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Degradation of DNA RNA Hybrids by Ribonuclease H and DNA Polymerases of Cellular and Viral Origin

(RNA tumor viruses/DNA synthesis)

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ABSTRACT **Ribonuclease H from human KB cells, chick embryos, calf thymus, avian myeloblastosis virus, and Rous associated virus specifically degrades the RNA of DNA·RNA hybrids, producing mono- and oligoribonucleotides terminated in 5′-phosphates. The cellular RNase H is an endonuclease, whereas the viral enzyme appears to be an exonuclease. Viral DNA polymerase and RNase H copurify through all separation steps. Therefore, RNase H activity is an intrinsic part of the viral DNA polymerase. DNA·RNA hybrids are also degraded by nucleases associated with cellular DNA polymerases and by exonuclease III. However, these nucleases differ from RNase H in their ability to degrade *both* strands of DNA·RNA hybrids.**

Ribonuclease H (RNase H), an enzyme originally discovered in calf-thymus tissue, specifically degrades the RNA strand of DNA·RNA hybrids to acid-soluble products (1, 2). Recently, Mölling *et al.* (3) demonstrated an RNase H activity in partially purified DNA polymerase preparations of avian myeloblastosis virus (AMV), and suggested a possible involvement of this nuclease in the RNA-directed synthesis of viral DNA. As we reported earlier (4), RNase H can remove 5′-terminal RNA, covalently linked to DNA, that had been synthesized *in vitro*. On this basis, we suggested a possible role of RNase H-type activities in RNA-primed DNA synthesis *in vivo*. In the model of Mölling *et al.* (3), RNase H was conceived to degrade the template RNA after its transcription into RNA·DNA hybrid, releasing single-stranded DNA, which could then be converted into double-stranded product DNA.

As a first step in testing this hypothesis, we determined the specificity and mode of action of the RNase H found in avian RNA tumor viruses and compared these properties with those of RNase H from uninfected cells. In addition, we attempted to decide whether the nuclease activity associated with the viral DNA polymerase constitutes an intrinsic part of this enzyme or whether it represents contaminating host-cell RNase H.

MATERIALS AND METHODS

Reagents. Unlabeled ribonucleoside triphosphates and deoxyribonucleoside triphosphates came from Calbiochem and Schwartz/Mann BioResearch, respectively; [α-^{32}P]ATP was purchased from New England Nuclear Corp., and [α-^{32}P]-dATP and [α-^{32}P]dTTP were from International Chemical and Nuclear Corp. Poly(dA)·oligo(dT) and poly(rA)·oligo(dT) were prepared by incubation of poly(dA) or poly(rA) (both from Miles Laboratories) at a concentration of 0.2 mg/ml with 0.05 mg/ml of oligo(dT) (chain length: 12–18 nucleotides, Collaborative Research, Inc.) in 0.1 M NaCl–1 mM Na-EDTA (pH 7.6), for 30 min at 25°.

Enzymes. DNA-Dependent RNA polymerase from *Escherichia coli* was purified by the procedure of Berg *et al.* (5), and was a gift from Dr. Michael Cashel. *Exonuclease III* from *E. coli* (6, 7) was a gift from Dr. Malcolm Gefter. *DNA polymerase I* from *E. coli* [fraction VII of Jovin *et al.* (8)] was purchased from Boehringer Mannheim Corp. *DNA polymerase I*, (9, 10), *DNA polymerase II*, (9, 11), and *RNase H* from KB cells, as well as RNase H from 9-day-old chick embryos (Truslow Farms Inc., Chestertown, Md.), were purified as described (4). Both preparations of RNase H were about 50% pure as judged by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (12). *DNA polymerase* (RNA-dependent) from avian myeloblastosis virus (kindly provided by Drs. Dorothy and J. Beard) was isolated and purified, essentially as described by Kacian *et al.* (13), by chromatography on DEAE-cellulose, phosphocellulose, and Sephadex G-150. *DNA polymerase* (RNA-dependent) from Rous associated virus (RAV-1) (a gift from Dr. John Bader) was extracted as described (13), and was purified by sucrose gradient sedimentation and chromatography on phosphocellulose. *RNase H* from calf thymus was a gift from Dr. Roy C. Haberkern.

