JOURNAL OF VIROLOGY, July 1987, p. 2273–2279
0022-538X/87/072273-07$02.00/0

Vol. 61, No. 7

Duck Hepatitis B Virus Replicates in the Yolk Sac of Developing Embryos

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Received 2 February 1987/Accepted 7 April 1987

Duck hepatitis B virus (DHBV) is the only member of the hepadnavirus family in which nearly 100% vertical transmission from carrier mother to embryo has been reported. Large quantities of maternally transmitted virus particles are present in the yolk prior to incubation of the eggs, and replicative forms of DHBV DNA are detectable in the liver at 6 days of incubation. Since the yolk sac is similar to the liver in its production of serum proteins, we examined the yolk sacs of developing embryos for signs of viral replication. We detected the supercoiled form of DHBV DNA, DHBV RNA transcripts similar to those in the virus-replicating liver, and DNA polymerase activity and viral DNA in corelike particles in extracts of yolk sac tissue of naturally infected eggs. DHBV core antigen was strongly stained in only the endodermal layer of the yolk sac by immunofluorescence. DHBV RNA was detectable in the yolk sac from 4 days of incubation until hatching, and a larger quantity of DHBV RNA was present in the yolk sac than in the liver during all the stages of embryogenesis. Our data indicate that DHBV replicates actively in the yolk sac from an earlier stage than that previously reported in studies of embryonic liver and that replication is limited to the endodermal cell layer, which is ontogenetically and functionally related to the liver. The yolk sac may support the vertical transmission of DHBV.

Duck hepatitis B virus (DHBV) is the only known avian hepadnavirus (16). While transmission of the mammalian members of this virus family most often appears to be perinatal, transmission of DHBV occurs predominantly through eggs (9). Viral DNA replication is first detectable in pooled embryos of eggs from virus-positive mothers at 6 days of incubation, coincident with the formation of the liver. Urban and co-workers detected large quantities of virus particles in the yolk and suggested that the virus is transported by the yolk sac from the yolk to the embryo, where the virus replicates in the liver (19).

The yolk sac of the avian embryo is an organ similar to the liver in its production of such serum proteins as albumin, prealbumin, transferrin, and α-globulin, which is homologous to the mammalian α-fetoprotein. Synthesis of these proteins is observed as early as the primitive streak stage (4). In chicken embryos, it has been determined that transcription of mRNA and protein synthesis occur in the endodermal layer of the yolk sac tissue (20, 21), while mesodermal cells of the yolk sac are associated with erythropoiesis, granulocytopoiesis, and vascular formation (13).

We determined that the similarity of the yolk sac to the liver extends to its support of the replication of DHBV. We compared viral replication in the yolk sac tissue of DHBV-infected developing embryos to that in DHBV-infected developing liver and found that viral replication in the yolk sac is detectable earlier and in greater amounts than in the developing liver.

MATERIALS AND METHODS

Experimental animals. Fertile eggs and adult animals were obtained from the flock of DHBV-infected Pekin ducks maintained at Stanford University. The presence of DHBV DNA in the sera of hatched ducks was confirmed by slot blot hybridization. The DHBV strain in this flock is similar but not identical in DNA restriction pattern to published cloned DHBVs (5, 15).

Extraction of nucleic acids and Southern and Northern blot analyses. Yolk sacs were rinsed with phosphate-buffered saline several times to remove adhering yolk and either used immediately for nucleic acid extraction or quick-frozen and stored at −70°C until use. DNA and RNA extractions, poly(A)$^+$ RNA isolation, agarose gel electrophoresis, and Southern or Northern transfer were carried out for yolk sac tissue by methods previously described for the liver (6, 18). A hot phenol-sodium dodecyl sulfate (SDS) procedure for the extraction of protein-free DNA was also carried out as described previously (14). Briefly, frozen samples were homogenized in 5 volumes of a 1:1 (vol/vol) mixture of phenol and 1.0% SDS–50 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–2 mM EDTA, and the DNA was extracted with phenol at 65°C and then precipitated with ethanol. DNA was prepared from isolated nuclei as previously described (10). A ^{32}P-labeled DNA probe was made by nick translation of DHBV DNA separated from plasmid vector pACYC184 by *Eco*RI digestion and gel electrophoresis. Subgenomic, plus-strand-specific RNA probes were prepared as follows. DHBV DNA fragments obtained from a pACYC184-DHBV head-to-tail dimer were cloned into the multiple cloning site of pSP65, and recombinant DNAs were linearized by restriction enzymes. In vitro RNA transcription with [α-^{32}P]CTP or [α-^{32}P]GTP by SP6 RNA polymerase was carried out under the conditions previously reported (8). Northern hybridization with these RNA probes was done at 55°C by using a hybridization mixture containing 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1 mM EDTA), 2× Denhardt solution, 0.5 mg of tRNA per ml, and 0.5 mg of salmon sperm DNA per ml; the blot was washed with 0.1× SSPE–0.1% SDS at 65°C.

