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Detection of an RNase H Activity Associated with Hepadnaviruses

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Replication of the hepadnavirus DNA genome is accomplished via reverse transcription of an intermediate, pregenomic RNA molecule. This process is likely to be carried out by a virally encoded, multifunctional polymerase which possesses DNA- and RNA-dependent DNA polymerase and RNase H activities. However, the nature of the product(s) of the polymerase gene predicted to mediate these functions is unclear. Biochemical studies of the polymerase protein(s) have been limited by its apparent low abundance in virus particles and, until recently, the inability to express active polymerase protein(s) heterologously. We have used activity gel assays to detect DNA- and RNA-dependent DNA polymerase activities associated with highly purified duck hepatitis B virus (DHBV) core particles (S. M. Oberhaus and J. E. Newbold, J. Virol. 67:6558–6566, 1993). Now we report that the same approach identifies a 35-kDa RNase H activity in association with highly purified DHBV core particles and crude preparations of virions from DHBV-infected ducks and woodchuck hepatitis virus-infected woodchucks. This is the first report of the detection of an hepadnavirus-associated RNase H activity. Its apparent size is smaller than any of the DNA polymerase activities that we detected previously and significantly smaller than the full-length protein predicted from the polymerase open reading frame (p85 for DHBV). These data suggest that the viral polymerase and RNase H activities are separable and that these enzymes may coordinate their activities in vivo by forming a complex.

Hepadnaviruses are characterized by their small (~3,000 bp), partially double-stranded DNA genomes and a replication scheme which involves reverse transcription of an intermediate RNA molecule (for reviews, see references 44 and 51). Members of the *Hepadnaviridae* family have been divided into two groups, those which infect mammalian species (e.g., human hepatitis B virus and woodchuck hepatitis virus [WHV]) and those which infect avian species (e.g., duck hepatitis B virus [DHBV]).

Several steps in the replication of the viral genome have been shown to take place within cytoplasmic core particles which encapsidate the pregenomic RNA molecule and a DNA polymerase (DNAp) activity (56). This process of reverse transcription includes synthesis of minus-strand DNA from the pregenomic RNA template, degradation of the RNA template as minus-strand synthesis proceeds, and synthesis of plus-strand DNA from the minus-strand template. These steps are predicted to be mediated by a virally encoded polymerase with RNA- and DNA-dependent DNAp and RNase H activities similar to those found in retroviruses.

RNases H hydrolyze only the RNA strand in RNA-DNA hybrids (for a review, see reference 67). In retroviruses an RNase H activity associated with the viral reverse transcriptase (RT) is required for replication (for a review, see reference 7). It degrades genomic RNA present in the RNA-DNA hybrid generated during first-strand DNA synthesis so that the resulting single-stranded DNA molecule can serve as template for second-strand synthesis. The RNase H activity also generates the RNA primer for second-strand synthesis and is responsible later for removing the tRNA and second-strand RNA primers prior to completion of the double-stranded DNA molecule. In hepadnaviruses, an RNase H activity is predicted to degrade the pregenomic RNA molecule during minus-strand synthesis and to generate an RNA oligomer that is used to prime plus-strand synthesis. This oligoribonucleotide remains covalently attached to the plus-strand of the genomic DNA molecule until the virion infects a cell and formation of the covalently closed, circular DNA molecule is initiated (32).

All hepadnaviruses contain similar versions of three genes encoding core, envelope, and polymerase proteins. The mammalian viruses contain a fourth gene, X, encoding a protein of unknown function. Expression of the structural core and envelope proteins is relatively well understood (for a review, see reference 13). The nature of *pol* gene expression is not. Unlike retroviral polymerases, the hepadnaviral polymerase is not expressed as a fusion protein (9, 49), although both core and polymerase proteins are translated from the same mRNA (21, 38). The predicted amino acid sequences derived from hepadnaviral *pol* genes contain motifs which are conserved among the hepadnaviruses and are similar to sequences known to be required for RT and RNase H activities in retroviruses (63). In addition, the amino-terminal region contains residues which are unique to and conserved among the hepadnaviral *pol* sequences (26). This region appears to be involved in priming minus-strand synthesis (2, 70). The similarities between hepadnaviral and retroviral *pol* genes and evidence that replication of the hepadnaviral DNA genome involves reverse transcription have indicated the existence of a virally encoded, multifunctional polymerase. In vitro transfection experiments which examined the effects of mutations in the *pol* gene on production of specific replicative intermediates suggested four domains within the polymerase: an amino-terminal primer, a spacer region, an RT, and a carboxy-terminal RNase H (8, 10, 31, 43).

Biochemical studies of the polymerase protein(s) have been limited by unsuccessful attempts to purify useful quantities of the protein(s) from virus particles. As a result, little is known

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about how the *pol* gene is expressed in vivo. Recently, several groups have succeeded in expressing full-length polymerase proteins in vitro which exhibit low levels of DNAp activity (20, 52, 59, 64). Two of these proteins have also been demonstrated to have the ability to prime minus-strand synthesis via a tyrosine residue near their amino termini (66, 70). None of these proteins has been demonstrated to have RNase H activity or to complete minus-strand synthesis.