Substrates. Poly(dT)·poly([^{32}P]rA) was synthesized in a reaction mixture containing in 0.5 ml: 0.05 M Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM [α-^{32}P]ATP (1.2 Ci/mmol), 50 μg of poly(dT), 50 μg of DNA-dependent RNA polymerase (*E. coli*), and 5% of glycerol.

Poly([^{32}P]dT·(rA) was synthesized in a reaction mixture containing in 0.5 ml: 0.05 M Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 0.02 M KCl, 1 mM dithiothreitol, 0.04 mM [α-^{32}P]dTTP (1 Ci/mmol), 5 μg of poly(rA)·oligo(dT), about 10 μg of RNA-dependent DNA polymerase (AMV), and 5% of glycerol.

Poly([^{32}P]dT·(dA) was synthesized in a reaction mixture containing in 0.5 ml: 0.05 M Tris·HCl (pH 7.9), 5 mM $MgCl_2$, 0.06 M KCl, 1 mM dithiothreitol, 0.04 mM [α-^{32}P]dTTP (1 Ci/mmol), 5 μg of poly(dA)·oligo(dT), 20 μg of KB DNA polymerase II, and 5% of glycerol.

After incubation for 30 min at 37°, the reaction mixtures were extracted with phenol (saturated with 1 M Tris·HCl,

Abbreviations: AMV, avian myeloblastosis virus; RAV, Rous associated virus.

pH 7.5) and chloroform–isoamylalcohol 24:1. The material in the aqueous phase was precipitated with ethanol, and was purified on a 1 × 25 cm column of Sephadex G-75 equilibrated with 0.05 M NaCl–0.01 M Tris·HCl (pH 7.5)–0.1 mM EDTA.

Covalently-closed, circular DNA of the colicinogenic factor E_1 (*Col* E_1) of *E. coli* was a kind gift of Drs. Peter Williams and Donald Helinski. The DNA was extracted from chloramphenicol-treated cells. About 70% of the molecules contained one or more ribonucleotides inserted at a single site in one of the two complementary strands (14). The *Col* E_1 DNA was labeled with ^{32}P and had a specific activity of about 100,000 cpm/μg.

RESULTS

Mode of Action of Cellular and Viral RNase H Activities. Purified RNase H from KB cells or chick embroys has a molecular weight of about 70–90,000, as determined by zone sedimentation in sucrose gradients. It requires Mg^{++} as a divalent cation; KCl or NaCl above a concentration of 0.15 M are strongly inhibitory. When assayed with poly(dT)·poly(rA) as substrate, our preparations degraded 2 to $6 \cdot 10^6$ pmol of poly(rA) per mg of protein in 10 min. The cellular RNase H was completely free of any DNA polymerase activities. [DNA polymerase assays were performed with 'activated' calf-thymus DNA, poly(dA)·oligo(dT), and poly(rA)·oligo(dT) as template-primers.] The RNase H activity associated with the RNA-dependent DNA polymerase of AMV and RAV also requires Mg^{++} and is inhibited by KCl or NaCl above 0.05 M. Our most purified preparations degraded 1 μmol of poly(rA) per mg of protein in 10 min.

When the synthetic DNA·RNA hybrid poly(dT)·poly([^{32}P]rA) was used as substrate for RNase H from KB cells, the pattern of fragments produced is shown in Fig. 1 (panel *1*). In this electrophoretic separation, oligonucleotides are distributed according to their chain length (5′-rAMP being the product of greatest mobility). The length of the fragments resolved with this technique ranged from mononucleotides to oligonucleotides containing up to eight bases. These oligonucleotides were not made with equal frequency; mononucleotides were always found in much lower molar quantities than oligonucleotides two, three, and four bases in length. This frequency distribution was independent of enzyme concentration and time of incubation. Upon prolonged treatment, all of the RNA of DNA·RNA hybrids was converted to oligonucleotides. As well as with RNase H from KB cells, we have observed this characteristic pattern of degradation products with RNase H from chick embryos and calf thymus and with the DNA polymerases from AMV and RAV (Fig. 1).

