Different Mode of Action of Ribonuclease H in Purified $\alpha$ and $\alpha\beta$ Ribonucleic Acid-directed Deoxyribonucleic Acid Polymerase from Avian Myeloblastosis Virus*

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SUMMARY

The mechanism of action of ribonuclease H (RNase H) associated with the two structurally different RNA-directed DNA polymerases of avian myeloblastosis virus was studied. Both the two-subunit enzyme, $\alpha\beta$, and the single subunit enzyme, $\alpha$, require free termini to initiate nuclease activity since they cannot cleave a covalently closed RNA-DNA duplex. The exoribonuclease activity of both $\alpha$ and $\alpha\beta$ RNase H can degrade RNA of an RNA-DNA hybrid in the 3'-5' and 5'-3' directions, as shown by the use of [3H, 3'- or 5'-32P]poly(A).poly(dT) as substrate. Two oligonucleotide species approximately 10 and 30 nucleotides long are generated by limited digestion of [3H]poly(A).poly(dT) by both enzymes. But $\alpha$ and $\alpha\beta$ possess a different mode of exoribonuclease activity. $\alpha\beta$ RNA-directed DNA polymerase, as shown previously, has processive exoribonuclease activity which degrades one polyribonucleotide chain completely prior to initiating hydrolysis of a second chain. In contrast, $\alpha$ RNA-directed DNA polymerase has random exoribonuclease activity which results in the release of the substrate molecule after each chain scission. This conclusion is supported by two types of experiments: (a) while the degradation of radioactive substrate by $\alpha\beta$ is unaffected by addition of unlabeled substrate after the reaction has been initiated, the degradation of the labeled polymer by $\alpha$ is immediately blocked, and (b) $\alpha\beta$ degrades 3' and 5' end terminally labeled substrate at the same rate as the whole molecule, while $\alpha$ degrades the end terminal nucleotides at a much faster rate than the whole molecule. The $\beta$ subunit of $\alpha\beta$ may facilitate tighter binding of the enzyme to the substrate resulting in a processive mode of nuclease activity.

RNase H, found initially in calf thymus (1, 2) and recently isolated in highly purified form from this tissue (3, 4), specifically digests the RNA of DNA-RNA hybrids to acid-soluble products. RNase H isolated from cells of human, bovine, and avian origin are endoribonucleases which can degrade covalently closed RNA sequences in an RNA-DNA hybrid (5). Like RNase H from eukaryotic cells, Escherichia coli RNase H (6), also extensively purified (7, 8), possesses endoribonucleolytic activity (7, 9).

Mölling et al. (10) discovered that the RNA-directed DNA polymerase of avian myeloblastosis virus possessed RNase H activity. Ten different RNA tumor viruses were subsequently demonstrated to possess RNase H activity (11). Both DNA polymerase and RNase H activity were demonstrated recently on the single subunit form, $\alpha$ (12), and on the major two subunit form, $\alpha\beta$, of the avian myeloblastosis virus RNA-directed DNA polymerase (5, 7, 12-14). Unlike bacterial and eukaryotic RNase H which are endoribonucleases, $\alpha\beta$ DNA polymerase was shown to be a processive exoribonuclease (5, 7). We have studied the mechanism of action of the RNase H activity associated with the $\alpha$ and $\alpha\beta$ forms of viral RNA-directed DNA polymerase. In this report, we show that the single subunit enzyme $\alpha$ is an exoribonuclease which possesses a random rather than processive mode of action. The function of the $\beta$ subunit associated with the $\alpha\beta$ DNA polymerase is discussed.

EXPERIMENTAL PROCEDURE

Materials—Avian myeloblastosis virus tissue culture and plasma were generously supplied by Dr. Joseph Beard and Dorothy Beard of Life Science, Inc., Florida. Sources of most radioactive and unlabeled compounds were described (11, 12). [\(\alpha\text{-}32P\)]ATP and [\(\gamma\text{-}32P\)]ATP were purchased from New England Nuclear, venom phosphodiesterase and bacterial alkaline phosphatase from Worthington, and polynucleotide kinase from Miles Laboratory. Escherichia coli DNA polymerase I (Fraction VII) was kindly provided by Drs. D. Helinski and P. Williams (University of California, San Diego).