We have used activity gel assays to detect RNA- and DNA-dependent DNAp activities in association with highly purified DHBV core particles (37). Unlike the polymerase proteins expressed in vitro, these activities were detected in preparations of core particles purified from infected animals and are likely to represent polymerase proteins active in vivo. Our results indicate that multiple polymerase proteins are expressed, perhaps functioning as a complex. We describe similar studies here in which we have used an activity gel assay to detect a single 35-kDa RNase H activity in association with highly purified DHBV core particles and virus particles derived from the sera of DHBV- and WHV-infected ducks and woodchucks, respectively. This is the first report describing detection of an RNase H activity associated with hepadnavirus particles. These data further support the suggestion that multiple *pol* gene products are expressed in vivo and raise questions about how they are synthesized, how they are packaged into core particles, and how they carry out the various functions attributed to them.

MATERIALS AND METHODS

Preparation of DHBV core particles. We have devised a protocol for the purification of DHBV core particles to near homogeneity (37). Briefly, each preparation of core particles was derived from 250 to 500 g of livers taken from congenitally DHBV-infected Pekin ducks or Toulouse geese sacrificed 2 to 10 weeks posthatch (livers are usually taken from animals sacrificed 2 to 3 weeks posthatch). Ducks were obtained as described previously (37) or hatched from eggs congenitally infected with DHBV strain p2.3 (62). Geese were also hatched from eggs congenitally infected with the DHBV p2.3 strain. Livers were homogenized by using a Dounce and loose-fitting pestle. Nuclei, large cellular material, and polysomes were removed by differential centrifugation. Core particles were concentrated by pelleting through a sucrose cushion and then fractionated from large proteins and protein complexes by precipitation with polyethylene glycol. Core particles were then isolated by sedimentation in one, two, or three sucrose gradients followed by, in some cases, banding twice in a buoyant CsCl density gradient. Fractions which contained core particles (density of 1.34 g/cm^3) were identified by the endogenous DNAp assay (37).

Preparation of virus particles from sera viremic for DHBV, Ross's goose hepatitis virus (RGHV), and WHV. Each preparation of DHBV was derived from 75 to 100 ml of viremic serum taken from congenitally infected Pekin ducks or Toulouse geese similar to those from which livers were taken for the purification of DHBV core particles, as described above. All steps were carried out at 5°C. Serum was cleared of cellular material and debris by low-speed centrifugation. Supernatants were pooled, and virus particles were pelleted in an SW27 rotor at 27,000 rpm for 28 h. Pellets were dissolved in virus buffer (VB) (0.15 M NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.4]) and layered over a 3-ml cushion of 20% sucrose–VB in an SW40 tube. Virus particles were pelleted through the cushion in an SW40 rotor at 32,000 rpm for 17 h and redissolved in VB. The sucrose cushion was diluted with VB and centrifuged in an SW40 rotor at 32,000 rpm for at least 8 h. The pelleted material, which contains some virions but is enriched for surface antigen particles (36), was redissolved in VB and pooled with the virion-enriched material which had been pelleted through the sucrose cushion. We refer to this material as a crude preparation of virions. Crude virions were further purified by mixing with CsCl in VB (0.235 g of CsCl per ml) and centrifuging in an SW56 rotor at 48,000 rpm for 48 h. Fractions (100 μl) were collected by bottom puncture and assayed for the presence of virions by using the endogenous DNAp reaction and Southern blotting.

A crude preparation of RGHV was prepared as described for DHBV. RGHV is an avian hepadnavirus whose genome sequence is distinct from that of DHBV (53); it was isolated in our laboratory from a Ross's goose. Serum (130 ml) was taken from Pekin ducks which were congenitally infected with RGHV and sacrificed 16 days posthatch. Material which had been pelleted through and from the sucrose cushion was pooled and tested for RNase H activity.

WHV was prepared from the viremic serum of chronically infected woodchucks (provided by J. M. Cullen). WHV particles were pelleted through a sucrose cushion as described for DHBV, redissolved in VB, mixed with CsCl in VB (0.4 g of CsCl per ml), and centrifuged in an SW56 rotor at 48,000 rpm for 48 h. Fractions were collected by bottom puncture and assayed for the presence of virions by using the endogenous DNAp reaction and Southern blotting. Fractions which contained virions were diluted with VB and centrifuged in an SW56 rotor at 40,000 rpm for at least 8 h to concentrate virions and remove CsCl. These fractions were then tested for RNase H activity.

