

Detection of DNA Polymerase Activities Associated with Purified Duck Hepatitis B Virus Core Particles by Using an Activity Gel Assay

STEPHANIE M. OBERHAUS AND JOHN E. NEWBOLD*

Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received 22 January 1993/Accepted 30 July 1993

Replication of hepadnaviruses involves reverse transcription of an intermediate RNA molecule. It is generally accepted that this replication scheme is carried out by a virally encoded, multifunctional polymerase which has DNA-dependent DNA polymerase, reverse transcriptase, and RNase H activities. Biochemical studies of the polymerase protein(s) have been limited by the inability to purify useful quantities of functional enzyme from virus particles and, until recently, to express enzymatically active polymerase proteins in heterologous systems. An activity gel assay which detects in situ catalytic activities of DNA polymerases after electrophoresis in partially denaturing polyacrylamide gels was used by M. R. Bavand and O. Laub (J. Virol. 62:626-628, 1988) to show the presence of DNA- and RNA-dependent DNA polymerase activities associated with hepatitis B virus particles produced in vitro. This assay has provided the only means by which hepadnavirus polymerase proteins have been detected in association with enzymatic activities. Since conventional methods have not allowed purification of useful quantities of enzymatically active polymerase protein(s), we have devised a protocol for purifying large quantities of duck hepatitis B virus (DHBV) core particles to near homogeneity. These immature virus particles contain DNA- and RNA-dependent DNA polymerase activities, as shown in the endogenous DNA polymerase assay. We have used the activity gel assay to detect multiple DNA- and RNA-dependent DNA polymerase proteins associated with these purified DHBV core particles. These enzymatically active proteins appear larger than, approximately the same size as, and smaller than an unmodified DHBV polymerase protein predicted from the polymerase open reading frame. This is the first report of the detection of active hepadnavirus core-associated DNA polymerase proteins derived from a natural host.

The *Hepadnaviridae* family is divided into two subgroups based on the host species that these viruses infect: the orthohepadnaviruses infect mammalian species (humans, woodchucks, ground squirrels), and the avihepadnaviruses infect avian species (ducks, herons) (15). The orthohepadnavirus genome is a partially double stranded, circular DNA molecule in which the plus and minus strands are covalently linked to an RNA oligomer and a protein moiety, respectively. The avihepadnavirus genome shares the same structural features except that it appears to be fully double stranded (23). The mammalian viruses contain four open reading frames (ORFs) encoding core (nucleocapsid), surface (envelope), X (unknown function), and polymerase (Pol) proteins. The avian viruses lack the X ORF but contain similar versions of the other three ORFs (for reviews, see references 35 and 38).

Studies using duck hepatitis B virus (DHBV) provided the first model for hepadnavirus replication (42). Summers and Mason (42) showed that an RNA intermediate was reverse transcribed and progressively degraded as first (minus)-strand DNA synthesis took place; full-length minus-strand DNA then served as template for second (plus)-strand synthesis. Synthesis of minus-strand DNA is primed by a protein encoded by the *pol* ORF (4, 10, 46). Plus-strand synthesis is primed by an RNA oligomer derived from the 5' terminus of the pregenomic RNA, presumably by an RNase H activity (22). In human hepatitis B virus (HBV) and by analogy in the other orthohepadnaviruses, the polymerase appears to have the additional

function of completing plus-strand synthesis prior to formation of the covalently closed circular DNA molecule from which the intermediate (pregenomic) RNA is transcribed (for a review, see reference 39).

Analysis of the predicted amino acid sequences derived from the mammalian and avian *pol* ORFs revealed regions which are highly conserved among hepadnaviruses (28). Some of these regions are also similar to sequences found in retroviral polymerases which are known to be required for reverse transcriptase (RT) and RNase H activities (44). The similarity in amino acid sequences between retrovirus and hepadnavirus *pol* ORFs and the observation that replication takes place within cytoplasmic core particles (42) suggested that the enzymatic steps involved in replication are carried out by a virally encoded DNA polymerase (DNAP). These steps require DNA-dependent DNAP (DDDP), RNA-dependent DNAP (RT), and RNase H activities, as well as the generation of the RNA oligomer and protein primers. In vitro transfection experiments which examined the effects of mutations in the *pol* ORF on production of specific replicative intermediates support the presence of a virally encoded polymerase with domains encoding a primer protein and an RT-RNase H separated by a nonessential spacer (11, 13, 21, 33).

Biochemical studies of the polymerase protein(s) have been limited by the inability to purify useful quantities of the protein(s) and, until recently, to express enzymatically active *pol* ORF products in heterologous systems (16, 43, 46). As a result, little is known about how the *pol* gene is expressed. Unlike retroviral polymerases, the hepadnavirus polymerase is not derived from a fusion protein but rather appears to be

* Corresponding author.

translated from a bicistronic mRNA at an internal initiation site (12, 31, 37). Proteins exhibiting DNA- and RNA-dependent DNAP activities have been detected in HBV particles secreted from a transfected hepatoma cell line in an activity gel assay (6, 7). Similar *in situ* gel assays have been used to detect a variety of enzyme activities in other systems (9, 41). This assay has provided the only means by which HBV Pol proteins from virus particles have been identified in association with specific enzymatic activities. Since products of the *pol* ORF appear to be present in very low abundance within virus particles, we have used the activity gel assay to detect DNA- and RNA-dependent DNAP activities associated with large quantities of purified DHBV core particles. These subviral particles, which have not yet been assembled with envelope proteins, were isolated from the livers of congenitally infected ducks, in which they are present in high concentrations. A protocol for the purification of large quantities of these particles to near homogeneity is described. This is the first report of the detection of hepadnavirus core-associated DNAP activities derived from a natural host.

MATERIALS AND METHODS

Preparation of DHBV core particles. A protocol for the purification of DHBV core particles to near homogeneity was devised. DHBV core particles were purified from 1 kg of liver taken from congenitally infected Pekin ducks which were sacrificed 10 to 18 days posthatch. Ducks were obtained from a commercial supplier and were of the same lineage as those from which DHBV 16 was cloned (26) and sequenced (25). The livers were used at once or stored at -70°C and thawed as needed. All steps of the purification scheme were carried out on ice or at 5°C .

