Vol. 110, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 27, 1983 Pages 470-476

RIBONUCLEASE H FROM CHICK EMBRYOS CLEAVES PRECISELY AT THE JUNCTION BETWEEN THE RNA AND DNA PORTION OF THE HYBRID HELIX

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Received November 15, 1982

SUMMARY; DNA polymerase from Micrococcus luteus and RNA polymerase from E. coli catalyze the synthesis of poly(dA) with poly(dT) template, in the presence of ATP and [α-^{32}P]dATP. The reaction is completely dependent on poly(A) primer synthesis. Poly(A) chains are covalently extended by DNA polymerase. Primer poly(A) is linked to the product poly(dA) via a 3':5'-phosphodiester bond, and can be specifically removed by ribonuclease H from chick embryos, leaving a 5'-phosphate end of poly(dA). The length of RNA and DNA products appears to be relatively variable. The size of the DNA is less than 3 000 nucleotides.

Nascent discontinuous DNA fragments, Okazaki pieces, are formed as intermediates in the process of DNA replication (1). These fragments have a covalent attached short RNA molecule which is presumed to serve as primer (2, 3) and has been characterized for mammalian cells both in vivo (4) and in vitro (5-7) as an 8-11-nucleotide RNA sequence. Recently, DNA polymerase associated with primase activity was shown to synthesize DNA following synthesis of initiator RNA in Drosophila melanogaster embryos (8), human lymphocytes (8, 9) and Ehrlich ascites tumor cells (10).

However, no enzyme capable of removing the RNA primer has been reported in eukaryotic cells, although a 5' → 3' exonuclease was shown to remove the RNA in E. coli. Earlier, we have shown that ribonuclease H (RNase H), which specifically degrades the RNA portion in RNA·DNA hybrid structures, might perform this function in enkaryotic cells (11-13). At least two RNases H are present in animal cells: a Mg^{2+}-dependent RNase H and a Mn^{2+}-dependent RNase H,

0006-291X/83/020470-07$01.50/0

distinguished from one another by chemical and physical properties (14, 15). Recently, we have purified these enzymes from chick embryo extracts, and have shown that the properties of these enzymes are similar to those of rat liver nuclei (16). This communication describes the mode of cleavage of the RNA part of the RNA-DNA chains, using a poly(A)-poly(dA) homopolymer and RNase H from chick embryos.

MATERIALS AND METHODS

[^{3}H]ATP (25 Ci/mmol) and [α-^{32}P]dATP (400 Ci/mmol) were from Radiochemical Centre. Poly(dT) was from Miles Laboratories, Inc.

RNA polymerase (17) from E. coli K-12 (specific activity 1 x 10^{3} units/mg protein) and DNA polymerase (18) from Micrococcus luteus (specific activity at least 150 units/mg protein) were purchased from Sigma Chemical Co. and Miles Laboratories Inc. respectively.

Mg^{2+}- and Mn^{2+}-dependent RNases H were purified from chick embryos as described previously (16). The most highly purified enzymes were used in each experiment.

Protein was determined by the method of Lowry et at. (19) with bovine serum albumin as standard.

Poly(dT)·poly(A)-[^{32}P]poly(dA) hybrid was prepared in 1.0 ml of a solution containing 9% glycerol, 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μg/ml of poly(dT), 18 μM [α-^{32}P]dATP (25 μCi), 65 μM ATP, 7.2 units/ml of RNA polymerase and 5.8 units/ml of DNA polymerase. After incubation at 37°C for 60 min, each reaction mixture was extraced with phenol-chloroform (1:1) saturated with 1 M Tris-HCl (pH 7.8). The phenol-chloroform was subsequently removed by extraction with ether. The hybrid isolated was thoroughly dialyzed against 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5) and 0.1 mM EDTA.

RESULTS AND DISCUSSION

When homopolymer template poly(dT) was incubated with ATP or [α-^{32}P]dATP, in the presence of DNA polymerase and RNA polymerase, poly(dA) synthesis showed a complete requirement for poly(A) synthesis, as shown in Table I. To detect RNA priming activity, RNA polymerase and DNA polymerase were examined for the capacity to form poly(A)-poly(dA) junctions. This was assayed by the transfer of ^{32}P label from incorporated [α-^{32}P]dATP to 2'(3')-[^{32}P]AMP after hydrolysis in alkali. With poly(dT) as template, transfer from [α-^{32}P]dATP was observed to 2'(3')-AMP as shown in Fig. 1. The only labeled spot corresponding to 2'(3')-AMP area by autoradiography after analyzing two-dimensional chromatography on polyethyleneimine thin layer plates was detected. These results indicate that the

Table I

Requirement for poly(dA) synthesis by DNA polymerase from Micrococcus luteus and RNA polymerase from E. coli with poly(dT)

Component	Poly(dA) synthesis
	cpm
Complete	43 901
-Poly(dT)	11
-ATP	8
-RNA polymerase	4

The complete reaction mixture (100 µl) contained 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 9% glycerol, 48.5 µM ATP, 18.2 µM/0.9 µCi [α-^{32}P]dATP, 2.5 µg of poly (dT), 0.67 unit of RNA polymerase and 0.5 unit of DNA polymerase. After incubation at 37°C for 60 min, 5% trichloroacetic acid precipital material was washed extensively on a glass filter (Whatman GF/C) with 5% trichloroacetic acid/10 mM sodium pyrophosphate and ethanol, the filters were counted in toluene-based scintillation fluid.

primer poly(A) is linked to the product poly(dA) via 3':5'-phosphodiester bond.

