from the 5'-triphosphate terminus. Participation of T7 gene 6 exonuclease in the removal of primer RNA in discontinuous replication of T7 DNA is discussed.

INTRODUCTION

Analyses performed in this laboratory of nascent short DNA in various prokaryotic systems have shown that RNA is linked to the 5' termini of the pieces (1, 2, 3, 4, 5, 6). This supports the assumption made earlier that synthesis of DNA pieces in discontinuous replication is primed by RNA that is removed prior to the joining of DNA pieces (7). Presence of RNA-linked DNA pieces has been also reported from other groups (8).

In E. coli, degradation of primer RNA may result from the nick translation activity of DNA polymerase I (9, 10, 11), because RNA-linked nascent short DNA accumulate when the 5'→ 3' exonuclease activity or/and polymerase activity of the enzyme are deficient (1, 2, 3). In support of this; DNA polymerase I degrades the RNA of RNA·DNA hybrids in vitro, i. e. it has RNase H activity (12, 13, 14).

Our previous study of T7 phage infected E. coli showed that RNA-linked nascent T7 DNA accumulates in the absence of functional T7 gene 6 (6). The accumulation is particularly pronounced when both gene 6 exonuclease and host DNA polymerase I are deficient, even though the deficiency of the latter

enzyme alone does not cause this accumulation. T7 gene 6 codes for a 5'→ 3' exonuclease that is specific for double-stranded DNA (15, 16). Because our results indicate that it removes T7 primer RNA, we have tested if it also has RNase H activity.

We find that purified gene 6 protein indeed degrades RNA in variety of RNA·DNA hybrid molecules. The exonucleolytic degradation from the 5' terminus releases ribonucleoside 5' monophosphates. The enzyme also digests 5'-triphosphate terminated RNA which is hydrogen bonded to DNA.

MATERIALS

Bacteria, Bacteriophages and Culture Media

Escherichia coli C-N3 (*polAexl*, *his*) was described previously (6). *E. coli* 011' (*suII*$^+$, *thy*) and a double mutant T7 *am*29, *am*28 phage which has amber mutation in genes 3 and 5 were provided by Dr. H. Ogawa. T7 *ts*136 phage which bears a temperature sensitive mutation in gene 6 was a gift of Dr. F. W. Studier. A triple mutant T7 *am*29, *am*28, *ts*136 (genes 3 am + 5 am + 6 ts) was constructed by crossing T7 *am*29, *am*28, against T7 *ts*136 as described by Studier (17). Stocks of amber mutants were grown on *E. coli* 011' in modified 3XD medium supplemented with 10 μg/ml thymine.

Culture media were modified 3XD medium (10.5 g Na_2HPO_4, 4.5 g KH_2PO_4, 1.0 g NH_4Cl, 0.3 g $MgSO_4$, 0.033 g $CaCl_2$, 30 ml glycerol, 0.01 g gelatine and 15.0 g Casamino acids in 1 l of water), and medium A supplemented with 1% Casamino acids (18).

Chemicals and Enzymes

[Methyl-^{3}H]thymine and [^{3}H (G)]ADP were purchased from New England Nuclear Corp.; [8-^{3}H]ATP, GTP, CTP, UTP and [methyl-^{3}H]dTTP were from Schwartz/Mann Research. [γ-^{32}P]ATP has been described previously (3). ^{14}C-labeled SP variant double-stranded RNA synthesized with SP RNA replicase was a gift of Mr. A. Aoyama (19). CPV double-stranded RNA was provided by Dr. K. Miura (20). Unlabeled poly(dT), poly(U) and poly(rA) were purchased from Sigma Chemical Co., Miles Laboratories Inc. and P-L Biochemicals, respectively. Unlabeled ATP and ADP were from Boeringer Mannheim GmbH; unlabeled AMP and dTTP were obtained from Sigma Chemical Co. DEAE-cellulose (DE23) and phosphocellulose (P11) were purchased from Whatman. Hydroxyapatite was prepared by Dr. F. Tamanoi as described (21). Sephadex G-200 and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals. *E. coli* RNA polymerase and calf thymus terminal deoxynucleotidyl transferase were provided by Dr. A.

Ishihama and Dr. N. Cozzarelli, respectively. E. coli polynucleotide phosphorylase was purified by Mr. T. Seki as described (22).

METHODS

Preparation of [^{3}H]thymine-labeled E. coli native DNA

E. coli B3 (thy) was grown in 500 ml of Medium A containing 1% Casamino acids and 10 μM [^{3}H]thymine (50 mCi/mmol) for 5.5 hours at 37°C. The cells were harvested by centrifugation at 6,000 rpm in a Sorvall GSA rotor. The labeled DNA was extracted by the method of Thomas et al. (23), except for a 4-fold greater DNA concentration, precipitated with ethanol, suspended in 30 ml of 1 x SSC and then dialyzed against 1 x SSC. After treatment with pancreatic RNase IA (50 μg/ml) and RNase T1 (10 μg/ml) for 150 min at 37°C, the DNA sample was incubated with pronase (1 mg/ml) in 1% SDS, 1 x SSC and 1 mM EDTA for 6 hours at 37°C and treated sequentially with phenol and ether. After precipitation with ethanol, the sample was suspended in 12 ml of 1 x SSC, and dialyzed against 1 x SSC. The specific activity of the labeled DNA was 4.09 μCi/mmol. The yield was 13.7 μmoles.