The fresh or thawed livers were minced and homogenized with a Dounce tissue homogenizer and a loose-fitting pestle in H buffer (0.02 M Tris [pH 7.4], 0.05 M NaCl, 0.007 M MgCl_2 , 0.1% 2-mercaptoethanol, 8% sucrose). The homogenate was centrifuged in an HB-4 rotor at 10,000 rpm for 20 min to remove nuclei, large cellular material, and unbroken cells. A surface layer of fat which formed at the top was removed, and the pooled supernatants were centrifuged in an SW27 rotor at 27,000 rpm for 90 min to pellet polysomes. These supernatants were pooled, and EDTA was added to a final concentration of 10 mM to convert any remaining polysomes to monosomes. 2-Mercaptoethanol was added to a final concentration of 0.1% to prevent oxidation. Core particles were concentrated from this pool by pelleting through a sucrose cushion. The pool was layered over 4 ml of 15% and 4 ml of 30% sucrose in core buffer (CB; 0.02 M Tris [pH 7.4], 50 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.01% Triton X-100) and centrifuged in an SW27 rotor at 20,000 rpm for 15 h. The pellets containing the crude cores were dissolved in CB, using a Dounce tissue homogenizer.

Large-molecular-weight proteins and protein complexes were removed by precipitation with 5% polyethylene glycol 8000. Following immediate centrifugation in an HB-4 rotor at 10,000 rpm for 20 min, core particles were concentrated from the supernatants by precipitation with 15% polyethylene glycol 8000 and 0.5 M NaCl in an ice bath for at least 4 h. Core particles were pelleted as before and dissolved in CB, using a Dounce tissue homogenizer. This crude preparation of core particles was sedimented in 30-ml continuous 15 to 30% sucrose gradients. Approximately 100 to 200 mg of protein was layered on each gradient, and centrifugation was carried out in an SW27 rotor at 27,000 rpm for 4 h. One-milliliter fractions were collected by bottom puncture and assayed for the pres-

ence of core particles, using the endogenous DNAP reaction (17, 42). In this assay, 20 μl of each fraction was incubated with 10 mM MgCl_2 , 0.05 mM each dATP, dCTP, and dTTP, and 0.95 μCi of $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ (800 or 3,000 Ci/mmol) for 30 min at 37°C . The reaction was terminated by the addition of 2 volumes of 0.1 M NaPP_i and 1% sodium dodecyl sulfate (SDS). The reaction was then transferred to a glass fiber disc, washed 10 times with 0.6 M HCl and 95% ethanol alternately, and dried. Incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$ was detected by scintillation counting. Fractions which showed significant endogenous DNAP activity (usually the bottom 13 to 15 fractions for the first gradients) were diluted with CB and pelleted in an SW27 rotor at 27,000 rpm overnight. These pellets were dissolved in CB by using a Dounce tissue homogenizer and sedimented again in sucrose gradients. Approximately 35 to 100 mg of protein was layered per gradient, and centrifugation, DNAP assays, and concentration of cores were repeated as described above. In some cases, cores pooled from the second set of sucrose gradients were layered onto a single sucrose gradient and isolated as before.

Core particles were further purified in buoyant CsCl density gradients. Partially purified core particles were mixed with CsCl in CB (0.4725 g of CsCl per ml) plus 2 μl of Triton X-100, brought to a final volume of 3 ml, and then centrifuged in an SW50.1 rotor at 40,000 rpm for 48 h. If Triton X-100 was omitted from core buffer, a quantitative loss of core particles was observed, apparently by adherence to the tube walls (data not shown). Fractions (100 μl) were collected by bottom puncture and assayed by using the endogenous DNAP assay. Fractions containing endogenous DNAP activity were pooled, and cores were banded a second time in buoyant CsCl. These fractions were brought to 1 ml with 30% sucrose in CB and stored at 4°C .

In a separate experiment, CsCl-banded core particles were shown to cosediment with partially purified core particles in sucrose gradients in the following way. It has been previously observed that HBV particles are associated with a protein kinase that transfers the ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to core protein and other proteins in the presence of MgCl_2 (1). An analogous activity has been observed in association with purified DHBV core particles (30). Core particles which had been purified by using the protocol described above (through two CsCl gradients) were radioactively labeled in a phosphorylation reaction mixture (3.3 mM MgCl_2 , 3.3 mM dithiothreitol, 250 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ [3,000 Ci/mmol]) at 37°C for 3 h. The labeled cores were then pelleted through a 1-ml 30% sucrose cushion in an SW56 rotor at 40,000 rpm at 5°C overnight. The pelleted cores were dissolved in CB and analyzed by polyacrylamide gel electrophoresis (PAGE) and autoradiography. The major band observed was core protein, as judged by its predicted molecular weight and characteristic multiple bands (32, 36). Approximately 200 g of DHBV liver was homogenized, and core particles which had been radiolabeled in the phosphorylation reaction were added. Core particles were then purified from the homogenate by using the protocol described above through three successive sucrose gradients. All sucrose gradient fractions were analyzed by Cerenkov counting and assayed by using the endogenous DNAP reaction. The ^{32}P -labeled cores were found to cosediment with unlabeled cores, indicating that these purified cores do not represent an aberrant population of particles selected for exclusively by banding in CsCl.

Analysis of purified DHBV core particles. Fractions from the final CsCl density gradient were assayed for endogenous DNAP activity and analyzed for the presence of core protein and other proteins by PAGE and silver staining (20, 47). Core

particles from fractions which contained endogenous DNAP activity and core protein were visualized by negative staining using uranyl acetate and electron microscopy.

In addition, the presence of viral DNA- and RNA-dependent DNAP activities were inferred from examination of the products of the endogenous DNAP reaction. In some cases, samples were preincubated in the presence of 100 μ g of actinomycin D per ml for 5 min prior to addition of the reaction mixture. Actinomycin D inhibits DNA-directed and, to a lesser extent, RNA-directed DNA synthesis, allowing for a more definitive evaluation of the enzymatic activities responsible for synthesis of the reaction products (42). Following the 30-min incubation at 37°C, the reaction was terminated and proteins were digested by addition of SDS to 0.4%, proteinase K to 0.4 mg/ml, and EDTA to 0.02 M and incubation at 37°C for 1 h. The reaction products were examined by agarose gel electrophoresis in E buffer (40 mM Tris HCl [pH 7.2], 20 mM sodium acetate, 1 mM EDTA) and autoradiography (26, 42).

