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SHORT COMMUNICATION

Preparations of Duck Hepatitis B Virions Contain Multiple DNA Polymerase Activities

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The hepadnaviral DNA genome is synthesized by a viral-encoded reverse transcriptase, but the nature of this protein(s) *in vivo* remains obscure. We have previously described studies in which activity gel assays identified multiple DNA polymerase (DNAp) activities associated with highly purified duck hepatitis B virus (DHBV) core particles. We now report that virions isolated from viremic sera are associated with DNA-dependent DNAp activities which are nearly identical to major DNAp activities detected with highly purified DHBV core particles. These results suggest that the virion-associated polymerases are the same as those which are detected with core particles and are likely to represent DHBV *pol* gene products involved in replication of the genome.

For hepadnaviruses, synthesis of the virion DNA occurs primarily in the liver within cytoplasmic core particles containing the pregenomic RNA and viral polymerase molecules. The RNA pregenome templates synthesis of the virion DNA by reverse transcription (*1*). This process includes polymerization of minus-strand DNA (via RNA-dependent DNA synthesis), degradation of the pregenome from an RNA:DNA heteroduplex (by a putative RNase H activity) as minus-strand DNA synthesis proceeds, and synthesis of plus-strand DNA from the minus-strand DNA template (via DNA-dependent DNA synthesis). Enzymatic activities responsible for these steps are encoded by the viral polymerase gene. Sequence analyses (*2, 3*) and mutational studies have indicated four domains within the polymerase open reading frame: an amino terminal primer, a spacer region, a reverse transcriptase (RT), and a carboxy terminal RNase H (*4–7*). However, little is known regarding expression of this viral gene and the nature of the protein(s) *in vivo* which carry out the enzymatic functions required during reverse transcription. Our previous studies using activity gel assays identified multiple DNA- and RNA-dependent DNA polymerase (DNAp) activities associated with highly purified duck hepatitis B virus (DHBV) core particles (*8*). In this report we show that multiple DNA-dependent DNAp (DDDP) activities are also detected in preparations of virions.

A crude preparation of virions was made (*9*) from 100 ml of serum taken from DHBV congenitally infected Pekin ducks. Viremic serum contains two kinds of viral particles, infectious virions, and surface antigen particles which are present in excess of virions. Surface antigen particles are composed of viral S and pre-S proteins, as well as host-derived lipids, but do not contain viral nucleic acids, core, or polymerase proteins. Virions and surface antigen particles have different densities, but are difficult to separate completely by banding in buoyant CsCl density gradients. A 40-μl sample from the crude virion preparation (900 μl) was mixed with CsCl in core buffer (CB) containing Triton X-100 and 2-mercaptoethanol as has been described for banding DHBV core particles (*8*). By using this procedure we hoped to isolate core particles derived from virions stripped of envelope (S and pre-S) proteins and to solubilize surface antigen particles. Fifty-one (100-μl) fractions were collected from the gradient and tested for the presence of virus particles using the endogenous DNAp assay and scintillation counting (*8*). Fractions exhibiting endogenous DNAp reactivity (EPR) were pooled and banded again in a second CsCl density gradient. Fractions (100-μl) were collected and analyzed for the presence of EPR and viral DNA by Southern blotting and hybridization with a radiolabeled plus-stranded RNA probe (*9*) and then diluted with CB and centrifuged. Pellets were dissolved in sample buffer and tested in the DDDP activity gel assay. The DDDP activity gel assay was done as described previously (*8*) with a modified washing protocol (*10*). After electrophoresis, each gel was incubated at 4° on a shaker with 1-liter buffer changes as follows: 1 hr with two changes of buffer 1:50 m*M* Tris–HCl (pH 8.0), 2 m*M* dithiothreitol and 20% glycerol; then 16 hr with two changes of buffer

