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Viral nucleic acid synthesis and antigen accumulation in pancreas and kidney of Pekin ducks infected with duck hepatitis B virus

(immunofluorescence/immunoperoxidase staining/agarose gel electrophoresis)

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ABSTRACT Liver, pancreas, and kidney from Pekin ducks infected with duck hepatitis B virus (DHBV) were assayed for the presence of both viral antigen and replication-specific forms of viral nucleic acid. In young congenitally infected ducks, antigen was detectable in hepatocytes and bile duct epithelia, in kidney glomeruli and tubular epithelia, and in cells localized to pancreatic acini. In older experimentally infected ducks, antigen was detectable in hepatocytes, in glomeruli and tubular epithelia, and in cells localized to presumptive pancreatic α-islets. All but the glomeruli-associated viral antigen appeared to be localized to the cytoplasm of antigen-positive cells. Much of the glomeruli-associated antigen appeared to be extracellular and was detected in glomeruli that were positive for the accumulation of immunoglobulin, observations suggestive of the deposition of viral antigen–antibody complexes. As analyzed with bulk tissue, replication-specific forms of viral nucleic acid were detectable in liver and pancreas from the young congenitally infected ducks and in liver and kidney from the older experimentally infected ducks.

The hepatitis B viruses constitute a family of DNA-containing viruses that exhibit a marked tropism for hepatocytes. The possibility that this tropism is not absolute was raised by early observations of Australia antigen in cells of extrahepatic tissues from several patients with human hepatitis B virus infection (1). More recently, hepatitis B surface antigen was found in pancreatic juice of patients with chronic and acute infection (2). Subsequently this antigen was detected in the cytoplasm of pancreatic cells, identified as acinar cells, from autopsied human subjects with antemortem hepatitis B surface antigenemia (3). The detection of virus-specific DNA and RNA in pancreas, as well as in liver, of Pekin ducks congenitally infected with duck hepatitis B virus (DHBV) (4–6) in turn provided direct evidence for an extrahepatic site of replication and prompted us to use the duck model to study the tissue localization of viral antigen and of replication-specific forms of viral nucleic acid. We report here the results of analyses of pancreas and kidney, as well as liver, from DHBV-infected Pekin ducks.

MATERIALS AND METHODS

Experimental Animals. Pekin ducks were obtained from a commercial supplier at 1 day after hatching. Congenitally infected ducklings, which constituted 10–20% of the total received, were identified by a DNA hybridization assay for viremia, as described (5). Thirteen congenitally DHBV-infected ducks were analyzed in the present study, and these are designated group I ducks.

Experimental infections were carried out not with the commercially obtained ducks but with 1-day-old ducklings from a small flock of apparently virus-free Pekin ducks that do not transmit DHBV to their progeny (unpublished observations). After injection with 8 μl of serum from a viremic duck, three ducklings developed a viremia that was present from 10 days after injection until sacrifice. A fourth duckling was not deliberately infected but was maintained in a cage with infected ducklings from 1 day after hatching. Viremia developed in this fourth duckling between 10 and 20 days after hatching and was present until sacrifice. All four ducks were analyzed, and these are designated group II ducks.

Preparation and Analysis of DNA and RNA from Duck Tissues. DNA was extracted from 0.07–0.2 g of tissue as described (5), subjected to electrophoresis in a horizontal slab gel of 1.5% agarose, and denatured and transferred to a nitrocellulose sheet by the method of Southern (7), essentially as modified by Wahl *et al.* (8). Transfer to nitrocellulose was carried out in 1 M ammonium acetate/0.02 M NaOH (9).

