Avian and Mammalian Hepadnaviruses Have Distinct Transcription Factor Requirements for Viral Replication†

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Hepadnavirus replication occurs in hepatocytes in vivo and in hepatoma cell lines in cell culture. Hepatitis B virus (HBV) replication can occur in nonhepatoma cells when pregenomic RNA synthesis from viral DNA is activated by the expression of the nuclear hormone receptors hepatocyte nuclear factor 4 (HNF4) and the retinoid X receptor α (RXR α) plus peroxisome proliferator-activated receptor α (PPAR α) heterodimer. Nuclear hormone receptor-dependent HBV replication is inhibited by hepatocyte nuclear factor 3 (HNF3). In contrast, HNF3 and HNF4 support duck hepatitis B virus (DHBV) replication in nonhepatoma cells, whereas the RXR α -PPAR α heterodimer inhibits HNF4-dependent DHBV replication. HNF3 and HNF4 synergistically activate DHBV pregenomic RNA synthesis and viral replication. The conditions that support HBV or DHBV replication in nonhepatoma cells are not able to support woodchuck hepatitis virus replication. These observations indicate that avian and mammalian hepadnaviruses have distinct transcription factor requirements for viral replication.

Hepadnaviruses are enveloped viruses that replicate in the liver of the host (11, 24, 35). The mammalian hepadnaviruses include hepatitis B virus (HBV), chimpanzee hepatitis B virus (13), orangutan hepatitis B virus (45), woolly monkey hepatitis B virus (16), woodchuck hepatitis virus (WHV) (40), and ground squirrel hepatitis virus (22). The avian hepadnaviruses include duck hepatitis B virus (DHBV) (23), heron hepatitis B virus (37), Ross goose hepatitis B virus (3), snow goose hepatitis B virus (3), and stork hepatitis B virus (28).

The hepadnaviruses all contain a small partially double-stranded DNA genome of 3.0 to 3.3 kb in length and replicate by reverse transcription of a pregenomic RNA that contains all the genetic information of the virus (11, 24, 35, 38, 47). Hepadnavirus replication is believed to be largely restricted to the liver because virus entry into hepatocytes is dependent on the presence of a receptor that is predominantly expressed on this cell type. However, it is likely that additional steps in the viral life cycle also contribute to the predominantly hepatocyte-specific tropism of hepadnaviruses.

HBV replication is restricted to hepatocytes in part because the liver-enriched nuclear hormone receptors hepatocyte nuclear factor 4 (HNF4) and retinoid X receptor α (RXR α) plus peroxisome proliferator-activated receptor α (PPAR α) are essential for pregenomic RNA synthesis (42). Therefore, viral transcription is a critical determinant of HBV tropism. The contribution of transcriptional regulation to the tropism of other hepadnaviruses is largely unknown due to the lack of extensive characterization of the factors regulating viral RNA

synthesis. In WHV, HNF1 and HNF4 bind to recognition sites located in the nucleocapsid promoter region of the viral genome and probably contribute to the expression of the pregenomic RNA and viral replication in the liver (10, 44). The DHBV genome contains an enhancer element upstream from the pregenomic RNA initiation site (8, 18, 33). This regulatory element may contribute to both the level and tissue-specific expression of pregenomic RNA synthesis and consequently to viral replication (8, 18, 33). The presence of binding sites for the liver-enriched HNF1, HNF3, and CCAAT/enhancer binding protein (C/EBP) transcription factors may influence the tissue-specific properties of this regulatory element (7, 17, 19).

In this study, the possible role of liver-enriched transcription factors in determining the tropism of additional hepadnaviruses was examined by determining their ability to support DHBV and WHV replication in nonhepatoma cells. As with HBV, HNF4 supported DHBV replication in nonhepatoma cells. However, contrary to the findings with HBV, HNF3 can also support DHBV replication in nonhepatoma cells. HNF3 can also synergistically activate DHBV pregenomic RNA synthesis and viral replication when expressed with HNF4. RXR α -PPAR α failed to support DHBV replication and inhibited HNF4-dependent DHBV DNA synthesis. This is the opposite of the effect of RXR α -PPAR α on HBV synthesis, where it was the most efficient activator of viral replication. WHV replication was not observed under any of the conditions that supported HBV and DHBV replication. These observations indicate that these three hepadnaviruses have distinct transcription factor requirements for viral replication.