Quantitation of DHBV transcripts. RNA samples were denatured in 7.4% formaldehyde–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 15 min and applied to nitrocellulose filters (BA85; Schleicher & Schuell,

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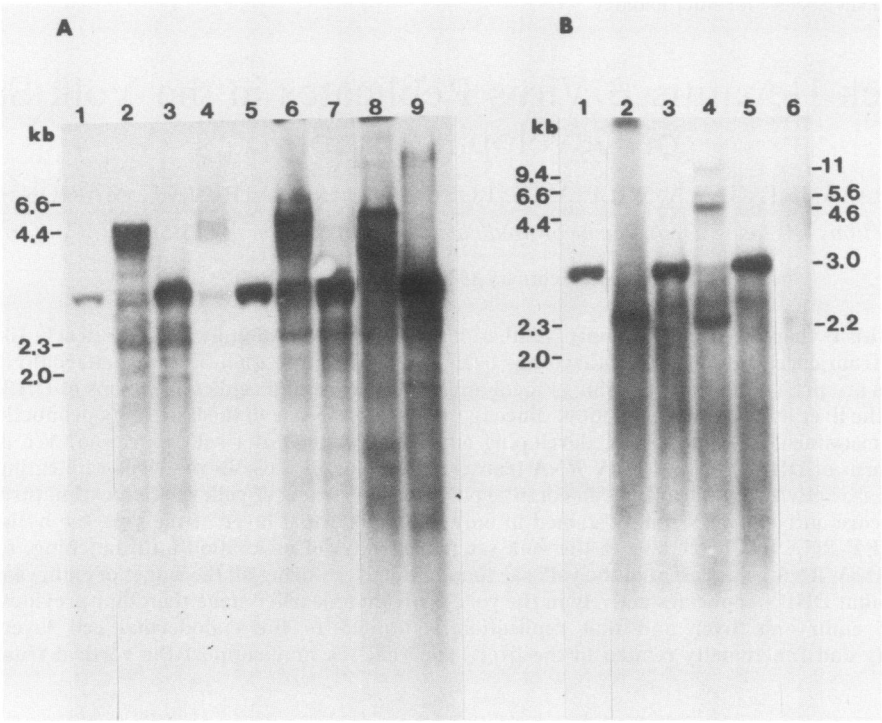

FIG. 1. Southern blot analysis of DHBV DNA sequences in duck yolk sac and liver DNAs after electrophoresis on a 1.4% agarose gel. ^{32}P-labeled DHBV DNA isolated from the vector pACYC184 by *Eco*RI digestion and gel electrophoresis was used as the hybridization probe. (A) Lanes: 1, 60 pg of cloned DHBV DNA; 2 and 3, 4 μg of adult duck liver DNA not digested and digested with *Eco*RI, respectively; 4 and 5, 20 μg of 4-day-old yolk sac DNA not digested and *Eco*RI digested, respectively; 6 and 7, 8 μg of 10-day-old yolk sac DNA not digested and *Eco*RI digested, respectively; 8 and 9, 4 μg of 26-day-old yolk sac DNA not digested and *Eco*RI digested, respectively. (B) Yolk sac DNA extracted by the hot phenol-SDS procedure described in Materials and Methods (lanes 2 to 5) and after purification of nuclei (lane 6). Lanes: 1, 100 pg of cloned DHBV DNA; 2 and 3, 20 μg of 10-day-old yolk sac DNA not digested and *Eco*RI digested, respectively; 4 and 5, 20 μg of 26-day-old yolk sac DNA not digested and *Eco*RI digested, respectively; 6, 3.4 μg of 26-day-old yolk sac DNA not digested.

Inc.) presoaked in 20× SSC by using a slot blot manifold (18). Known amounts of unlabeled DHBV RNA synthesized in vitro were treated in the same way and used as standards. The autoradiographic density of each sample was scanned with a Helena Laboratories densitometer, and the concentration of DHBV RNA in each sample was calculated by comparison with known standards.

Endogenous DNA polymerase assay of immature cores (replicative complexes) from yolk sac and liver tissues. Immature cores were prepared from fresh tissue (liver from two embryos and yolk sac from one embryo after 22 days of incubation) by homogenizing the tissue in 4 ml of buffer containing 20 mM Tris hydrochloride (pH 7.5), 7 mM $MgSO_4$, 50 mM NaCl, 0.1% 2-mercaptoethanol, 100 μg of bovine serum albumin per ml, and 25 M sucrose. The same amount of serum from a chronically infected adult duck was used as the control. After sedimentation through 33 ml of a 15 to 30% sucrose gradient at 25,000 rpm (81,500 × *g*) for 7 h at 4°C in a Beckman SW27 rotor (17), 1.5 ml of each fraction was collected from the bottom. Aliquots (30 μl) of fractions were incubated at 37°C for 2 h with 100 mM Tris hydrochloride (pH 7.5)–40 mM $MgCl_2$–120 mM NH_4Cl–200 μM dATP–200 μM TTP–3.2 μM [α-^{3}H]dCTP–3.2 μM [α-^{3}H]dGTP and with or without 1% Nonidet P-40 (NP-40). The reaction mixture was spotted on Whatman 3MM cellulose filters, and the acid-precipitable radioactivity was counted as described previously (11). Each fraction (10 μl) was tested for the presence of DHBV DNA by slot blot hybridization.