Total RNA was isolated from *ca.* 0.3 g of tissue by extraction with 2.5 ml of 4 M guanidinium thiocyanate according to the method of Chirgwin *et al.* (10) as modified by Hsu *et al.* (11). The RNA was then collected by precipitation with ethanol and poly(A)$^+$ RNA was selected by two passages through an oligo(dT)-cellulose column (12). Poly(A)$^+$ and poly(A)$^-$ fractions were then concentrated by precipitation with ethanol. Samples containing the indicated amounts of RNA were resuspended for electrophoresis in a 1% agarose gel containing formaldehyde (13). After electrophoresis, the RNA was blotted to nitrocellulose. Detection of DHBV-specific RNA and DNA was achieved by hybridization with a ^{32}P-labeled DNA probe, prepared from double-stranded cloned viral DNA (5).

Preparation of Rabbit Serum Reactive with DHBV. Each rabbit immunization was carried out with immunogen composed of the pooled yield of surface antigen particles and virions, as purified from the sera of several ducks on either CsCl or sucrose density gradients (4). Approximately 140–200 μg of immunogen was adjusted to a concentration of 0.1% Nonidet P-40 before use, mixed with Freund's adjuvant, and injected subcutaneously. Rabbit number 365 received five injections over a period of 4 months, whereas number 314 received three injections over the same period. The two antisera yielded equivalent results in both the immunofluorescence and immunoperoxidase assays.

Histological Assays. All tissue sections were prepared as described (14) by a slight modification of the method of Sainte-Marie (15). The immunofluorescence assay was based on the indirect method as described (16). The immunoperoxidase as-

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Abbreviations: DHBV, duck hepatitis B virus; L chain, immunoglobulin light chain.

say was based on the peroxidase–anti-peroxidase method as described (17).

RESULTS

Virus-Related Antigen Expression in Tissue from DHBV-Infected Pekin Ducks. Immunofluorescence and immunoperoxidase methods were used to assay liver, pancreas, and kidney from 13 10- to 20-day-old congenitally DHBV-infected ducks (group I) and from 4 5-month-old experimentally DHBV-infected ducks (group II) for the accumulation of antigen recognized by rabbit anti-DHBV sera. Whereas only background levels of peroxidase and fluorescence staining (defined in assays with normal rabbit sera) were detected with tissue from age-matched, non-DHBV-infected ducks, positive staining indicative of antigen accumulation was detected with all three tissues from both groups of DHBV-infected ducks. Unless otherwise indicated, the pattern of staining for a given tissue was reproduced for all ducks of the respective groups.

Antigen in the livers of the group I ducks (Fig. 1*A*; cf. Fig. 1*B*) and group II ducks (data not shown) was present in hepatocytes and appeared to be localized to the cytoplasm. Virtually all of the hepatocytes were antigen positive, but the variable intensity of the staining among hepatocytes in an individual liver suggested different levels of antigen accumulation; on the average, the level of hepatocyte-associated staining was greater in liver from the group I ducks. In addition, most, if not all, of the bile duct epithelial cells in liver of the group I ducks were antigen positive (Fig. 1 *A* and *C*). The intensity of staining of these epithelial cells, which are developmentally related to hepatocytes, appeared uniform. Antigen was not detectable in bile duct epithelia of the group II ducks (Fig. 1*D*).

Antigen in pancreas of the group I ducks was present in a small subpopulation of scattered cells (Fig. 1*E*; cf. Fig. 1*F*). These cells were intensely stained in the immunofluorescence assay. As resolved in the immunoperoxidase assay, which afforded better definition of pancreas morphology, the antigen-positive cells were associated with acini (Fig. 1*H*). Individual acini generally contained zero to three antigen-positive cells, and no observed acini consisted of only antigen-positive cells.

The antigen-positive cells in sections of pancreas from the group II ducks (Fig. 2*A*) were not scattered throughout the section, as was observed with the group I ducks, but were confined to a small number of discrete clusters (fewer than five per section). These cells exhibited a lower level of staining in comparison to the level of staining exhibited by the antigen-positive cells in pancreas from the group I ducks. Oblique illumination with visible light of sections stained with hematoxylin and eosin (Fig. 2 *C* and *D*) or histologically nonstained (data not shown) indicated that the antigen-positive cells in group II pancreas were present in structures that had different light-scattering properties than the acini (these structures were resolved by oblique illumination whether or not antigen staining was carried out). Direct illumination of sections stained with hematoxylin and eosin indicated that the cells in these structures had nonpolarized nuclei and a granular eosinophilic cytoplasm with indistinct borders (Fig. 2*D*).