Preparation of [^{3}H]poly(A) and [5'-^{32}P],[^{3}H]poly(A)

[^{3}H]poly(A) was synthesized using polynucleotide phosphorylase in a reaction mixture (500 μl) containing 150 mM Tris·HCl (pH 8.1), 10 mM $MgCl_2$, 0.5 mM EDTA, 25 mM [^{3}H]ADP (34 mCi/mmol), 0.2 mM poly(A) and 0.025 unit of E. coli polynucleotide phosphorylase. After incubation for 6.5 hours at 30°C, the reaction was diluted with 1.5 ml of 150 mM Tris·HCl (pH 8.1), 5 mM EDTA and treated with phenol. The sample was then passed through a Sephadex G-100 column (1.2 x 20 cm) in 5 mM potassium phosphate buffer (pH 7.4), 50 mM NaCl, 5 mM EDTA and dialyzed against 10 mM Tris·HCl (pH 7.4). [^{3}H]poly(A) was partially degraded by incubation in 0.1 M NaOH at 20°C for 25 min and after neutralization, dialyzed against 10 mM Tris·HCl (pH 7.4). Average chain length of alkali-treated [^{3}H]poly(A) was 460 nucleotides.

To label the 5' termini of the [^{3}H]poly(A), a part of the alkali-treated [^{3}H]poly(A) was first incubated with 0.6 U/ml E. coli alkaline phosphatase in 1 mM $MgCl_2$ at 37°C for 45 min. Then, the sample was phosphorylated in a reaction mixture containing 67 mM Tris·HCl (pH 8.0), 17 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1.5 mM potassium phosphate buffer (pH 8.0), 16 μM [γ-^{32}P]ATP (3 cpm/fmol) and 67 units of T4 polynucleotide kinase per ml at 37°C for 30 min. After treatment with phenol, the sample was passed through a Sephadex G-100 column (1.2 x 20 cm) in 5 mM potassium phosphate buffer (pH 7.4), 50 mM

NaCl, 5 mM EDTA and dialyzed against 10 mM Tris·HCl (pH 7.4), 0.1 mM EDTA.

Preparation of φX174 DNA·RNA hybrids with E. coli RNA polymerase

φX174 viral DNA was prepared as described (24). RNA synthesis was carried out in a reaction mixture (2 ml) containing 40 μM φX174 DNA, 100 μg of E. coli RNA polymerase, 42 mM Tris·HCl (pH 8.0), 12 mM $MgCl_2$, 0.1 M KCl, 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin (Sigma, crystalized and lyophilized), 150 μM each of [^{3}H]GTP, [^{3}H]CTP, [^{3}H]UTP, and [^{3}H],[γ-^{32}P]ATP (^{3}H of each nucleoside triphosphate, 0.17 Ci/mmol; ^{32}P of ATP, 0.63 Ci/mmol). After incubation for 6 hours at 37°C, the reaction was stopped by the addition of 20 μmoles of EDTA and treated with phenol. The sample was then passed through a Sephadex G-100 column (1.5 x 25 cm) equilibrated with 10 mM Tris·HCl (pH 7.5), 0.15 M NaCl, 10 mM EDTA to remove acid-soluble radioactive materials. The [5' γ-^{32}P],[^{3}H] RNA·DNA hybrid product was isolated from the excluded portion of Sephadex G-100 chromatography by equilibrium density gradient centrifugation in Cs_2SO_4 as follows: 1.15 mmoles of Tris·HCl (pH 7.5), 0.116 mmoles of EDTA and 6.9 g of solid Cs_2SO_4 were added to the sample and a final volume was adjusted to 11.5 ml with distilled water. The density of the solution was 1.44 g per ml. The mixture was centrifuged at 36,000 rpm for 42 hours at 15°C in a Beckman No. 50 Ti rotor in a polyallomer tube. After fractionation, the radioactive material banding at the hybrid density (1.50 ~ 1.54 g per ml) was collected and dialyzed overnight against 10 mM Tris·HCl (pH 7.5), 0.1 M KCl. The yield was 10 pmol of ^{32}P and 8.5 nmol of ^{3}H.

Preparation of [^{3}H]poly(dT) and [^{3}H]poly(dA)

[^{3}H]poly(dT) was synthesized using terminal deoxynucleotidyl transferase in a reaction mixture (500 μl) containing 200 mM potassium cacodylate buffer (pH 7.1), 40 mM KCl, 1 mM mercaptoethanol, 2 mM $CoCl_2$, 2 mM [^{3}H]dTTP (25 μCi/μmol), 0.13 mM oligo(dT), 2.15 mg terminal deoxynucleotidyl transferase, and 0.95 mg inorganic pyrophosphatase. After incubation for 22.5 hr at 35°C, the reaction was treated with phenol, and then passed through a Sephadex G-100 column (1.4 x 23 cm) in 0.15 M NaCl, 10 mM Tris·HCl (pH 8.0), 5 mM EDTA. The [^{3}H]poly(dT) sample was concentrated and dialyzed against 10 mM Tris·HCl (pH 8.0). The average chain length was 600 nucleotides and specific activity was 6.3 μCi/μmol. [^{3}H]poly(dA) was synthesized in a reaction mixture containing 97 mM potassium cacodylate buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM mercaptoethanol, 1 mM [^{3}H]dATP (100 μCi/μmol), 0.1 mM oligo(dA), 2 mg of terminal deoxynucleotidyl transferase and 0.95 mg of inorganic pyrophosphatase. The average chain length and specific activity of the final sample was about 200 nucleotides and 37 μCi/μmol, respectively.