MATERIALS AND METHODS

Plasmid construction. The steps in the cloning of the plasmid constructs used in the transfection experiments were performed by standard techniques (32). HBV DNA sequences in these constructions were derived from plasmid pCP10, which contains two copies of the HBV genome (subtype *ayw*) cloned into the

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*Eco*RI site of pBR322 (9). Two tandem copies of the HBV genome were cloned into the *Eco*RI site of pSP65 to generate the pHBV2E construct. Two tandem copies of the replication-competent DHBV 16 genome were cloned into the *Eco*RI site of pSP65 to generate the pSPDHBV2X5.1 construct (20, 27). The pCMVDHBV construct contains the cytomegalovirus (CMV) immediate-early promoter (region -522 to -4) (1) located directly upstream of the DHBV 16 sequence from nucleotide coordinates 2527 to 3021 plus 1 to 3021 plus 1 to 19. In this construct, the expression of the DHBV 3.3-kb pregenomic RNA is controlled by the CMV immediate-early promoter. Two tandem copies of the replication-competent WHV 2 genome were cloned into the *Hind*III site of pSP65 to generate the pSPWHV2X5.2 construct (15, 34).

The pMTHNF1 α , pMTHNF1 β , pCMVHNF3 α , pCMVHNF3 β , pCMVHNF4, pRS-hRXR α and pCMVPPAR α -G vectors express HNF1 α , HNF1 β , HNF3 α , HNF3 β , HNF4, RXR α , and PPAR α -G polypeptides, respectively, from the rat HNF1 α , mouse HNF1 β , rat HNF3 α , rat HNF3 β , rat HNF4, human RXR α , and mouse PPAR α -G cDNAs, respectively, using the mouse metallothionein I promoter (pMT), the CMV immediate-early promoter (pCMV), or the Rous sarcoma virus long terminal repeat (pRS) (4, 21, 26, 29–31). The PPAR α -G polypeptide contains a mutation in the PPAR α cDNA that changes Glu²⁸² to Gly and may decrease the affinity of the receptor for the endogenous ligand. Consequently, this mutation increases the peroxisome proliferator-dependent (i.e., clofibrate acid-dependent) activation of transcription from a peroxisome proliferator response element-containing promoter (26) and was used in this study to demonstrate the peroxisome proliferator-dependent transcriptional transactivation of the nucleocapsid promoter.

Cells and transfections. The human hepatoma HepG2 cell line and the mouse NIH 3T3 fibroblast cell line were grown in RPMI 1640 medium and 10% fetal bovine serum at 37°C in 5% CO₂-air. Transfections for viral RNA and DNA analysis were performed as previously described (25) with 10-cm plates containing approximately 10⁶ cells. DNA and RNA isolation was performed 3 days posttransfection. The transfected DNA mixture was composed of 15 μ g of pHBV2E DNA, pSPDHBV2X5.1 DNA, pCMVDHBV DNA, or pSPWHV2X5.2 DNA plus 1.5 μ g of the liver-enriched transcription factor expression vectors pMTHNF1 α , pMTHNF1 β , pCMVHNF3 α , pCMVHNF3 β , pCMVHNF4, pRS-hRXR α , and pCMVPPAR α -G as required (4, 21, 26, 29–31, 42). Controls were derived from cells transfected with pHBV2E DNA, pSPDHBV2X5.1 DNA, or pSPWHV2X5.2 DNA and the pCMV expression vector lacking a liver-enriched transcription factor cDNA insert (30). All-trans retinoic acid and clofibrate acid at 1 μ M and 1 mM, respectively, were used to activate the nuclear hormone receptors RXR α and PPAR α (42).

Characterization of HBV, DHBV, and WHV transcripts and viral replication intermediates. Transfected cells from a single plate were divided equally and used for the preparation of total cellular RNA and viral DNA replication intermediates as described previously (39) with minor modifications. For RNA isolation (5), the cells were lysed in 1.8 ml of 25 mM sodium citrate (pH 7.0)–4 M guanidinium isothiocyanate–0.5% (vol/vol) Sarcosyl–0.1 M 2-mercaptoethanol. After addition of 0.18 ml of 2 M sodium acetate (pH 4.0), the lysate was extracted with 1.8 ml of water-saturated phenol plus 0.36 ml of chloroform-isoamyl alcohol (49:1). After centrifugation for 30 min at 3,000 rpm in a Sorval RT6000, the aqueous layer was precipitated with 1.8 ml of isopropanol. The precipitate was resuspended in 0.3 ml of 25 mM sodium citrate (pH 7.0)–4 M guanidinium isothiocyanate–0.5% (vol/vol) Sarcosyl–0.1 M 2-mercaptoethanol and precipitated with 0.6 ml of ethanol. After centrifugation for 20 min at 14,000 rpm in an Eppendorf 5417C microcentrifuge, the precipitate was resuspended in 0.3 ml of 10 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–0.1% (wt/vol) sodium lauryl sulfate and precipitated with 45 μ l of 2 M sodium acetate plus 0.7 ml of ethanol.