As resolved with the hematoxylin and eosin staining, the antigen-positive structures in pancreas of the group II ducks resembled α-islets, which in fowl pancreas are separated from β-islets (18). Direct evidence that the analogous (antigen-negative) structures in pancreas from uninfected ducks age-matched with the group II ducks (Fig. 2 *E* and *F*) were α-islets derived from the positive staining of these structures in the argyrophyl reaction (Fig. 2 *G* and *H*), which is commonly used to histologically identify α-islets (18, 19). In comparison to the α-islets

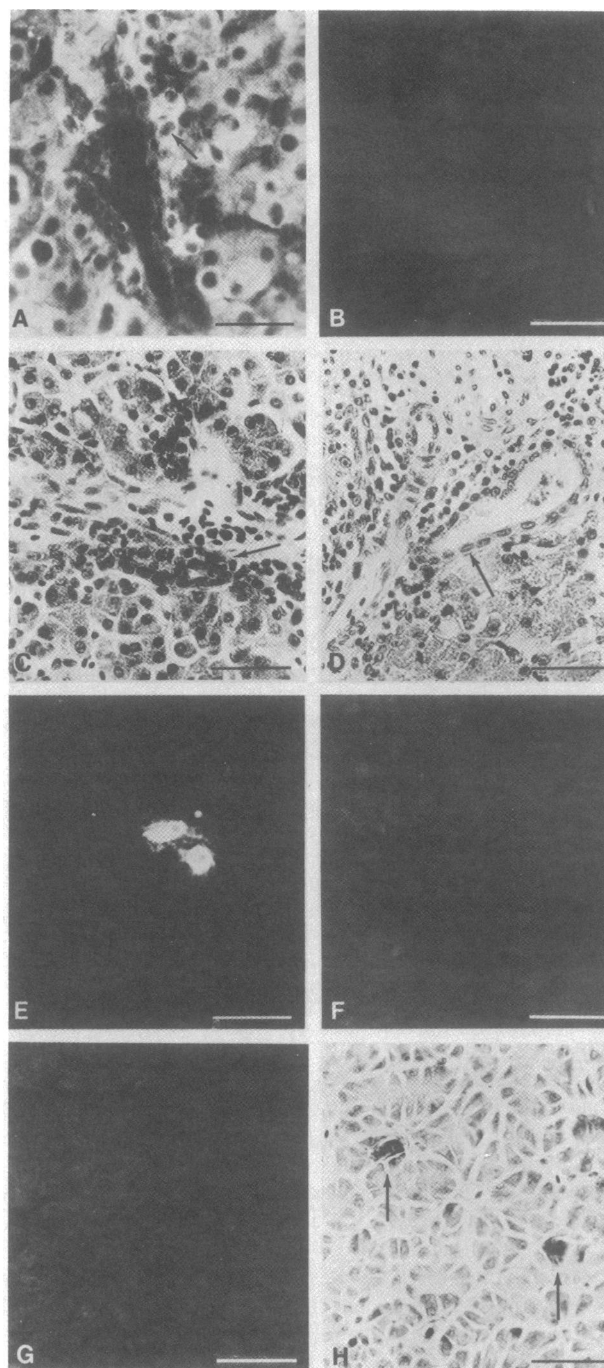

FIG. 1. Reactivity of anti-DHBV sera for sections of liver and pancreas. (*A*) Liver from a group I duck, immunofluorescence assay; (*B*) liver from a noninfected duck age-matched with the group I ducks, immunofluorescence assay; (*C*) liver from a group I duck, immunoperoxidase assay; (*D*) liver from a group II duck, immunoperoxidase assay; (*E*) pancreas from a group I duck, immunofluorescence assay; (*F*) pancreas from a noninfected duck age-matched with the group I ducks, immunofluorescence assay; (*G*) same as *E* except assay was carried out in the presence of 100 μg of a purified preparation of DHBV protein; (*H*) pancreas from a group I duck, immunoperoxidase assay. The sections in *C*, *D*, and *H* were stained with hematoxylin. The cells in *H* indicated by the arrows were positively stained in the immunoperoxidase assay; the somewhat dark appearance