Preparation of ^{14}C-labeled linear δA DNA

[^{14}C]thymine-labeled δA DNA, single-stranded circle DNA, was prepared with a specific activity of 770 cpm/nmol nucleotide (25). The labeled δA was cut into linear fragments by incubation in 0.2 N KOH at 95°C for 15 min. Average chain length of the linear DNA was about 4,000 nucleotides.

Assay of RNase H activity

Assay of RNase H activity was carried out as follows: A reaction mixture (0.05 ml) containing 30 μM [^{3}H]poly(A) (14.4 cpm/pmol, 460 nucleotides in length), 90 μM poly(dT) (about 2,000 nucleotides in length), 50 mM Tris·HCl (pH 8.1), 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, 0.5 mg/ml crystalline bovine serum albumin and various amounts of gene 6 exonuclease were incubated at 30°C for 15 min. In the case of thermo-sensitive enzyme, reactions were carried out at 20°C for 10 min. The reaction was stopped by the addition of 0.1 ml of 1 mg/ml salmon sperm DNA and 0.15 ml of 10% trichloroacetic acid. After centrifugation for 5 min at 10,000 rpm, radioactivity of the acid-soluble supernatant fluid was measured.

By definition, one unit of enzyme activity produces 1 nmol of acid-soluble material in 15 min at 30°C. Enzyme fractions were diluted in a solution containing 50 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, and 0.5 mg/ml of crystalline bovine serum albumin.

Assay of other enzyme activities

DNase active on a double-strand substrate, DNase active on a single stranded substrate and RNase active on single strand were assayed with *E. coli* native [^{3}H]DNA (100 μM, 4.09 μCi/mmol), *E. coli* [^{3}H]DNA (50 μM) heated at 100°C for 4 min and [^{3}H]poly(A) (30 μM) as substrates, respectively. The assay procedure and the definition of a unit of enzyme were the same as described in the RNase H assay except for the substrate.

Separation on DEAE-Sephadex of products generated from poly(A) or φX174 complementary RNA

[^{3}H]poly(A)·poly(dT) or φX174 RNA·DNA hybrid was treated with gene 6 exonuclease and the reaction was stopped by the addition of 0.3 mg/ml salmon sperm DNA and 0.3 N perchloric acid. After centrifugation for 10 min at 10,000 rpm, the supernatant containing acid soluble material was collected, neutralized with potassium hydroxide and perchloric acid was removed as potassium perchlorate. The samples were diluted twenty times with 5 mM Tris·HCl (pH 8.0), 7 M urea, and, after addition of 45 nmoles AMP and 44 nmoles ATP as charge markers, applied at room temperature to a DEAE-Sephadex A-25 column (0.2 x 6.4 cm) equilibrated with 5 mM Tris·HCl (pH 8.0), 7 M urea.

The adsorbed nucleotides were eluted with a 20-ml linear NaCl gradient (0 to 0.35 M or 0 to 0.3 M) in the same buffer at a flow rate of 2 ml per hour.

RESULTS

(1) Purification of the gene 6 exonuclease from T7 am 3+5 infected E. coli polAexl cells

Gene 6 exonuclease was purified by a modification of the procedure of Kerr and Sadowski (15). Enzyme was purified from T7 am 3+5 phage-infected E. coli polAexl cells, in which the 5'→ 3' exonuclease activity of E. coli DNA polymerase I is very low and heat labile (26). The digestion step with pancreatic RNase was eliminated, so that the same procedure could be used to purify the very unstable gene 6 exonuclease induced by T7 am 3+5, ts 6 phage. The omission of this step also avoided contamination by RNase. The procedure adopted is summerized in Table 1. All steps were performed at 4°C, and all buffers (except for the "lysis buffer") contained 20% glycerol (v/v), 1 mM dithiothreitol, and 1 mM EDTA.

Preparation of phage-infected cells

E. coli C-N3 was grown in 3XD medium (3 liters) to a density of 1.5 x 10^9 cells per ml at 30°C, and infected with T7 am 3+5 at a multiplicity of infection of 10. In the case of the triple mutant T7 am 3+5, ts 6, a multiplicity of infection of 20 was adopted according to Kerr and Sadowski (15). After 24 min, the cultures were poured into a vessel containing 600 g of crushed ice at -20°C, and phage-infected cells were collected by centrifugation at 8,000

Table 1. Purification of T7 gene 6 exonuclease from T7 am 3+5 phage-infected E. coli C-N3 (polAexl)

Fraction	RNase H activity			dsDNase activity			Ratio of RNase H to dsDNase
	Total activity	Specific activity	Purification	Total activity	Specific activity	Purification	
	(units x 10^{-4})	(units/mg protein)		(units x 10^{-4})	(units/mg protein)		
Crude extract	8.32	98	1	11.1	131	1	0.75
S100	7.68	153	1.6	9.48	189	1.4	0.81
DEAE-cellulose I	8.18	200	2.0	9.94	243	1.9	0.82
DEAE-cellulose II	6.81	1,750	18	5.86	1,500	11	1.16
Phosphocellulose	4.46	31,400	320	2.86	20,100	154	1.56
Hydroxyapatite	2.82	88,800	906	1.70	53,400	408	1.66

Methods were described in the text.

rpm for 10 min in a Sorvall GSA rotor. Cells were suspended in 200 ml of 0.85% NaCl, resedimented and stored at -20°C. The yield of cells was 15 g.