Detection of virion DNA by Southern blotting. Fractions from CsCl density gradients in which viremic sera from DHBV- or WHV-infected animals were banded were assayed for the presence of viral DNA by Southern blotting and hybridization with radiolabeled RNA probes. Samples (2 to 10 μl from 100-μl fractions) were incubated at 37°C for 1 h in the presence of 0.4% sodium dodecyl sulfate (SDS)–0.4 mg of proteinase K per ml 0.02 M EDTA and then electrophoresed in a 1% agarose gel in E buffer (37). The gel was soaked in 0.1 M NaOH–1.5 M NaCl for 30 min with shaking to denature DNA and hydrolyze RNA and then neutralized by soaking in two changes of 0.1 M Tris-HCl and 1.5 M NaCl for 30 min each. DNA was transferred to a nylon membrane (Biotrans; ICN Biomedicals) by upward capillary transfer from the gel (54). The membrane was baked, prehybridized, and hybridized with a ^{32}P-labeled DHBV plus-stranded RNA probe for DHBV samples as described previously (37). For WHV samples, a WHV plus-stranded RNA probe was prepared from a plasmid construct similar to that described for DHBV probes, containing a full-length copy of the WHV genome (11) as described previously (37). Washes were carried out at 52°C as described previously (37), and membranes were analyzed by autoradiography.

RNase H activity gel assay. RNase H activity gel assays were carried out essentially as described previously (55). Polyacrylamide gels (10% except where noted otherwise, 0.75 mm thick) were prepared as described by Laemmli (28) except for the addition of a M13 DNA–(^{32}P)-labeled RNA hybrid to the gel mix prior to polymerization. Samples were dissolved in, or diluted with, sample buffer (final concentrations: 62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 1% SDS, 5% 2-mercaptoethanol, 2 mM EDTA, 0.01% bromophenol blue) and electrophoresed at 4°C. All samples were heated for 4 min in a boiling water bath, except where noted otherwise. Gels were then soaked in a series of buffers as described previously (55), except that the 16- and 8-h incubations were done at 4°C instead of room temperature. After the final wash with trichloroacetic acid and $NaPP_i$, gels were stained with Coomassie blue for 40 min in 0.006% Coomassie blue G250–7.5% methanol–5% acetic acid, destained for 20 min in 7.5% methanol–5% acetic acid, dried under vacuum, and analyzed by autoradiography. RNase H activity is observed as a clear area in the gel where the enzyme has digested the ^{32}P-labeled RNA in the DNA–(^{32}P)-labeled RNA hybrid and the products of the digestion have been washed out of the gel.

Ultrapure polyacrylamide was obtained from Bethesda Research Laboratories (BRL), ultrapure SDS and dithiothreitol were obtained from Boehringer Mannheim Biochemicals, and glycerol was obtained from Fisher Scientific. All other chemicals were purchased from Sigma. Exonuclease III was obtained from Promega or BRL. Protein molecular weight standards were from Promega. A preparation of the p66 subunit of human immunodeficiency virus type 1 (HIV-1) RT, which was purified from *Escherichia coli* expressing this polypeptide (29), was provided by P. Furman, Burroughs Wellcome.

Preparation of DNA–^{32}P-labeled RNA hybrid substrate. M13mp18 plus-strand DNA was purified from the supernatant of infected *E. coli* JM101 (15). RNA polymerase reactions were carried out in the presence of 4 μg of purified M13 DNA as described previously (19) with the addition of 10 μm of UTP. Total and acid precipitable cpm in 1-μl aliquots were determined by scintillation counting. Between 40 and 60% incorporation was generally observed. RNAp reactions were terminated by adding EDTA to 20 mM, and the mixtures were then stored at 4°C without removing unincorporated [α-^{32}P]UTP. A total of 100 μl of the RNA polymerase reaction mixture was added per 15 ml of polyacrylamide gel, and the unincorporated [α-^{32}P]UTP was removed during electrophoresis.

RNA polymerase and ribonucleotides were obtained from Pharmacia. [α-^{32}P]UTP was from ICN Biomedicals or New England Nuclear, and all other chemicals were from Sigma.

Immunoprecipitation of DHBV core particles. DHBV core particles were immunoprecipitated with a rabbit antiserum generated against recombinant DHBV core protein (23). Core particles, which had been isolated by sedimentation in two sucrose gradients, were incubated on a rocker at 4°C for at least 12 h with 2 μl of preimmune or immune serum or in the absence of serum in 500 μl of STE buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100). Each immunoprecipitation reaction mixture contained 3% of the total preparation of cores. A 50% slurry of IgGsorb (The Enzyme Center) in STE buffer (50 μl) was then added to each immunoprecipitation reaction mixture, and the mixtures were incubated on a 4°C rocker for 1 to 1.5 h. The reaction mixtures were pelleted in a microcentrifuge, and the pellets were washed three times with 1 ml of STE buffer each time. The pellets were resuspended in 50 μl of sample buffer and heated in a boiling water bath for 4 min. IgGsorb was pelleted by microcentrifugation for 5 min at room temperature, and the supernatants were tested in the RNase H activity gel assay.