For the isolation of viral DNA replication intermediates, the cells were lysed in 0.4 ml of 100 mM Tris hydrochloride (pH 8.0)–0.2% (vol/vol) NP-40. The lysate was centrifuged for 1 min at 14,000 rpm in an Eppendorf 5417C microcentrifuge to pellet the nuclei. The supernatant was adjusted to 6.75 mM magnesium acetate plus 200 μ g of DNase I per ml and incubated for 1 h at 37°C to remove the transfected plasmid DNA. The supernatant was readjusted to 100 mM NaCl, 10 mM EDTA, 0.8% (wt/vol) sodium lauryl sulfate, and 1.6 mg of pronase per ml and incubated for an additional 1 h at 37°C. The supernatant was extracted twice with phenol, precipitated with 2 volumes of ethanol, and resuspended in 30 or 100 μ l of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA for DHBV DNA and HBV DNA, respectively. RNA (Northern) and DNA (Southern) filter hybridization analyses were performed with 10 μ g of total cellular RNA and 30 μ l of viral DNA replication intermediates, respectively, as described before (32).

FIG. 1. Transcription and replication of DHBV in duck liver and HepG2 cells. RNA and DNA were isolated from infected duck liver (lane 1), HepG2 cells transiently transfected with the pCMVDHBV construct (lane 2), and HepG2 cells transiently transfected with the pSPDHBV2X5.1 construct (lane 3). (A) RNA (Northern) filter hybridization analysis of DHBV transcripts. The locations of the DHBV 3.3-, 2.1-, and 1.8-kb transcripts are indicated. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for RNA loading in each lane. (B) DNA (Southern) filter hybridization analysis of DHBV replication intermediates. The locations of the DHBV relaxed circular (RC) and single-stranded (SS) DNA replication intermediates are indicated.

RESULTS AND DISCUSSION

DHBV transcripts and replication intermediates in human hepatoma cells. The transcripts and replication intermediates synthesized from a replication-competent DHBV genome were initially characterized in the human hepatoma cell line HepG2 and compared with the viral products present in infected duck liver (Fig. 1). The pCMVDHBV construct directed the expression of the DHBV 3.3-kb pregenomic RNA from the cytomegalovirus immediate-early promoter in HepG2 cells (Fig. 1A, lane 2). The pSPDHBV2X5.1 construct, which contains a dimer of the DHBV genome, also directed the expression of the DHBV 3.3-kb RNA in HepG2 cells (Fig. 1A, lane 3). These transcripts comigrated with the DHBV 3.3-kb RNA present in infected duck liver (Fig. 1A, lane 1). Expression of the DHBV 3.3-kb pregenomic RNA was associated with the synthesis of DHBV viral replication intermediates (Fig. 1B). The pCMVDHBV and pSPDHBV2X5.1 constructs supported similar levels of DHBV replication intermediates in HepG2 cells (Fig. 1B, lanes 2 and 3). The relative levels of the DHBV relaxed circular and single-stranded replication intermediates present in the transfected HepG2 cells were similar to those observed in the infected duck liver (Fig. 1B, lane 1).

HepG2 cells transfected with the pCMVDHBV construct synthesized very low levels of the subgenomic RNAs encoding the envelope polypeptides (Fig. 1A, lane 2). The pSPDHBV2X5.1 construct encoded a significant level of the DHBV 2.1-kb transcript but a very low level of the DHBV 1.8-kb RNA in HepG2 cells (Fig. 1A, lane 3). This contrasted with the infected duck liver, where the DHBV 1.8-kb RNA was expressed at a higher level than the DHBV 2.1-kb RNA (Fig. 1A,

FIG. 2. Effect of liver-enriched transcription factors on DHBV and HBV replication in mouse NIH 3T3 fibroblasts. Cells were transiently transfected with (A) the pSPDHBV2X5.1 DNA construct or (B) the pHBV2E DNA construct plus liver-enriched transcription factors as indicated. All-*trans* retinoic acid and clofibric acid at 1 μ M and 1 mM, respectively, were used to activate the nuclear hormone receptors RXR α and PPAR α . The locations of the DHBV and HBV relaxed circular (RC) and single-stranded (SS) DNA replication intermediates are indicated.

lane 1). The reason for these findings probably reflects, in part, the difference in the relative levels of transcription factors that regulate DHBV transcription in HepG2 cells and duck liver.

