Woodchuck Hepatitis Virus X Protein Is Required for Viral Infection In Vivo

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The X gene of the mammalian hepadnaviruses is believed to encode a protein of 17 kDa which has been shown to transactivate a wide range of viral and cellular promoters. The necessity for X gene expression during the viral life cycle in vivo has recently been suggested (H.-S. Chen, S. Kaneko, R. Girones, R. W. Anderson, W. E. Hornbuckle, B. C. Tennant, P. J. Cote, J. L. Gerin, R. H. Purcell, and R. H. Miller, *J. Virol.* 67:1218–1226, 1993). We have independently constructed two variants of woodchuck hepatitis virus (WHV) with mutations in the X coding region. Transient transfection of two different hepatoma cell lines showed that these WHV X gene mutants were competent for virus replication in vitro. To determine whether X expression was required for viral replication in vivo, we injected mutant and wild-type genomes into the livers of susceptible woodchucks. While the wild-type WHV genomes were infectious in all animals examined, the mutant genomes did not initiate a WHV infection in woodchucks. These results indicate that the X gene of the hepadnaviruses plays a major role in viral replication in vivo.

The X gene of mammalian hepadnaviruses encodes a protein of 17 kDa whose function during viral infection is not known (7, 12). Nevertheless, the fact that a significant proportion of individuals infected with hepatitis B virus (HBV) show an anti-X-protein antibody response (17) suggests that the X gene is indeed expressed at least during certain stages of viral infection. In vitro studies have shown that the X protein can act as a transactivator for transcription not only from HBV promoters and enhancers (5, 27) but also from a wide range of other viral and cellular RNA polymerase II and III promoters (14, 27, 29, 30). Many studies have shown that the level of transactivation by X depends on the cell lines used (25) and varies from 2- to 10-fold stimulation of transcription, depending on the conditions selected (5, 25). Because of this observation and the apparent lack of DNA-binding activity of the X gene product, it has been suggested that the X protein acts either through interaction with other cellular transcription factors or through a signal transduction pathway controlled by protein kinase C (9, 16, 24). As a consequence of one or more of its activities, the X protein appears capable of inducing transformation of NIH 3T3 cells (26) and liver tumors in a selected strain of mice that express the X protein from a transgene (11). Nevertheless, the role of the X gene product during the viral life cycle in vivo remains elusive. Although analysis of viral replication after transfection of tissue culture cells with HBV genomes bearing mutations in the X gene indicated that X is not required for DNA replication, the results did not show whether X is actually necessary for the production of infectious progeny virus (2, 13, 31). Recently, Chen et al. (4) have observed that woodchuck hepatitis virus (WHV) genomes bearing mutations in the X gene, in contrast to wild-type WHV genomes, were not able to initiate a viral infection in susceptible woodchucks, suggesting that the WHV X gene is important for virus replication in its natural host. However, the role of the X gene in the viral infection could not be demonstrated definitely, since the ability of these mutant

genomes to replicate in cultured cells was not demonstrated, and no evidence that plasmid DNA transfects hepatocytes, the natural host cells of hepadnaviruses, was found. Moreover, the susceptibility of the animals previously inoculated with the mutant genomes to wild-type virus infection was not examined.

To clarify the role of the X protein, we have examined, independently of the work of Chen et al. (4), whether a functional X gene is necessary for WHV infection in vivo. We have, therefore, constructed two variants of WHV with mutations in the X coding region. We used plasmid pCMW82 as a substrate for the construction of the WHV mutants; this plasmid directs the expression of infectious WHV in tissue culture cells and can initiate productive WHV infection when transfected into the livers of susceptible woodchucks (19). In the first construct, called pΔAUG, we changed the first AUG initiation codon of the X open reading frame at position 2892 to UUG (Fig. 1) in the wild-type plasmid, pCMW82. A second mutant, called pΔC, had a single base change at position 3162, converting a UGG codon to an UGA stop codon (Fig. 1). Thus, this variant could support the expression of a truncated p17. Both mutations did not change conserved DNA sequences known to be required for the expression of the overlapping polymerase gene (pΔAUG) or for the regulation of viral transcription or DNA synthesis (12, 21, 22, 32).