Preparation of crude extract and S100 fraction

Frozen T7 am 3+5 phage-infected E. coli cells (7.5 g) were thawed and suspended in 27 ml of 50 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol and 1 mM EDTA, and then lysed in a pressure cell (crude extract, 30 ml). The lysed cells were centrifuged at 100,000 g for 90 min (S100 fraction, 26 ml).

DEAE cellulose I

The supernatant was brought to 20% glycerol (v/v) and 0.3 M $(NH_4)_2SO_4$ by the dropwise addition of equal volume of 40% glycerol (v/v), 0.6 M $(NH_4)_2SO_4$, 50 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, and 1 mM EDTA. The sample was applied to a column of DEAE-cellulose (1.8 x 28 cm) previously equilibrated with 50 mM Tris·HCl (pH 7.5), 0.3 M $(NH_4)_2SO_4$. The protein not adhering to the column was collected (DEAE cellulose I fraction, 66 ml). About 85% of the nucleic acids were removed in this step.

DEAE cellulose II

The DEAE-cellulose I fraction was dialyzed for 6 hours against 1 liter of 50 mM Tris·HCl (pH 7.5), and was applied to a column of DEAE-cellulose (1.7 x 30 cm) equilibrated with the same buffer. After washing the column with 70 ml of the same buffer, the proteins were eluted with a 700-ml linear gradient from 0 to 0.4 M $(NH_4)_2SO_4$ in the same buffer, and fractions of 9 ml were collected. The flow rate was maintained at 25 ml per hour during the sample adsorption and the column washing, and at 40 ml per hour in the protein elution. Major portion of both the RNase H and dsDNase activities were eluted between 0.19 and 0.26 M $(NH_4)_2SO_4$ (DEAE-cellulose II fraction, 144 ml). This major peak was missing in uninfected E. coli cell extract. Two minor peaks with RNase H activities were eluted prior to this peak, which were also present in uninfected E. coli cell extract. Most of ssRNase and ssDNase activities were removed in this step.

Phosphocellulose

The DEAE-cellulose II fraction was dialyzed for 5 hours against 1 liter of 20 mM potassium buffer (pH 6.5), and was applied to a column of phosphocellulose (1.3 x 24 cm) equilibrated with the same buffer. After a 30-ml buffer wash, the proteins were eluted with a 300-ml linear gradient from 0 to 0.4 M $(NH_4)_2SO_4$ in the same buffer. Of the applied RNase H activity, 65% was eluted in a peak between 0.08 and 0.12 M $(NH_4)_2SO_4$, and 3% was eluted between 0.25 and 0.28 M $(NH_4)_2SO_4$. Fractions eluted between 0.08 and 0.12 M were

pooled (Phosphocellulose fraction, 17 ml). All the ssRNase and ssDNase activities which had remained in DEAE-cellulose II fraction were removed from gene 6 exonuclease fraction in this step.

Hydroxyapatite

The phosphocellulose fraction was dialyzed for 4 hours against 500 ml of 20 mM potassium phosphate (pH 6.5), and applied to a column of hydroxyapatite (0.8 x 10 cm) previously equilibrated with the same buffer. After washing the column with 10 ml of the same buffer, the proteins were eluted with a 50 ml linear gradient from 0.02 to 0.4 M potassium phosphate buffer (pH 6.5). As shown in Fig. 1, both RNase H and dsDNase activities were co-purified in a single, sharp peak between 0.16 and 0.21 M potassium phosphate (pH 6.5), and no ssRNase and ssDNase activities were detected. The peak fractions were pooled, dialyzed for 6 hours against 300 ml of 20 mM potassium phosphate (pH 6.5), 50% glycerol, 1 mM dithiothreitol and 1 mM EDTA, and then stocked at -20°C (Hydroxyapatite fraction, 1.3 ml). This fraction was used for all further experiments except the thermal-inactivation experiment, and was stable at least for 6 months. Analysis by electrophoresis on 5% polyacrylamide gel in the presence of sodium dodecyl sulfate showed that 70% of the coomassie blue-positive material was in protein band with the molecular weight of gene 6 exonuclease (data not shown).

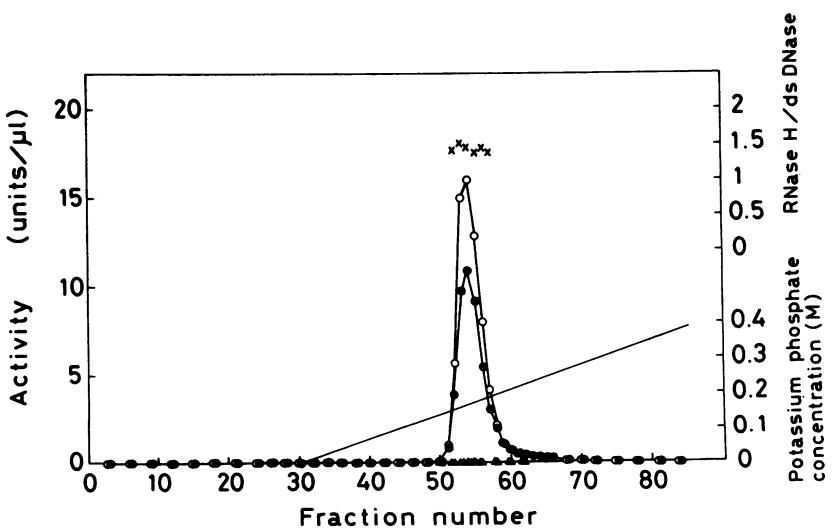

Fig. 1. Hydroxyapatite column chromatography of gene 6 exonuclease. The dialyzed phosphocellulose fraction was applied to a column of hydroxyapatite and eluted with a gradient of 0.02 M to 0.4 M potassium phosphate (pH 6.5). Fraction of 0.8 ml were collected. o, RNase H; ●, dsDNase; Δ, ssRNase; ▲, ssDNase; x, RNase H/dsDNase ratio.