Immunofluorescence staining of immature core antigen. Frozen sections (7 μm thick) were fixed with acetone for 10 min at −20°C. The sections were rehydrated in phosphate-buffered saline and incubated for 30 min at 37°C with rabbit antiserum to immature core antigen (DHBcAg) (a generous gift from W. S. Mason and M. S. Halpern) (2) diluted 1:40 with 2% bovine serum albumin–2% normal duck serum in phosphate-buffered saline. After being washed with phosphate-buffered saline, the sections were incubated with fluorescein-labeled goat anti-rabbit serum diluted 1:240 (2) for 30 min at 37°C. The stained sections were examined with a Zeiss microscope with epi-illumination and photographed with Kodak Tri-X pan film.

RESULTS

DHBV DNA in yolk sac tissue. The forms of DHBV DNA in yolk sac tissue were analyzed by Southern blot hybridization (Fig. 1). A viral DNA band with the electrophoretic mobility of 4.4-kilobase (kb) linear double-stranded DNA, representing relaxed circular double-stranded 3.0-kb DHBV DNA, similar to virion DNA, was detected in the yolk sac tissue of eggs after 4 days of incubation (Fig. 1A, lane 4). This band disappeared and a single band in the position of 3.0-kb linear viral DNA appeared when the DNA was digested with *Eco*RI, which recognizes one site on this DHBV genome. Large quantities of DHBV DNA, including bands with higher electrophoretic mobilities and a smear between the positions of 4.4- and 0.5-kb DNAs, were detected in 10- and 26-day-old yolk sacs (Fig. 1A, lanes 6 and