To examine whether plasmids pΔAUG and pΔC were competent for DNA replication, we transfected two human hepatoma cell lines, HepG2 and Huh7, and examined virion DNA expressed after transfection. Figure 2 shows the analysis of WHV DNA purified from virions in the supernatant of the HepG2 (Fig. 2A) and Huh7 (Fig. 2B) cell lines. In both cases, virions released after 24 days were enriched in mature forms of viral DNA (relaxed circular and double-stranded linear DNAs as opposed to single-stranded DNA) in comparison with virions collected in the culture medium 9 days after transfection. In HepG2 cells transfected with pΔC and pΔAUG, the levels of virion DNA were approximately 15% of the levels obtained with the wild-type genome, pCMW82. In Huh7 cells transfected with pΔC and pΔAUG, the amount of virion DNA at 24 days posttransfection was decreased by about 60% relative to the amount in Huh7 cells transfected with pCMW82. Furthermore, analysis of the viral core DNA in the

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FIG. 1. Physical maps of the X gene of the WHV wild-type genome (pCMW82) and two mutant genomes (pΔAUG and pΔC). In the wild type, pCMW82, WHV DNA from clone pWHV81-2 was fused with pBC12/CMV/IL2 to direct the synthesis of WHV pregenomic RNA from the cytomegalovirus immediate-early promoter on pBC12/CMV/IL2 (19). Variants of pCMW82 were prepared by oligomer-directed site-specific mutagenesis as described by Taylor et al. (28). The mutations were verified by nucleotide sequence or restriction site analysis. The different mutations are indicated in boldface type and with a black arrowhead, and the conserved AUGs are shown. The first nucleotide of the precore open reading frame AUG is taken as position 1.

cytoplasm of the same cells showed a comparable decrease in viral DNA synthesis in the cells transfected with the X gene mutants relative to the cells transfected with the wild-type plasmid (data not shown). Thus, our results indicated that the two mutations introduced into the X gene did not interfere with WHV DNA replication but did reduce the levels of viral DNA in transfected cells.

Although intrahepatic inoculation of cloned DNA can initiate viral infection, the cellular target for uptake of naked DNA has not yet been identified (6); i.e., it is not known whether hepatocytes actually are infected by the cloned DNA or are infected through the spread of virus from other liver cells such as endothelial cells or Kupffer cells. Therefore, it was important to rule out the possibility that X⁻ mutants might have lost their infectivity under the conditions selected for in vivo DNA transfection because of their inability to replicate in cells other than hepatocytes. To examine whether wild-type and mutant WHV genomes can actually direct the expression of viral RNA in hepatocytes following the injection of viral DNA into the liver, we injected the wild-type plasmid, pCMW82, and the mutant plasmid pΔC into the livers of Fisher 344 rats, as described for the experimental inoculation of the woodchucks (see below). Four days after the injection, the rats were sacrificed, and two liver specimens, one excised at the site of injection and the other excised at a distal site, were fixed, embedded in paraffin wax, and sectioned. The tissue samples were then analyzed for WHV core antigen by standard immunofluorescence staining (8). Figure 3 shows the detection of viral core antigen at the site of DNA injection in the cytoplasm of liver cells from animals that were given injections of pCMW82 or pΔC. WHV core antigen was not detected at a

FIG. 2. Expression of WHV with X variants in tissue culture cells. HepG2 and Huh7 cells were transfected with plasmid DNA (22 μg/100-mm-diameter petri dish) by the calcium phosphate procedure. Culture medium from transfected cells was collected at 3-day intervals over a period of 27 days after transfection and assayed for virion DNA as described previously (21). Nucleic acids were subjected to electrophoresis through a 1.5% agarose slab gel and transferred to a nylon membrane (Hybond-N; Amersham). WHV DNA was detected by hybridization with a ³²P-labelled DNA probe containing the entire WHV genome (22). Membranes were exposed to Kodak XAR 5 film at -70°C, and radioactivity was quantitated with an AMBIS radioanalytic imaging system. The figure shows the DNA extracted from virions in the supernatant of HepG2 (A) and Huh7 (B) cells 9 and 24 days posttransfection (DPT) with pCMW82, pΔAUG, and pΔC. RC, relaxed circular WHV DNA; DL, double-stranded linear WHV DNA; SS, single-stranded WHV DNA.

site distal from the injection site or in control animals which were given injections of phosphate-buffered saline or plasmid DNA free of WHV sequences. These results indicated that uptake of viral DNA does occur in hepatocytes and that wild-type and X mutant genomes are competent for viral gene expression in vivo.