(2) Co-purification of RNase H and dsDNase activities coded by T7 phage

The major portion of RNase H activity in T7 am 3+5 infected E. coli polAexl cells was co-purified with dsDNase activity throughout the purification procedure as shown in Table 1. The 5-fold lower RNase H activity in uninfected E. coli cell extract was eluted prior to the phage-coded RNase H activity in the second DEAE-cellulose chromatography. Another unidentified minor RNase H activity was removed in the phosphocellulose step; the RNase H/dsDNase ratio was nearly constant after the phosphocellulose step (Table 1). In the hydroxyapatite step, RNase H and dsDNase activities were eluted in a single, sharp peak and the ratio of the two activities was constant across the peak fractions (Fig. 1). A part of the Hydroxyapatite fraction was subjected to gel filtration chromatography (Fig. 2) and again both RNase H and dsDNase activities chromatographed in a single peak. These results suggest that the two enzyme activities reside in the same T7 encoded protein.

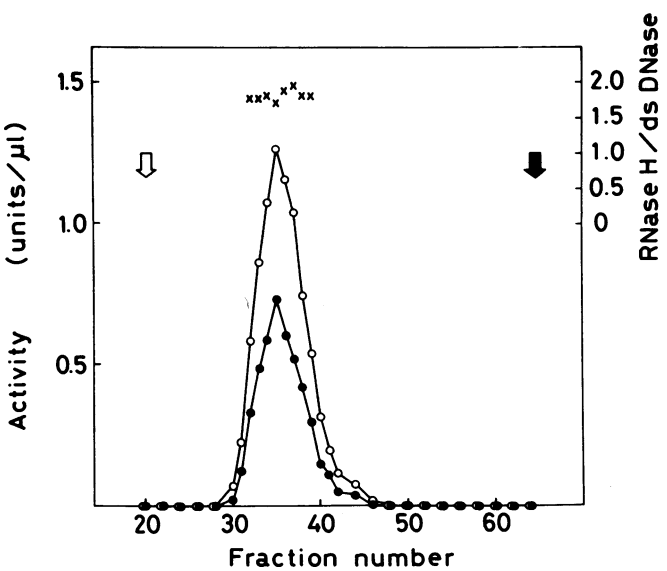

Fig. 2. Gel filtration of purified gene 6 exonuclease. The hydroxyapatite fraction containing 70 μg protein was dialyzed for 2 hours against 100 ml of 0.2 M potassium phosphate (pH 7.5), 20% glycerol, 1 mM dithiothreitol and 1 mM EDTA and applied to a column of Sephadex G-200 (0.9 x 35 cm) equilibrated with the same buffer. Fraction of 0.4 ml were collected at a rate of 2 ml per hour. Recoveries of both RNase H and dsDNase activities were 50%. o, RNase H; ●, dsDNase; x, RNase H/dsDNase ratio; ⇩ , Blue dextran marker; ⬇ , p-nitrophenol marker.

(3) Temperature sensitive T7 mutant of gene 6 induces heat-labile RNase H and dsDNase activities

RNase H and dsDNase activities also increase following infection of E. coli polAexl cells with T7 am 3+5, ts 6 phage, which carries a temperature sensitive mutation in gene 6. In the S100 fraction, both activities induced by this phage were more thermo labile than those induced by T7 am 3+5 phage. However, the results obtained with crude S100 fraction are not conclusive, since the fraction also contains RNase and DNase activities other than gene 6 exonuclease. Thus, we purified gene 6 exonuclease from E. coli polAexl cells infected with T7 am 3+5, ts 6 phage up to the phosphocellulose step to remove contaminating ssRNase and ssDNase activities. Because the enzyme was unstable at 30°C, enzyme reactions were performed at 20°C. The recoveries of RNase H and dsDNase activities during the purification were 7% and 10%, respectively, and about one hundred-fold purification of each activity was achieved. The ratio of the RNase H to the dsDNase activity in the phosphocellulose fraction was 1.74. As shown in Fig. 3(a), the mutant enzyme immediately lost RNase H activity as well as dsDNase activity during incubation at 30°C, while the wild-type enzyme was stable for 30 min at 30°C. The mutant enzyme was unstable even at 20°C (Fig. 3(b)). These results with mutant enzyme strongly support the previous conclusion that gene 6 exonuclease has an RNase H activity together with a dsDNase activity.