In Fig. 2, the specificity of the cellular and viral RNase H activities has been compared with that of exonuclease III from *E. coli* with the synthetic polymers poly(dT)·poly([^{32}P]rA), poly([^{32}P]dT·poly(rA), and poly([^{32}P]dT)·poly-

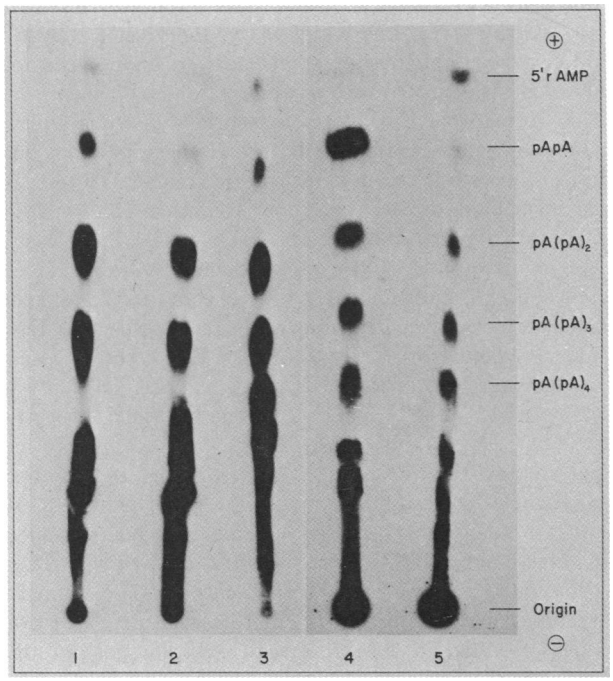

FIG. 1. Products formed after treatment of poly(dT)·poly([^{32}P]rA) with RNase H from various sources. Reaction mixtures contained in a total volume of 25 μl: 0.05 M Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 M NaCl, about 10^4 cpm of poly(dT)·poly([^{32}P]rA), and enzyme as indicated below. After incubation at 37° for 30 min the reaction mixtures were spotted on 20 × 40 cm polyethyleneimine cellulose thin-layer sheets (Brinkmann Inc.) The sheets were sprayed with 0.1 M K-PO_4–7 M urea (pH 7.5) and electrophoresed at 1500 V for 2 hr under Varsol. (A detailed description of this procedure will be published elsewhere.) Panel *1* = 0.2 μg of KB RNase H, panel *2* = 0.35 μg of chick-embryo RNase H, panel *3* = 0.5 μg of calf-thymus RNase H, panel *4* = about 0.1 μg of AMV DNA polymerase, panel *5* = 0.01 units of RAV DNA polymerase [1 unit = 1 nmol TMP incorporated in 30 min at 37° when assayed with poly(dAT) as template].

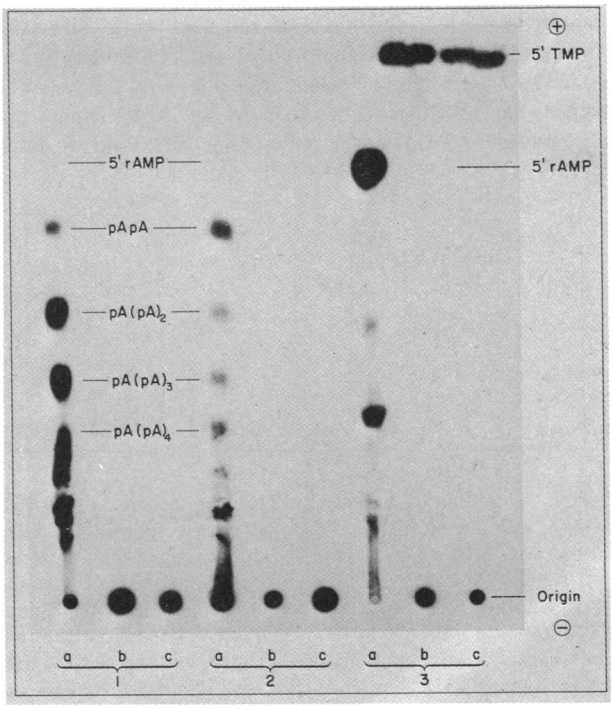

FIG. 2. Specificity of cellular and viral RNase H. Reaction conditions were as described in the legend to Fig. 1. Reaction mixtures contained in panels (*a*): poly(dT)·poly([^{32}P]rA), in panels (*b*): poly([^{32}P]dT)·poly(rA), and in panels (*c*): poly([^{32}P]-dT·poly(dA). Group 1 = KB RNase H, group 2 = AMV DNA polymerase, group 3 = *E. coli* exonuclease III.