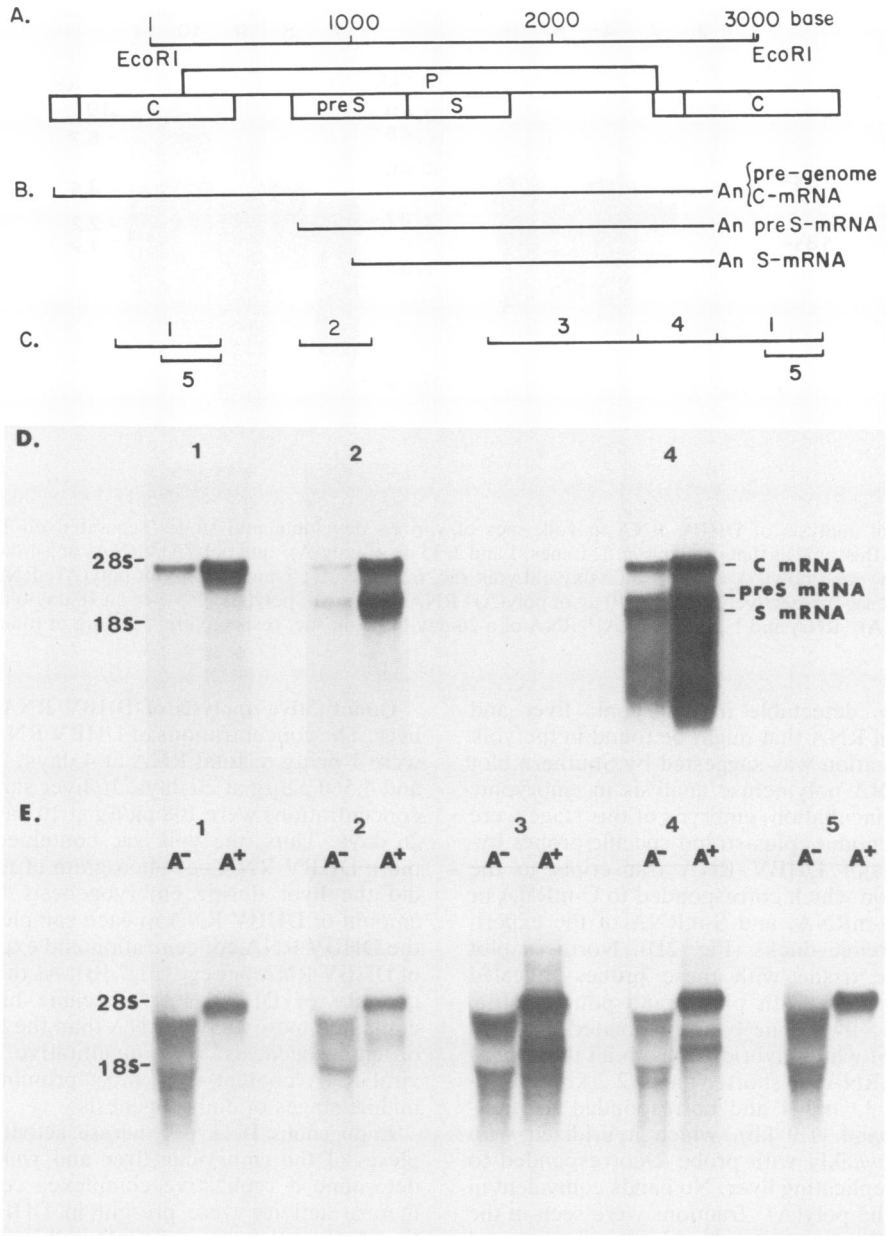

FIG. 2. (A) Open reading frames of cloned DHBV DNA (5, 15). (B) Major RNA transcripts of 6-week-old experimentally infected ducks, taken from Buscher and co-workers (1). The following numbers correspond to the location on the DHBV genomic map. C-mRNA or pregenome: (5′) 2530 or 2531 ± 1, (3′) 2800 ± 15; preS-mRNA: (5′) 732 or 740 ± 5, (3′) 2800 ± 15; S-mRNA: (5′) 985 ± 2, (3′) 2800 ± 5; An, polyadenylation site. (C) Probes used for the Northern blot analysis shown in panels D and E. DHBV DNA fragments were cloned into pSP65, and these areas were transcribed and labeled with ^{32}P by SP6 RNA polymerase. As these probes have minus-strand polarity, they detect plus-stranded DHBV RNA. DHBV sequences covered by the probes are as follows: 1, 2808 to 320; 2, 718 to 1068; 3, 1658 to 2410; 4, 2410 to 2808; 5, 14 to 320. (D) Northern blot analysis of embryonic liver RNA after 22 days of incubation. One microgram (left lane) and five micrograms (right lane) of poly(A)$^+$ RNA were hybridized with probes 1, 2, and 4. (E) Northern blot analysis of RNA from a 6-day-old yolk sac. Fifteen micrograms of poly(A)$^-$ RNA (left lane) and two micrograms of poly(A)$^+$ RNA (right lane) were hybridized with probes 1 to 5.

8). To test for the presence of supercoiled DNA, we used the hot phenol-SDS procedure and nuclear isolation to enrich for this DNA form. When DNA was extracted from both 10- and 26-day-old yolk sacs by the hot phenol-SDS procedure, a viral DNA band with the mobility of 2.2-kb linear DNA appeared as the predominent species of DNA (Fig. 1B, lanes 2 and 4). Consistent with it being supercoiled viral DNA, this band migrated in the position of 3.0-kb DNA following digestion with *Eco*RI (Fig. 1B, lanes 3 and 5). A 2.2-kb band was also observed in DNA isolated from the nuclei of yolk sacs incubated for 26 days (Fig. 1B, lane 6). Multimers of DHBV DNA were detected in the 26-day-old yolk sac.

DHBV RNA in embryonic liver and yolk sac tissues. The major DHBV RNA transcripts in the liver of 6-week-old experimentally infected ducks have been precisely analyzed and mapped by Buscher and co-workers (1). These are shown in Fig. 2 below a map of the DHBV genome and the major open reading frames. We wished to determine the

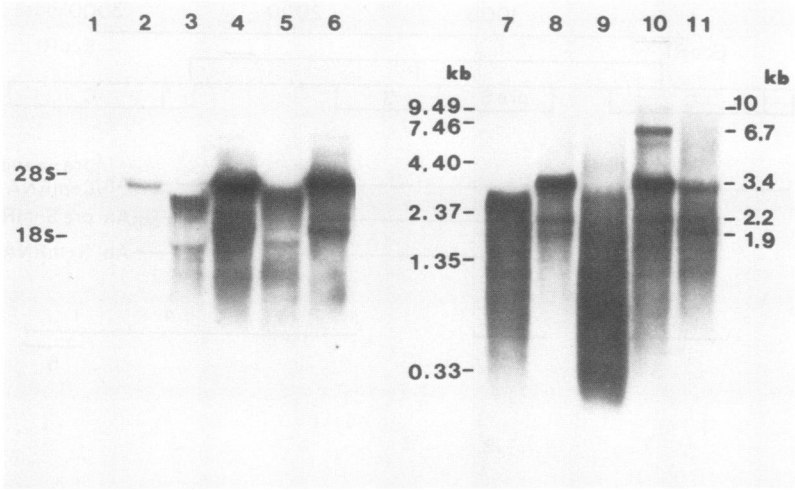

FIG. 3. Northern blot analysis of DHBV RNA in yolk sacs of various developmental stages separated on 1.1% agarose gels. The hybridization probe was the same as that used in Fig. 1. Lanes: 1 and 2, 15 μg of poly(A)$^-$ and poly(A)$^+$ RNAs of a 4-day-old sac, respectively; 3 and 4, 15 μg of poly(A)$^-$ and poly(A)$^+$ RNAs of a 5-day-old yolk sac, respectively; 5 and 6, 15 μg of poly(A)$^-$ RNA and 2 μg of poly(A)$^+$ RNA of a 6-day-old yolk sac, respectively; 7 and 8, 10 μg of poly(A)$^-$ RNA and 1 μg of poly(A)$^+$ RNA of an 18-day-old yolk sac, respectively; 9 and 10, 10 μg of poly(A)$^-$ RNA and 1 μg of poly(A)$^+$ RNA of a 26-day-old yolk sac, respectively; 11, 5 μg of total RNA of an 18-day-old liver.