Incubation of the poly(dT)·poly(A)-[^{32}P]poly(dA) homopolymer with Mg^{2+}-dependent RNase H from chick embryos resulted in a marked decrease in the labeled 2'(3')-AMP. These results show that RNase H attacked a phosphodiester link between AMP and dAMP, leaving 5'-phosphate-poly(dA).

Poly(dT)·[^{3}H]poly(A)-poly(dA) or poly(dT)·poly(A)-[^{3}H]poly(dA) was synthesized with RNA- and DNA polymerases. The partial hybrid was then purified by phenol-chloroform extraction. When the products were denatured and subjected to electrophoresis in 4% polyacrylamide slab gels, the polynucleotides synthesized with [^{3}H]ATP had the same mobility as those synthesized with [^{3}H]dATP (Fig. 2, lines 1 and 3). On treatment with Mg^{2+}-dependent RNase H, the polynucleotides synthesized with [^{3}H]ATP was no longer detectable by autoradiography, while those synthesized in the presence of [^{3}H]dATP are smaller than those synthesized in the absence of the RNase

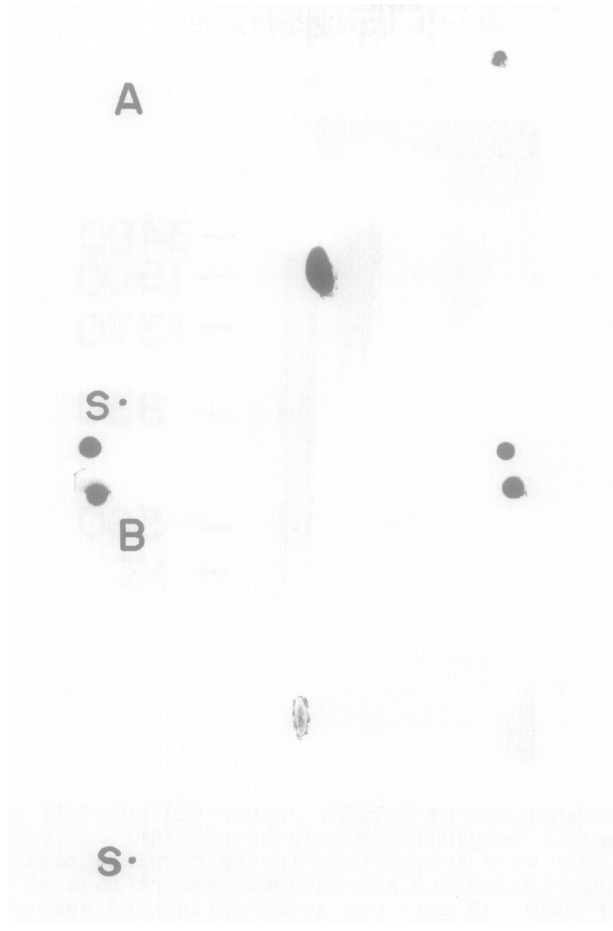

Fig. 1. Radioautogram of two dimensional chromatography of acid-soluble material after alkaline hydrolysis of poly(dT)·poly(A)-[^{32}P]poly(dA) incubated with Mg^{2+}-dependent RNase H. Poly(dT)·poly(A)-[^{32}P]poly(dA) containing 3 x 10^6 cpm of ^{32}P, was incubated with 100 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 300 µg of bovine serum albumin, with or without 5 units of Mg^{2+}-dependent RNase H from chick embryos, for 2 h at 37°C in total volume of 3.0 ml containing 0.1 M NaCl. The reaction was stopped by the addition of 0.1 M EDTA, 0.1 ml of bovine serum albumin (10 mg/ml) and 5 ml of 10% trichloroacetic acid. After centrifugation, the precipitate was suspended in 0.8 ml of 0.5 M KOH and the mixture was shaken. After the addition of 10 ml of 5% trichloroacetic acid, the precipitate was resedimented by centrifugation and the process was repeated twice more. The acid-washed precipitate was incubated for 18 h at 37°C in 3.0 ml of 0.3 M KOH. 50 nmol each 2'- and 3'-AMP was added to the supernatant and 300 µl of 3 M perchloric acid was added to get the acid soluble fraction. The resulting supernatant after centrifugation was neutralized with KOH. The material was subjected to two-dimensional chromatography on a polyethyleneimine-cellulose thin-layer (20 x 20 cm sheet) (20). The sample was applied to the starting point S, and the chromatogram was developed in the first dimension (from left to right) with 1.0 M acetic acid, up to 2 cm, and with 1.0 M acetic acid-3.0 M LiCl (9:1, v/v) up to 16 cm, and in the second dimension (from bottom to top) with the solution (100 ml) containing 6g of $Na_2B_4O_7 \cdot 10H_2O$, 3g of H_3BO_3 and 25 ml ethylene glycol. The chromatogram was exposed for 2 days to Kodak RPR X-ray film. Dark spot and the dotted line indicate the position of the corresponding 2'(3')-AMP marker. A, no RNase H; B, plus RNase H.