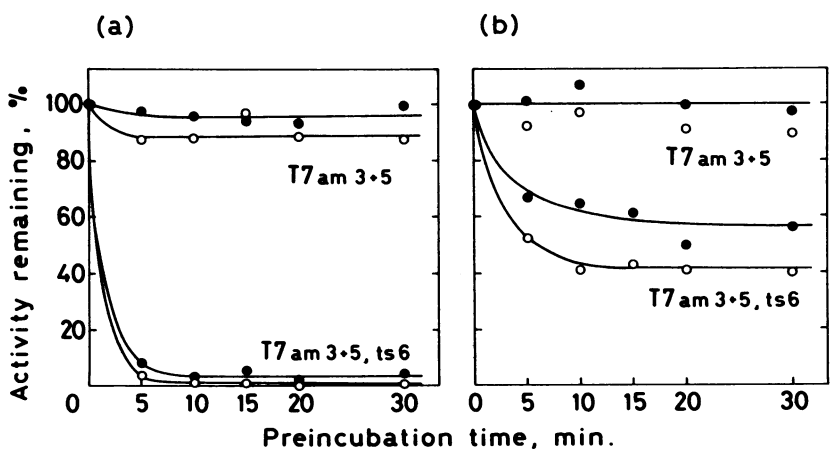

Fig. 3. Thermal inactivation of RNase H and dsDNase activities of gene 6 exonuclease purified from T7 am 3+5, ts 6- or T7 am 3+5-phage infected cells. The phosphocellulose fractions of gene 6 exonuclease were diluted 5 fold with the dilution buffer and incubated at 30°C (a) and at 20°C (b). At the times indicated samples were removed and assayed at 20°C for RNase H activity (o) and dsDNase activity (•).

(4) Reaction mechanism of the RNase H activity of gene 6 exonuclease

To determine whether gene 6 RNase H degrades exonucleolytically from the 5' terminus as does the dsDNase activity, we measured the production of acid-soluble radioactivity from [5'-^{32}P], [^{3}H]poly(A)·unlabeled poly(dT) hybrid polymer (Fig. 4). The ^{32}P label on the 5' terminus was rendered acid-soluble much faster than ^{3}H label; after a 5-min incubation at 30°C, 70% of the ^{32}P label become acid soluble, while only 10% of the ^{3}H label was rendered acid soluble. Therefore, the RNase H activity as well as the DNase activity of gene 6 exonuclease begins its exonucleolytic attack at the 5' terminus.

After 30-sec incubation at 30°C, both the ^{32}P and ^{3}H label released from the [5'-^{32}P], [^{3}H]poly(A)·unlabeled poly(dT) hybrid molecules were identified as 5' ribonucleoside monophosphates by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea (Fig. 5). The same result was obtained with the acid-soluble product obtained after 5-min incubation at 30°C where 87% of the ^{32}P and 21% of the ^{3}H were acid-soluble. Thus, all the ribonucleotides released from 5'-phosphorylated poly(A)·poly(dT) hybrid molecules are 5' ribonucleoside monophosphates.

We also used as substrates for the RNase H activity of gene 6 exonuclease φX174 RNA·DNA hybrid molecules that were produced by the in vitro transcrip-

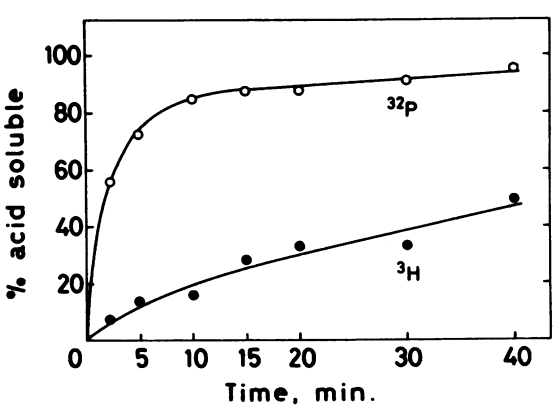

Fig. 4. Mode of action of gene 6 exonuclease on [5'-^{32}P],[^{3}H]poly(A)·poly(dT) hybrid polymer. Eight 50 µl reaction mixtures contained 50 mM Tris·HCl (pH 8.1), 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM KCl, 0.5 mg/ml bovine serum albumin, 30 µM [5'-^{32}P], [^{3}H]poly(rA) (^{32}P, 3 cpm/pmole; ^{3}H, 14.4 cpm/pmole; 460 nucleotides in average length), 90 µM poly(dT) (about 2,000 nucleotides in length), and 0.46 U RNase H. At the indicated times after incubation at 30°C, the acid-soluble radioactivity released was measured. Radioactivity (100%) for ^{32}P was 2,900 cpm and for ^{3}H was 19,000 cpm. o, ^{32}P; •, ^{3}H.

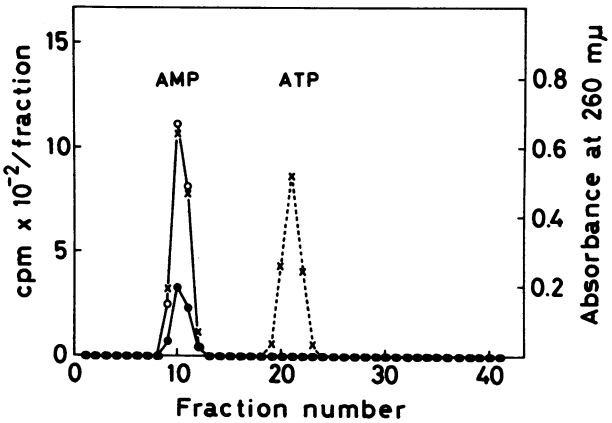

Fig. 5. DEAE-Sephadex column chromatography of acid-soluble products released from [5'-^{32}P], [^{3}H]poly(A)·poly(dT) hybrid molecules by gene 6 exonuclease. Reaction mixtures had the same composition as those in Fig. 4 except the enzyme concentration was 2.76 U of RNase H in a 300 μl reaction mixture. After 30-sec incubation at 30°C, reactions were stopped by the addition of 0.3 mg/ml salmon sperm DNA and 0.3 N perchloric acid. Acid-soluble materials and AMP and ATP absorbance markers were applied to a DEAE-Sephadex A-25 column, and eluted with a linear NaCl gradient (0 to 0.35 M) in 7 M urea as described in Methods. o, ^{32}P (19% acid soluble); •, ^{3}H (1.4% acid soluble); x, absorbance at 260 mμ (AMP and ATP).