DHBV RNA species detectable in embryonic liver and compare them to viral RNA that might be found in the yolk sac. Since viral replication was suggested by Southern blot hybridization and DNA polymerase analysis in embryonic liver after 22 days of incubation, embryos of this stage were examined (19). Subgenomic, plus-strand-specific probes hybridized to three major DHBV RNA transcripts in the poly(A)$^+$ RNA fraction which corresponded to C-mRNA or the pregenome, preS-mRNA, and S-mRNA of the experimentally infected viremic ducks (Fig. 2D). Northern blot analysis of yolk sac tissue with these probes revealed poly(A)$^+$ RNA transcripts with plus-strand polarity (Fig. 2E). As in embryonic liver, one band (estimated with another gel to be 3.4 kb) which hybridized with all the probes corresponded to C-mRNA. A shorter band (2.2 kb) hybridized with probes 2, 3, and 4 and corresponded to preS-mRNA. The third band (1.9 kb), which hybridized with probes 3 and 4 and weakly with probe 2 corresponded to S-mRNA of DHBV-replicating liver. No bands equivalent in mobility to those in the poly(A)$^+$ fractions were seen in the poly(A)$^-$ fractions. RNase treatment of both poly(A)$^+$ and poly(A)$^-$ fractions eliminated all hybridization with the probes (data not shown). The smears of hybridizable material seen in the poly(A)$^-$ fractions are most likely degraded RNA, as described by Buscher et al. (1).

DHBV RNA transcription in yolk sac tissue of various developmental stages. To investigate different stages of embryogenesis for DHBV transcription in the yolk sac, we analyzed by Northern blot hybridization the yolk sac tissue of various incubation times. Poly(A)$^+$ RNA from a 4-day-old yolk sac already contained C-mRNA (3.4 kb), a small amount of S-mRNA (1.9 kb), and probably preS-mRNA (2.2 kb) (Fig. 3). DHBV RNA was not detected at 3 days or at earlier stages (data not shown). Major RNA transcripts were continuously present from 4 through 26 days of incubation, 2 or 3 days before hatching. Greater-than-pregenome-length RNA transcripts were more easily detected in yolk sac tissue than in the liver, especially in the later stages. In the poly(A)$^+$ fraction of the 26-day-old yolk sac, 6.7- and 10-kb bands were clearly visible (Fig. 3, lane 10).

Quantitative analysis of DHBV RNA in the yolk sac and liver. The concentrations of DHBV RNA in yolk sac samples were 1 pg/μg of total RNA at 4 days, 227 pg/μg at 10 days, and 4,560 pg/μg at 26 days. In liver samples, the viral RNA concentrations were 108 pg/μg at 10 days and 1,707 pg/μg at 26 days. Thus, the yolk sac contained two to eight times more DHBV RNA per microgram of total tissue RNA than did the liver during embryogenesis (Fig. 4A). The total amount of DHBV RNA in each sample was calculated from the DHBV RNA concentration and expressed as nanograms of DHBV RNA per egg (Fig. 4B). As the liver developed, the quantity of DHBV RNA became higher. The yolk sac contained more DHBV RNA than the liver during all stages of embryogenesis. The quantitative differences between viral RNA content were most prominent at the early and middle stages of embryogenesis.

Endogenous DNA polymerase activity in replicative complexes of the embryonic liver and yolk sac. We wished to determine if replicative complexes containing DNA polymerase activity were present in DHBV-infected yolk sac tissue as well as in virus-infected liver tissue. Samples of virus-containing serum and homogenates of liver and yolk sac from infected embryos were centrifuged through identical 15 to 30% sucrose gradients, and aliquots of each fraction were tested for DNA polymerase activity with and without the addition of 1.0% NP-40. The removal of the virion surface antigen envelope with NP-40 (12) exposes the DNA polymerase-containing cores to the reaction mixture and allows the incorporation of radiolabeled nucleotides into viral DNA. In the serum analysis, a peak of polymerase activity appeared in fraction 6 in the presence of NP-40, while in the absence of the nonionic detergent, much less enzyme activity was observed. In the liver sample, which contained nonenveloped immature cores (replicative complexes) and few complete virions, a peak of DNA polymerase activity was detected with or without 1% NP-40. The yolk sac samples showed very high activity at almost the same fraction as the liver samples without 1% NP-40, indicating the presence in the yolk sac of immature cores like those found in the liver and not complete virions (Fig. 5).

