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Woodchuck Hepatitis Virus X Protein Is Present in Chronically Infected Woodchuck Liver and Woodchuck Hepatocellular Carcinomas Which Are Permissive for Viral Replication

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The woodchuck hepatitis virus (WHV) X gene (WHx) is required for infectivity of WHV in woodchucks, and the gene encodes a broadly acting transcription factor. Several lines of evidence from cell culture and transgenic mice suggest that X proteins can promote hepatocarcinogenesis. To determine whether WHx-encoded proteins are present during persistent infection and hepatocellular carcinoma (HCC) in woodchucks, we surveyed livers and HCCs from a panel of WHV carrier woodchucks for the presence of WHx by utilizing an immunoprecipitation-Western blot (immunoblot) procedure. We detected a single 15.5-kDa WHx gene product in 100% of the persistently infected livers but not in livers from animals which had recovered from acute infection or in those of uninfected woodchucks. Analysis of HCCs revealed that all of the tumors which contained WHV replication intermediates were also positive for WHx. In contrast, WHx was undetectable in HCCs which did not contain replicative intermediates. Subcellular localization studies detected WHx in the cytoplasm but not in the nuclei of primary woodchuck hepatocytes. Comparative immunoprecipitation experiments revealed that there were 4×10^4 to 8×10^4 molecules of WHx per primary woodchuck hepatocyte. Four lines of WHx transgenic mice did not develop HCC spontaneously. However, when one line was treated with diethylnitrosamine, the occurrence of precancerous lesions was enhanced compared with that in diethylnitrosamine-treated nontransgenic controls. The apparent absence of WHx in some woodchuck HCCs indicates that WHx may not be required to maintain the tumor phenotype, whereas its presence in all persistently infected livers leaves open the possibility that it plays a role in hepatocarcinogenesis.

Woodchuck hepatitis virus (WHV) is a member of the hepadnavirus family, and like the other members of the family, it infects the liver of its host and causes both acute and persistent infections (60). Persistent infections usually last for the life of the animal and the inevitable consequence of lifelong infection with WHV is primary hepatocellular carcinoma (HCC). In fact, the epidemiological link between persistent WHV infection and HCC is nearly 100% in woodchucks, making this the strongest example of a specific viral infection leading to a specific malignancy (42, 43, 56).

In light of this link, the mechanisms by which viral infection causes HCC are of great interest. Hepatocarcinogenesis is a multistage process driven by progressive genetic changes in hepatocytes, leading to the selection of hepatocytes with increasingly malignant phenotypes (5, 46). Hepatocarcinogenesis in WHV-infected woodchuck liver also proceeds through a series of stages marked by the sequential occurrence of precancerous lesions, termed altered hepatic foci (AHF), neoplastic nodules, benign hepatocellular adenomas, and finally malignant HCCs, which occur in both the highly differentiated and poorly differentiated phenotypes (1, 2, 46, 50). Molecular genetic studies have revealed that the cellular N-*myc* and C-*myc* protooncogenes and the fetal liver growth factor insulin-like growth factor 2 are both overexpressed in nearly all woodchuck HCCs of the poorly differentiated phenotype (16, 17, 24, 70, 71).

In light of the complex series of pathogenic changes in the liver, WHV might be expected to exert a carcinogenic influence through more than one mechanism. Indeed, persistent infection appears to confer at least a triple threat for malignant transformation of hepatocytes. The first threat is accomplished as a result of the limited immune response, which allows the virus to persist in the liver, leading to a continuous cycle of hepatocyte death and regeneration mediated by various degrees of inflammation (11). This provides the mitogenic driving force which would be expected to fix mutations in the host genome which occur partly as a result of the continuous production of toxic oxygen radicals during persistent infection (32, 33).

The second threat is directly mediated by the viral DNA in the form of viral DNA integrations into host chromosomes. WHV DNA integration occurs in some woodchuck hepatocytes during persistent infection, even though integration is not needed for viral replication (39, 47). WHV integration into host DNA in the vicinity of either the N-*myc* or C-*myc* protooncogene activates the expression of this gene by an enhancer insertion mechanism (16, 21, 68). The high frequency of activated transcription of the N- and C-*myc* genes has established them as important contributors to hepatocarcinogenesis in woodchucks.