tion of φX174 viral DNA with E. coli RNA polymerase. The RNA was labeled with ^{32}P at the γ position of 5' triphosphate end, and with ^{3}H in four bases. Gene 6 exonuclease degraded the RNA region of φX RNA·DNA hybrid up to 80%. Following heat denaturation of the φX174 RNA·DNA, the RNA was no longer attacked (Table 2). The radioactivity released from the φX174 [5' γ-^{32}P], [^{3}H] RNA·DNA hybrid was analyzed by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea (Fig. 6). Approximately 70% of the ^{32}P label co-chromatographed with an ATP marker, and 20% of the label eluted slightly earlier than ATP. Both components were desalted and further analyzed with paper electrophoresis in sodium citrate buffer (pH 3.5). The 70% portion of the label co-migrated with ATP and the 20% portion with pyrophosphate (data not shown). We did not detect any ATPase activity, which degrade ATP to AMP and pyrophosphate, in the hydroxyapatite fraction (data not shown). It is not clear, however, whether gene 6 exonuclease has polynucleotide pyrophosphatase activity or not.

(5) Substrate specificity of T7 gene 6 exonuclease

The specificity of T7 gene 6 exonuclease for various substrates was examined (Table 3). We tested labeled synthetic homopolymers alone or after

Table 2. Action of T7 gene 6 exonuclease on φX174 RNA·DNA hybrid molecule.

enzyme (μg)	% made acid soluble	
	φX174 RNA·DNA hybrid	heated φX174 RNA·DNA*
0.08	22	-
0.2	56	-
0.4	76	0.9
0.8	-	2.4

Reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 8.1), 1 mM dithiothreitol, 20 mM KCl, 0.5 mg/ml bovine serum albumin, heated or unheated φX174 [5' γ-^{32}P], [^{3}H] RNA·unlabeled DNA hybrid; 54.0 pmole of RNA and 270 pmole of DNA, and the indicated amounts of enzyme. After 15-min incubation at 30°C, the reaction was stopped and acid soluble radioactivity was measured.

* Heated at 90°C for 4 min and chilled quickly.

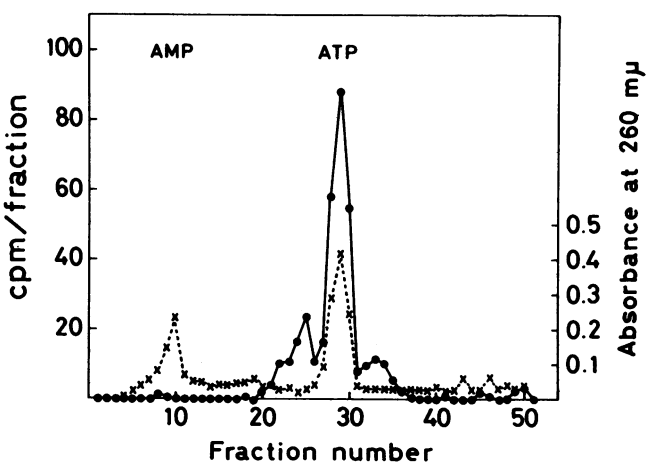

Fig. 6. Separation of acid-soluble products released from φX174 RNA·DNA hybrid by gene 6 exonuclease on DEAE-Sephadex in 7 M urea. The reaction mixture (2 ml) was the same as those in Table 2 except the enzyme concentration was 3.92 mg protein per ml. After 2-min at 30°C, the reaction was stopped by the addition of 0.3 mg/ml salmon sperm DNA and 0.3 N perchloric acid. Acid-soluble materials and AMP and ATP absorbance markers were applied to a DEAE-Sephadex A-25 column, and eluted with a linear NaCl gradient (0 to 0.3 M) in 7 M urea as described in Methods. ^{32}P radioactivity in each fraction was determined by measuring the Čerenkov radiation in a spectrophotometer. •, ^{32}P (55% made acid soluble); x, absorbance at 260 mμ (AMP and ATP).

Table 3. Substrate specificity of T7 gene 6 exonuclease.