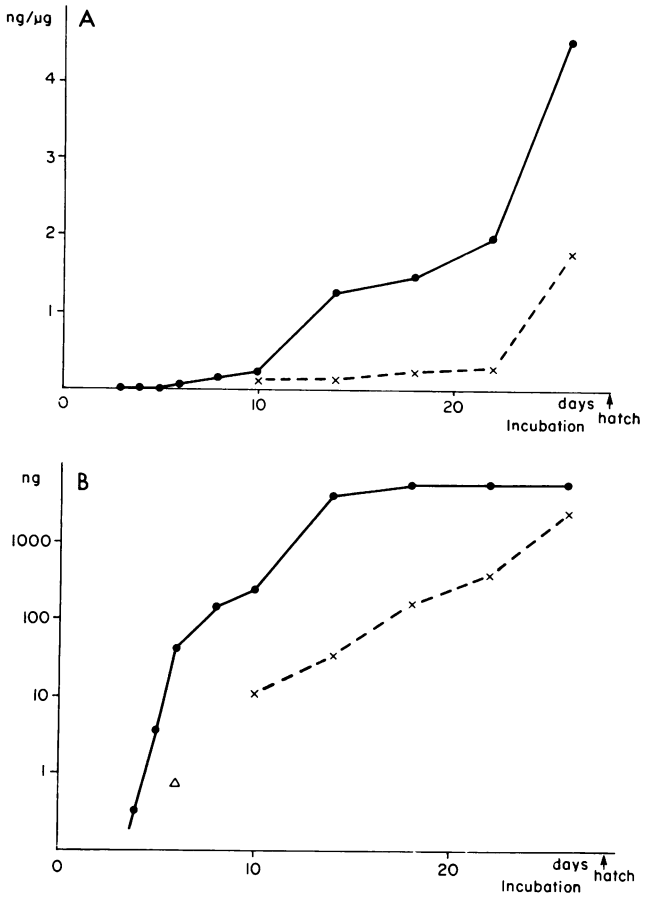

FIG. 4. Quantitative analysis of DHBV RNA in the yolk sac (●) and liver (×). (A) Concentration of DHBV RNA (nanograms of DHBV RNA per microgram of total RNA extracted) as a function of days of incubation. (B) Total amount of DHBV RNA per egg (nanograms of DHBV RNA per egg) as a function of days of incubation. △, Embryo.

Slot blot hybridization with the cloned DHBV DNA probe showed that the quantity of DHBV DNA in each fraction of liver and yolk sac gradients was proportional to the DNA polymerase activity (data not shown).

Immunofluorescence staining of DHBcAg in yolk sac tissue. To determine which cell types of yolk sac tissue are involved in viral replication, we made sections of DHBV-infected and uninfected yolk sac tissues and stained them for DHBV core antigen by using rabbit antiserum to DHBcAg and fluorescein-labeled anti-rabbit immunoglobulin G. There are three cell types in yolk sac tissue: (i) endodermal cells, which face the yolk, (ii) mesodermal cells, including hemangioblasts or blood islands, and (iii) ectodermal cells. The cytoplasm of endodermal cells is filled with many yolk granules, especially at the later stages, and nuclei are present at peripheral parts of the cell. In 8-day-old yolk sacs, individual fluorescein-stained cells were scattered in the endodermal layers of DHBV-infected samples but were not present in hemangioblasts or blood islands (mesodermal cells) or ectodermal cells (Fig. 6A). After 10 days of incubation, increasing numbers of individual, nonadjacent stained cells were seen in the endodermal cells only, and after 13 days of incubation, the whole endodermal cell layer was strongly stained (Fig. 6B).