Purification of Viral DNA Polymerase—The $\alpha$ and $\alpha\beta$ DNA polymerases were purified through phosphocellulose column chromatography, glycerol gradient centrifugation, or non dissociating gel electrophoresis as previously described (12). Phosphocellulose purified enzymes were used unless otherwise indicated. Escherichia coli RNA polymerase was purified through glycerol gradient centrifugation (16).

TEMED, N,N,N',N'-tetramethylethylenediamine; CoE1, coliogenic factor E1 of E. coli.

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‡ The abbreviations used are: RNase H, ribonuclease H;
Synthesis of \([H, 3'\sigma^P]Poly(A)\)—The elongation of \([H]poly(A)\) with \([\sigma^P]ATP\) using \(E. coli\) RNA polymerase was performed as described (13). The specific activity of \([H]poly(A)\) was 40 cpn per pmole and that of \([\sigma^P]ATP\) was 375 cpn per pmole. With the limiting concentration of ATP used, \(E. coli\) RNA polymerase is primer dependent (17), and the average chain length of \([H]poly(A)\) was increased by approximately 10 \([\sigma^P]AMP\) residues. Virtually no RNA synthesis occurred in the absence of \([H]poly(A)\), as previously reported (13).

Synthesis of \([H, 5'\sigma^P]Poly(A)\)—The 5' end of \([H]poly(A)\) (10 or 40 cpn per pmole) was dephosphorylated with bacterial alkaline phosphatase and rephosphorylated using \([\gamma^P]ATP\) and polynucleotide kinase. \([H]poly(A)\) (5.5 A) was dephosphorylated with bacterial alkaline phosphatase (pH 8.0) at 37° for 20 min. An additional 3.2 mg per ml of phosphatase was added, the incubation was continued for another 30 min, the product was extracted with 1:1 phenol-chloroform (phenol was saturated with 0.1 M Tris-HCl, pH 9.1), and dialyzed against 10 mM Tris-HCl (pH 8.0). The polynucleotide kinase reaction mixture (0.2 ml) contained 60 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10 mM MgCl₂, 20 μg bovine serum albumin, 40 mmoles of dephosphorylated \([H]poly(A)\), 0 mmoles of \([\gamma^P]ATP\) (1.2 X 10⁵ cpum per pmole) and 10 units of polynucleotide kinase. After a 30-min incubation at 37°, 4.5 mmoles of \([\gamma^P]ATP\) and 10 units of polynucleotide kinase were added, the reaction was continued for an additional 30 min, and terminated by the addition of 100 mmoles of unlabeled ATP. \(poly(dT)\) was added to the reaction mixture and was passed through a Sephadex G-50 column (1 X 55 cm). The addition of poly(dT) increased the recovery of labeled polymer during purification. After extraction with phenol (saturated with 0.1 M Tris-HCl, pH 9.1), the product was dialyzed against 10 mM Tris-HCl (pH 8.0). The specific activity with the \([\sigma^P]ATP\) in the \([H, 5'\sigma^P]poly(A)\) was 16 cpum per pmole of \(poly(A)\). A less than 1/2% of the \([\sigma^P]ATP\) label remained acid insoluble after incubation for 60 min with 1 unit of phosphatase.

**R**\(Nase H Assay**—The reaction mixture (0.1 ml) using \([H]poly(A)\)-\(poly(dT)\) as substrate contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5 mM dithiothreitol, 20 mM NaCl, 500 mmoles of \([H]poly(A)\) (10 or 40 cpn per pmole), and 2000 pmoles of \(poly(dT)\). Samples were incubated at 37° for various times and acid-solubilized counts were determined as described (11).

