Expression of Infectious Woodchuck Hepatitis Virus in Murine and Avian Fibroblasts

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The liver is the primary site for replication of the hepadnavirus genome. We asked whether the posttranscriptional phase of the viral replication cycle would depend on hepatocyte-specific functions. For this purpose, we assayed a previously constructed chimera between sequences of the cytomegalovirus immediate-early promoter-enhancer region and woodchuck hepatitis virus (WHV) (C. Seeger and J. Maragos, *J. Virol.* 63:1907-1915, 1989) for its ability to direct the synthesis of infectious WHV in hepatoma cells and in murine and avian fibroblast cells. Viruslike particles containing WHV DNA were produced transiently in transfected hepatoma cells and in fibroblasts. Inoculation of woodchucks with culture medium from hepatoma cells or fibroblasts transfected with viral DNA led to productive WHV infection, as observed following infection of woodchucks with serum from WHV-infected animals. These results demonstrate that posttranscriptional events of the hepadnavirus replication cycle are not dependent on hepatocyte-specific functions.

Tissue tropism is a major attribute of hepadnavirus infection. Woodchuck hepatitis virus (WHV), like other hepadnaviruses, primarily replicates in the livers of infected animals (for a review, see reference 10). To a lower and variable degree, WHV replication has been detected in spleen and peripheral blood cells of WHV-infected woodchucks (16, 17, 19). Hepatocyte-specific functions may be required during several steps of virus replication. Among these are virus uptake, delivery of the viral genome into the nucleus, transcription of viral RNAs, and assembly and secretion of virus particles.

The molecular mechanisms that govern hepatotropism of hepadnaviruses are not well understood. Based on *in vitro* tissue culture experiments, restriction could occur on at least two levels, the tissue specificity of the pregenomic RNA promoter and the inability to infect cultured cells with virus. Pregenomic RNA with correct 5' ends could not be detected in tissue culture cells transfected with cloned hepadnavirus DNA (10, 11), and attempts to infect hepatoma and other permanent cell lines with infectious virus were not successful (26). Recently, several human hepatoma cell lines were identified that support the synthesis of correctly initiated pregenomic RNA and that allow the production of infectious human hepatitis B virus (HBV) (1, 4, 27, 28, 30) and duck HBV (9, 13, 20). Infection of cells *in vitro* has been demonstrated only with primary hepatocytes which have been in culture for no more than 5 to 7 days (12, 18, 29; C. E. Aldrich, L. Coates, T.-T. Wu, J. Newbold, C. Seeger, and W. S. Mason, *Virology*, in press).

The aim of this study was to test whether posttranscriptional events in the WHV replication cycle depend on hepatocyte-specific functions. We adopted the strategy of expressing WHV pregenomic RNA with the help of the cytomegalovirus immediate-early (CMVIE) promoter-enhancer region in human hepatoma cells and in murine and avian fibroblasts and of assaying WHV produced in these cells for infectivity in woodchucks susceptible to WHV infection. Our results demonstrate that infectious WHV can be produced in hepatoma cells and in murine and avian

fibroblast cells provided that transcription of pregenomic RNA is controlled by the CMVIE promoter.

MATERIALS AND METHODS

Molecular clones. The construction of pCMW82 has been described previously (24). Briefly, WHV clone pWHV81-2 was fused with pBC12/CMV/IL2 (5) to direct the synthesis of WHV pregenomic RNA from the CMVIE promoter on pBC12/CMV/IL2. pCMW1710 is identical to pCMW82 except that the *SspI* site at position 3227 on the WHV genome has been fused to the *SsrI* site of pBC12/CMV/IL2 (5).