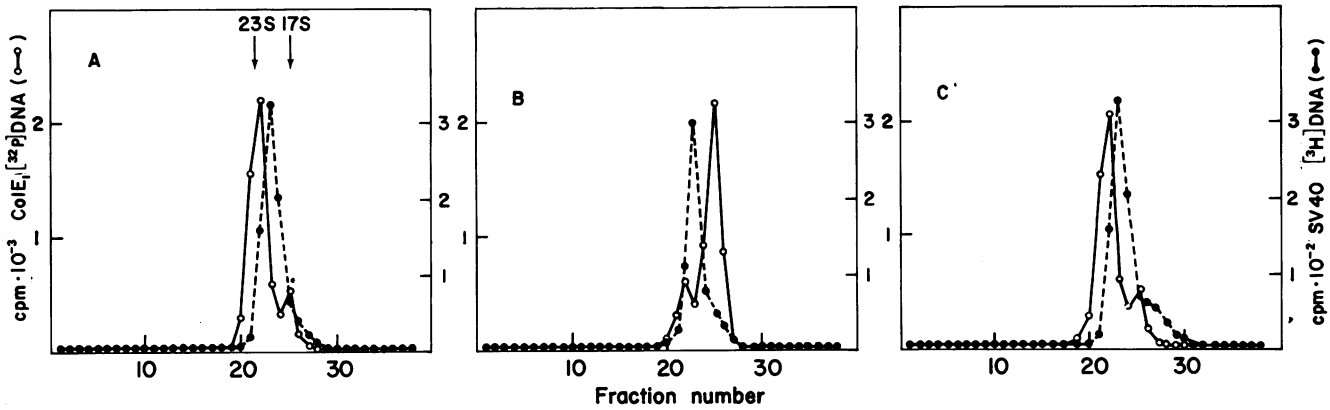

FIG. 3. Sedimentation analysis of the DNA of the plasmid *Col* E_1 after treatment with cellular and viral RNase H. Incubation mixtures contained in 0.1 ml: 0.05 M Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 20 mM NaCl, about 3000 cpm [^{32}P]DNA of *Col* E_1 from *E. coli* grown in the presence of chloramphenicol (14), about 600 cpm of SV40 [^{3}H]DNA, and enzyme. After incubation for 30 min at 37°, 0.1 ml of 0.1 M Na-EDTA (pH 7.5) was added and the mixtures were layered on 5–20% sucrose gradients containing 0.05 M Tris·HCl (pH 7.9)–0.55 M NaCl–55 mM EDTA. The gradients were centrifuged for 3 hr at 45,000 rpm and 15° in a Spinco SW56 rotor. Fractions were collected from the bottom of the centrifuge tube and counted in 'Aquasol'. Panel *A:* no enzyme, panel *B:* incubation with 0.1 μg of chick-embryo RNase H, panel *C:* incubation with 8 μg of AMV DNA polymerase.

(dA) as substrates. The RNase H from KB cells and AMV exclusively degraded the RNA strand of synthetic hybrids. Exonuclease III, in contrast, could attack *both* the DNA and the RNA of these hybrids. In addition, exonuclease III produced mainly mononucleotides as products.

To determine whether the cleavage by RNase H activities occurs at the 5′ or the 3′ side of the phosphodiester bond, we subjected the oligonucleotides formed after incubation of poly(dT)·poly(rA) to alkaline hydrolysis and subsequent chromatography. As illustrated in Table 1, the oligonucleotides generated by both cellular and viral RNase H were terminated with a 5′-phosphate, as revealed by the formation of pAp and Ap upon alkaline hydrolysis. The production of 5′-phosphate-terminated oligonucleotides by RNase H activities has also been demonstrated by D. Baltimore and D. F. Smoler for AMV DNA polymerase (21a) and by R. C. Haberkern and G. L. Cantoni for the calf-thymus enzyme (personal communications). Using instead of the homopolymer hybrid poly(dT)·poly(rA), DNA·RNA hybrid made with DNA of phage φX174 as template, we found that RNase H could cleave phosphodiester bonds next to any of the four ribonucleotides (unpublished results).