With \([H, 5'\sigma^P]P\) or \([H, 3'\sigma^P]poly(A)\)-\(poly(dT)\) as substrate for \(\alpha\) and \(\beta\) \(R\)\(Nase H\), the reaction mixture (0.1 ml) contained 50 mM Tris-HCl (pH 8.0), 20 mM NaCl, 10 mM MgCl₂, 5 mM dithiothreitol, and either 193 pmoles of \([H, 3'\sigma^P]poly(A)\) plus 300 pmoles of \(poly(dT)\), or 200 pmoles of \([H, 5'\sigma^P]poly(A)\) and 240 pmoles of \(poly(dT)\). The activity of snake venom phosphodiesterase on \([H, 5'\sigma^P]poly(A)\) was assayed using a reaction mixture (0.1 ml) containing 6 mM MgCl₂, 60 mM glycine NaOH (pH 9.2), and 1 mg of enzyme. The assay for nuclease activity of \(E. coli\) DNA polymerase I and \(E. coli\) \(R\)\(Nase H\) was performed in a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 5 mM dithiothreitol, 5 mM MgCl₂, and the concentration of \([H, 5'\sigma^P]poly(A)\)-\(poly(dT)\) described above. After incubation at 37° for various times, reactions were terminated by the addition of 15 μl of 0.2 M EDTA (pH 7.4), 100 μl of 0.2 M sodium pyrophosphate, 250 μl of 10% trichloroacetic acid, and 50 μg of calf thymus DNA. After 20 min in an ice bath, acid-insoluble radioactivity was collected on membrane filters and counted in a liquid scintillation spectrometer.

**Sedimentation Analysis of \(E. coli\) DNA**—\(R\)\(Nase H\) hybrid was prepared using \(E. coli\) RNA polymerase with adenovirus type 2 \([14C]DNA\) as template, and was used as substrate as previously described (11).

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**Polynacrylamide Gel Electrophoresis**—Electrophoresis on polynacrylamide gels containing 7 M urea was used for examining the size distribution of \([H]poly(A)\)-\(poly(dT)\) after digestion by different \(R\)\(Nase H\) enzymes. After incubation, samples were denatured by heating for 3 min at 70° in 7 M urea prior to analysis on a discontinuous gel system. \([H]poly(A)\) itself or the hybrid treated with \(R\)\(Nase H\) (100 μg per ml) for 1 hour at 37° have the same profile as urea-treated hybrid. A slightly modified procedure described by Dr. Sherman Weisman of Yale Medical School was used to prepare the upper spacer gel and lower analytical gel. The lower gels (0.6 X 11 or 13 cm) containing either 7 or 15% acrylamide were prepared by mixing an appropriate volume of Stock B with 1 ml of Tris-borate buffer, 0.6 ml of TEMED (1.6%, v/v), and 4.2 g of urea. The solution was degassed for 10 min, 0.1 ml of ammonium persulfate (0.8%, w/v) was added, the volume was adjusted to 10 ml, and dispensed into glass tubing. The gels were allowed to polymerize overnight at 4° and 1.5 cm of 5% acrylamide spacer gel (prepared by adding 0.25 ml of riboflavin (0.05%, w/v) to 10 ml of Stock A) was added, and the gel was placed 2 cm from a fluorescein light for 1 hour. Samples prepared in 15% sucrose were subjected to electrophoresis at 5 ma per gel for 1.5 to 3 hours at 25°. Gels were cut into 2-mm slices and incubated in closed scintillation vials with 300 μl of 30% H₂O₂ at 60° overnight. The caps were removed, and the samples were aerated for 1 hour and then counted in Aquasol (10 ml).

**Results**

**Properties of \(\alpha\) and \(\beta\) \(R\)\(Nase H\)** Activity—The degradation of a ribopolymer by \(\alpha\) and \(\beta\) \(R\)\(Nase H\) requires the presence of a complementary decoxyribopolymer. As shown in Table I, the digestion of \([H]poly(A)\) by both \(\alpha\) and \(\beta\) \(R\)\(Nase H\) was increased from 1% to 40 to 50% by the addition of poly(dT). The \(R\)\(Nase H\) activity of DNA polymerase I and \(E. coli\) \(R\)\(Nase H\) also required the presence of a complementary decoxyribopolymer (Table I), in agreement with previous reports (7, 13).

To demonstrate that only RNA and not DNA of a RNA-DNA hybrid is degraded by the viral \(R\)\(Nase H\) enzymes, \([14C]poly(dT)\) which formed a complex with \([H]poly(A)\) was used as substrate. Neither \(\alpha\) nor \(\beta\) \(R\)\(Nase H\) degraded the \([14C]poly(dT)\), but both enzymes solubilized over 40% of the \([H]poly(A)\) present (Fig. 1). In addition, no detectable DNA of an adenovirus \([14C]DNA\)-\(H\)RNA hybrid was solubilized by either enzyme at a time in the reaction when 30% of the RNA was digested.