Identification of liver-enriched transcription factors required for DHBV replication in mouse fibroblasts. HBV and DHBV can replicate in hepatoma cells transfected with greater-than-genome-length constructs (2, 6, 12, 36, 41, 43, 48). However, HBV and DHBV replication cannot be detected in nonhepatoma cells (Fig. 2, lane 1) (42). In the case of HBV, it has been demonstrated that expression of the nuclear hormone receptors HNF4 and RXR α -PPAR α activates pregenomic RNA synthesis (Fig. 3B, lanes 6 and 7) and viral replication in nonhepatoma cells (Fig. 2B, lanes 6 and 7) (42). As DHBV is often used as a model system to investigate the mechanisms involved in regulating various steps in the hepadnavirus life cycle, it was of interest to determine the similarities and differences in the transcriptional regulation of HBV and DHBV replication.

The role of liver-enriched transcription factors in regulating DHBV replication in mouse NIH 3T3 fibroblasts was investigated and compared with their effect on HBV replication (Fig. 2). C/EBP α , C/EBP β , C/EBP δ , and HNF6 do not support detectable DHBV or HBV replication (H. Tang and A. McLachlan, unpublished data) (42). HNF1 α , HNF1 β , and RXR α -PPAR α also failed to support detectable DHBV replication in mouse fibroblasts (Fig. 2A, lanes 2, 3, and 7). This result is significantly different from those observed with HBV, in which viral replication was activated to the greatest extent by

FIG. 3. Effect of liver-enriched transcription factors on DHBV, HBV, and WHV transcription in mouse NIH 3T3 fibroblasts. Cells were transiently transfected with (A) the pSPDHBV2X5.1 DNA construct, (B) the pHBV2E DNA construct, or (C) the pSPWHV2X5.2 DNA construct plus liver-enriched transcription factors as indicated. All-*trans* retinoic acid and clofibric acid at 1 μ M and 1 mM, respectively, were used to activate the nuclear hormone receptors RXR α and PPAR α . The locations of the DHBV 3.3-, 2.1-, and 1.8-kb transcripts are indicated. The locations of the HBV 3.5-, 2.1-, and 0.7-kb transcripts are indicated. The locations of the WHV 2.1- and 0.7-kb transcripts are indicated. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was used as an internal control for RNA loading in each lane.

RXR α -PPAR α (Fig. 2B, lane 7). Although both HBV and DHBV replication could be activated by HNF4, RXR α -PPAR α not only failed to activate DHBV replication but also inhibited HNF4-dependent DHBV replication (Fig. 2A, lane 12). Therefore, DHBV replication is negatively regulated by RXR α -PPAR α , which is the opposite of the effect of this transcription factor on HBV replication (Fig. 2, lanes 7 and 12). The nuclear hormone receptor binding sites in the DHBV genome responsible for modulating viral replication have not been defined.

HNF3 also modulated HBV and DHBV replication in mouse fibroblasts in very different manners. HNF3 α and HNF3 β supported DHBV replication (Fig. 2A, lanes 4 and 5), whereas HNF3 could not support detectable HBV replication (Fig. 2B, lanes 4 and 5). In addition, HNF3 synergistically activated DHBV replication in combination with HNF4 (Fig.

2A, lanes 10 and 11). In contrast, HNF3 inhibited nuclear hormone receptor-dependent HBV replication (Fig. 2B, lanes 10 and 11) (42). Therefore, it is apparent that the transcriptional regulation of HBV and DHBV replication in nonhepatoma cells is significantly different for these two hepadnaviruses. Although HNF4 activated both HBV and DHBV replication, DHBV replication was also activated by HNF3 and repressed by RXR α -PPAR α , whereas HBV replication was activated by RXR α -PPAR α and repressed by HNF3 under identical conditions in mouse fibroblasts. These observations indicate that avian and mammalian hepadnaviruses have distinct transcription factor requirements for viral replication.