DNAP activity gel assays. DNAP activity gel assays were done essentially as described by Bavand et al. (6, 7), with minor modifications. PAGE was carried out in the presence of 0.02% SDS with the addition of 100 μ g of nicked, duplex DNA per ml for the detection of DNA-directed DNA synthesis or 118 μ g of poly(rC · oligo(dG))₁₂₋₁₈ at a molar ratio of 1:10 for detection of RNA-directed DNA synthesis (RT activity) prior to gel polymerization. Core particles, purified to near homogeneity, were pelleted and dissolved in sample buffer (50 mM Tris [pH 6.8], 5% glycerol, 0.67% SDS, 0.1 μ M 2-mercaptoethanol, 0.33 mM EDTA, 0.002% bromophenol blue); in some cases, 15 μ g of bovine serum albumin (BSA) which had been heated at 65°C for 4 h was added. Samples were not heated prior to loading. Electrophoresis in 0.75-mm, 8% discontinuous polyacrylamide (acrylamide/bisacrylamide, 30:0.8) gels was carried out at 4°C at 100 V for approximately 12 to 15 h. The gels were then washed with six 1-liter changes of chilled 50 mM Tris (pH 7.5) per 15- by 15-cm gel on a shaker at 4°C over a 20-h period. Gels were placed in a sealed bag with a reaction mixture (20 ml per 15- by 15-cm gel) containing 70 mM KCl, 10 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, and 4 μ Ci of [α -³²P]dGTP (3,000 Ci/mmol) per ml for RT gels; and for DDDP gels, 13.3 μ M each dATP, dTTP, and dCTP were also added. The bags were submerged and incubated in a 37°C water bath for 20 h. To stop the reaction and remove unincorporated deoxynucleoside triphosphates (dNTPs), gels were washed with six 1-liter changes of chilled 5% trichloroacetic acid–1% NaPP₃ on a shaker at 4°C over a 24-h period. The gels were dried and analyzed by autoradiography.

Reagents for activity gel assays were obtained as follows. DNA used as substrate in DDDP activity gels was salmon sperm DNA from Sigma dissolved in 10 mM Tris–1 mM EDTA (pH 8.0), phenol extracted, and ethanol precipitated but not treated with nuclease; poly(rC) was from Pharmacia, oligo(dG))₁₂₋₁₈ was from Midland, and ultrapure SDS was from BRL-GIBCO. All other chemicals were obtained from Sigma, and [α -³²P]dGTP and [γ -³²P]ATP came from NEN or ICN. Moloney murine leukemia virus (MMLV) RT from Bethesda Research Laboratories and *Escherichia coli* DNAP I from Boehringer Mannheim Biochemicals were used as positive controls and molecular weight markers.

Southern blot analysis of a DNAP activity gel. An 8% polyacrylamide activity gel containing salmon sperm DNA was prepared as described above. Cores isolated through two successive sucrose gradients were loaded in duplicate lanes, and electrophoresis was carried out as described above. The gel was rinsed briefly in water and then equilibrated in two 500-ml changes of NAQ buffer (ISS) for 15 min each time. The

gel was electroblotted to MagnaGraph positively charged nylon membrane (MSI), using an ISS semidry electroblotter according to the manufacturer's instructions. The membrane was baked at 80°C for 1 h and then prehybridized at 42°C for 3 h in 50% formamide–5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% SDS–100 μ g of denatured salmon sperm DNA per ml–1 mg of BSA per ml–0.1% polyvinylpyrrolidone–0.1% Ficoll. Prehybridization solution was replaced with hybridization solution (50% formamide, 5 \times SSC, 0.2% SDS, 50 μ g of denatured salmon sperm DNA per ml, 0.2 mg of BSA per ml, 0.02% polyvinylpyrrolidone, 0.02% Ficoll) containing 2 \times 10⁶ cpm of either a plus- or minus-strand DHBV RNA probe per ml and incubated at 42°C for 15 h.

Plus- and minus-strand DHBV RNA probes were prepared from plasmid constructs containing a full-length copy of the DHBV genome in both orientations: pSP65.DHBV5.1 for plus-strand polarity and pSP65.DHBV5.2 for minus-strand polarity (45). ³²P-labeled RNA was synthesized by using SP6 polymerase (Promega) as described by Melton et al. (27). Labeled RNA was separated from free [α -³²P]UTP by gel filtration over a Sephadex G-75 column.

Blots were washed for 5 min in 2 \times SSC–0.5% SDS at room temperature with shaking, then for 15 min in 2 \times SSC–0.1% SDS at room temperature with shaking, and finally for 2.5 h in three changes of 0.1 \times SSC–0.5% SDS at 52°C. Membranes were analyzed by autoradiography.

RESULTS

Analysis of purified DHBV core particles. (i) Endogenous DNAP activity. All fractions from the final CsCl density gradient were assayed for the presence of core particles, using the endogenous DNAP reaction (Fig. 1A). Fractions 24 to 32 showed significant activity compared with the negative controls (in which core buffer was substituted for fraction samples). Peak endogenous DNAP activity was observed in fractions 26, 27, and 28. The density of CsCl in these fractions was 1.34 g/cm³.

(ii) SDS-PAGE and silver staining. A subset of these fractions was also analyzed by SDS-PAGE and silver staining (Fig. 1B). The predominant species detected in these gels was core protein, as judged by the characteristic multiple bands at and greater than its predicted molecular weight of 31,000 (32, 36). Core protein was evident in fractions 25 to 32 and most abundant in fractions 27 to 30. In addition, several fainter-staining bands at approximately 110, 98, 90, 83, and 78 kDa were observed in fractions 26 to 31. These bands are resistant to treatment with DNase after core particles are disrupted by boiling (data not shown). They represent protein species which are closely associated with core particles and may be of viral origin.

(iii) Electron microscopy. Fractions from the final CsCl density gradient which were shown to contain core particles were examined by electron microscopy (Fig. 1C). The previously characterized (42) electron-dense and spiked, 35- to 37.5-nm spherical core particles were seen in the absence of other material in all fields viewed. These particles appeared to be intact and fully dispersed. Together, the electron microscopy and SDS-PAGE analyses indicate that these core particles have been purified to near homogeneity while maintaining their apparent native structure.