The \(R\)\(Nase H\) activity of \(\alpha\) and \(\beta\) was independent of DNA synthesis when \([H]poly(A)-poly(dT)\) was used as a substrate (Fig. 2, A and B). But DNA synthesis, that is, the requirement for TTP, was necessary for maximum activity when poly(dT) was replaced by oligo(dT) (Fig. 2, C and D). The increase in \(R\)\(Nase H\) activity with TTP was caused by the synthesis of complementary poly(dT) which formed a complex with \([H]poly(A)\), thus making more hybrid available for digestion.

The optimum divalent metal ion was Mg²⁺ (12 mM) for both viral enzymes with \([H]poly(A)-poly(dT)\) as substrate. Mn²⁺ (0.4 mM) could replace Mg²⁺, but was only one-third as effective.

**Nonsusceptibility of Covalently Closed RNA-DNA Hybrids to \(\alpha\) and \(\beta\) \(R\)\(Nase H\)**—To test whether \(\alpha\) \(R\)\(Nase H\) requires free end termini for enzymatic activity, the circular, superhelical decynucleic acid, \(CoE\)O; of \(E. coli\), was used as a substrate. When this plasmid replicates in the presence of chloramphenicol one or

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1 Personal communication.

2 D. Grandgenett and M. Green, unpublished observation.
more ribonucleotides are covalently inserted into one of the DNA strands, as shown by Blair et al. (15). The intact, covalently closed ColEI DNA (molecular weight of 4.2 × 10^6) sediments at 23 S (Fig. 3A), and the open circular molecule sedimented at 17 S. As demonstrated in Fig. 3, B and D, α and αβ RNase H did not nick this plasmid DNA. The αβ RNase H previously was shown not to affect the sedimentation profile of the plasmid superhelical DNA (5). Upon treatment with E. coli RNase H (an endoribonuclease), about 50% of the ColEI DNA was converted into a form that sedimented at 17 S (Fig. 3C). Thus, α like αβ, requires free end termini for the initiation of RNase H activity.

**Products of [3H]Poly(A) Digestion by α and αβ RNase H**—The RNase H activity associated with αβ DNA polymerase digests [3H]poly(A)-poly(dT) in the 5' → 3' and 3' → 5' directions to oligonucleotides with an average length of 5 to 12 AMP residues (Y like cup, requires free end termini for the initiation of RNase H activity. A, degradation of [3H]poly(A).poly(dT) by α RNase H. Catalysis of [3H]poly(A) degradation by poly(dT) (Fig. 4, A and B). Under conditions of limited digestion, electrophoresis in 7 M urea-7% polyacrylamide gels detected large pieces near the origin approximately 400 nucleotides in length and oligonucleotides at the position of the marker dye (18) (Fig. 4, A and B). When samples were subjected to electrophoresis on 7 M urea-15% acrylamide gels, two species of oligonucleotides, of approximately 10 and 30 nucleotides, were resolved (Fig. 5C). α RNase H formed relatively more 30 nucleotide species than did αβ RNase H (Fig. 5C). In contrast to the two [3H]poly(A) degradation products of α and αβ RNase H (Fig. 5C), E. coli RNase H generated numerous intermediate species (Fig. 5D). About 60% of the degradation products formed by E. coli RNase H migrated between Fractions 10 and 37 (Fig. 5D) while α and αβ RNase H produced no detectable products between Fractions 13 to 52 (Fig. 5C). These data showing that α and αβ RNase H do not generate oligonucleotides of intermediate length are consistent with the requirement for free termini and with the conclusion that both enzymes are exoribonuclease.

**Comparison of the gel profiles of samples which were digested to the same extent show that approximately three times as many oligonucleotides, as revealed by product at the position of the marker dye were found with α then with αβ RNAse H (Fig. 4, A and B).** The same results were obtained over a range of limited solubilization (5 to 30%).