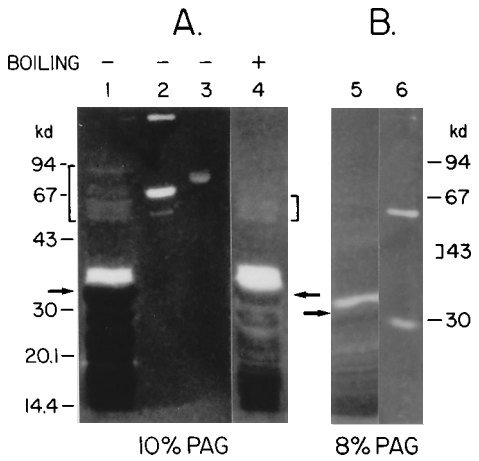

FIG. 1. RNase H activity gel assays with crude preparations of DHBV core particles. (A) DHBV core particles before (lane 1) and after (lane 4) boiling. Faint RNase H activities between 50 and 91 kDa are bracketed. Lane 2 contained ~1 μg of purified HIV RT (p66); weaker RNase H activities were also detected in this preparation at 54 kDa (an apparent degradation product) and ~130 kDa (an apparent homodimer of p66). Lane 3 contained 1,000 U of Moloney murine leukemia virus RT (p80). Lanes 1 to 3 are from the same gel, and lane 4 is from a gel run in parallel. (B) DHBV core particles (lane 5) were tested in the RNase H activity gel assay using an 8% polyacrylamide gel to separate the RNase H activity from core protein. Lane 6 contained ~ 1 μg of purified HIV RT (p66) and exonuclease III (p28, 8 U from BRL) which also exhibits RNase H activity. Lanes 5 and 6 were from the same gel. Arrows point to the dark, hybrid-binding bands composed of core protein. Molecular size markers (in kilodaltons [kd]) are represented by lines beside the gels and were used to align lanes taken from different gels. PAG, polyacrylamide gel.

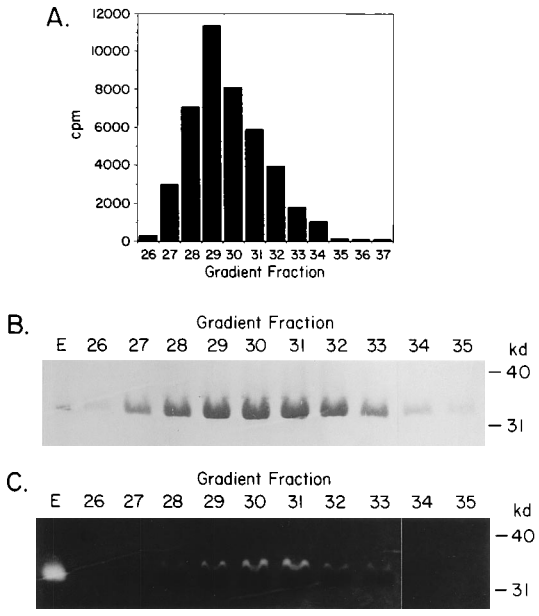

FIG. 2. Endogenous DNAp and RNase H activities associated with CsCl gradient fractions containing DHBV core particles purified to near homogeneity. Cores were banded twice in CsCl gradients, pelleted from the final gradient fractions, and resuspended in 100 μl of core buffer (37). Fractions are numbered from the bottom of the gradient to the top. (A) Fractions were assayed using the endogenous DNAp reaction. Incorporation of [α-^{32}P]dGTP was detected by scintillation counting. Fractions (20 μl of samples) were also tested in the RNase H activity gel assay in which the activity gel was stained with Coomassie blue (B) and then analyzed by autoradiography (C). Regions of the gel above and below the 35-kDa RNase H activity have been omitted from the figure since no activity was detected in these areas. Lane E contained 250 U of exonuclease III from Promega. Molecular size markers (in kilodaltons [kd]) are represented by lines beside the gels and were used to align lanes taken from different gels.

RESULTS

RNase H activity in crude preparations of DHBV core particles. DHBV core particles were prepared by sedimentation in one sucrose gradient. Fractions containing significant endogenous DNAp activity were pooled, and cores were pelleted by centrifugation. Samples from this pool (equivalent to 2.6% of the total preparation of cores) were tested in the RNase H activity gel assay with and without boiling prior to electrophoresis. In this assay, RNase H activity is detected as a clear band in the gel, representing a protein which has digested the radiolabeled RNA of a DNA–^{32}P-labeled RNA hybrid in situ. A large, clear band indicative of RNase H activity was discerned in the autoradiograph at approximately 34 to 36 kDa for both samples (Fig. 1A, lanes 1 and 4). Several fainter clear bands were also observed between 50 to 91 kDa and between 20 to 30 kDa. Dark bands represent nucleic acid-binding proteins which appear to retain labeled polymer during incubations in the various buffers (22). These proteins do not concentrate labeled polymer during electrophoresis since the dark bands were not observed immediately after electrophoresis (22). The RNase H activity at 34 to 36 kDa was located just behind and in contact with a dark band identified as core protein by its characteristic size and prominence in core preparations (37, 42, 48). Its appearance as a dark band in this assay is consistent with its property of binding nucleic acid moieties (34, 39). These results indicate that the major RNase H activity is efficiently renatured after the sample is boiled in SDS. They also suggest that the RNase H released from core particles is not associated noncovalently with nucleic acid or other proteins that would retard its mobility in the gel.