(iv) Endogenous DNAP reaction in the presence and absence of actinomycin D. The presence of DNA- and RNA-directed DNA syntheses in fractions from the final CsCl gradient was determined by examining the endogenous DNAP

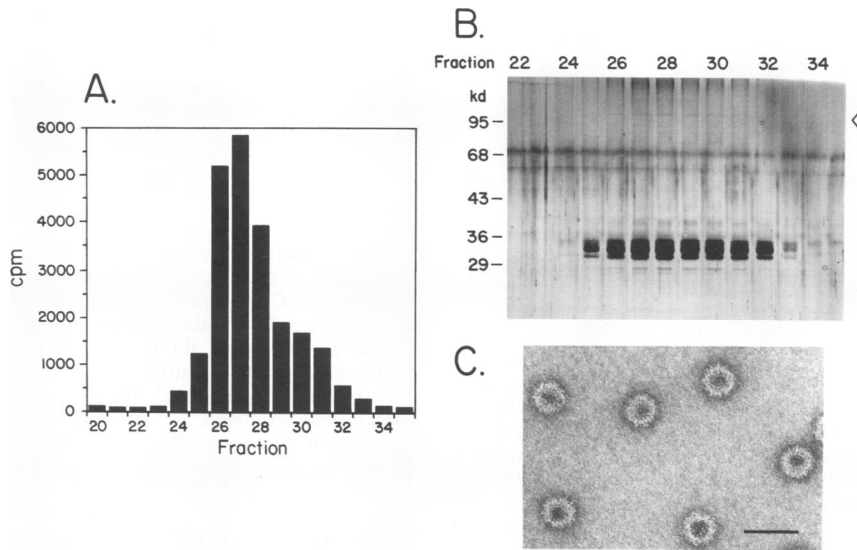


FIG. 1. Analysis of purified DHBV core particles. Fractions are numbered from the bottom of the gradient to the top. (A) Fractions from the final CsCl gradient were assayed by using the endogenous DNAP assay. Incorporation of [α - 32 P]dGTP was detected by scintillation counting. (B) Silver-stained SDS-12% polyacrylamide gel of fractions from the final CsCl gradient. Core protein is represented by the heaviest-staining bands seen between the 29- and 36-kDa protein standards. Arrow points to DNase-resistant protein at 110 kDa. (C) Electron micrograph of DHBV core particles purified to near homogeneity. The bar represents 50 nm.

reaction products in the presence and absence of actinomycin D (Fig. 2). The greatest incorporation was observed in fractions 27 and 28. Both plus- and minus-strand DNA syntheses appear to be taking place in all of these fractions except fraction 24, which showed plus-strand synthesis only. The replicative intermediates detected in the fractionated core particles exhibited a previously observed density-dependent profile (29). Cores containing replicative intermediates representing early steps in replication were found in fractions less dense than those showing peak endogenous DNAP activity (fractions 30 to 32), while cores undergoing later steps in replication were found in more dense fractions (fractions 24 and 25). These results indicate the presence of both DNA- and RNA-dependent DNAP activities within these core particles.

Recovery of endogenous DNAP activities during purification. Core particles were assayed for endogenous DNA- and RNA-dependent DNAP activities before and after centrifugation in CsCl. Previous observations have indicated that exposure to CsCl may result in a loss of endogenous DNAP activities (4). Both plus- and minus-strand species were easily detected in core particles prior to centrifugation in CsCl (Fig.

3A). In core particles assayed after centrifugation in CsCl, plus-strand synthesis was easily detected, but minus-strand synthesis appeared to be less than that in pre-CsCl cores (Fig. 3B). These results suggest that the endogenous RT activity is more adversely affected than the endogenous DDDP activity by a single banding in CsCl.

DNAP activity gel assays. (i) Core particles before banding in CsCl. Core particles (purified through two successive sucrose gradients) were assayed in DNAP activity gels before centrifugation in CsCl (Fig. 4A). Three major bands were detected in the DDDP activity gel assay: a very strong band at 80 kDa, a slightly less intense band at 63 kDa (the molecular mass of this band was calculated by extrapolation because of the lack of an appropriate marker), and a weaker band at 109 kDa. Three bands were also detected in the RT activity gel assay: a strong band at 80 kDa, a less intense band at 63 kDa, and a very faint band at 109 kDa. The three RT-active bands appear to have the same apparent molecular masses as the three major DDDP-active bands, 109, 80, and 63 kDa, suggest-

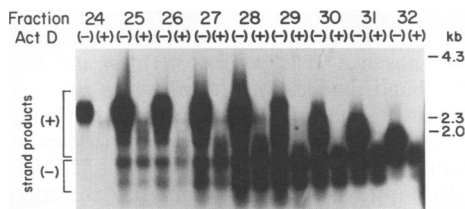


FIG. 2. Fractions from the final CsCl gradient assayed in the endogenous DNAP reaction in the absence (-) and presence (+) of actinomycin D (Act D). Fractions are numbered from the bottom of the gradient to the top. Radioactively labeled plus- and minus-strand products were detected by autoradiography and identified by size and actinomycin D sensitivity (42).

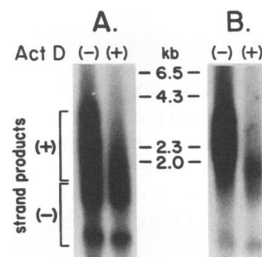


FIG. 3. Endogenous DNAP reaction products from DHBV core particles purified through two successive sucrose gradients before (A) and after (B) a single banding in CsCl. Radioactively labeled plus- and minus-strand products were detected by autoradiography and identified by size and actinomycin D (Act D) sensitivity (42).

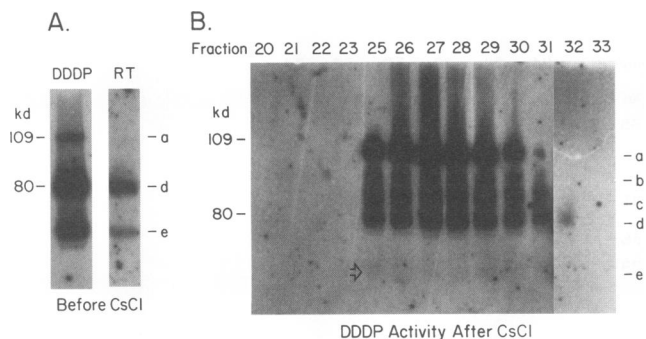


FIG. 4. (A) Activity gel assays for DNA- and RNA-dependent DNAP activities associated with DHBV core particles before banding in CsCl. (B) Fractions from the final CsCl gradient in an activity gel assay for the detection of DDDP proteins. The arrow points to faint DDDP band at approximately 63 kDa. Fractions are numbered from the bottom of the gradient to the top. *E. coli* DNAP I (p109) and MMLV RT (p80) were included as molecular weight markers and positive controls for activity. They are represented by the lines beside the gels and were used to align the DDDP and RT gels in panel A. DDDP and RT bands are identified by the letters a to e; their approximate sizes are 109 (a), 90 (b), 85 (c), 80 (d), and 63 (e) kDa.

ing that these three proteins exhibit both DNA-dependent and RNA-dependent DNAP activities.