The third threat is the one which this study addressed. It is the expression of proposed hepadnavirus oncoprotein X during persistent infection and in HCCs. From the time of its discovery as an open reading frame (ORF) in all of the mammalian hepadnaviruses, the function of X has proved difficult to determine (19, 64). Genetic evidence has revealed a requirement for WHx in the establishment of infection (8, 72). Carcinogenesis studies have demonstrated that HBx can function as an oncoprotein when it is overexpressed in simian virus 40 (SV40) large-T antigen-immortalized hepatocyte cell lines, in

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NIH 3T3 cells (23, 53), or in the livers of transgenic mice (26, 28). A large number of studies have shown that all of the mammalian X proteins can function as transacting factors when expressed in a wide variety of cell culture systems and tested for activity on a wide range of viral and cellular promoters (27, 37, 49, 52, 57, 63, 69).

In light of the growing body of evidence that X is a promiscuous transcription factor which may play a role in both viral infection and hepatocarcinogenesis, evidence to firmly establish the presence of X proteins during acute or persistent infection or in HCCs is an urgent need. The presence of antibodies to X proteins in the serum of persistently infected individuals has led researchers to conclude that X must be expressed at some time during infection (31, 40). The presence of X proteins during persistent infection and in HCCs has also been proposed on the basis of immunohistochemical data (65, 67). However, data describing the natural history of X expression during hepadnavirus infections are incomplete.

In this study, we began to determine the natural history of WHx expression during WHV infections. We developed a protocol to detect WHx in woodchuck liver and isolated primary hepatocytes. Our protocol is based on initial immunoprecipitation of WHx followed by Western blot (immunoblot) analysis. By using this protocol, we detected a 15.5-kDa WHx protein in 100% of the persistently infected woodchuck livers in our panel. Comparative immunoprecipitation studies with isolated primary woodchuck hepatocytes revealed 4×10^4 to 8×10^4 molecules of WHx per hepatocyte, and cell fractionation studies detected WHx only in the cytoplasmic fraction of primary woodchuck hepatocytes. WHx was also present in HCCs which remained permissive for viral replication, whereas it was undetectable in HCCs which were nonpermissive for viral replication. Studies with WHx transgenic mice suggest that WHx may promote precancerous progression in mice. These studies provide a foundation for further investigations of the mechanism of action of WHx in hepatocytes.

MATERIALS AND METHODS

Source of material. The liver and tumor samples used in this study were collected from 24 woodchucks (Table 1). Our sample population included (i) six uninfected animals negative for all WHV markers, (ii) five woodchucks which had recovered from acute WHV infection (anti-WHV surface antigen positive [anti-WHs$^+$] and anti-WHV core antigen positive [anti-WHc$^+$]), (iii) five woodchucks with persistent WHV infection and no HCC (WHsAg$^+$/anti-WHc$^+$), and (iv) eight woodchucks with persistent infection and primary HCC (WHsAg$^+$/anti-WHc$^+$). In this study, we examined 12 tumors from the latter eight animals for the presence of WHx protein and viral DNA. The tumor samples were carefully dissected to ensure removal of all nonmalignant peritumor liver tissue, and sections were also analyzed histologically to confirm the malignant phenotype.

All of the recovered animals and two chronically infected animals (J49 and J40) had been experimentally inoculated with pools of WHV-positive sera from infected woodchucks at 1 week after birth. All of the other persistently infected woodchucks were captured as young adults with preexisting WHV infections and were housed at the Penrose Research Laboratory of the Philadelphia Zoological Society (17). Serologic analysis of WHV markers (WHsAg, anti-WHs and anti-WHc) were performed as previously described (13, 41). The woodchucks were 2 to 5 years old when sacrificed. Tumors and normal liver tissues were snap-frozen in a dry-ice–isopentane bath immediately after surgical removal and stored in liquid nitrogen until further processing. For detection of WHx protein and WHV DNA analysis, frozen tissue samples were crushed in a mortar under liquid nitrogen and divided into two parts. The first part was immediately homogenized in protein lysis buffer as described below, and the second part was processed for DNA extraction.