Cell culture. HepG2 (2) and NIH 3T3 cells were cultured in minimal essential medium and Dulbecco modified Eagle medium (DMEM) (GIBCO Laboratories), respectively, supplemented with 10% bovine calf serum (HyClone Laboratories, Inc.) in the presence of penicillin (90 U/ml) and streptomycin (90 µg/ml) at 37°C and 3% CO₂. Secondary turkey fibroblasts were received from Volker Vogt (Cornell University) and maintained in DMEM supplemented with 10% fetal calf serum. Cells were transfected with plasmid DNA as described by Chen and Okayama (4). Procedures for isolation of virion DNA and primer extension analysis were used as described previously (24), except that DNA oligomer CS3, corresponding to positions 3229 to 3248 on the WHV genome, was used for the primer extension reaction.

Experimental woodchuck studies. Juvenile woodchucks were trapped in Tompkins County (New York) and were negative for serologic markers of WHV infection. Woodchucks were inoculated with supernatant from tissue culture cells, collected 3 to 9 days following DNA transfection with pCMW82. Woodchucks were anesthetized with ketamine and xylazine, and cell supernatant (100 to 150 µl) was injected into the sublingual vein. Serum samples were obtained at biweekly intervals for serologic analysis. Liver specimens for histologic examination were obtained under ketamine-xylazine general anesthesia by an ultrasound-directed needle biopsy technique.

Immunologic assays. The occurrence of WHV surface antigen (WHsAg) in serum was monitored by using the heterologous AUSZYME and AUSRIA II radioimmunoassay kits (Abbott) for the detection of HBV surface antigen

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FIG. 1. (A) 5' ends of the 3.5-kb-long WHV RNA. The figure shows the positions of the 5' ends of the 3.5-kb RNA transcripts identified in vivo (top) (7) and the positions of the 5' ends of the RNA transcripts directed by pCMW82 and pCMW1710 (bottom) (24). The precore and core antigen genes are depicted by open bars, and the AUG initiation codons for the two genes are indicated. RNA is symbolized with solid lines. The nucleotide sequence numbering of the WHV genome was arranged to begin with the first AUG of the precore gene (position 1931 in reference 15). (B) Virion DNA in the supernatant of HepG2 cells transfected with pCMW82 and pCMW1710. The products of the primer extension reaction from DNA purified from virions in the supernatant of HepG2 cells transfected with pCMW82 (lane 2) and pCMW1710 (lane 3) are shown. Primer extension analysis was performed as described previously with DNA oligomer CS3 as a primer (22, 24). A nucleotide sequence ladder of known origin provided size markers (lane 1). nt, Nucleotides.

(HBsAg). Assays were performed according to the manufacturer's protocol.

Histology. Tissues were fixed in 10% buffered Formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin.

RESULTS

Expression of WHV with foreign promoter. We have previously fused sequences containing the CMVIE promoter region to a cloned WHV genome, which directs the synthesis of a 3.5-kilobase (kb)-long pregenomic RNA, the template for reverse transcription of virion DNA (Fig. 1A) (24). Transfection of HepG2 cells with this CMVIE-WHV hybrid, termed pCMW82, leads to packaging and reverse transcription of pregenomic RNA and to secretion of DNA containing viruslike particles (24). Transcription of WHV RNA from the natural DNA template, covalently closed circular DNA, leads to the synthesis of two additional 3.5-kb-long RNA species (7). Except for their 5' ends, which initiate at nucleotides -25 and -16 relative to the first AUG codon of the precore gene, they are identical to pregenomic RNA (Fig. 1A) (7). We asked whether these 3.5-kb-long WHV transcripts could also direct synthesis of virion DNA. Consequently, we constructed a second CMVIE-WHV chimera, pCMW1710, which leads to the synthesis of an RNA species initiating at nucleotide -30 relative to the first AUG codon of the precore gene, corresponding to position 3291 on the WHV genome (Fig. 1A). HepG2 cells were transfected with both pCMW82 and pCMW1710, supernatant from transfected cells was collected, and DNA was purified from concentrated virus. To detect virion DNA, we performed primer extension analysis on minus-strand DNA purified from virions in the culture medium of transfected cells and analyzed the reaction products by electrophoresis through acrylamide. Figure 1B shows the DNA products extended on virion DNA purified from the supernatant of HepG2 cells transfected with pCMW82 (lane 2) and pCMW1710 (lane 3), respectively. The length of the DNA product obtained with virion DNA from cells transfected with pCMW82 was char-