(ii) **Core particles after banding in CsCl.** Selected fractions from the final CsCl gradient were assayed in DNAP activity gels. Major bands at approximately 63, 80, 85, and 109 kDa were detected in fractions 25 to 31 in the DDDP activity gel assay (Fig. 4B). Fraction 32 showed only a very faint band at 80 kDa. All other fractions tested were negative for DDDP activity. Fractions 25 to 32 were also the only fractions found to contain significant endogenous DDDP activity, core protein, and DNase-resistant silver-stained bands similar in size to some of the DDDP proteins, as shown in Fig. 1B. Three of the DDDP bands detected in the CsCl banded core particles appeared to be identical in size to the three bands detected in core particles prior to banding in CsCl (109, 80, and 63 kDa). The 63-kDa band is faint but can be seen in fractions 25 to 31 upon close inspection. It may represent a protein species which is not closely associated with core particles and is mostly lost after banding in CsCl, or it may represent a polymerase which is more inactivated by banding in CsCl, while the other DDDP species are less so.

The same selected fractions were also tested in RT activity gels in which two very faint bands 109 and 80 kDa could be discerned in fractions 27 to 30 (data not shown). These bands appeared only in fractions which also exhibited DDDP activity in the gel assay, endogenous DNA- and RNA-dependent DNAP activities, and the presence of core protein. They also appeared to be the same size as two of the DDDP and RT activities (109 and 80 kDa) detected in core particles prior to centrifugation in CsCl (Fig. 4A).

These results were somewhat different from those seen with another preparation of core particles purified from 1 kg of liver by using the same protocol. In this case, the core-containing fractions showed only one DDDP band (109 kDa) and no RT bands in the gel assay. However, these fractions were not assayed until 2 months after collection, and only 5% of each fraction was tested in the gel assay. (The results shown in Fig. 4B were obtained with 30% of each fraction assayed immediately after collection.) Similar activity gel results were obtained with these core particles by adding BSA to the samples prior to loading the activity gels as described below.

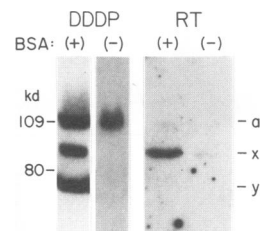


FIG. 5. Activity gel assays for DNA-dependent (A) and RNA-dependent (B) DNAPs associated with DHBV core particles after banding in CsCl. Samples were tested in the presence (+) and absence (-) of 15 μ g of BSA added just prior to loading the gels. *E. coli* DNAP I (p109) and MMLV RT (p80) were included as molecular weight markers and positive controls for activity. They are represented by the lines beside the gels. MMLV RT was used to align the individual gels. DDDP and RT bands are identified by the letters a, x, and y; their approximate sizes are 109 (a) (as in Fig. 4), 86 (x), and 75 (y) kDa.

(iii) **Addition of BSA to activity gel samples.** It has been observed by others that the addition of exogenous protein(s) to samples prior to SDS-PAGE sometimes enhances detection of enzyme activities in the activity gel assay (18). BSA was added to CsCl-purified core particles which showed only a single DDDP band and no RT bands in the activity gel assays, and the assays were repeated (Fig. 5). Three DDDP bands of approximately the same intensity were detected: 109, 86, and 75 kDa. Two RT bands were detected: a very faint band at 109 kDa and a strong band at 86 kDa. A faint 86-kDa band was also detected when 10-fold more of the core-containing fraction for which RT activity could not be detected before was tested. Since the detection of DDDP and RT activities must be carried out in separate gels, it is somewhat difficult to compare DDDP and RT active bands with respect to size. This analysis is complicated further by the scarcity of DDDP and RT molecular weight markers. The molecular masses of the DDDPs and RTs reported here were extrapolated by using the known molecular masses of the *E. coli* DDDP and MMLV RT controls and therefore are approximations. All of these bands were approximately the same size as DDDP and RT activities detected in cores prior to centrifugation in CsCl and after two bandings in CsCl in which a third of the CsCl fractions were tested in the gel assays.

The BSA which was added to the samples was tested in both DDDP and RT activity gels to determine whether it contained DDDP and RT activities (data not shown). This BSA was found to contain a weak DDDP activity at 109 kDa but no RT activity. Since the core-associated 109-kDa activity appeared the same in DDDP activity gels in the presence and absence of BSA, the BSA was clearly not the source of this band. The two additional DDDP bands detected in the presence but not in the absence of BSA (86 and 75 kDa) are similar in size to DDDP bands detected in association with a larger sample of cores in the absence of BSA (Fig. 4B), 85 and 80 kDa. The 86-kDa RT band detected in the presence of BSA was also detected in its absence by loading more of the sample. These results indicate that the addition of BSA to cores banded in CsCl enhances detection of at least some DDDP and RT proteins. In contrast, the addition of BSA to cores prior to banding in CsCl did not have any effect on the detection of DDDP and RT proteins (data not shown). This may be due to the presence of proteins in these crude preparations which may act similarly to exogenously added BSA but are removed when cores are banded in CsCl.

(iv) **DNAP activity gel assay in the absence of substrate.** It

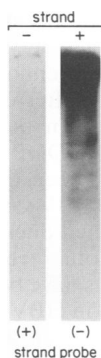


FIG. 6. Southern blot analysis of an activity gel. Sucrose gradient-purified DHBV core particles were loaded into duplicate lanes of a DNAP activity gel. The gel was blotted to a positively charged nylon membrane without hydrolysis of RNA and probed with a plus (+)- or minus (-)-strand DHBV RNA probe, as indicated.

has been observed by others that some DNAPs detected in the activity gel assay do not always utilize the primer-template provided in the gel (8, 40). These polymerases are thought to be bound to a part of their native substrate which then comigrates with the polymerase molecules. During the activity gel assay, the polymerase acts on the native and perhaps some of the exogenous primer-template as well, using the radiolabeled dNTP supplied. This type of enzyme-substrate complex can be detected in the activity gel assay by omitting the primer-template from the gel.

Core particles were assayed for polymerase activity in the absence of substrate to determine whether core-associated polymerase activities were acting on the exogenously supplied or comigrating, core-associated substrates. Core particles isolated through two successive sucrose gradients and cores twice banded in CsCl were tested. None of the cores samples which showed polymerase activity in gels containing primer-template substrates showed activity in their absence (data not shown). These results indicate that the core-associated polymerase activities are acting on the exogenously supplied substrates and not viral nucleic acids which are likely to be present.