Preparation and storage of woodchuck hepatocytes. Hepatocytes from a WHV-positive woodchuck were provided by S. Gupta and P. Rajvanshi, Liver Research Center of Albert Einstein College of Medicine, Bronx, N.Y., by the two-step in situ collagenase cell perfusion method (20, 54). Purification of primary hepatocytes from nonparenchymal cells was accomplished by differential centrifugation at $50 \times g$ and 4°C. Woodchuck hepatocyte viability was 95% according to the trypan blue exclusion test. Aliquots of 10^7 hepatocytes were frozen in a freezing medium composed of 70% Wisconsin medium (used for liver transplants), 20% fetal bovine serum, and 10% dimethyl sulfoxide, and the frozen cells were stored in liquid nitrogen.

TABLE 1. Presence of WHV X proteins and WHV DNA in woodchuck livers and HCCs

Group and woodchuck designation	Liver pathology	WHx protein status	WHV DNA status[a]
Uninfected (no WHV markers)			
CW873	None	−	−
CW806	None	−	−
CW750	None	−	−
CW521	None	−	−
CW527	None	−	−
CW833	None	−	−
Recovered from acute infection (anti-WHVs$^+$/anti-WHc$^+$)			
J36	None	−	−
J37	None	−	−
J39	None	−	−
J28	None	−	−
J53	None	−	−
Persistent infection, no HCC (WHsAg$^+$/anti-WHc$^+$)			
CW811	CPH[b]	+	+
CW813	CPH	+	+
CW825	CAH[c]	+	+
J49	CAH	+	ND
CW522	CAH	+	+
Persistent infection and HCC (WHsAg$^+$/anti-WHc$^+$)			
Peritumor liver sources:			
CW824	CAH	+	+
CW796	CAH	+	+
CW819	CAH	+	+
J40	CAH	+	+
CW1193	CAH	+	+
Tumor sources:			
CW824-T1	HCC	−	−(INT)
CW796-T1	HCC	−	−
CW819-T2	HCC	+	+(INT)
CW819-T5	HCC	+	ND
J40-T1	HCC	+	+(INT)
CW1193-T2	HCC	+	+
CW153-T1	HCC	+	+
CW153-T2	HCC	+	+
CW156-T1	HCC	+	+
CW156-T2	HCC	+	+
CW605-T1	HCC	−	−(INT)
CWW605-T4	HCC	−	−(INT)

[a] −, no WHV replication forms detectable; +, WHV replication forms detectable; INT, WHV integrations detected in high-molecular-weight DNA; ND, not determined.
[b] CPH, chronic persistent hepatitis.
[c] CAH, chronic active hepatitis.

Cytoplasmic and nuclear fractionation of woodchuck hepatocytes. Frozen WHV-positive hepatocytes were rapidly thawed, resuspended in phosphate-buffered saline, checked for viability by trypan blue staining, and collected by centrifugation ($50 \times g$, 5 min). Hepatocytes (10^7) were resuspended in approximately 10 volumes of hypotonic buffer (20 mM Tris [pH 7], 1 mM $MgCl_2$, 5 mM KCl, 2 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40), incubated on ice for 15 min, and then homogenized in a loose-fitting Dounce homogenizer (15 to 20 strokes). After cell lysis, cytoplasmic and nuclear (nuclear pellet) fractions were prepared by centrifugation at $1,000 \times g$ for 15 min at 4°C. The cytoplasmic fraction (supernatant) was brought to 150 mM NaCl and 1% Nonidet P-40 and used for the immunoprecipitation assay.

The crude nuclear pellet was either (i) resuspended in protein lysis buffer, homogenized in a tight-fitting Dounce homogenizer, and used directly for the immunoprecipitation assay or (ii) resuspended in sucrose buffer (1 M sucrose in 20 mM Tris [pH 7], 10 mM $MgCl_2$, 25 mM KCl, 2 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol) and subjected to further purification by homogenization in a loose-fitting Dounce homogenizer, followed by the addition of 2 volumes of 2.3 M sucrose buffer. This mixture was gently layered onto a 2-ml cushion of 2.3 M sucrose and centrifuged at $120,000 \times g$ for 45 min at 4°C. The nuclear pellet was resuspended in 0.5 ml of protein lysis buffer (see below),

homogenized in a tight-fitting Dounce homogenizer, and used for immunoprecipitation of WHx protein. Cytoplasmic and nuclear lysates from the WHV-negative WC-3 woodchuck liver epithelial cell line were used as a negative control in our experiments (29).