A sample from the same DHBV core particle preparation described for Fig. 1A taken just prior to sedimentation in sucrose gradients was tested in the RNase H activity gel assay by using an 8% polyacrylamide gel in an attempt to separate the RNase H activity from core protein. A sample equivalent to 0.3% of the total volume of cores at this stage of purification was tested. A single clear band almost identical in size (35 kDa) to the one shown in Fig. 1A was discerned as a separate moiety from the dark band of core protein (Fig. 1B, lane 5). As an additional control, RNase H activity was not detected when recombinant DHBV core protein (produced in *E. coli* and provided by A. Jilbert) was tested in the RNase H activity gel assay (data not shown). These data indicate that the core-associated RNase H activity is not an integral part of, or dependent upon, contact with core protein.

RNase H activity in preparations of DHBV core particles purified to near homogeneity. To more rigorously define the association of the 35-kDa RNase H with DHBV core particles, their further purification was carried out by two different methods: (i) banding in buoyant CsCl density gradients (37) and (ii) immunoprecipitation of DHBV cores by an antiserum specific for DHBV core protein.

(i) RNase H activity is associated with liver-derived DHBV core particles purified to near homogeneity by banding in CsCl. DHBV core particles were purified to near homogeneity by buoyant density centrifugation in two CsCl gradients, and fractions from the final CsCl gradient were analyzed for the presence of core protein, endogenous DNAp, and RNase H activities. Endogenous DNAp activity was recovered in fractions 27 to 34, with peak activity in fraction 29 (Fig. 2A). Core protein was the predominant protein in all fractions tested but was most abundant in fractions 29 to 31 (Fig. 2B). It was identified in the Coomassie-stained activity gel by its charac-

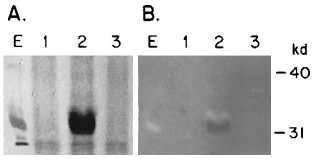

FIG. 3. RNase H activity gel assay on DHBV core particles which were immunoprecipitated with an antiserum directed against DHBV core protein. The activity gel was stained with Coomassie blue (A) and then analyzed by autoradiography (B). Lanes: 1, immunoprecipitation reaction mixture to which preimmune serum was added; 2, reaction mixture with anti-core antiserum; 3, reaction mixture without serum. Regions of the gel above and below the 34-kDa RNase H activity have been omitted from the figure since no activity was detected in these areas. Lane E contained exonuclease III (250 U; Promega). Molecular size markers (in kilodaltons [kd]) are represented by lines beside the gels.

teristic size (32 to 34 kDa) (42, 48). RNase H activity was detected as a single band with an apparent molecular mass of 35 kDa in fractions 28 to 33, with maximal activity in fractions 30 and 31 (Fig. 2C). The fainter clear bands at 50 to 91 kDa and 20 to 30 kDa which were present in the crude preparation of core particles (Fig. 1A) were not detected in association with core particles purified through two CsCl density gradients. These bands may represent RNase H activities which are not derived from DHBV and are lost upon banding in CsCl, or they may be DHBV polymerase proteins which have RNase H activity but are sensitive to exposure to CsCl.

The peak of RNase H activity in the CsCl gradient is overlapping, but not precisely concordant with maximal endogenous DNAp activity. The fractionation of RNase H activity is more precisely concordant with the distribution of core protein and is maximal in fractions which are found on the slightly less dense side of the peak endogenous DNAp activity.

These data identify a single RNase H associated with highly purified DHBV core particles. RNase H activity was not detected in the absence of markers for core particles (endogenous DNAp activity and core protein) or in identically prepared material from the livers of DHBV-negative ducks (data not shown).

(ii) RNase H activity is associated with liver-derived DHBV core particles which have been immunoprecipitated. DHBV core particles were prepared by sedimentation in two sucrose gradients and immunoprecipitated with a rabbit antiserum generated against recombinant DHBV core protein. The immunoprecipitate was tested for RNase H activity in the gel assay to determine whether core particles isolated by immunoprecipitation were associated with the same RNase H activity observed with core particles isolated by banding in CsCl. Figure 3A shows the Coomassie-stained activity gel in which core protein can be seen in lane 2, the material immunoprecipitated with anti-core antiserum, but not in lanes 1 (preimmune serum) or 3 (no serum). The autoradiograph of this gel shows a single RNase H activity at 34 kDa, just above the dark band of core protein in the anti-core antiserum reaction mixture (Fig. 3B, lane 2) but not in the presence of preimmune serum (Fig. 3B, lane 1) or in the absence of serum (Fig. 3B, lane 3). This activity appears to be identical to the major RNase H active band detected in association with crude and highly purified core particles (Fig. 1 and 2). In all three cases a single RNase H activity was discerned at ca. 34 kDa, just above a dark band of core protein. The fainter RNase H activities at 51 to 94 kDa and the dark, hybrid-binding protein bands other than core protein which were seen with the crude preparation of cores were not seen with CsCl-banded or immunoprecipitated cores. These data indicate that the 34- or 35-kDa RNase H activity is closely associated with core particles and is not adversely affected by exposure to CsCl.