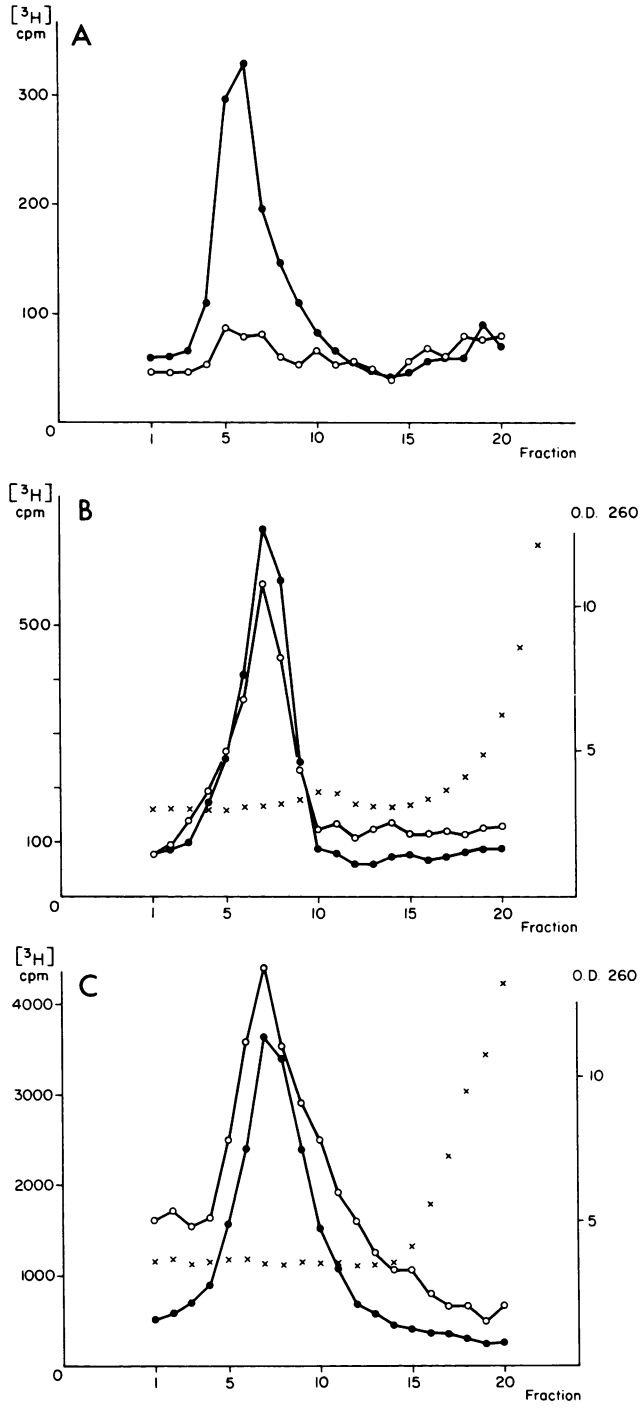

FIG. 5. Endogenous DNA polymerase activity in serum (A), liver (B), and yolk sac extract (C) sedimented through a 15 to 30% sucrose gradient. Each fraction (30 μl) was incubated in the reaction mixture with (●) or without (○) 1% NP-40, and ^{3}H incorporation was counted. ×, Optical density at 260 nm (O.D. 260).

DISCUSSION

Past studies of the tissue tropism of all members of the hepadnavirus family have always led to the same conclusion: although there is limited viral replication in other tissues, the liver is the major site of viral replication. DHBV, the avian

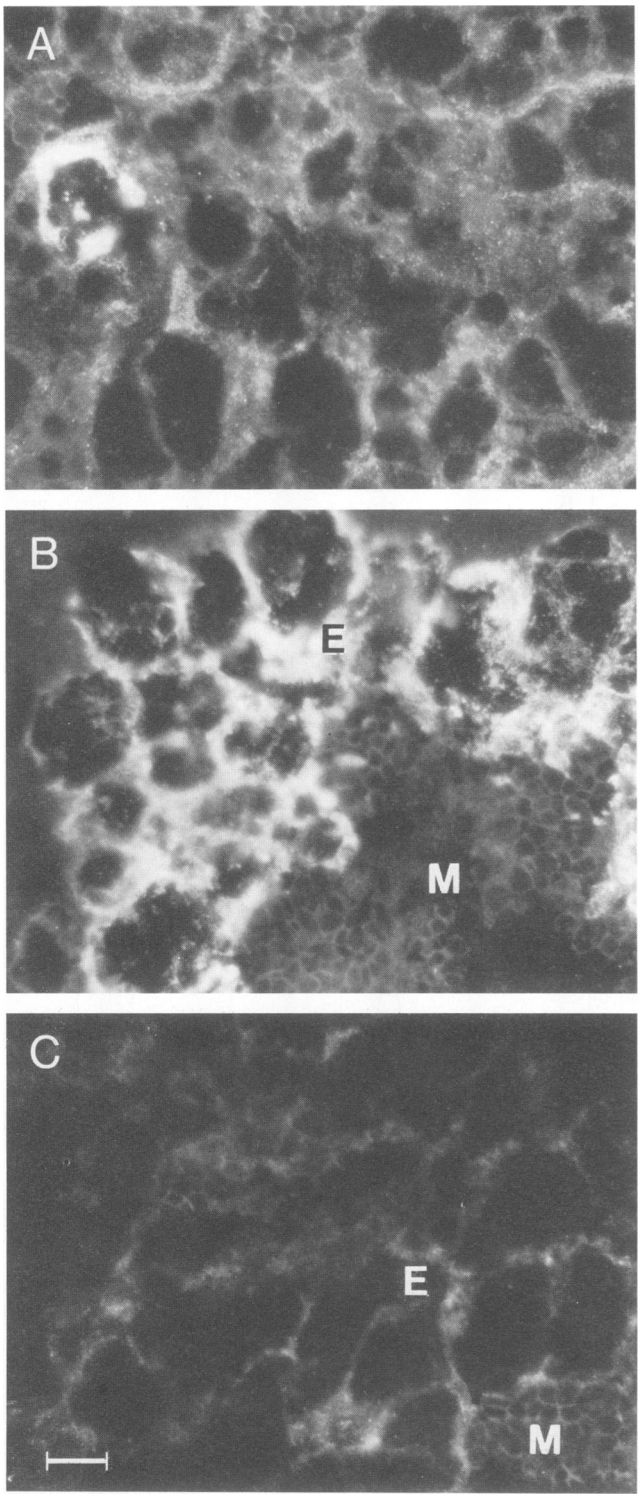

FIG. 6. Immunofluorescence staining of DHBcAg in yolk sac tissue. (A and B) Yolk sac tissue of naturally infected 8- and 13-day-old embryos, respectively. (C) Uninfected 13-day-old embryo. E, Endodermal cells; M, mesodermal cells. Bar, 20 μm.

hepadnavirus, differs from the mammalian hepadnaviruses in that a greater amount of viral replication is observed in the presence of DHBV-infected animals than in that of hepadnavirus-infected mammalian hosts. DHBV and the other hepadnaviruses also differ in the way the virus is transmitted. While the mammalian hepadnaviruses appear to be transmitted most often perinatally or later in a horizontal fashion, DHBV is transmitted vertically from the DHBV carrier mother to the eggs and is present in yolk synthesized by maternal liver. Urban and co-workers (19) have shown that DHBV replication in the liver of such naturally infected embryos is first detected after 6 days of incubation, coinciding with the formation of the liver. Another study has shown that viral antigen can be seen in the pancreas of naturally infected embryos by immunofluorescence staining, with the endocrine islets of the pancreas becoming viral antigen positive after 10 to 12 days of incubation (3).