Effect of liver-enriched transcription factors on DHBV transcription in mouse fibroblasts. The absence of detectable DHBV replication in mouse fibroblasts reflects the failure of the DHBV 3.3-kb pregenomic RNA to be transcribed from the nucleocapsid promoter (Fig. 3A, lane 1). Transcription of the DHBV 3.3-kb pregenomic RNA and viral replication in mouse fibroblasts are dependent on the expression of HNF3 and HNF4 (Fig. 2A and 3A, lanes 4 to 6 and 8 to 11). The level of DHBV 3.3-kb pregenomic RNA synthesis in the presence of HNF3 α , HNF3 β , or HNF4 was very low (Fig. 3A, lanes 4 to 6). In the presence of HNF3 α plus HNF4 or HNF3 β plus HNF4, the DHBV 3.3-kb pregenomic RNA was readily detectable (Fig. 3A, lanes 10 and 11), and the level correlated with the observed level of viral replication (Fig. 2A, lanes 10 and 11).

The liver-enriched transcription factors also appeared to influence the expression of the DHBV subgenomic transcripts (Fig. 3A). HNF1 and RXR α -PPAR α expression increased the level of expression of the DHBV 2.1-kb RNA (Fig. 3A, lanes 2, 3, and 7). HNF3 increased the level of expression of the DHBV 1.8-kb RNA (Fig. 3A, lanes 4 and 5). This observation is consistent with the presence of an HNF3 binding site in the DHBV major surface antigen promoter, which is a critical regulatory element within this promoter (46).

Avian and mammalian hepadnaviruses have distinct transcription factor requirements for viral replication. Hepadnavirus replication is dependent on transcription of the viral pregenomic RNA. The synthesis of pregenomic RNA is controlled by the activity of the nucleocapsid promoter. The regulatory sequence elements that control the level of transcription from the nucleocapsid promoter for the different hepadnaviruses have been characterized to various extents. For HBV, nuclear hormone receptors are essential for transcription of pregenomic RNA and viral replication (42). HNF3 antagonizes nuclear hormone receptor-dependent viral replication (42). The observation that HNF3 mediates DHBV replication in mouse fibroblasts is consistent with the observation that the nucleocapsid promoter regulatory sequences contain recognition elements that bind this transcription factor (7, 19). However HBV also contains HNF3 recognition elements within the nucleocapsid promoter (14), but replication of this hepadnavirus is negatively regulated by HNF3 in mouse fibroblasts (42). Therefore, the presence of HNF3 recognition sites within the nucleocapsid promoter is not sufficient to predict the role that this transcription factor might have in viral replication. The presence of additional HNF3 sites in the viral genome or their relationship to other transcription factor regulatory elements must influence the effect of HNF3 on viral transcription and replication.

DHBV replication is positively regulated by HNF4 but negatively regulated by RXR α -PPAR α (Fig. 2A). The nuclear hormone receptor recognition sequences that might mediate these alterations in viral replication in the DHBV genome have not been identified. However, the opposing effects of these transcription factors contrast with the similar effects they have on HBV replication (Fig. 2B) (42). Therefore, it is apparent that although the same transcription factors are the major regulators of HBV and DHBV pregenomic RNA synthesis and replication in mouse fibroblasts, the effects of HNF3 and RXR α -PPAR α are distinct.

Transcription and viral DNA synthesis derived from a replication-competent WHV genome were also examined in mouse fibroblasts (Fig. 3C). WHV subgenomic 2.1-kb RNA was transcribed in mouse fibroblasts in the absence of liver-enriched transcription factors (Fig. 3C, lane 1). RXR α -PPAR α expression increased the level of expression of the WHV 2.1-kb and 0.7-kb RNAs (Fig. 3C, lane 7). However, WHV pregenomic RNA synthesis and viral replication were not observed with any of the combinations of transcription factors demonstrated to activate HBV or DHBV replication (Fig. 3C) (H. Tang and A. McLachlan, unpublished data). Consequently it appears that avian and mammalian hepadnaviruses have evolved distinct modes of transcriptional regulation as they have adapted to their highly specific host organisms.