**Processive versus Random Exoribonuclease Activity of αβ and α RNase H**—The αβ RNAse H is a processive exoribonuclease (7), i.e. once bound to a polynucleotide, it completely degrades the chain to enzyme-resistant oligonucleotides before it is released (7, 10, 20). With a processive mechanism, the addition of unlabeled poly(A)-poly(dT) after initiating the reaction should not affect the rate of degradation of labeled poly(A). If the enzyme is not processive, acid-solubilization of substrate would immediately cease. To test these alternative mechanisms, [3H]poly(A)-poly(dT) was incubated with limiting amounts of α and αβ RNase H. As shown in Fig. 5, the addition of a 17-fold excess

**TABLE I**

Catalysis of [3H]poly(A) degradation by poly(dT)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Poly(A) solubilized</th>
<th>Poly(A)-poly(dT) solubilized</th>
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</thead>
<tbody>
<tr>
<td>α RNase H</td>
<td>92</td>
<td>41</td>
</tr>
<tr>
<td>αβ RNase H</td>
<td>1.3</td>
<td>48</td>
</tr>
<tr>
<td>E. coli RNase H</td>
<td>3.3</td>
<td>71</td>
</tr>
<tr>
<td>E. coli DNA polymerase</td>
<td>3.4</td>
<td>73</td>
</tr>
</tbody>
</table>

**Fig. 1 (left).** Degradation of RNA and not of DNA in a RNA-DNA hybrid by α and αβ RNAse H. [3H]poly(A) (500 pmol). and [3H]poly(A) (600 pmol) were incubated under standard nuclease assay conditions with α (7 μg) and αβ (3.2 μg) RNase H. Solubilized H (●, △) and αβ (○, △) counts were determined.

**Fig. 2 (center).** Effect of DNA synthesis on α and αβ RNAse H activity. A, degradation of [3H]poly(A)-poly(dT) by α RNase H (5.5 μg). Standard RNase H assay conditions were employed as described under “Experimental Procedure” except TTP (8 × 10^-4 M) was (●) or was not (○) added. B, degradation of [3H]-poly(A)-poly(dT) by αβ RNase H (1.6 μg) using conditions described in A. C and D, same as A and B except [3H]poly(A)-oligo(dT) (12 to 18 nucleotides), 500 and 125 pmol, respectively, was used as substrate.

**Fig. 3 (right).** Sedimentation analysis of the plasmid ColEI DNA of Escherichia coli after treatment with different RNase H enzymes. Details are described under “Experimental Procedure.” A, no enzyme. B, α (2 μg) and αβ (3 μg) RNase H (purified by nondissociating gels). C, Escherichia coli RNase H (0.45 μg).
of unlabeled poly(A)-poly(dT) at 1 min after the start of the reaction had no effect on the rate of degradation of [3H]poly(A)-poly(dT) by α RNase H (7). In contrast, the rate of solubilization with μ RNase H was inhibited 90% by the addition of unlabeled hybrid. No significant solubilization was observed with either enzyme when unlabeled substrate was added prior to the addition of enzyme. Thus, α RNase H does not exhibit the properties of a processive exoribonuclease but instead behaves like a random exoribonuclease which releases the substrate molecule after each bond scission.

To further substantiate the above data indicating that α RNase H is a random exoribonuclease, such as snake venom phosphodiesterase (19), the degradation of [3H]poly(A) specifically labeled with 32P at either the 5' or 3' end was investigated. In this manner, the extent of hydrolysis of the labeled end could be compared with that of the entire molecule. To test for the 5’→3’ exonuclease activity, [3H, 5'-32P]poly(A) was synthesized using polynucleotide kinase. As a control, E. coli DNA polymerase I was used because of the nonprocessive, 5’→3’ nuclease activity associated with this polymerase which degrades RNA complexed to DNA (7, 13). As shown in Fig. 6A, E. coli DNA polymerase I released over 80% of the 32P of 5’ end labeled poly(A) when only 10% of the 3H label was solubilized. α RNase H gave results similar to E. coli DNA polymerase I (Fig. 6B). The preferential release of 32P over 3H was observed over a 4-fold range of protein concentrations with both phosphocellulose or glycerol gradient purified α RNase H. No significant solubilization of 32P or 3H was observed with either α or μ RNase H if the hybrid was digested with DNase I prior to assay indicating that α and μ RNase H were not contaminated with phosphatases. These data indicate that α is a random exonuclease. Unlike α, μ RNase H at limiting concentrations released both 32P and 3H at the same rate (Fig. 6D), consistent with its processive exonuclease activity (7). Escherichia coli RNase H releases both labels at the same rate because of its endonucleolytic action (Fig. 6C) (7, 9).