of the cytoplasm of the other cells in the section was indistinguishable from the appearance of the cytoplasm in the absence of peroxidase staining and is a consequence of the staining with hematoxylin. Bile duct epithelium is indicated by the arrows in *A*, *C*, and *D*. The bars represent 20 μm.

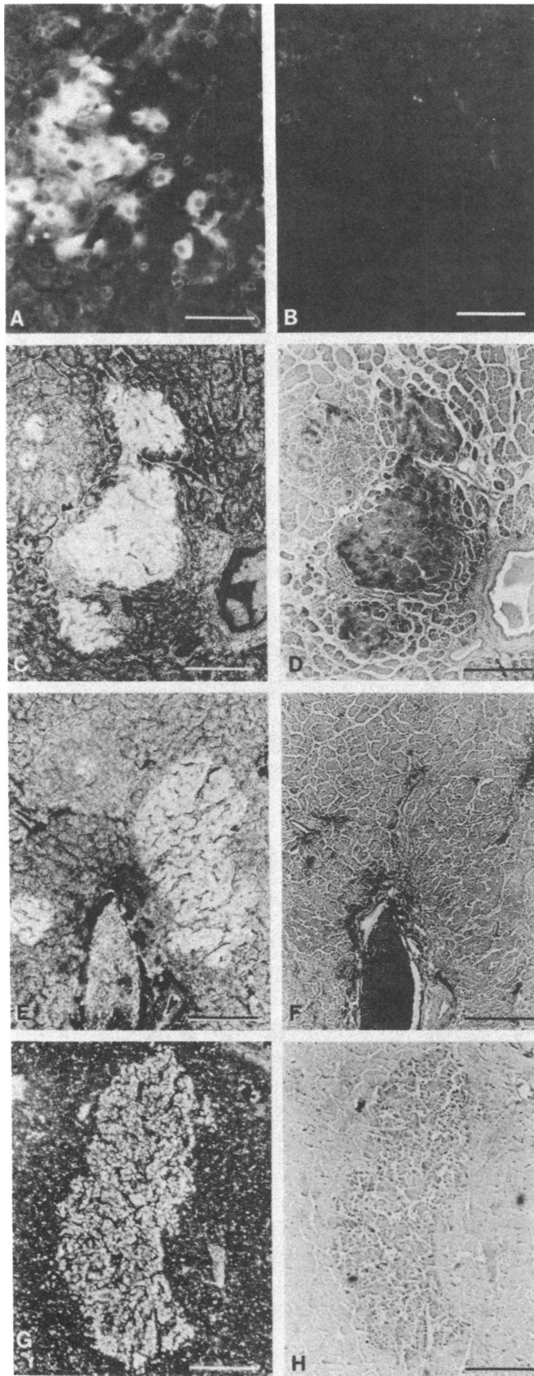

FIG. 2. Histological analysis of pancreas from group II and age-matched, noninfected ducks. (*A*) Section from a group II duck, immunofluorescence assay with anti-DHBV sera; (*B*) same as *A* except assay carried out in the presence of 100 μg of a purified preparation of DHBV protein; (*C*) section from a group II duck, immunoperoxidase assay with anti-DHBV sera, oblique illumination; (*D*) same field as in *C*, direct illumination; (*E*) section from a noninfected duck, immunoperoxidase assay with anti-DHBV sera, oblique illumination; (*F*) same field as in *E*, direct illumination; (*G*) argyrophyl reaction with section from a noninfected duck age-matched with the group II ducks, oblique illumination; (*H*) same field as in *G*, direct illumination. As viewed with oblique illumination, the fields shown in *A* and *B* had the same light-scattering properties as the structure positively stained in the argyrophyl reaction as resolved in *G*. Both the section in *C* and *D* and the section in *E* and *F* were stained with hematoxylin and eosin. The bars in *A* and *B* represent 20 μm; the bars in *C–H* represent 100 μm.