(v) **Southern blot analysis of an activity gel.** The presence of core-associated viral nucleic acids in the activity gel was determined by Southern blot analysis. Although viral nucleic acids do not appear to be used as substrates in the gel assay, they are likely to be present and may still be associated with viral polymerase protein(s). A typical DDDP activity gel containing the DNA substrate and duplicate lanes of cores isolated through two successive sucrose gradients was analyzed by Southern blotting with plus- and minus-strand DHBV RNA probes (Fig. 6). Both probes hybridized with material (both smeared and discrete bands) from the top of the gel to approximately 109 kDa, but hybridization with the minus-strand probe was much stronger. In addition, the minus-strand probe bound to material migrating faster in the gel (<109 kDa). These results suggest that viral nucleic acids are present in the activity gels and migrate within regions where polymerase activities have been detected. If polymerase molecules are bound to these nucleic acids, their apparent molecular masses would be aberrantly represented in the gel assay. These nucleic acids may be single-stranded RNA or DNA species, since an alkaline denaturation step was not included in the Southern blot procedure. The strong hybridization of the minus-strand probe and the previously demonstrated endoge-

nous RT activity taking place within these core particles suggests the presence of significant amounts of viral RNA in these gels. Since minus-strand DNA has been shown to be covalently attached to a Pol-related protein (4), the presence of the plus-strand probe at the top of the gel may represent minus-strand DNA-Pol protein complexes which are unable to migrate into the separating gel.

In a similar experiment in which an alkaline denaturation step was carried out, minus-strand DNA was detected at the top of the gel, but no plus-strand DNA was detected. This result together with the Southern blot analysis in the absence of an alkaline denaturation step suggests that viral RNA is present in the sample lanes of these activity gels, including regions where polymerase activities have been detected. These experiments do not determine whether the polymerases that we detect in the activity gel assays are associated with viral nucleic acids, but they suggest that it is possible.

DISCUSSION

The apparent low abundance of *pol* ORF products in virus particles has limited detection and characterization of these proteins from naturally infected hosts. We have purified and concentrated large quantities of DHBV core particles from the livers of congenitally infected ducks and shown that they are associated with proteins which exhibit DNA- and RNA-dependent DNAP activities in activity gel assays.

Strong DNA- and RNA-dependent DNAP activities were detected with cores assayed prior to banding in CsCl at 109, 80, and 63 kDa. A similar profile was observed for cores after banding in CsCl, except that RT activity appeared to be preferentially decreased. This phenomenon was also observed in the endogenous DNAP assay. These results suggest that the viral polymerase is sensitive to CsCl even when it is contained within core particles (examination of CsCl banded cores by electron microscopy does not indicate that core particles are being disrupted). The addition of BSA was shown to enhance detection of some DDDP and RT proteins in the activity gel assay.

The assignment of molecular weights to the DNAP proteins detected in the activity gel assay has been done by comparison with standard proteins. However, the conditions under which these gels are run do not preclude the possibility that proteins may remain associated with nucleic acid or even other proteins. In addition, posttranslational modifications (i.e., phosphorylation) or other factors may alter the expected mobility of a protein whose molecular weight has been predicted from amino acid composition alone. Southern blot analyses of DDDP activity gels indicate that viral nucleic acid is present in sample lanes, including regions where DNAP activities are detected. Minus-strand DNA appears to be restricted to the top of the gel. Plus-strand species were detected at the top of the gel and throughout the sample lane. These species may represent various lengths of pregenomic RNA generated by RNase H digestion during minus-strand synthesis. These RNA molecules would have to be free from nascent minus-strand DNA to show up as a range of sizes as observed, but this denaturation has been observed to occur under relatively mild conditions (29, 42). If Pol proteins detected in the activity gel assay are covalently or otherwise associated with these nucleic acids, their apparent molecular weights will appear greater than predicted for the unmodified protein(s). These data are consistent in part with observations from two groups who have expressed hepadnavirus polymerases in vitro. Wang and Seeger used an in vitro translation system to express DHBV polymerase as a single protein predicted from the *pol* ORF

(46). When this protein was incubated in the presence of a polymerase reaction mixture including ^{32}P -labeled dGTP, it became covalently bound to the labeled dGTP and then continued to incorporate unlabeled dNMPs. The resulting Pol-DNA complex was analyzed by SDS-PAGE and was observed as a smear from the predicted molecular mass of 90 kDa to the top of the gel. The more slowly migrating species are believed to represent the Pol protein covalently attached to minus-strand DNA molecules increasing in length as dNMPs are added. These species were found to be unaffected by digestion with RNase A but could be resolved as a single 90-kDa band after digestion with micrococcal nuclease. Bartenschlager et al. have expressed the HBV *pol* gene as a single protein in hepatoma cells by using recombinant vaccinia viruses (3). This construct included the addition of target sites for protein kinase C so that the Pol protein could be tagged by in vitro phosphorylation. They also detected a smear of material from 90 kDa to the top of the gel when they immunoprecipitated the labeled Pol protein and analyzed it by SDS-PAGE. This smear was also unaffected by digestion with RNase A but was reduced to a more homogeneous band at 90 kDa following digestion with micrococcal nuclease. Although the viral polymerase is not thought to be associated covalently with pregenomic RNA, an association of some kind is required for encapsidation of both the RNA and polymerase molecules, and a continued association must exist during minus-strand synthesis (2, 5, 14, 19). Since both of these studies described detection of Pol-DNA complexes following heat denaturation, the results indicate that viral RNA is not associated covalently with the polymerase, but these data do not preclude other kinds of complexes. Our Southern blot analyses were done without heating samples prior to SDS-PAGE so that any association of pregenomic RNA with Pol and minus-strand DNA might be preserved.

Our experiments do not distinguish whether the DNAP proteins detected in the activity gel assay are bound to viral nucleic acid. However, these viral nucleic acid species do not appear to act as substrates in the DNAP gel assays, since DNAP activities were not detected in the absence of exogenously added substrates. In contrast to a previous report in which DHBV polymerase did not act on exogenous templates added to permeabilized core particles (34), our results indicate that DHBV core-associated DNA- and RNA-dependent DNAP proteins are able to utilize a nonviral, exogenously added substrate in the activity gel assay. This feature of the activity gel assay allows detection and characterization of the specific enzymatic functions ascribed to the product(s) of the *pol* gene.