acteristic of initiation of minus-strand DNA near position 14 on the WHV map, as determined previously (22). No extension products were obtained from reactions with DNA purified from the culture medium of cells transfected with pCMW1710. This result suggested that transfection of HepG2 cells with this construct did not lead to the secretion of DNA containing virus particles into the culture medium. Successful transfection of HepG2 cells with pCMW1710 was confirmed by Northern blot analysis of mRNA purified from transfected cells. Both the 3.5- and 2.3-kb RNA species were present in cells transfected with pCMW82 and pCMW1710 (data not shown). In summary, these results indicate that the position of the 5' end of the 3.5-kb RNA species expressed with the CMVIE promoter is critical for the accumulation of DNA containing virions in the supernatant of transfected HepG2 cells.

Replication of WHV in hepatoma and fibroblast cells. To investigate whether WHV could be produced in cells other than differentiated hepatoma cells, we examined virus production in HepG2 and mouse NIH 3T3 cells transfected with either the CMVIE-WHV chimera or with infectious clones of WHV and ground squirrel hepatitis virus (GSHV). Cells were transfected with pCMW82, pWHV-82, and pGSHV-3, and the amount of virion DNA accumulation in the culture medium was determined by primer extension analysis. pWHV-2 contains a head-to-tail dimer of an infectious WHV genome (15, 23) and pGSHV-3 contains a head-to-tail trimer of GSHV clone pBA131 (23). Figure 2A shows the products of the primer extension reactions with DNA purified from virions in the supernatant of HepG2 cells transfected with pCMW82 (lane 5), pWHV-2 (lane 6), and pGSHV-3 (lane 7). The major extension products, as expected, were 105 nucleotides in length and identical to the major primer extension product obtained with DNA purified from virions in the serum of a WHV-infected woodchuck (lane 4). Extension reactions with virion DNA purified from the supernatant of cultured cells exhibited an additional DNA product absent from reactions with virion DNA purified from the serum of the WHV-infected woodchuck (Fig. 2A, compare lanes 4

FIG. 2. Virion DNA in the supernatant of HepG2 and NIH 3T3 cells transfected with pCMW82, pWHV-2, and pGSHV-3. Primer extension analysis was performed as described in the legend to Fig. 1B. The figure shows the DNA products obtained from primer extension reactions with DNA purified from virions in the supernatants of HepG2 cells (A) and NIH 3T3 cells (B) transfected with pCMW82 (lanes 5), pWHV-2 (lanes 6), and pGSHV-3 (lanes 7). The primer extension products obtained from reactions with virion DNA from WHV-infected woodchuck W1451 are shown in lanes 4. A nucleotide sequence ladder derived from pCMW82 primed with DNA oligomer CS3 provided size markers (lanes 1 and 2). nt, Nucleotides.

and 5). While no qualitative difference was observed between the primer extension products from virion DNA produced in HepG2 cells transfected with pCMW82, pWHV-2, or pGSHV-3, quantitative differences were clearly apparent. HepG2 cells transfected with the CMVIE-WHV recombinant produced approximately 10-fold more virion DNA than cells transfected with either pWHV-2 or pGSHV-3 (Fig. 2A, lanes 5 to 7).

The results obtained following transfection of NIH 3T3 cells with the pCMW82, pWHV-2, or pGSHV-3 construct were different from the results obtained following transfection of HepG2 cells. Virion DNA production was strictly dependent on the presence of the CMVIE sequences, as judged from the products of the primer extension reactions (Fig. 2B). The expected 105-nucleotide-long extension product was observed exclusively in NIH 3T3 cells transfected with pCMW82 (Fig. 2B, lane 4). These results indicate that NIH 3T3 cells will support production of virion DNA if the synthesis of pregenomic RNA is directed by the CMVIE promoter.