To determine whether α can degrade [3H]poly(A) also from the 3’ end, [3H]poly(A) was extended about 5% with [32P]AMP residues by using E. coli RNA polymerase with a low concentration of [γ32P]ATP (13, 17). As a control for random 3’→5’ exonuclease activity, snake venom phosphodiesterase (19) was used (Fig. 7A). This enzyme released the 32P end label approximately twice as rapidly as it released 3H from the rest of the
molecule, consistent with a previous study with this polymer and enzyme (13). The α RNase H released the 5'-P label three times faster than the 3'-label (Fig. 7C), these rates being comparable to that found with the 5'-3'P end labeled [32P]poly(A) (Fig. 6B). In contrast, both αβ and E. coli RNase H enzymes released 3'-P and 3'-H from [32P, 3'-32P]poly(A) at the same rate (Fig. 7, B and D) (7, 9, 13).

**Discussion**

In agreement with previous reports (5, 7), we find that the αβ DNA polymerase associated RNase H is an exonuclease. Its mode of action is processive like that of E. coli RNase H (19). In contrast, the α RNase H, a single subunit with a molecular weight of 65,000 (12), is not a processive but rather a random exonuclease, such as snake venom phosphodiesterase (19). The enzyme releases the substrate after bond scission and randomly attacks another substrate molecule. Both α and αβ RNase H lack the ability to nick a covalently closed RNA-DNA hybrid, demonstrating the requirement for free end termini to initiate enzymatic activity. Both α and αβ RNase H can degrade [32P]poly(A)·poly(dt) from either 5'-phosphate or 3'-OH termini, generating two different size classes of oligonucleotides under conditions of limited digestion. α RNase H preferentially forms the larger of the two species. We did not determine whether a free 5'-hydroxyl group or a 3'-terminal phosphate would interfere with the exonuclease activities of α and αβ RNase H.

The α subunits of α and αβ DNA polymerase have identical molecular weights and common antigenic determinants (12). Whether or not the α subunit of αβ has DNA polymerase or RNase H (or both) activity is not known. The major property that distinguishes between the RNase H activity of α and αβ is the processive versus random mechanism of exonuclease action. Two explanations can be offered for the different mode of action of α and αβ RNase H. The first possibility assumes that both DNA polymerase and RNase H activity reside on the α subunit of αβ, and that the α polypeptides of both enzymes are identical. The α subunit could provide the site on the αβ enzyme which binds tightly to the substrate distal to the cleavage site and facilitates a processive mechanism (21). The second possibility is that the α subunit of α and αβ are different enzyme molecules. The further characterization of the α component of αβ and its comparison to the α subunit should clarify these alternatives.

α and αβ DNA polymerase appear to have identical template specificities (12). However, the rate of both DNA-directed and RNA-directed DNA synthesis catalyzed by each molecule of αβ is approximately 7 times greater than that catalyzed by α DNA polymerase. Thus the β subunit may affect an increased rate of transcription of the viral RNA genome. The possible effect of β on the kinds of RNA sequences that are copied is under study.

Several lines of evidence support a role for the RNA-directed DNA polymerase in the synthesis of DNA early during infection and cell transformation by RNA tumor viruses. These include the increased content of virus-specific DNA in transformed cells (see Ref. 22 for a review), the demonstration that mammalian cells transformed by Rous sarcoma virus contain infectious viral DNA (23), and the diminished RNA-directed DNA polymerase activity of defective and mutant RNA tumor viruses (24, 25). The role of RNase H in synthesis of viral DNA, if any, is only speculative at present (5, 7, 10). The specificity of the viral RNase H towards the RNA moiety of DNA-RNA hybrids suggests that it may play a role in the biosynthesis of DNA, either by its activity on the viral RNA genome or on the RNA primer (26-30).

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**References**

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