in pancreas of the noninfected ducks, the presumptive α-islets in group II pancreas stained much more weakly in the argyrophyl reaction (data not shown); however, because these structures were indistinguishable from the α-islets in the noninfected ducks on the basis of staining with hematoxylin and eosin, we would tentatively identify these antigen-positive structures as α-islets.

As measured in both the immunofluorescence and immunoperoxidase assays, the reactivity of the anti-DHBV serum for liver and pancreas was abrogated by incubation with sera from DHBV-infected ducks but not with sera from noninfected ducks (data not shown). Fractionation of the sera from infected ducks by equilibrium density gradient centrifugation, which is the standard method of DHBV purification (4), indicated that the inhibition of antibody reactivity was mediated by antigen that purified with viral protein (Fig. 1*G*; cf. Fig. 1*E*; Fig. 2*B*; cf. Fig. 2*A*); the greatest degree of inhibition (as measured with different dilutions of the respective gradient fractions) was achieved with the virus fraction maximally enriched for surface antigen particles, as visualized by electron microscopy (data not shown). No inhibition was detectable with virus-negative serum fractionated by isopycnic gradient centrifugation or with a purified preparation of an unrelated avian enveloped virus, Rous sarcoma virus, adjusted to the same protein concentration (data not shown). The inhibition mediated by the purified DHBV protein fraction appeared to be a competition of antibody reactivity rather than a generalized degradation or inactivation of rabbit immunoglobulin; this was evidenced by the observation that incubation of the purified DHBV protein fraction with a rabbit antiserum reactive to avian retroviral envelope glycoprotein did not inhibit reactivity as measured in a standard immunofluorescence assay (16).

Assays of kidney revealed two sites of antigen accumulation. The kidneys from both groups of ducks exhibited glomeruli-associated antigen, much of which appeared to be extracellular (Fig. 3*A*). In addition, kidney from all of the group II ducks and several (3 of 13) of the group I ducks exhibited antigen localized to a subpopulation of tubular cells (Fig. 3*C*), which were resolved as low columnar, eosinophilic, epithelial cells with an irregular luminal surface (Fig. 3 *E* and *F*). The presence in the sections of linear as well as circular arrays of antigen-positive epithelial cells suggested that antigen accumulation extended lengthwise in the tubules and was not confined to a single cell layer. The reactivity for the glomeruli and tubular epithelial cells was abrogated (data not shown) by incubation of the anti-DHBV sera with the purified preparation of viral protein used in the earlier competition assays (Figs. 1*G* and 2*B*).

Experiments were then carried out to assay for kidney-associated immunoglobulin deposits. Double immunofluorescence assays indicated that DHBV-related antigen-positive glomeruli in the kidneys of both groups of infected ducks were reactive with a goat anti-chicken L chain serum (Fig. 3*B*), which had been shown to recognize duck immunoglobulin. Kidney from age-matched noninfected ducks exhibited little, if any, reactivity for the anti-L chain serum. Because the fluorescence mediated by the anti-L chain serum (but not by the anti-DHBV sera) was abrogated by competition with purified preparations of duck immunoglobulin (data not shown), we concluded that the anti-L chain serum was detecting glomeruli-associated duck immunoglobulin. By contrast, no reactivity of the anti-L chain serum was detectable for tubular epithelial cells, including that subpopulation reactive with the anti-DHBV sera (Fig. 3*D*).