Infectivity of WHV produced in hepatoma cells and in murine and avian fibroblasts. In a second step, we asked whether WHV produced in hepatoma cells and in fibroblasts transfected with pCMW82 was infectious in woodchucks. HepG2 cells, NIH 3T3 cells, and secondary turkey fibroblasts were transfected with pCMW82, and supernatants from these cells were collected 3 to 9 days later for inoculation into woodchucks. Two woodchucks were inoculated intravenously with 100 to 150 μ l of supernatant from trans-

TABLE 1. WHsAg in sera of inoculated woodchucks^a

Time after inoculation (wk)	P/N ratio							
	HepG2		NIH 3T3				STF	
	W2171	W2172	W1781	W1783	W1796	W1908	W2501	W2509
0	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—
8	—	2.1	—	2.2	—	—	—	—
10	3.0	—	—	4.1	—	4.1	1.9	—
12	—	—	2.0	3.6	2.4	3.4	3.8	6.9
14	—	—	—	3.0	2.8	2.7	—	8.0
16	—	—	—	2.1	1.9	2.9	—	—
18	—	—	—	ND ^b	ND	1.9	—	—

^a Animals were inoculated with 100 to 150 μ l of supernatant from HepG2 cells, NIH 3T3 cells, and secondary turkey fibroblasts (STF) transfected with pCMW82. Serum samples, obtained from the femoral vein from each woodchuck at the indicated times after inoculation, were analyzed with the AUSRIA II assay kit. Results are expressed as P/N ratios, where P is cpm of ¹²⁵I-labeled anti-HBsAg bound to beads incubated with test serum and N is cpm of ¹²⁵I-labeled anti-HBsAg bound to beads incubated with control serum negative for WHsAg. P/N ratios greater than 1.8 are considered positive; ratios below 1.8 are shown as dashes.

^b ND, Not done.

fected HepG2 cells, four animals were inoculated with supernatant from transfected NIH 3T3 cells, and two woodchucks were inoculated with supernatant from transfected turkey fibroblasts. Two woodchucks that served as negative controls each received intravenous injections of 20 μ g of purified pCMW82 plasmid DNA.

Woodchucks were monitored for the appearance of surface antigen in their sera. The results of this experiment are summarized in Table 1. Both animals that received supernatant from transfected HepG2 cells became seropositive for WHsAg after an incubation time of 8 and 10 weeks, respectively. All four woodchucks inoculated with supernatant from transfected NIH 3T3 cells and both animals that received supernatant from transfected turkey cells developed surface-antigenemia after incubation periods of 8 to 12 weeks. Neither of the two animals inoculated with pCMW82 plasmid DNA became WHsAg positive during the surveillance period of 28 weeks (data not shown). These results indicate that WHV produced in NIH 3T3 cells and in turkey fibroblasts transfected with pCMW82 is infectious in woodchucks.

To confirm that WHV produced in NIH 3T3 fibroblasts displayed pathogenic properties similar to WHV produced in infected woodchucks, we assayed alanine aminotransferase and aspartate aminotransferase activities in sera of the inoculated woodchucks at biweekly intervals. Hepatic biopsies were obtained from three woodchucks (W1783, W1796, and W1908) at 14 and 18 weeks following inoculation when all three had 4- to 10-fold increases in the activity of alanine aminotransferase and aspartate aminotransferase in serum. In all three animals, there was mild to moderate portal hepatitis characterized by mononuclear cell infiltrates in portal areas, increased numbers of sinusoidal cells, and various degrees of single-cell necrosis (Fig. 3). In summary, histologic analysis of liver samples from woodchucks infected with WHV produced in mouse NIH 3T3 cells revealed characteristics attributed to WHV infection as would be expected in woodchucks infected with serum from a WHV-producing animal.