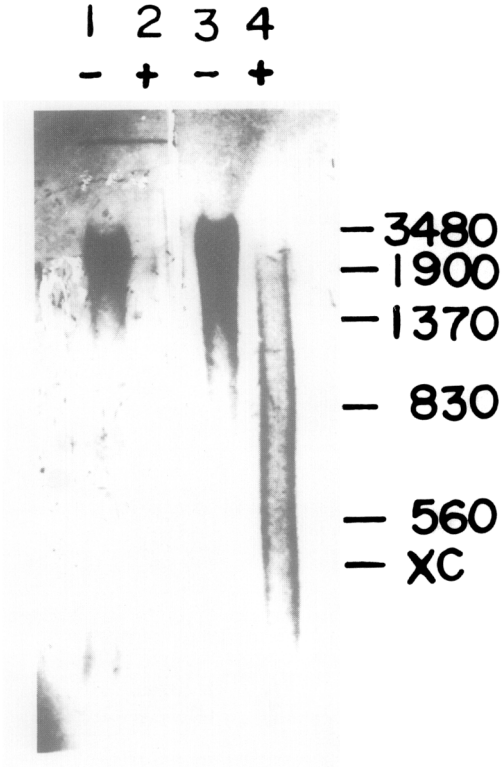

Fig. 2. Size analysis of poly(A) primer and poly(dA) on polyacrylamide gels. Poly(dT)·[^{3}H]poly(A)-poly(dA) and poly(dT)·poly(A)-[^{3}H]poly(dA) were synthesized in the complete reaction mixture described under "MATERIALS AND METHODS" except that 65 μM ATP (25 μCi) or 18 μM dATP (25 μCi) was added as labeled substrates, and purified by phenol-chloroform extraction. Each product was treated with Mg^{2+}-dependent RNase H as described in Fig. 1. After incubation at 37°C for 2 h, the reaction was stopped by heating at 100°C for 5 min and dialyzed against 1 mM EDTA, then the samples were dried, and dissolved in 20 μl of 36 mM Tris/32 mM KH_2PO_4/1 mM EDTA (adjusted the pH to 7.8 by KOH), 7 M urea and 0.05% xylene cyanol (XC) and heated at 100°C for 1 min. The denatured sample was electrophoresed on a 4% polyacrylamide gel in 7 M urea. Chain length markers were prepared from λDNA by digestion with EcoR1 and HindIII and detected by staining with ethidium bromide.
Lanes 1 and 2, poly(dT)·[^{3}H]poly(A)-poly(dA); lanes 3 and 4, poly(dT)·poly(A)-[^{3}H]poly(dA). In 3, and 4, 3-times more radioactivity than that in 1, and 2, was applied. +, with RNase H; -, no RNase H.

H (Fig. 2). These findings suggested that polyriboadenylate synthesis provide a 3'-hydroxyl terminated primer that can be elongated by DNA polymerase action, and confirmed the results in Fig. 1 regarding the action of RNase H. Same results were obtaine when Mn^{2+}-dependent RNase H was used.

The primer RNA synthesized by the associated primase was shown to be an oligonucleotide 7-11 nucleotides long by several investi-

gators studying chromosomal DNA replication in intact cells (4) and in isolated nuclei (6), during papovavirus DNA replication in isolated nuclei of infected cells (5, 7), in Ehrlich ascites tumor cells (10) and in human lymphocytes (9). RNase H from rat liver (14, 21) and brain (15), plant cells (22) and chick embryos (16) cleaves the RNA endonucleolytically, producing a series of oligonucleotides three to 9 bases long having a 5'-phosphate and a free 3'-hydroxyl end groups. The 5'-termini of the DNA, after removal of priming RNA, also carry the 5'-phosphate. Thus RNase H shows precisely the characteristics expected of a nuclease whose *in vivo* function is to remove priming RNA pieces from nascent DNA chains. From these results, RNase H might be involved in the removal of the RNA primer portion of newly synthesized DNA chains, allowing elongation by the DNA polymerase and DNA ligase.

ACKNOWLEDGEMENTS

This investigation was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

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