Viral Nucleic Acid Synthesis in Tissue from DHBV-Infected Ducks. The detection of virus-related antigen in pancreas and kidney, as well as in liver, of infected ducks raised the question whether synthesis of viral nucleic acid occurs in the extrahe-

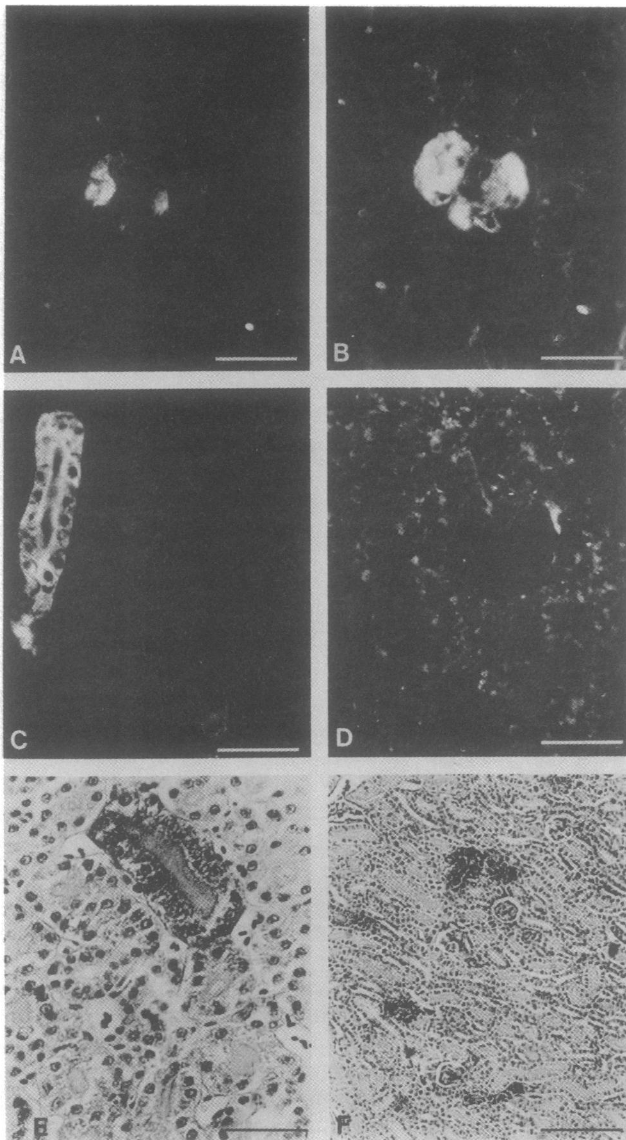

FIG. 3. Histological analysis of kidney from DHBV-infected ducks. (A) Section from a group I duck, immunofluorescence assay with anti-DHBV sera; (B) same field as in A, immunofluorescence assay with antiserum to chicken immunoglobulin light (L) chain; (C) section from a group II duck, immunofluorescence assay with anti-DHBV sera; (D) same field as in C, immunofluorescence assay with antiserum to chicken L chain; (E and F) section from a group II duck, immunoperoxidase assay with anti-DHBV sera (lower magnification view in F). The section in E and F was stained with hematoxylin and eosin. The bars in A–E represent 20 μm; the bar in F represents 100 μm.