If the DHBV *pol* gene was expressed as a single, full-length, unmodified protein, it would have a predicted molecular mass of ~85 kDa. Although it is difficult to assign precise sizes to the DNAP proteins that we have detected, they correspond approximately to full-length (85 kDa), greater than full-length (109 kDa), and less than full-length DHBV *pol* (80, 75, and 63 kDa) gene products. Some of these proteins were difficult to detect after cores were banded in CsCl. The addition of BSA appeared to enhance the activity of some DDDP and RT proteins and facilitate their detection after cores were banded in CsCl. It is not certain whether this loss of activity is due to inactivation of these activities in CsCl or loss of nonviral DNAP activities which have copurified with core particles. It seems likely that these activities represent proteins which reside within the core particles, since (i) core particles are isolated by fractionation through two successive sucrose gradients and two successive bandings in CsCl, (ii) examination of these core particles by electron microscopy shows them to be

intact and free of protein species attached to them or present in the preparation, (iii) RT activity, as judged by the elongation of minus-strand DNA in the endogenous DNAP assay, also shows a significant decrease after cores were banded in CsCl with no significant decrease in the amount of core protein (data not shown), and (iv) DDDP proteins at approximately 109 and 85 kDa have also been detected in the activity gel assay, in association with DHBV virions fractionated from the serum of congenitally infected ducks (data not shown).

There have been two other reports in which less than full-length *pol* gene products have been detected. Bavand et al. identified a 70-kDa protein in HBV particles produced in vitro which showed RT activity in an activity gel assay (6). This protein was precipitated by a mixture of antisera directed against the carboxy terminus of the predicted Pol protein but not by an antiserum against the amino terminus. Mack et al. detected a 65-kDa protein in HBV virions from the serum of an infected patient by Western blotting (immunoblotting) with an antiserum generated against a tribrid fusion protein which included 143 amino acids from the predicted Pol protein (24). These findings and the detection of less than full-length DHBV core associated DNAP proteins in this report suggest that expression of the *pol* gene may involve mechanisms for the generation of subgenomic Pol proteins. Wu et al. have made mutations in the spacer region of the DHBV *pol* gene and evaluated their effects on replication in an in vitro transfection system (48). Their results indicate that expression of enzymatically active, truncated Pol proteins is possible in vitro. If the *pol* gene is expressed as multiple proteins in vivo, a strategy for packaging all of them required for replication would have to exist. They suggest a packaging signal present in each separate polypeptide or formation of a protein complex which is packaged as a single moiety. The 109 kDa DDDP and RT activities detected in our activity gel assay may represent a protein complex or a posttranslationally modified full-length *pol* gene product.

Three groups have recently reported heterologous expression of enzymatically active hepadnavirus Pol proteins (16, 43, 46). All three reports describe expression of a single full-length Pol protein predicted from the *pol* ORF with minimal levels of activity. The data described in this report and the detection of a 35-kDa RNase H in association with DHBV core particles and virions (30a) suggest that the *pol* ORF gives rise to multiple proteins which may function together as a protein complex in vivo. Our data suggest that the Pol proteins expressed as described in these other reports may lack structural features, perhaps the presence of the 35-kDa RNase H or its absence from the full-length Pol protein, which prevent efficient synthesis of complete minus-strand DNA.

Despite the difficulties in studying the hepadnavirus Pol protein(s), we have shown that it is possible to detect and characterize virally associated enzyme activities in the activity gel assay. We are using this assay in conjunction with a panel of antisera directed against *pol* ORF-derived synthetic peptides to confirm that the DNAP activities that we detect are derived from the *pol* ORF. In addition, preliminary evidence indicates that this assay will be useful for testing the effects of various enzyme inhibitors on the core-associated DNA- and RNA-dependent DNAP activities.

ACKNOWLEDGMENTS

This work was supported in part by grant 87-G-01009 from the North Carolina Biotechnology Center and by Public Health Service grant AI26052 from the National Institute of Allergy and Infectious Diseases.

We thank Millbrey Cate Starnes for many useful discussions.