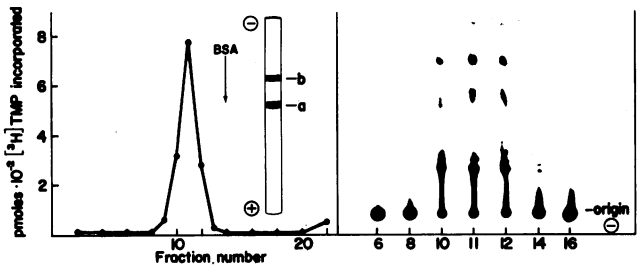

FIG. 4. Cosedimentation of viral DNA polymerase and RNase H. About 0.2 mg of AMV DNA polymerase (phosphocellulose fraction) was layered on a gradient containing 5–20% sucrose, 0.2 M KPO_4 (pH 7.5), 2 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol and centrifuged for 19 hr at 55,000 rpm in a Spinco SW56 rotor at 4°. 0.2-ml Fractions were collected from the bottom of the tube. DNA-polymerase activity was assayed (4) with poly(rA)·oligo(dT) as template. RNase H was detected in 2-μl aliquots as described in the legend to Fig. 1. A 0.2-ml aliquot of the combined peak fractions was subjected to polyacrylamide (5%) gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (12). The gel pattern obtained is shown in the *insert* of the left panel. *BSA* indicates the position of bovine-serum albumin, centrifuged in a separate tube.

The formation of oligoribonucleotides as degradation products by cellular and viral RNase H suggested that both activities operate in an endonucleolytic manner. To test this mechanism more directly, we took advantage of the closed-circular superhelical DNA of the plasmid *Col* E_1 as a substrate for RNase H. When this plasmid replicates in the presence of chloramphemicol, its DNA contains one or more ribonucleotides covalently inserted in one of the two DNA strands, as shown by Blair *et al.* (14). Fig. 3 shows a sedimentation analysis of *Col* E_1 DNA that had been treated with chick-embryo RNase H (panel *B*) or the RNase H associated with AMV DNA polymerase (panel *C*). The intact, covalently-closed *Col* E_1 DNA (4.2×10^6 daltons) sediments at 23 S, slightly faster than superhelical SV40 DNA (3×10^6 daltons), used as a marker (Fig. 3, panel *A*). Upon treatment with chick-embryo RNase H, about 70% of the *Col* E_1 DNA was converted into a form that sedimented at 17 S, which is characteristic of open circles. Incubation with RNase H from KB cells resulted in the same sedimentation pattern (not shown).

(Alkali treatment of the same *Col* E_1 DNA preparation gave the identical result, indicating that 70% of the DNA molecules contained ribonucleotides.) In contrast to the cellular RNase H, the nuclease associated with viral DNA polymerase did not convert any of the *Col* E_1 DNA into the open-circular form even when used at very high concentrations. On this basis we conclude that the viral RNase H requires RNA with free ends to initiate its nucleolytic action and appears, therefore, to be an exonuclease. Leis *et al.* (28) using a different approach, came to the same conclusion. The results illustrated in Fig. 3 also indicate that both cellular and viral enzymes are completely free of DNase (endonuclease) activity that would convert superhelical SV40 DNA into a slower-sedimenting open-circular form.

TABLE 1. *The presence of 5′ phosphate in oligoribonucleotides produced by treatment of poly(dT)·poly([^{32}P]rA) with RNase H from various sources*

Source of RNase H	Radioactivity moving from origin after digestion of poly(dT)·poly(rA) (%)	Fraction of ^{32}P in pAp (%)
KB cells	97	37
Calf thymus	99	33
Chick embryos	99	29
AMV	51	8
RAV	46	4
None	0	0

The reaction conditions were as described in Fig. 1, except that the concentration of enzyme from KB cells, calf thymus, and chick embryos was tripled. After 30 min at 37°, 5 μl of 4 N KOH was added to the reaction mixtures and the samples were incubated at 37° for 18 hr. After neutralization of the mixture with 4 N $HClO_4$, Ap was separated from pAp by two-dimensional chromatography on polyethyleneimine thin layers by the LiCl-NaCOOH method (27). The compounds were localized by autoradiography and their radioactivity was measured in a liquid scintillation counter.