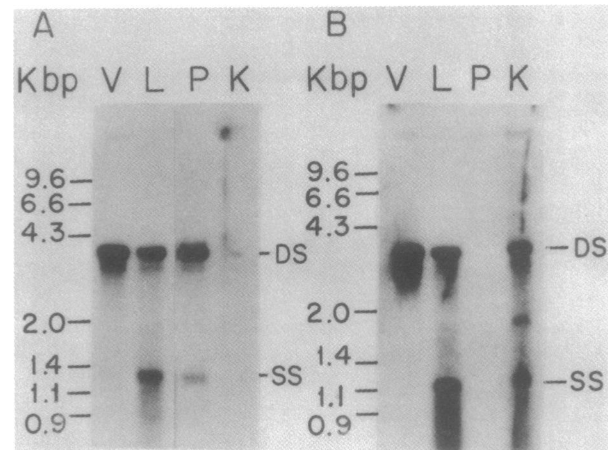

FIG. 4. Agarose gel electrophoresis of DNA extracted from virions, liver, pancreas, and kidney of DHBV-infected Pekin ducks. (A) DNA extracted from the tissues of a single group I duck. Virion DNA from 2.5 μl of serum (lane V), 0.5 μg of liver DNA (lane L), 5 μg of pancreas DNA (lane P), and 10 μg of kidney DNA (lane K) were electrophoresed into a 1.5% agarose gel, transferred to a nitrocellulose filter, and detected with a DHBV-specific probe. As normalized to total cell DNA, the pancreas contained approximately 1% as much virus-specific DNA as did the liver. (B) DNA extracted from tissues of a single group II duck. Virion DNAs from 400 μl of serum (lane V), 1 μg of liver DNA (lane L), 10 μg of pancreas DNA (lane P), and 10 μg of kidney DNA (lane K) were electrophoresed into a 1.5% agarose gel and analyzed as above. The kidney contained approximately 10% as much virus-specific DNA as did the liver. DS, full-length double-stranded DNA; SS, full-length single-stranded DNA; Kbp, kilobase pairs of double-stranded DNA markers.

patic antigen-positive tissues. To investigate this question, DNA extracted from liver, pancreas, and kidney of group I and group II ducks was analyzed for the presence of single-stranded species of virus-specific DNA, which are indicative of ongoing viral DNA replication (5, 20). As quantitated by densitometry of the autoradiographs of electrophoretic patterns, DNA from liver of the infected ducks examined here contained similar amounts of full-length (3-kilobase-pair) double-stranded and full-length (3-kilobase) single-stranded virus-specific species. In virion DNA, the amount of full-length single-stranded DNA was less than 5% the amount of full-length double-stranded DNA. Nucleic acid extracted from tissue of noninfected ducks showed no virus-specific forms.

Fig. 4A shows representative DHBV DNA patterns of virions, liver, pancreas, and kidney from group I ducks whose kidneys were negative for antigen accumulation in the tubular epithelia. The detection of a significant quantity of single-stranded DNA in pancreas (20–25% of double-stranded DNA; lane P) and in liver (same as double-stranded DNA; lane L) indicated that viral DNA replication had occurred in both tissues. The kidney exhibited low amounts of double-stranded DNA, presumably virion-associated, but no detectable single-stranded DNA (lane K) even on longer exposure. Kidneys from group I ducks exhibiting antigen-positive tubular epithelia were unavailable for nucleic acid analysis. Representative DHBV DNA patterns of virions, liver, pancreas, and kidney from group II ducks are shown in Fig. 4B. Although little or no virus-specific DNA was detected in pancreas (lane P), significant quantities of single-stranded DNA were detected in both kidney (24% of double-stranded DNA; lane K) and in liver (50% of double-stranded DNA; lane L).

As analyzed in a previous study (6) with young congenitally infected ducks, both liver and pancreas were found to contain at least three distinct species of virus-specific poly(A)$^+$ RNA. A similar analysis was carried out in the present study to ascertain if these results generalized for kidney, as well as for liver, of group II ducks. As shown in Fig. 5, three comparably sized species of poly(A)$^+$ RNA were extracted from kidney and liver from the three group II ducks examined. Much less virus-specific material was detected in the poly(A)$^-$ RNA fraction from the same tissues, implying that the species in the poly(A)$^+$ fractions were not poly(A)$^-$ contaminants (Fig. 5).

DISCUSSION

Antigen recognized by rabbit anti-DHBV sera is present in liver, kidney, and pancreas from DHBV-infected ducks. The absence of reactivity of the anti-DHBV sera for tissue from uninfected ducks indicates that the antigen recognized in tissue from infected ducks is expressed as a consequence of DHBV infection.