We have presented evidence that DHBV replicates in the yolk sac of developing embryos from eggs produced by DHBV-infected mothers. Supercoiled viral DNA, not seen in virions, was detected in yolk sac tissue. This viral DNA form is thought to be the template for transcription of the RNA intermediate (pregenome) (7, 17). Using plus-strand-specific subgenomic probes and Northern blot analysis, we found RNA transcripts of DHBV DNA in yolk sac tissue. These transcripts were the same sizes as C-mRNA, preS-mRNA, and S-mRNA of virus-replicating liver. They were not detectable in the allantoic membrane of 18-day-old embryos, another well-vascularized extraembryonic membrane (data not shown). Particles from yolk sac tissue that sediment in sucrose gradients, like the immature core particles from virus-infected liver, also had a strong DNA polymerase activity and contained hybridizable DHBV DNA, like the liver particles. The presence of these particles indicates that there is not only viral transcription in the yolk sac but also viral replication, like that in the liver.

As shown by the immunofluorescence staining of DHBcAg, DHBV replication is limited to the endodermal layer of the yolk sac, which has metabolic functions that are similar to those of the liver. Serum protein synthesis in the yolk sac endoderm begins at the primitive streak stage (less than 24 h of incubation), and the yolk sac remains the main organ of protein synthesis until the liver is well developed. The scattered cell pattern of viral antigen staining in the 8-day-old yolk sac changed as increased numbers of cells had detectable antigen at 10 days, and confluent staining was observed at 13 days. This staining pattern suggests that the endodermal cells of the yolk sac are individually infected, presumably by the DHBV present in the yolk. There is no indication of the spread of virus from cell to cell, as no patches of adjacent stained cells were observed.

Transcription of C-mRNA and S-mRNA was detectable as early as 4 days of incubation, 2 days before replicative forms of viral DNA were seen in pooled, newly formed embryonic liver by Urban and co-workers (19). In our study, all major RNA transcripts were easily detectable from 5 to 26 days of incubation. DHBV replication in naturally infected eggs, therefore, starts after 4 days of incubation or earlier. The liver primodium of the duck becomes visible after 3 days of incubation. It is formed by vertical or oblique evagination of the endoderm to the embryo and is linked to the endodermal layer of the yolk sac at the ventral end (13). The liver primodium and the endodermal layer of the yolk sac are closely related ontogenetically and develop at the same time. The DHBcAg staining pattern of the developing yolk sac resembled that of the developing liver, as reported by Halpern et al. (3). They showed that only scattered hepatocytes were DHBcAg positive at 8 days, with confluent staining observed at 12 days. Thus, DHBcAg accumulation occurs in the hepatocytes and yolk sac at approximately the

same rate and in the same fashion. From this evidence, we suggest that DHBV replication begins at almost the same time in the endodermal cells of the yolk sac and the liver primodium and at an earlier stage than that previously reported.

If the quantity of viral RNA present in a tissue is representative of the amount of viral replication, the yolk sac is actually the primary site of DHBV replication in infected embryos. Quantitative analysis showed that yolk sac tissue contains more DHBV RNA than does liver tissue during all stages of embryogenesis examined. There is also more viral RNA per microgram of total tissue RNA in yolk sac than in liver from the earliest period assayed (10 days) through the latest period (26 days), indicating that DHBV is transcribed in more individual cells or more actively in each cell of the yolk sac (or both) than of embryonic liver.

A yolk sac in which viral replication takes place may play a more important role in the vertical transmission of DHBV than simply moving virions from the yolk to the bloodstream of the developing embryo. Virus-infected endodermal yolk sac cells may deliver newly synthesized infectious virus particles into the bloodstream. Even if virus in the yolk sac reaches the liver during embryogenesis, synthesis of virus by the yolk sac might elevate the virus titer in the blood to cause a more massive infection of the liver. It remains to be determined whether DHBV replication in the yolk sac is a required step in vertical transmission or an artifact of the similarity of endodermal cells to hepatocytes.

ACKNOWLEDGMENTS

We thank William Mason and Michael Halpern for providing antiserum to DHBcAg, Doug Tapper for helpful discussions, Dorothy Schafer and Naoto Aoki for advice on immunofluorescence staining, Roberto Azcarraga for technical assistance, Lucy Rasmussen for critical reading of the manuscript, and Paula Fairchild for assistance in manuscript preparation.

This work was supported by Public Health Service grants CA34514 and AI20551 from the National Institutes of Health.

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