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REFERENCES

1. Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate-early gene of human cytomegalovirus. *Cell* **41**:521-530.
2. Chang, C., K. Jeng, C. Hu, S. J. Lo, T. Su, L.-P. Ting, C.-K. Chou, S. Han, E. Pfaff, J. Salfeld, and H. Schaller. 1987. Production of hepatitis B virus *in vitro* by transient expression of cloned HBV DNA in a hepatoma cell line. *EMBO J.* **6**:675-680.
3. Chang, S. F., H. J. Netter, M. Bruns, R. Schneider, K. Frölich, and H. Will. 1999. A new avian hepadnavirus infecting snow geese (*Anser caerulescens*) produces a significant fraction of virions containing single-stranded DNA. *Virology* **262**:39-54.
4. Chen, D., G. Lepar, and B. Kemper. 1994. A transcriptional regulatory element common to a large family of hepatic cytochrome P450 genes is a functional binding site of the orphan receptor HNF-4. *J. Biol. Chem.* **269**:5420-5427.
5. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
6. Condreay, L. D., C. E. Aldrich, L. Coates, W. S. Mason, and T.-T. Wu. 1990. Efficient duck hepatitis B virus production by an avian liver tumor cell line. *J. Virol.* **64**:3249-3258.
7. Crescenzo-Chaigne, B., J. Pillot, and A. Lilienbaum. 1995. Interplay between a new HNF3 and the HNF1 transcriptional factors in the duck hepatitis B virus enhancer. *Virology* **213**:231-240.