FIG. 3. Induction of hepatitis with WHV produced in NIH 3T3 fibroblasts. Hepatic biopsy of woodchuck W1908 was obtained 14 weeks after inoculation with supernatant from NIH 3T3 cells transfected with pCMW82. The section of liver from W1908 shows marked infiltration of the portal area by mononuclear cells (a) compared with that of an uninfected control woodchuck (b). Magnification, $\times 400$.

DISCUSSION

Infectious HBV and duck HBV have been obtained following transfection of selected differentiated hepatoma cell lines with recircularized or tandemly repeated viral genomes (1, 9, 13, 20, 26). The results described in this study extend this observation to WHV expressed in human HepG2 cells. Most importantly, our results show that infectious WHV can be produced in both murine and avian fibroblasts, provided that pregenome RNA synthesis is directed by the CMVIE promoter-enhancer region.

While *in vivo* transcription leads to the synthesis of 3.5-kb RNA species with heterogeneous 5' ends, expression of RNA with the CMVIE promoter leads to synthesis of RNA with a distinct 5' end initiating 30 nucleotides downstream of the CMVIE TATA box sequence (25). Our data show that the position of the 5' end of the 3.5-kb WHV RNA is critical for virus production. Expression of an RNA species corresponding to the longest RNA transcript present in WHV-infected livers does not lead to accumulation of DNA containing virus particles in the supernatant of transfected HepG2 cells (Fig. 1B). At least two mechanisms could account for this observation. The 5' end of the 3.5-kb RNA could contain signals for packaging of viral RNA, leading to selective packaging of the shortest species of pregenomic RNA (8, 22). Alternatively, the RNA synthesized by pCMW1710 could lead preferentially to the synthesis of the precore gene product (e antigen) and thus interfere with the synthesis of core antigen or with the release of virus particles from the transfected cells. Junker et al. engineered chimeras between the metallothionein promoter and HBV sequences directing the expression of HBV particles with endogenous polymerase activity in transfected HepG2 cells (14). Minus-strand DNA synthesis has been observed with a construct similar to pCMW1710 except for a mutation in the first methionine codon of the precore gene (M. Niepmann and H.

Schaller, personal communication). Therefore, it seems likely that the major impediment for virus production with pCMW1710 is initiation of translation at the precore AUG, which might interfere with translation of the core gene. The apparent dependence of virus production for correctly initiating pregenomic RNA might also explain why HBV replication has not previously been observed in fibroblasts and other cells transfected with cloned viral DNA, although pregenomic RNA-like transcripts have been detected in these cells (11, 31).

While production of WHV and the related GSHV in HepG2 cells can be demonstrated with concatemeric viral genomes, production of WHV in fibroblasts was only observed upon transfection of cells with the CMV-WHV hybrid pCMW82, confirming that synthesis of pregenomic RNA depends on cell type-specific transcriptional factors. In contrast, the promoter for the surface transcript exhibits activity in tissue culture cells of different origin, including fibroblasts and cells of epithelial origin (6, 21).

These results also suggest that synthesis of the pre-S1 and X proteins, for which no mRNA species have yet been identified in WHV-infected liver tissue, is either independent of hepatocyte-specific transcripts other than pregenomic RNA or is not required for the production of infectious virus.

Infection of woodchucks with WHV produced in HepG2 cells showed an incubation time of 8 to 12 weeks and self-limited WHsAg antigenemia as observed upon infection of woodchucks with WHV of animal origin. Inoculation of woodchucks with supernatant from NIH 3T3 cells or turkey fibroblasts transfected with pCMW82 led to productive infection in all six animals after a similar incubation time. The development of histologically confirmed hepatitis in three animals infected with virus from murine fibroblasts demonstrated the pathogenic potential of this virus.

The data presented in this study provide direct evidence that posttranscriptional events of the hepadnavirus replication cycle do not depend on hepatocyte-specific functions. This implies that the determinants directing hepatotropism displayed by hepadnaviruses, other than pregenome RNA synthesis, function during the initial phase of the virus replication cycle, which includes virus uptake, uncoating, and delivery of viral DNA into the nucleus of the infected cell.

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