RNase H activity in preparations of hepadnavirus particles from viremic sera. Since hepadnaviral core particles are precursors to infectious virions, it seemed likely that DHBV in viremic serum would also display the RNase H activity found associated with the liver-derived DHBV core particles. Thus, a crude preparation of virions pelleted from sera viremic for DHBV and a CsCl gradient fraction containing buoyant DHBV were tested in the RNase H activity gel assay. Both samples contained a single RNase H activity nearly identical in size (ca. 35 kDa) to that detected in association with DHBV core particles (Fig. 4, lanes 3 and 4). Additionally, a crude preparation of virions from sera viremic for RGHV and also a CsCl gradient fraction containing WHV were similarly assayed. Both the RGHV (Fig. 4, lane 5) and WHV (Fig. 4, lane 6) preparations displayed a single RNase H activity nearly identical in size to the RNase H activity associated with the serum- and liver-derived DHBV particles. These results indicate that both mammalian and avian hepadnaviruses are associated with RNase H activities which are very similar, if not identical, in size.

The RNase H associated with DHBV is more active with magnesium than manganese ions. Virions from sera viremic for hepatitis B virus have been shown to exhibit greater endogenous DNAp activity when magnesium rather than manganese ions are added to the reaction mixture (25). We have observed with DHBV core particles that synthesis of both minus- and plus-strand DNA, as measured in the endogenous DNAp reaction, is stronger in the presence of magnesium ions than with manganese ions (data not shown). DHBV core particles isolated by sedimentation in three sucrose gradients and a crude preparation of virions from serum viremic for DHBV were tested in the RNase H activity gel assay in the presence of 8 mM $MgCl_2$ or 8 mM $MnCl_2$. Sample sizes were both equivalent to 2% of each of the total core and virion preparations, respectively. The 35-kDa RNase H activity, which was detected in association with both DHBV cores and crude virions in the presence of $MgCl_2$, was not detected in the preparation of core particles when $MnCl_2$ was substituted for $MgCl_2$ (Fig. 5). The RNase H activity associated with serum-derived virus particles was significantly reduced in the presence of $MnCl_2$ but not completely inactive. These results support the idea that the RNase H activities associated with both DHBV cores and crude virions are the same and, like the endogenous DNAp activity, are more active with magnesium rather than manganese ions under these conditions.

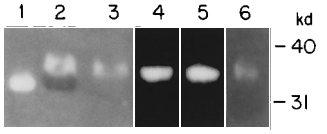

FIG. 4. RNase H activity gel assays with preparations of DHBV, RGHV, and WHV from viremic sera. Lanes: 2, 2% of a preparation of DHBV core particles isolated through two sucrose gradients; 3, DHBV from viremic serum which was banded in a CsCl gradient and pelleted from one fraction in which significant amounts of viral DNA and endogenous DNAp activity were detected; 4 and 5, each contained 1% of crude preparations of DHBV and RGHV, respectively; both were pelleted from viremic sera; 6, 10% of a CsCl fraction in which significant amounts of WHV DNA and endogenous DNAp activity were detected; 1, exonuclease III (250 U; Promega). None of the serum-derived samples was heated prior to electrophoresis. Lanes 1 to 3, 4 and 5, and 6 were taken from three separate gels, respectively. Molecular size markers (in kilodaltons [kd]) represented by lines beside the gels, were used to align lanes taken from separate gels.

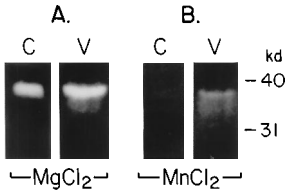

FIG. 5. RNase H activities associated with DHBV cores and crude virions in the presence of magnesium or manganese ions. Cores which had been isolated by sedimentation in sucrose gradients (lanes marked C) and crude virions (lanes marked V) were tested in RNase H activity gel assays in the presence of $MgCl_2$ (A) or $MnCl_2$ (B). None of these samples was heated prior to electrophoresis. Lanes C and V in panel A were from the same gel, while lanes C and V in panel B were from a separate gel run in parallel. Molecular size markers (in kilodaltons [kd]) are represented by lines beside the gels and were used in align the lanes from separate gels.

DISCUSSION

We have previously described detection of RNA- and DNA-dependent DNAp activities associated with highly purified DHBV core particles, using activity gel assays (37). The requirement for an RNase H activity in the hepadnaviral replication scheme and the likelihood of its existence based on genetic studies and amino acid sequence analyses prompted us to use the same approach to look for RNase H activity in preparations of virus particles.