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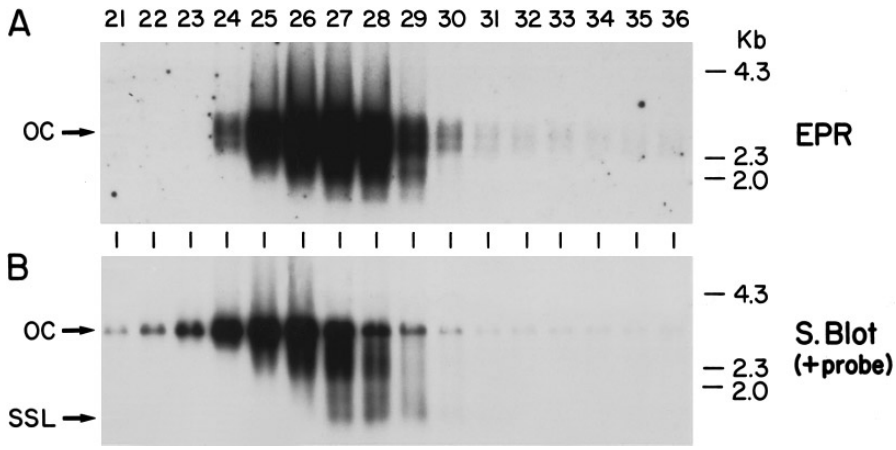

FIG. 1. Fractions from the second CsCl gradient in which serum-derived virions were banded and tested for the presence of nucleocapsids using the endogenous DNAp reaction (EPR) and Southern blotting (S. blot). Products of the EPR were analyzed by agarose gel electrophoresis (A). DHBV DNA was detected using a radiolabeled plus-stranded RNA probe (+ probe) (B). Fractions were numbered from the bottom of the gradient to the top. Open circular (OC) and single-stranded linear (SSL) molecules are designated by arrows.

2:buffer 1 with the addition of 70 m*M* KCl and 10 m*M* $MgCl_2$; and finally 8 hr with two changes of buffer 2 without glycerol.

Fractions 22 through 28 from the first CsCl gradient contained peak amounts of EPR and were pooled for rebanding in the second CsCl gradient. These fractions were from the middle of the gradient and ranged in density from 1.2 to 1.3 g/ml, approximately halfway between the density of virions (1.16 g/ml) and core particles (1.34 g/ml). Western blots of these fractions indicated that these particles contained core protein, a much reduced level of the viral pre-S protein (p36), none of the S envelope protein (p17), and a significant amount of a species (p28) presumed to be derived from p36 (data not shown). Fractions 20 to 38 from the final CsCl gradient were tested using the endogenous DNAp reaction and the products were analyzed by agarose gel electrophoresis (Fig. 1A). EPR was detected in fractions 24 to 38, but was strongest in fractions 26 to 28. The same fractions were also assayed for the presence of DHBV DNA by Southern blot analysis. Viral DNA was detected in all fractions tested, but was most abundant in fractions 24 to 27 (Fig. 1B). The density of fractions 24 to 28 was between 1.26 and 1.28 g/ml, nearly identical to the range observed for fractions containing most of the EPR in the first CsCl gradient. Fractions 21 to 36 were tested, without heating prior to electrophoresis, in a Laemmli-type DDDP activity gel assay. DDDP activities were detected in fractions 22 to 36, but were greater in number and intensities in fractions 25 to 31 (Fig. 2). Five DDDP active bands were discernible in these fractions with approximate molecular masses of 155, 98, 78, 70, and 68 kDa. These DDDP activities were divided into two size groups, I and II, based on similar sizes and migration patterns seen with DHBV core particle-associated DDDPs detected in Laemmli-type DDDP activity gels (unpublished observations). Although the strongest DDDP activities were concordant with peak amounts of viral markers (DHBV DNA and EPR) in fractions 25 to 29, significant DDDP activity was also detected in fractions containing less abundant amounts of these viral markers (fractions 23, 24, and 30–35). However, DDDP activity was not detected in the absence of either viral DNA or EPR. The minor discordance between peak amounts of viral markers with peak DDDP activities detected in the activity gel assay probably reflects differences in the sensitivity of detection among these three assays. In a separate experiment, crude virions were banded once in a CsCl gradient and selected fractions were tested in a partially denaturing RT activity gel assay. The profile of DHBV DNA and EPR for fractions 20 to 38 was nearly identical to those shown in Figs. 1A and 1B and a very faint RT activity with an approximate molecular mass of 80 kDa (group II) was detected in fractions 25 to 36 (data not shown).