Relationship of Viral RNase H to the Host Enzyme. As shown above, the cellular RNase H differs from the viral enzyme by its ability to cleave superhelical *Col* E_1 DNA containing inserted ribonucleotides. To demonstrate further that the viral RNase H activity was not simply due to contamination by cellular enzyme, we purified the virus-associated nuclease by chromatography on DEAE-cellulose and phosphocellulose, and by sucrose gradient sedimentation, essentially following the procedure of Kacian *et al.* (13) for the purification of AMV DNA polymerase. Throughout these steps, the profile of RNase H activity followed closely that of the DNA polymerase (Fig. 4). Upon analysis by polyacrylamide electrophoresis in the presence of 0.1% sodium dodecyl sulfate, our purified enzyme preparation revealed two polypeptides with molecular weights of about 100,000 and 70,000, in accord with the report of Kacian *et al.* (13) (Fig. 4). These authors presented strong evidence that the two polypeptides represent subunits of the viral DNA polymerase. Therefore, it is very likely that the viral RNase H activity also resides within one of these two subunits. The cellular and viral RNase H activities could also be clearly distinguished by their different sedimentation rates in sucrose gradients. The viral DNA polymerase–RNase H complex sediments at 7 S, whereas the host RNase H sediments at 5 S (results not shown). We can thus conclude that the RNase H activity constitutes a specific component of viral DNA polymerase.

Degradation of DNA·RNA Hybrids by Cellular DNA Polymerases. The discovery that AMV DNA polymerase is associated with RNase H (3), and our finding that the host RNase H and the DNA polymerase-associated nuclease from AMV produce similar products, led us to investigate other DNA polymerases for RNase H activity. Fig. 5 shows that DNA polymerase I of *E. coli* and DNA polymerases I and II from KB cells were all capable of degrading the RNA strand

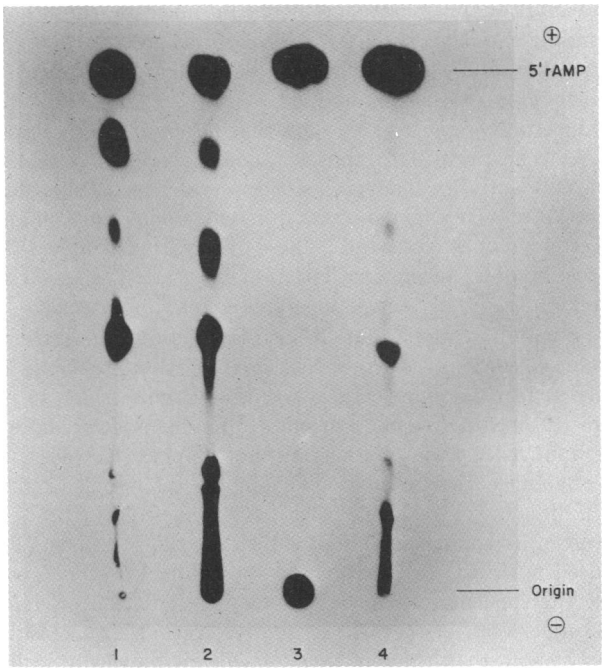

FIG. 5. Degradation of poly(dT)·poly([^{32}P]rA) by various DNA polymerases and exonuclease III. Reaction mixtures were as described in the legend to Fig. 1. Panel *1* = 2 μg of *E. coli* DNA polymerase I, panel *2* = 5 μg of KB DNA polymerase I, panel *3* = 5 μg of KB DNA polymerase II, panel *4* = 5 μg of *E. coli* exonuclease III.

of poly(dT)·poly([^{32}P]rA). It is interesting to note that *E. coli* DNA polymerase I and KB DNA polymerase I generated, in addition to mononucleotides as main products, oligoribonucleotides up to four bases long. The ability of *E. coli* DNA polymerase I to degrade DNA·RNA hybrids has also been observed by L. Bertsch and A. Kornberg (personal communication). The degradative processes observed with cellular DNA polymerases differed from those observed with cellular and viral RNase H in two important aspects: (*i*) these enzymes were not specific for the RNA strand of DNA·RNA hybrids, but degraded the DNA strand as well (results not shown) and (*ii*) the products formed were mainly mononucleotides. The latter finding suggests an exonucleolytic mode of action, similar to that shown for exonuclease III of *E. coli* (Figs. 2 and 5).