REFERENCES

1. **Albin, C., and W. S. Robinson.** 1980. Protein kinase activity in hepatitis B virus. *J. Virol.* **34**:297-302.
2. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. *J. Virol.* **64**:5324-5332.
3. **Bartenschlager, R., C. Kuhn, and H. Schaller.** 1992. Expression of the P-protein of the human hepatitis B virus in a vaccinia virus system and detection of the nucleocapsid-associated P-gene product by radiolabelling at newly introduced phosphorylation sites. *Nucleic Acids Res.* **2**:195-202.
4. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. *EMBO J.* **7**:4185-4192.
5. **Bartenschlager, R., and H. Schaller.** 1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *EMBO J.* **11**:3413-3420.
6. **Bavand, M., M. Feitelson, and O. Laub.** 1989. The hepatitis B virus-associated reverse transcriptase is encoded by the viral Pol gene. *J. Virol.* **63**:1019-1021.
7. **Bavand, M. R., and O. Laub.** 1988. Two proteins with reverse transcriptase activities associated with hepatitis B virus-like particles. *J. Virol.* **62**:626-628.
8. **Beckman, L. D., and G. D. Frenkel.** 1976. Detection of DNA and RNA polymerase activities in situ following electrophoresis in polyacrylamide gels. *Nucleic Acids Res.* **3**:1727-1737.
9. **Bertazzoni, U., A. I. Scovassi, M. Mezzina, A. Sarasin, E. Franchi, and R. Izzo.** 1986. Activity gels for analyzing DNA processing enzymes. *Trends Genet.* **2**:67-72.
10. **Bosch, V., R. Bartenschlager, G. Radziwill, and H. Schaller.** 1988. The duck hepatitis B virus P-gene codes for protein strongly associated with the 5'-end of the viral DNA minus strand. *Virology* **166**:475-485.
11. **Chang, L.-J., R. C. Hirsch, D. Ganem, and H. E. Varmus.** 1990. Effects of insertional and point mutations on the functions of the duck hepatitis B virus polymerase. *J. Virol.* **64**:5553-5558.
12. **Chang, L.-J., P. Pryciak, D. Ganem, and H. E. Varmus.** 1989. Biosynthesis of the reverse transcriptase of hepatitis B viruses involves de novo translational initiation not ribosomal frameshifting. *Nature (London)* **337**:364-368.
13. **Faruqi, A. F., S. Roychoudhury, R. Greenberg, J. Israel, and C. Shih.** 1991. Replication-defective missense mutations within the terminal protein and spacer/intron regions of the polymerase gene of human hepatitis B virus. *Virology* **183**:764-768.
14. **Hirsch, R. C., J. E. Lavine, J.-L. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. *Nature (London)* **344**:552-555.
15. **Howard, C. R.** 1991. Family Hepadnaviridae, p. 111-116. *In* R. I. B. Fauquet, D. L. Knudson, and F. Brown (ed.), *Classification and nomenclature of viruses, fifth report of the international committee on taxonomy of viruses.* Springer-Verlag, New York.
16. **Howe, A. Y. M., J. F. Elliott, and D. L. J. Tyrrell.** 1992. Duck hepatitis B virus polymerase produced by in vitro transcription and translation possesses DNA polymerase and reverse transcriptase activities. *Biochem. Biophys. Res. Commun.* **189**:1170-1176.
17. **Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson.** 1973. DNA polymerase associated with human hepatitis B antigen. *J. Virol.* **12**:995-1005.
18. **Karawya, E., J. A. Swack, and S. H. Wilson.** 1983. Improved conditions for activity gel analysis of DNA polymerase catalytic polypeptides. *Anal. Biochem.* **135**:318-325.
19. **Köchel, H. G., M. Kann, and R. Thomssen.** 1991. Identification of a binding site in the hepatitis B virus RNA pregenome for the viral pol gene product. *Virology* **182**:94-101.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
21. **Li, J.-S., L. Cova, R. Buckland, V. Lambert, G. Deleage, and C. Trépo.** 1989. Duck hepatitis B virus can tolerate insertion, deletion, and partial frameshift mutation in the distal pre-S region. *J. Virol.* **63**:4965-4968.
22. **Lien, J.-M., C. E. Aldrich, and W. S. Mason.** 1986. Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand DNA synthesis. *J. Virol.* **57**:229-236.
23. **Lien, J.-M., D. J. Petcu, C. E. Aldrich, and W. S. Mason.** 1987. Initiation and termination of duck hepatitis B virus DNA synthesis during virus maturation. *J. Virol.* **61**:3832-3840.
24. **Mack, D. H., W. Bloch, N. Nath, and J. J. Sninsky.** 1988. Hepatitis B virus particles contain a polypeptide encoded by the largest open reading frame: a putative reverse transcriptase. *J. Virol.* **62**:4786-4790.
25. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. *J. Virol.* **49**:782-792.
26. **Mason, W. S., C. Aldrich, J. Summers, and J. M. Taylor.** 1982. Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus-strand DNA. *Proc. Natl. Acad. Sci. USA* **79**:3997-4001.
27. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, R. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035-7057.
28. **Miller, R. H., and W. S. Robinson.** 1986. Common evolutionary origin of hepatitis B virus and retroviruses. *Proc. Natl. Acad. Sci. USA* **83**:2531-2535.
29. **Newbold, J. E.** Unpublished data.
30. **Newbold, J. E., W. S. Mason, and J. Summers.** 1984. Purification and characterization of DHBV liver cores, p. 653. *In* G. N. Vyas, J. L. Dienstag and J. H. Hoofnagle (ed.), *Viral hepatitis and liver disease, proceedings of the 1984 International Symposium on Viral Hepatitis.* Grune & Stratton, Inc., Orlando, Fla.
- 30a. **Oberhaus, S. M., and J. E. Newbold.** Unpublished data.
31. **Ou, J.-H., H. Bao, C. Shih, and S. M. Tahara.** 1990. Preferred translation of human hepatitis B virus polymerase from core protein- but not from precore protein-specific transcript. *J. Virol.* **64**:4578-4581.
32. **Pugh, J., A. Zweidler, and J. Summers.** 1989. Characterization of the major duck hepatitis B virus core particle protein. *J. Virol.* **63**:1371-1376.
33. **Radziwill, G., W. Tucker, and H. Schaller.** 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. *J. Virol.* **64**:613-620.
34. **Radziwill, G., H. Zentgraf, H. Schaller, and V. Bosch.** 1988. The duck hepatitis B virus DNA polymerase is tightly associated with the viral core structure and unable to switch to an exogenous template. *Virology* **163**:123-132.
35. **Raney, A. K., and A. McLachlan.** 1991. The biology of hepatitis B virus, p. 1-37. *In* A. McLachlan (ed.), *Molecular biology of the hepatitis B virus.* CRC Press, Boca Raton, Fla.
36. **Schlicht, H.-J., R. Bartenschlager, and H. Schaller.** 1989. The duck hepatitis B virus core protein contains a highly phosphorylated C terminus that is essential for replication but not for RNA packaging. *J. Virol.* **63**:2995-3000.
37. **Schlicht, H.-J., G. Radziwill, and H. Schaller.** 1989. Synthesis and encapsidation of duck hepatitis B virus reverse transcriptase do not require formation of core-polymerase fusion proteins. *Cell* **56**:85-92.
38. **Schödel, F., T. Weimer, D. Fernholz, R. Schneider, R. Sprengel, G. Wilder, and H. Will.** 1991. The biology of avian hepatitis B viruses, p. 53-80. *In* A. McLachlan (ed.), *Molecular biology of the hepatitis B virus.* CRC Press, Boca Raton, Fla.
39. **Seeger, C.** 1991. Hepadnavirus replication, p. 213-226. *In* A. McLachlan (ed.), *molecular biology of the hepatitis B virus.* CRC Press, Boca Raton, Fla.
40. **Spanos, A., and U. Hubscher.** 1983. Recovery of functional proteins in sodium dodecyl sulfate gels. *Methods Enzymol.* **91**:263-277.
41. **Spanos, A., S. G. Sedgwick, G. T. Yarranton, U. Hubscher, and G. R. Banks.** 1981. Detection of the catalytic activities of DNA polymerases and their associated exonucleases following SDS-

- polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9**:1825–1839.
42. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* **29**:403–415.
 43. **Tavis, J. E., and D. Ganem.** 1993. Expression of functional hepatitis B virus polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription. *Proc. Natl. Acad. Sci. USA* **90**:4107–4111.
 44. **Toh, H., H. Hayashida, and T. Miyata.** 1983. Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus. *Nature (London)* **305**:827–829.
 45. **Tuttleman, J. S., C. Pourcel, and J. Summers.** 1986. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* **47**:451–460.
 46. **Wang, G.-H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* **71**:663–670.
 47. **Wray, W., T. Bouliskas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203.
 48. **Wu, T.-T., L. D. Condeelis, L. Coates, C. Aldrich, and W. S. Mason.** 1991. Evidence that less-than-full-length *pol* gene products are functional in hepadnavirus DNA synthesis. *J. Virol.* **65**:2155–2163.