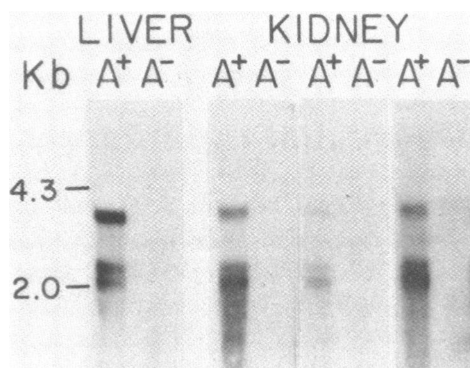

FIG. 5. Agarose gel electrophoresis of RNA extracted from liver or kidney of group II ducks. RNA was extracted from frozen tissue and passed twice through oligo(dT)-cellulose columns to separate poly(A)$^+$ and poly(A)$^-$ RNA. Poly(A)$^+$ RNAs (liver, 9 μg; kidneys, 0.5–1.5 μg) and poly(A)$^-$ RNAs (20–25 μg) were electrophoresed into a formaldehyde-containing gel, transferred to nitrocellulose, and detected with a DHBV-specific probe. As normalized to total cellular RNA the kidneys contained in the range of 0.6–6.0% as much virus-specific poly(A)$^+$ RNA as did the liver. Kb, kilobases of single-stranded DNA markers.

Competition experiments indicated that the tissue-associated antigen is crossreactive with antigen present in high concentration in the sera of infected ducks. Because the crossreactive serum material purified with viral protein, the most straightforward interpretation of our data is that the tissue-associated antigen represents one or more viral proteins. Further work will be required both to determine the relationship of these proteins to viral core and surface antigen and to define the basis for the differences in antigen accumulation observed with tissue from the two groups of infected ducks analyzed here.

The resolution of tissue structure was sufficient to unambiguously identify the liver bile duct epithelia and hepatocytes and the kidney glomeruli and tubular epithelia as sites of antigen accumulation. The presence of immunoglobulin in antigen-positive glomeruli is, in turn, suggestive that glomeruli-associated viral antigen is, at least in part, bound in immune complexes. On the basis of the morphology of the antigen-positive tubular epithelial cells, as well as their localization to the cortex and proximity to glomeruli, we would tentatively identify these cells as proximal tubular cells. Additional histologic analysis, based on detection of specific cell markers, will be required for identification of the antigen-positive cells localized to the acini and α-islets in pancreas.

The detection of replication-specific forms of viral nucleic acid in bulk tissue established that viral nucleic acid synthesis is not confined to liver but extends to pancreas of the group I ducks and to kidney of the group II ducks. An immediate question raised by these results is the identity of the cells that are the sites of this synthesis. The apparent cytoplasmic localization of viral antigen in sections of pancreas and kidney is suggestive that viral nucleic acid synthesis proceeds in the antigen-positive cells themselves. Nevertheless, kidney proximal tubular epithelia, in particular, are known to readily phagocytose protein across their luminal border, and the accumulation of antigen in these cells may simply reflect uptake of urinary DHBV protein.

The nucleic acid results, coupled with the observations of the cytoplasmic localization of antigen in group II pancreas and in group I bile duct epithelia, raise two additional questions pertaining to cellular sites of viral nucleic acid synthesis: (*i*) whether the absence of detectable virus-specific DNA in group II pancreas reflects the absence of such synthesis or, given the lower levels of staining in antigen-positive cells from group II as compared to group I pancreas, the insensitivity of the viral nucleic acid assay; and (*ii*) whether viral nucleic acid synthesis in group I liver is restricted to hepatocytes or occurs in bile duct epithelia as well. The combined use of immunofluorescence with anti-DHBV sera and *in situ* hybridization with viral cDNA may permit a definitive assessment to be made as to the congruence of antigen expression and viral nucleic acid synthesis.

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