These results show that DHBV isolated from viremic serum is associated with DDDP activities nearly identical to two major size groups of DDDP activities detected with liver-derived DHBV core particles, suggesting that they are specifically packaged into nucleocapsids which are later secreted into the circulation as virions. A study measuring the specific infectivity of DHBV finds that it is essentially one, indicating that defective virions are rare in viremic serum (Jilbert and Burrell, personal communication). Since the DDDPs we detected are not likely to be derived from defective virions, it follows that they are not derived from defective or aberrant core particles, but rather from replication-competent core particles which eventually mature into infectious virions. The *pol* gene origin of at least three of the DNAp activities is supported by previous studies with human hepatitis B virus (HBV).

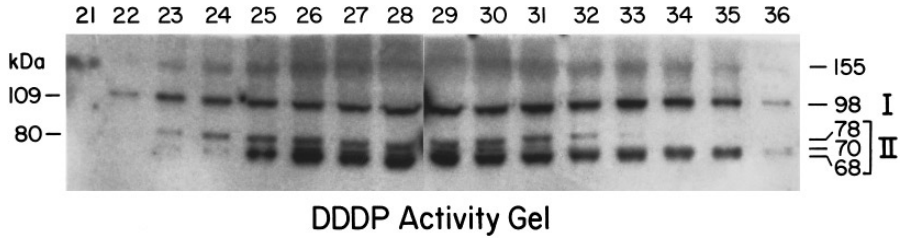

FIG. 2. Fractions from the second CsCl gradient in which serum-derived virions were banded and tested for DDDP activities in Laemmli-type, 8% PA DDDP activity gel assays (the same fractions described in the legend of Fig. 1). Samples were not heated prior to electrophoresis. DDDP activities were designated I and II based on their estimated molecular masses (in kDa) shown on the right side of the gels. *E. coli* DNAp I (p109) and Moloney murine leukemia virus (MMLV) RT (p80) are represented by the bars on the left side of the gels and were included as molecular mass markers and positive controls for the assay as described previously (*8*). The two separate gels shown here were run and assayed in parallel. They were aligned using the *E. coli* DNAp I and MMLV RT molecular mass markers.

HBV particles from patient sera and from a cell line stably transfected with HBV genomes contained, respectively, 100-kDa (*11*) and 70-kDa (*12*) proteins with DDDP and RT activities which were identified as *pol* gene products by specific antisera. These two DNAp species are nearly identical in size to the Group I (98 kDa) and II (68 to 78 kDa) DDDPs we detected in preparations of virions and similar to two of the RTs (p109 and p80) detected with highly purified core particles from DHBV-infected ducks (*8*). Two other studies identified a 65-kDa *pol* gene product solubilized from preparations of HBV from patient serum (*13*) and liver-derived woodchuck hepatitis virus (WHV) core particles (*14*), in Western blots using Pol-specific antisera. We have also detected a similarly sized protein (p63) with DDDP and RT activities in preparations of DHBV core particles (*8*) and virions (p68 in Fig. 2). Since the predicted full-length protein encoded by the *pol* gene is 94 kDa for HBV and WHV and 85 kDa for DHBV, the 65- to 70-kDa proteins likely represent less-than-full-length proteins. The reactive antisera in the WHV study were directed at the carboxy-terminal sequences of the WHV polymerase and also detected a 31-kDa protein in WHV core particles which is nearly identical in size to the RNase H activity (p35) associated with both DHBV and WHV viremic sera and purified DHBV liver cores (*9*). The similarities in sizes and activities of the HBV and WHV Pol proteins identified by immunostaining with many of the DNAp and RNase H activities associated with DHBV core particles and virions suggest that these activities represent products of the viral *pol* gene present within virus particles and, although not covalently linked to the viral genome, were nonetheless involved in its replication. It remains possible that the DHBV-associated DNAp and RNase H activities we have detected are host proteins incorporated into viral nucleocapsids; however, their consistent detection in virus particle preparations suggests that their association is not adventitious, but functionally relevant. Our findings support the contention that multiple *pol* gene products are expressed and packaged within core particles which are the precursors to infectious virions.

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