DISCUSSION

Mölling *et al.* (3) found that when an extract of AMV was chromatographed on DEAE-sephadex or subjected to zone

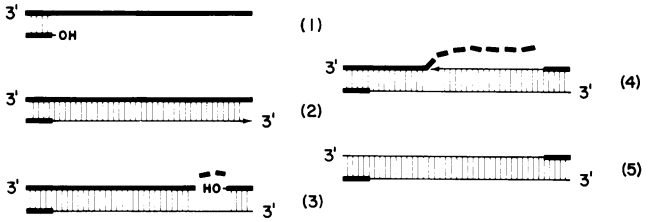

FIG. 6. Hypothetical involvement of RNase H activities in the synthesis of double-stranded DNA on viral RNA as template. See text for explanations. *Thick-stroke* lines represent RNA, *thin-stroke* lines represent DNA.

sedimentation in sucrose gradients, a nuclease capable of degrading the RNA of DNA·RNA hybrids accompanied the peaks of viral DNA polymerase. These activities however, were not more extensively purified.

Our results presented here show that DNA polymerase from AMV, when purified to apparent homogeneity, still retains RNase H activity. The viral nuclease can be distinguished from the cellular enzyme by its different mode of action and its faster sedimentation rate. These observations support the suggestion of a specific association of RNase H-type activity with viral DNA polymerase, as proposed by Mölling *et al.*

The inability of the viral RNase H to cleave the internal ribonucleotides of *Col* E_1 DNA suggests that its mode of action must be exonucleolytic i.e., the enzyme seems to require RNA with free ends to initiate its degradative action. The finding that the products are not mononucleotides, but are oligonucleotides of various chain lengths, seems at first sight difficult to reconcile with an exonucleolytic process. However, as has recently been shown for the coli-phage T5-induced nuclease (15) and for the *rec* BC nuclease of *E. coli* (16), oligonucleotides can be the main degradation products of exonucleases. As for the cellular RNase H, our results clearly show that this enzyme *can* act endonucleolytically, but we cannot exclude the possibility that it also has an exonucleolytic activity. It is conceivable that the enzyme makes endonucleolytic cleavages and then proceeds along its substrate as an exonuclease.

It is not clear where the RNase H that is associated with the viral DNA polymerase originates. It is conceivable that it is derived from the host RNase H, possibly in a manner analogous to Qβ-replicase, where only one of the four subunits of the enzyme is specified by the phage, the other three subunits being host proteins [see Blumenthal *et al.* (17) for references]. If this mechanism is correct, we must assume that the host RNase H loses its endonuclease function as a result of association with the viral DNA polymerase. On the other hand, it is equally possible that the viral RNase H is a new activity, unrelated to the host enzyme. We are currently doing reconstitution experiments on the subunits of the viral and cellular enzyme in order to obtain a more accurate correlation.

The mechanism of double-stranded DNA synthesis in virus-infected cells is still not understood. How could RNase H-type activities be involved in this process? Experiments with detergent-treated virus have shown that the reaction proceeds in two stages. First, the viral RNA is transcribed into a RNA·DNA hybrid (18–20). Low molecular weight RNA, complementary to the 70S viral RNA, seems to serve as a primer in this reaction (21*b*, 22). In the second stage, the newly synthesized DNA strand is copied, resulting in double-stranded DNA as final product. It is at this stage of the reaction that we would suggest the participation of both cellular and viral RNase H activities. As shown in the diagram of Fig. 6, cellular RNase H could introduce a gap (gaps) near the 5′-end of the template RNA after it has been copied into an RNA·DNA hybrid. This would create new primers with free 3′-hydroxyl groups for the viral DNA polymerase. As the viral DNA polymerase proceeds to copy the template DNA, it could in a simultaneous reaction remove any remaining viral RNA via its RNase H activity, resulting in double-stranded DNA. This is formally analogous to the 'nick-translation' reaction of *E. coli* DNA polymerase I (23, 24).

We made no attempt to further characterize the nucleases associated with *E. coli* DNA polymerase I and KB DNA polymerases I and II that are responsible for the degradation of poly(dT)·poly(rA) (Fig. 5), but it seems likely that these are exonuclease activities that have been described (24–26). The absence of these exonucleases in viral DNA polymerases provides an additional criterion for the distinction of these 'reverse transcriptase' activities from ordinary DNA polymerases.

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