To examine whether WHV genomes with mutations in X could replicate in the livers of susceptible animals, we inoculated three woodchucks with the mutant genome pΔC and one animal with pΔAUG and examined their sera for the appearance of serologic markers characteristic of natural WHV infections. As a positive control, a cohort of six woodchucks was inoculated with the wild-type plasmid, pCMW82. All animals, obtained from North-Eastern Wildlife (South Plymouth, N.Y.), were negative for serologic markers of WHV infection prior to inoculation. Each woodchuck received 40 μg of plasmid DNA dissolved in phosphate-buffered saline at 50 ng/μl by injection into the surgically exposed liver as described previously (20, 23). Serum samples for serologic analyses were obtained from the femoral veins of anesthetized animals prior to inoculation with DNA, 4 weeks after inoculation, and subsequently at 3-week intervals up to 9 months postinoculation. Examination of the samples for the appearance of WHV surface antigen (WHsAg) showed that five of the six animals that were given an injection of wild-type plasmid pCMW82 produced WHsAg after an incubation period of 7 to 10 weeks (Table 1). This incubation time is consistent with our previous findings with ground squirrels and woodchucks infected with

FIG. 3. Detection of WHV core antigen in the livers of rats after intrahepatic inoculation of wild-type and X mutant WHV genomes. WHV core antigen in the cytoplasm of liver cells was detected by immunofluorescence staining with rabbit anti-WHV core antigen antibody. Cells from a WHV-infected woodchuck (A), a rat inoculated with phosphate-buffered saline (B), a rat inoculated with pCMW82 (C), and a rat inoculated with pΔC (D) are shown. Magnification, ca. $\times 33$. Experiments were done in duplicate for each construct.

ground squirrel hepatitis virus and WHV DNA, respectively (20, 23). The sera of all five animals were positive for viral DNA, as determined by PCR amplification of viral DNA directly from serum samples (1). DNA became detectable 2 to 3 weeks after the appearance of WHsAg. Finally, five of the six animals, including one animal that remained negative for WHsAg, exhibited detectable levels of anti-WHV core (anti-WHc) antibodies. Thus, six of six woodchucks were found positive for at least one marker of WHV infection (Table 1). By contrast, none of the four animals inoculated with pΔC or pΔAUG became positive for either WHsAg or anti-WHc antibodies during the surveillance period of 23 to 36 weeks (Table 1). To examine whether the four animals inoculated with X mutant genomes were still susceptible to WHV infection after the observation period, they were challenged with a WHV-containing serum known to be infectious. Four to six weeks postinoculation, four of four animals had detectable levels of anti-WHc antibodies and three of four became positive for WHsAg (Table 1). This experiment thus showed that the woodchucks remained susceptible to WHV infection after inoculation with mutants pΔC and pΔAUG.

In summary, our results showed that mutations leading to the truncation of the carboxy terminus of the X protein or preventing its expression from the first AUG completely abolished infectivity of WHV DNA in susceptible woodchucks. Although the number of animals used for this study was small, the observed results are statistically significant ($P < 0.05$; chi-square test). Our observation that a replication-competent hepadnavirus with a mutated X gene cannot initiate viral infection *in vivo* is in good agreement with the recent report of Chen et al. (4). Furthermore, we have performed the control experiments required to examine the importance of the X gene in the infectivity process. First, we have shown that our plasmids are competent for replication *in vitro* and therefore provide valid substrates for *in vivo* inoculations. Interestingly, a reduction of viral DNA synthesis was observed after trans-

TABLE 1. Serum markers of WHV infection in woodchucks after experimental infection

Animal no.	Plasmid DNA	DNA inoculation ^a						Challenge ^b			
		WHsAg		Anti-WHc Ab		WHV DNA		WHsAg		Anti-WHc Ab	
		Presence	Incubation time (wk)	Presence	Incubation time (wk)	Presence	Incubation time (wk)	Presence	Incubation time (wk)	Presence	Incubation time (wk)
W100	pCMW82	+	10	+	10	+	13				
W101	pCMW82	+	7	+	10	+	10				
W102	pCMW82	+	7	+	10	+	10				
W116	pCMW82	+	7	+	9	+	9				
W117	pCMW82	—	16 ^c	+	10	ND ^d					
W118	pCMW82	+	7	—	9 ^c	+	9				
W104	pΔC	—	35 ^c	—	35 ^c	ND		+	6	+	6
W106	pΔC	—	35 ^c	—	35 ^c	ND		—	16 ^c	+	6
W107	pΔC	—	35 ^c	—	35 ^c	ND		+	4	+	6
W115	pΔAUG	—	22 ^c	—	22 ^c	ND		+	4	+	5