8. Crescenzo-Chaigne, B., J. Pillot, A. Lilienbaum, M. Levrero, and E. Elfassi. 1991. Identification of a strong enhancer element upstream from the pre-genomic RNA start site of the duck hepatitis B virus genome. *J. Virol.* **65**:3882–3886.
9. Dubois, M. F., C. Pourcel, S. Rousset, C. Chany, and P. Tiollais. 1980. Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proc. Natl. Acad. Sci. USA* **77**:4549–4553.
10. Fourel, G., F. Ringeisen, M. Flajolet, F. Tronche, M. Pontoglio, P. Tiollais, and M. A. Buendia. 1996. The HNF1/HNF4-dependent We2 element of woodchuck hepatitis virus controls viral replication and can activate the *N-myc2* promoter. *J. Virol.* **70**:8571–8583.
11. Ganem, D., and H. E. Varmus. 1987. The molecular biology of the hepatitis B viruses. *Annu. Rev. Biochem.* **56**:651–693.
12. Hirsch, R., R. Colgrove, and D. Ganem. 1988. Replication of duck hepatitis B virus in two differentiated human hepatoma cell lines after transfection with cloned viral DNA. *Virology* **167**:136–142.
13. Hu, X. L., H. S. Margolis, R. H. Purcell, J. Ebert, and B. H. Robertson. 2000. Identification of hepatitis B virus indigenous to chimpanzees. *Proc. Natl. Acad. Sci. USA* **97**:1661–1664.
14. Johnson, J. L., A. K. Raney, and A. McLachlan. 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. *Virology* **208**:147–158.
15. Kodama, K., N. Ogasawara, H. Yoshikawa, and S. Murakami. 1985. Nucleotide sequence of a cloned woodchuck hepatitis virus genome: evolutionary relationship between hepadnaviruses. *J. Virol.* **56**:978–986.
16. Lanford, R. E., D. Chavez, K. M. Brasky, R. B. Burns III, and R. Rico-Hesse. 1998. Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proc. Natl. Acad. Sci. USA* **95**:5757–5761.
17. Lilienbaum, A., B. Crescenzo-Chaigne, A. A. Sall, J. Pillot, and E. Elfassi. 1993. Binding of nuclear factors to functional domains of the duck hepatitis B virus enhancer. *J. Virol.* **67**:6192–6200.
18. Liu, C., L. D. Condeelis, J. B. E. Burch, and W. Mason. 1991. Characterization of the core promoter and enhancer of duck hepatitis B virus. *Virology* **184**:242–252.
19. Liu, C., W. S. Mason, and J. B. E. Burch. 1994. Identification of factor-binding sites in the duck hepatitis B virus enhancer and in vivo effects of enhancer mutations. *J. Virol.* **68**:2286–2296.
20. 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.
21. Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**:224–229.
22. Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard, and W. S. Robinson. 1980. A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. *Proc. Natl. Acad. Sci. USA* **77**:2941–2945.
23. Mason, W. S., G. Seal, and J. Summers. 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* **36**:829–836.
24. McLachlan, A. 1991. Molecular biology of the hepatitis B virus. CRC Press, Boca Raton, Fla.
25. McLachlan, A., D. R. Milich, A. K. Raney, M. G. Riggs, J. L. Hughes, J. Sorge, and F. V. Chisari. 1987. Expression of hepatitis B virus surface and core antigens: influences of pre-S and precore sequences. *J. Virol.* **61**:683–692.
26. Muerhoff, A. S., K. J. Griffin, and E. F. Johnson. 1992. The peroxisome proliferator-activated receptor mediates the induction of *CYP4A6*, a cytochrome P450 fatty acid omega-hydroxylase, by clofibrate. *J. Biol. Chem.* **267**:19051–19053.
27. Pult, I., N. Abbott, Y. Y. Zhang, and J. Summers. 2001. Frequency of spontaneous mutations in an avian hepadnavirus infection. *J. Virol.* **75**:9623–9632.
28. Pult, I., H. J. Netter, M. Bruns, A. Prassolov, H. Sirma, H. Hohenberg, S. F. Chang, K. Frölich, O. Krone, E. F. Kaleta, and H. Will. 2001. Identification and analysis of a new hepadnavirus in white storks. *Virology* **289**:114–128.
29. Raney, A. K., A. J. Easton, D. R. Milich, and A. McLachlan. 1991. Promoter-specific transactivation of hepatitis B virus transcription by a glutamine- and proline-rich domain of hepatocyte nuclear factor 1. *J. Virol.* **65**:5774–5781.
30. Raney, A. K., J. L. Johnson, C. N. A. Palmer, and A. McLachlan. 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. *J. Virol.* **71**:1058–1071.
31. Raney, A. K., P. Zhang, and A. McLachlan. 1995. Regulation of transcription from the hepatitis B virus large surface antigen promoter by hepatocyte nuclear factor 3. *J. Virol.* **69**:3265–3272.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. Schneider, R., and H. Will. 1991. Regulatory sequences of duck hepatitis B virus C gene transcription. *J. Virol.* **65**:5693–5701.
34. Seeger, C., P. L. Marion, D. Ganem, and H. E. Varmus. 1987. In vitro recombinants of ground squirrel and woodchuck hepatitis viral DNAs produce infectious virus in squirrels. *J. Virol.* **61**:3241–3247.
35. Seeger, C., and W. S. Mason. 2000. Hepatitis B virus biology. *Microbiol. Mol. Biol. Rev.* **64**:51–68.
36. Sells, M. A., M.-L. Chen, and G. Acs. 1987. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. USA* **84**:1005–1009.
37. Sprengel, R., E. F. Kaleta, and H. Will. 1988. Isolation and characterization of a hepatitis B virus endemic in herons. *J. Virol.* **62**:3832–3839.
38. 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.
39. Summers, J., P. M. Smith, M. Huang, and M. Yu. 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. *J. Virol.* **65**:1310–1317.
40. Summers, J., J. M. Smolec, and R. Snyder. 1978. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl. Acad. Sci. USA* **75**:4533–4537.
41. Sureau, C., J.-L. Romet-Lemonne, J. I. Mullins, and M. Essex. 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell* **47**:37–47.
42. Tang, H., and A. McLachlan. 2001. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proc. Natl. Acad. Sci. USA* **98**:1841–1846.
43. Tsurimoto, T., A. Fujiyama, and K. Matsubara. 1987. Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. *Proc. Natl. Acad. Sci. USA* **84**:444–448.
44. Ueda, K., Y. Wei, and D. Ganem. 1996. Cellular factors controlling the activity of woodchuck hepatitis virus enhancer II. *J. Virol.* **70**:4714–4723.
45. Warren, K. S., J. L. Heeney, R. A. Swan, Heriyanto, and E. J. Verschoor. 1999. A new group of hepadnaviruses naturally infecting orangutans (*Pongo pygmaeus*). *J. Virol.* **73**:7860–7865.
46. Welsheimer, T., and J. E. Newbold. 1996. A functional hepatocyte nuclear factor 3 binding site is a critical component of the duck hepatitis B virus major surface antigen promoter. *J. Virol.* **70**:8813–8820.
47. Will, H., W. Reiser, T. Weimer, E. Pfaff, M. Buscher, R. Sprengel, R. Cattaneo, and H. Schaller. 1987. Replication strategy of human hepatitis B virus. *J. Virol.* **61**:904–911.
48. Yaginuma, K., Y. Shirakata, M. Kobayashi, and K. Koike. 1987. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. *Proc. Natl. Acad. Sci. USA* **84**:2678–2682.