An RNase H activity at 34 to 36 kDa was detected in a crude preparation of DHBV core particles. This activity was detected both with and without boiling, indicating that it can be renatured in the activity gel assay and that it is not associated noncovalently with nucleic acid or other proteins. A nearly identical RNase H activity at 35 kDa was detected in CsCl gradient fractions containing DHBV core particles purified to near homogeneity. This RNase H activity was not detected in the absence of endogenous DNAp activity or core protein, nor was it detected in similar preparations from uninfected ducks. It was also detected in association with core particles which had been immunoprecipitated with antiserum generated against recombinant DHBV core protein. The CsCl gradient fractions in which the RNase H activity was detected were nearly identical to those in which RNA- and DNA-dependent DNAp activities were detected in a similar preparation of core particles described previously (37). These activities were concordant with peak amounts of core protein and endogenous DNAp activity, suggesting an association of all three activities with core particles. We have also detected a single RNase H activity at 35 kDa in preparations of DHBV, RGHV, and WHV from viremic sera. Although these virus particles were not purified to near homogeneity, the detection of an RNase H activity nearly identical to that identified with highly purified DHBV core particles suggests that these RNase H activities are the same. Although it is possible that the 35-kDa RNase H is encoded by the host genome, detection of nearly identical RNase H activities associated with cytoplasmic core particles isolated from avian liver tissue and extracellular virus particles prepared from the sera of both avian and mammalian hosts suggests that this RNase H activity is derived from the viral *pol* gene which is conserved among all members of the *Hepadnaviridae* family. Since it was detected in preparations of both immature core particles and crude virions, the RNase H activity appears to be retained after its predicted function in degrading the pregenomic RNA template is apparently completed. Perhaps in virions this RNase H activity is involved in removal of the RNA oligomer covalently attached to the 5′ end of the plus-strand prior to completion of plus-strand synthesis and formation of the covalently closed, circular DNA molecule. If so, the RNase H activity, like the polymerase activity appears to be restrained during secretion of mature virions.

The RNase H activity that we have detected is markedly smaller than the predicted full-length product of the *pol* open reading frame (ORF) (p85 for DHBV and p90 for WHV) and might arise by any of several mechanisms. In retroviruses and caulimoviruses, RT is expressed as a precursor protein with little or no activity until proteolytic processing by the viral protease (14, 57). The 35-kDa RNase H might arise by proteolytic cleavage from a larger molecule either prior to or after its association with the hepadnaviral nucleocapsid. Although expression of a full-length *pol* ORF product which is then proteolytically processed has not been ruled out, there is no evidence for a viral protease in hepadnaviruses. Initiation of translation at internal in-frame AUG codons allows for the synthesis of more than one protein from a single ORF. In general, this is not a common stratagem for protein synthesis in eucaryotes, but at least half of all hepadnaviral proteins are generated in this way (for a review, see reference 13). The *pol* ORF contains numerous in-frame ATG codons, some of which are conserved among all members of the *Hepadnaviridae* family. Translation beginning at a conserved methionine codon at 480 in the DHBV *pol* gene and at 551 in the WHV *pol* gene would produce a 34- to 36-kDa protein containing the conserved amino acid motifs essential for activity in characterized RNase H proteins. This protein would be predicted to lack RT activity since it does not contain all of the residues known to be required for RT activity. The multiple core-associated RNA- and DNA-dependent DNAp activities that we detected using activity gel assays are larger than (109 kDa), the same size as (85 kDa), and smaller than (80, 75, and 63 kDa) the predicted full-length DHBV *pol* gene product (37). These data suggest that the 35-kDa RNase H activity does not also have DNAp activity. However, like the endogenous DNAp activity observed with DHBV core particles and virions, the RNase H activities detected in preparations of DHBV core particles and crude virions are stronger in the presence of magnesium rather than manganese ions.

It is unclear why we did not detect the activities of RNase H proteins of the same sizes as the DNAp proteins found in the DNAp activity gel assays, since at least the larger proteins would be expected to contain the RNase H domain. If larger *pol* ORF products have RNase H activity in vivo, they may not be detected by using this assay because of difficulties in renaturation (45) or a requirement for formation of a protein complex, such as a dimer. Alternatively, RNase H activity may be restricted to the 35-kDa protein and is inactive as part of a larger polymerase protein. It is not apparent whether this 35-kDa RNase H activity acts alone, in concert with viral polymerase activities, or in addition to a putative RNase H activity associated with larger *pol* ORF products. Three of the best-characterized RTs (avian myeloblastosis virus, Moloney murine leukemia virus, and HIV-1) possess an RNase H activity which is physically linked to the polymerase as part of the same polypeptide (14, 30, 58). If the 35-kDa RNase H activity that we have detected is involved in virus replication, it would be the first example of an RNase H activity which must coordinate its activity with the RT as a separate entity.

In light of the similarities among reverse-transcribing elements, it is tempting to look at recurring themes among the better-characterized retroviral RTs to make sense of what little is known about the hepadnaviral RT. The three best-characterized retroviral RTs (avian myeloblastosis virus, Moloney murine leukemia virus, and HIV-1) share the ability to form and function as dimeric moieties (14, 60, 61). For HIV-1,

reverse transcription is carried out by a p66-p51 heterodimer which is formed initially as a p66 homodimer. One copy of p66 within the homodimer appears to be vulnerable to cleavage by the viral protease, while a conformational difference protects the other copy from proteolysis (27). The resulting p51 molecule does not appear to have RT or RNase H activities within the heterodimer, but it does play a key role in positioning the tRNA primer for first-strand synthesis. It may also help to stabilize interactions between the enzyme and its substrate, thereby increasing processivity. There have been reports that p15 is present within virion particles and exhibits RNase H activity in activity gel assays (16, 50). It has been suggested that this RNase H activity may act randomly because of its small size and "unlinking" from the polymerase domain (47). It is possible that this RNase H activity may have an accessory role in replication as a nonprocessive RNase H. The association of less-than-full-length DNAp and RNase H activities with hepadnavirus particles suggests that the replication process is carried out by a protein complex, perhaps a dimer as seen with the retroviral RTs. As with the HIV-1 p15 RNase H, the role of the p35 RNase H activity that we have detected has yet to be defined.