Substrate	Activity (nmoles(P) released/mg protein)
[^{3}H] poly(A) + poly(dT)	88,800
[^{3}H] poly(A) + poly(U)	undetectable
[^{3}H] poly(A)	13
[^{3}H] poly(dA) + poly(dT)	363
[^{3}H] poly(dA)	19
[^{3}H] poly(dT) + poly(A)	15,400
[^{3}H] poly(dT)	16
ϕX174 [^{3}H] RNA·DNA hybrid	117
Heated ϕX174 [^{3}H] RNA·DNA	1
[^{3}H] E. coli native DNA	53,400
Heated [^{3}H] E. coli DNA	977
[^{14}C] δA alkali-treated DNA	48
[^{14}C] SP variant double-stranded RNA	10
CPV double-stranded RNA	undetectable

T7 gene 6 exonuclease was incubated with various polynucleotides at 30°C for 15 min, and acid-soluble radioactivity was measured. In experiment with unlabeled CPV RNA, absorbance at 260 mμ of acid-soluble material was measured. The concentration of substrates expressed as nucleotides in each case was the following: [^{3}H]poly(A) 30 μM, poly(dT) 90 μM, poly(U) 90 μM, [^{3}H]poly(dA) 30 μM, [^{3}H]poly(dT) 30 μM, poly(dA) 90 μM, ϕX174 [^{3}H] RNA·DNA 0.85 μM of RNA and about 4 μM of DNA, [^{3}H] E. coli DNA 100 μM, [^{14}C] δA alkali-treated DNA 50 μM, [^{14}C] SP variant RNA 60 μM, CPV RNA 100 μM.

annealing to its complementary unlabeled ribo- or deoxyribohomopolymers for sensitivity to T7 gene 6 exonuclease. A high activity was detected with poly(A) in the presence of poly(dT), while little activity was detected with poly(A) alone or poly(A) annealed to poly(U). Poly(dA) and poly(dT) were degraded in the presence of poly(dT) and poly(A), respectively. Little activity was detected with poly(dA) and poly(dT) alone. Thus, the T7 gene 6 exonuclease was shown to cleave DNA strand as well as RNA strand of RNA·DNA hybrids. With various substrates other than homopolymers, T7 gene 6 exonuclease was also shown to have nuclease activities to degrade double-stranded DNA and RNA strand of RNA·DNA hybrid, while little activity was detected with double-

stranded RNA, single-stranded RNA and single-stranded DNA (Table 3). Rather low RNase H activity detected with ϕX174 RNA·DNA hybrid may be due to the low concentration of RNA termini in the reaction. When 5'-^{32}P labeled T7 nascent short DNA containing RNA-linked and free DNA pieces in 1 to 2 ratio were annealed to T7 phage DNA (3, 32) and digested with the enzyme, [^{32}P] nucleotides were released from alkali-labile and alkali-resistant 5'-termini at similar rate. Using the short pieces alone, the rate of release of ^{32}P from the both termini decreased more than 20 fold, suggesting that the linked RNA (primer RNA) is in a complementary structure to the template strands.

DISCUSSION

Our previous studies have indicated that RNA primes the synthesis of nascent T7 DNA pieces and that the gene 6 exonuclease removes the RNA primer (6). The predicted RNase H activity of this enzyme has now been found as shown by the following observations. (1) The major portion of an RNase H activity was co-purified with a dsDNase activity in each step of purification (Table 1). (2) These dsDNase and RNase H activities chromatographed on Hydroxyapatite and on Sephadex G-200 column with a constant ratio of activity (Fig. 1 and Fig. 2). (3) T7 gene 6 exonuclease purified from cells infected with T7 phages carrying a temperature sensitive mutation in gene 6 had a thermo labile RNase H activity as well as a dsDNase activity (Fig. 3).

The reaction mechanism of the RNase H activity of T7 gene 6 exonuclease resembles that of the dsDNase activity: both enzyme activities initiate at the 5' terminus and liberate mononucleoside 5' monophosphates (Fig. 4 and Fig. 5; 16). This suggests that a single active site of the enzyme catalyzes the two types of enzymatic reactions. Gene 6 exonuclease can also degrade a 5'-triphosphate terminated RNA hydrogen bonded to DNA (Table 2) to a ribonucleoside 5' triphosphate (Fig. 6). The DNA strand of RNA·DNA hybrid molecule is also susceptible to nuclease attack while double-stranded RNA is not (Table 3). A previous study of the dsDNase activity showed that the initial products released from a phosphorylated 5' terminus and a 5' hydroxyl terminus are a deoxynucleoside 5' monophosphate and a dinucleoside monophosphate, respectively (16). The enzyme probably recognizes a nucleoside 5' phosphate located on the 5' terminus of a duplex DNA or DNA·RNA hybrid and hydrolyses the phosphodiester linkage at the 3' side.

Three RNase H activities in E. coli cell have been reported: One is an endonuclease specific to the RNA region in RNA·DNA hybrid molecule (27, 28, 29); it cannot cleave the phosphodiester bond joining ribonucleotides to DNA

(29). This enzyme might operate in the initial stage of removal of the E. coli RNA primer, but no direct evidence is available at present (3). Another RNase H activity is associated with DNA polymerase I and its action is exonucleolytic starting from 5' termini (14, 29, 10). In E. coli and P2 phage-infected E. coli, DNA polymerase I has been shown to function in the removal of the primer RNA (1, 2, 3, 30). The similarlity of the T7 gene 6 exonuclease and the 5'→ 3' exonuclease of DNA polymerase I can explain the partial compensation by DNA polymerase I for gene 6 exonuclease in the removal of T7 RNA primer (6). The third RNase H in E. coli is exonuclease III (31, 14). Since it digests in the 3'→ 5' direction, it is probably not involved in the removal of the primer RNA.

Under restrictive conditions for T7 gene 6 exonuclease and E. coli DNA polymerase I mutants, intact T7 primer RNA molecules were found (32). The T7 RNA primer is at most tetra to penta nucleotides in size and very rich in A and C residues (32, 33) and its synthesis starts with pppApC (32).

Abbreviations

dsDNase, DNase acting on double-strands; ssDNase, DNase acting on single-strands; ssRNase, RNase acting on single-strands.

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