^a Susceptible woodchucks were inoculated with the mutant genome pΔC or pΔAUG or with the wild-type genome, pCMW82. DNA inoculations into the liver were performed under general anesthesia induced with ketamine and maintained with xylazine. The sera, obtained before and after the experimental infection, were tested for WHsAg with a heterologous AUSRIA II radioimmunoassay kit (Abbott Laboratories) for the detection of HBV surface antigen. Assays were performed according to the manufacturer's protocol. Results were expressed as P/N ratios, where P is counts of ^{125}I -labeled anti-hepatitis B surface antigen bound to beads incubated with test serum per minute and N is counts of ^{125}I -labeled anti-hepatitis B surface antigen bound to beads incubated with control serum negative for WHsAg per minute. P/N ratios greater than 2.1 are considered positive. The detection of anti-WHc antibody (Ab) was performed by a radioimmunoassay as described by Ponzetto et al. (18). WHV DNA was detected after PCR amplification with two DNA oligomers which spanned positions 76 to 96 and positions 504 to 524 on the WHV genome, as described elsewhere (1).

^b After a follow-up period of 23 to 36 weeks, the animals inoculated with X mutant genomes were challenged with a 100- μl aliquot of infectious serum containing approximately 10^{10} WHV particles per ml (obtained from K. Kajino) by intravenous injection. Sera obtained after the challenge were tested for WHsAg and anti-WHc antibody as described in footnote *a*.

^c Follow-up period rather than incubation period.

^d ND, not determined.

fection of tissue culture cells with the WHV X mutant genomes, in agreement with the results obtained by Koike et al. and Yaginuma et al. with HBV X mutants (13, 31) but in contrast to the study by Blum et al., in which no difference between wild-type and X mutant HBV genomes in viral DNA synthesis and virion export was observed (2). Second, the failure to establish a productive viral infection after inoculation of the X mutant genomes cannot be explained by the absence of DNA uptake in hepatocytes. Indeed, viral core proteins could be detected in hepatocytes at the site of intrahepatic DNA inoculation, and by analogy with the tissue culture system, one can postulate that after DNA uptake in hepatocytes, viral production occurred at the site of DNA injection. Third, we actually demonstrated that the woodchucks inoculated with X mutant genomes remained susceptible to WHV infection and hence that X mutant genomes cannot initiate even a low-level infection that could not be detected by serologic assays, which have been proved useful for the monitoring of natural and experimental infections with WHV (18). This control was important, since HBV replication in the absence of any conventional serologic marker for HBV has been found in patients with chronic liver disease (3).

Although we have demonstrated the importance of the X protein for viral infection, the functional role of the X protein for viral replication *in vivo* remains enigmatic. One possibility is that the X protein is a more potent transactivator in normal hepatocytes and in the liver than in hepatoma cell lines and may therefore have a key function during the viral replication cycle by increasing the level of pregenomic RNA, as suggested by Colgrove et al. (5). If the role of the X protein is limited to the transactivation of viral transcription, X mutant genomes should still be able to induce a low-level viremia and at least a weak serologic response in the susceptible woodchucks. The X⁻ variants could therefore exhibit an infection profile similar to the one observed with Nef⁻ or Vpr⁻ variants of simian immunodeficiency virus (10, 15). However, our results indicated that this is not the case (Table 1). Another possibility is that the X protein is involved in maturation of viral core particles or plays a role during the early steps of the viral replication cycle, which include uncoating and transport of viral DNA into the nucleus. Finally, it is conceivable that the X protein modulates the immune response *in vivo* and allows the virus to circumvent host antiviral defenses. However, support for this hypothesis has diminished, since the X protein appears to stimulate the expression of beta interferon and HLA class I genes in certain tissue culture cells (29, 33).

In conclusion, we have shown that the X protein is a critical component of the infectivity process *in vivo*. Thus, this polypeptide should provide a new target for the development of antiviral agents for treatment of patients with chronic hepatitis B.

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