If the 35-kDa RNase H activity that we have detected is a product of the *pol* ORF and involved in replication, a mechanism must exist for packaging it along with the DNAp active protein(s). Little is known about how the *pol* gene product(s) is packaged into core particles. In vitro studies with full-length Pol proteins expressed from plasmid constructs or by in vitro translation indicate that polymerase is required as a structural protein for packaging of (preferentially) the pregenomic RNA from which it is translated (1, 18). It appears to interact with an encapsidation signal, epsilon, at the 5′ end of the pregenomic RNA, but the nature of this interaction is unknown (24, 41, 65). Mutations made in all three functional domains have been shown to interfere with packaging of pregenomic RNA and polymerase (1, 46). However, there have been no reports of studies in which Pol proteins truncated from the amino terminus and corresponding to the minimal predicted RT or RNase H domains have been tested for the ability to be packaged. Wu et al. have reported that a DHBV *pol* mutant generated from a frameshift mutation in the spacer region apparently packaged the terminal protein domain separately from the RT and RNase H domains and was able to carry out reverse transcription of the pregenomic RNA, although at a reduced rate compared with that of the wild-type virus (68). Their data indicate that packaging of subgenic *pol* proteins is possible, perhaps via individual packaging signals or formation of a protein complex. The presence of a second copy of the encapsidation signal at the 3′ end of the pregenomic RNA suggests that a second Pol protein or complex might bind there and perhaps form a dimeric complex with the 5′ Pol protein. Since the 3′ epsilon is not sufficient by itself for packaging, it has been suggested that differences in the sequences adjacent to the 3′ and 5′ epsilons affect their abilities to direct packaging (3, 40). Perhaps the 3′ epsilon represents a second, but distinct, binding site for a Pol protein which is different from that which binds to the 5′ copy. This might provide the means by which subgenic Pol proteins are packaged and interact to form an enzyme complex. This possibility is supported by the findings of Wang and Seeger (65). Their RNA template from which a 90-kDa DHBV polymerase protein is expressed in vitro lacks the 5′ epsilon; yet, in some cases, initiation of minus-strand DNA took place at the 3′ epsilon, indicating an interaction between polymerase protein and the 3′ epsilon.

It is also unclear to what extent core protein interacts with the polymerase protein(s) and the pregenomic RNA during and after formation of core particles. Several studies have suggested that core protein may play a role in positioning and/or condensing replicative intermediates during replication (17, 35, 69). We have observed a strong interaction between core protein and the 35-kDa RNase H activity. DHBV core particles, treated with 5% SDS in an attempt to dissociate core and polymerase proteins, were subjected to immunoprecipitation with an antiserum generated against recombinant DHBV core protein. The immunoprecipitated material was tested in the RNase H activity gel assay. We observed the characteristic dark band of core protein and the 35-kDa RNase H activity (data not shown). This observation suggests a strong interaction between viral core and Pol proteins, in addition to the interactions observed between Pol protein and viral DNA (2, 6) and core protein and viral DNA (5, 12, 24, 39). A better understanding of these associations may be a key factor in overcoming the technical difficulties encountered by us and others in studying the viral Pol protein(s) in vivo and in expressing active Pol proteins.

Activity gel assays have allowed us to detect all three of the enzymatic activities predicted to be encoded by the *pol* ORF and required for hepadnaviral replication. We have argued that they most likely represent *pol* gene products, but studies demonstrating a direct relationship have been unsuccessful so far. The identification of Pol proteins from virus particles by using immunoprecipitation or Western blot (immunoblot) analyses has largely been unsuccessful. There have been two reports in which Pol proteins were identified from virus particles. Bavand et al. used antisera generated against peptides derived from the predicted Pol protein to immunoprecipitate a 70-kDa protein from HBV particles produced in vitro which exhibited RT activity in an activity gel assay (4). Mack et al. identified a 65-kDa protein in hepatitis B virus virions from an infected patient by Western blotting with an antiserum generated against a tribrid fusion protein which included 143 amino acids from the predicted Pol protein (33). These results and our own strongly suggest that enzymatically active subgenic Pol proteins are expressed and packaged into virus particles. It is unclear why such Pol proteins have not been detected in any of the in vitro systems used recently to express the full-length *pol* ORF. Possibly, the conditions under which subgenic Pol proteins are generated in vivo are not accurately reproduced in these systems. The DNAp and priming activities exhibited by the full-length Pol proteins further support the idea that the *pol* ORF encodes these functions required during virus replication. However, these results do not preclude the possibility that these functions may be carried out more efficiently in virus particles by a complex including less-than-full-length Pol proteins.

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