Immunoprecipitation and Western blot detection of WHx-encoded proteins. WHx-encoded protein in woodchuck primary hepatocytes, liver tissues, and tumors was detected with a rabbit antiserum raised against a recombinant WHx-encoded polypeptide. Recombinant WHx antigen and the corresponding antiserum were kindly provided by Kazunori Kajino and William S. Mason, Institute for Cancer Research, Philadelphia, Pa. Recombinant WHx polypeptides (recWHx) were prepared with the pQE-9 expression vector, in which an *Nco*I-*Hin*dIII fragment of WHV DNA containing the WHx open reading frame was inserted. recWHx was slightly larger than full-length WHx because of additional amino acids which served as a protease cleavage site during recWHx purification (35a).

Protocol for detection of WHx from woodchuck liver and HCC tissues. Liver and HCC tissues pulverized in liquid nitrogen were homogenized in 10 volumes of protein lysis buffer (20 mM Tris [pH 7], 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM KCl, 2 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 1% Nonidet P-40) by using a Teflon pestle homogenizer (Thomas Instruments). The cell extracts were clarified by centrifugation (12,000 × *g*, 20 min, 4°C), and the supernatants were used for immunoprecipitation.

The lysates were incubated with anti-WHx rabbit serum (1:100 dilution) or normal rabbit serum (1:100 dilution; Pierce or Sigma) with gentle rocking at 4°C for 2 h, a 10% (vol/vol) slurry of protein A-Sepharose beads (CL-4B Pharmacia) was added, and the mixture was gently rocked for an additional 2 h. Immunocomplexes were collected by centrifugation (4 min in a microcentrifuge at room temperature), and the pellets were washed three times with lysis buffer and then another three times in isotonic saline buffer (20 mM Tris [pH 7], 150 mM NaCl). Laemmli sample buffer (6×, containing 0.5 M Tris-HCl [pH 6.8], 10% sodium dodecyl sulfate [SDS], 6% 2-mercaptoethanol, 30% glycerol, and 1 mg of bromophenol blue per ml) was added to the pellets, and samples were boiled for 3 min. After centrifugation (microcentrifuge) to remove the protein A beads, the supernatants were collected and subjected to electrophoresis through an SDS–15% polyacrylamide gel and the resolved proteins were transferred to a polyvinylidene difluoride–Immobilon-P transfer membrane (Millipore) at a constant voltage (50 V) overnight by using a semidry system (LKB) and soaking Whatman 3MM filter paper in transfer buffer (50 mM Tris base, 192 mM glycine, 20% methanol). Transfer efficiency was assessed by visualization of the transfer of prestained protein molecular weight markers.

For Western blot analysis, the filters were fixed in 50% methanol for 30 min, briefly rinsed in TBS-T (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Tween 20), and then incubated for 2 h in blocking buffer containing 10% nonfat dry milk in TBS-T. WHx proteins were detected after incubating the blot in a 1:1,000 dilution of anti-WHx serum for 2 h at room temperature in TBS-T with 2% nonfat dry milk. Bound immunocomplexes were detected by exposure to horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (diluted 1:5,000; Amersham) for 30 min in TBS-T with 2% nonfat dry milk and visualized by the ECL-Western blot chemiluminescence detection system (Amersham).

Preparation of nucleic acids and Southern and Northern (RNA) blot analyses. Frozen tissues from tumors and liver were crushed in a mortar under liquid nitrogen, and genomic DNAs were prepared as previously described (39). Approximately 10 μg of DNA from each sample, both undigested and digested with the appropriate restriction endonuclease, was used for Southern blot analysis with nylon membranes (Zeta Bind; Cuno Lab). Total liver RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (12). For Northern blot analysis, 20 μg of total RNA was loaded per lane and processed as previously reported (17).

A genome length 3.3-kb WHV DNA fragment was used to prepare a ^{32}P-labeled probe for Northern and Southern blot analyses as previously reported (39). Northern and Southern blots were prehybridized for 2 h at 42°C in prehybridization buffer (10× Denhardt's solution plus 0.5% SDS) and then hybridized with the ^{32}P-labeled WHV DNA probe at high stringency (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 2.5× Denhardt's solution, 0.2% SDS, 42°C) for 16 to 24 h. After hybridization, the filters were washed at high stringency (39) and then exposed to Kodak XAR-5 film for 16 to 24 h at −70°C with intensifying screens.

MUP-WHx transgenic mice. The major urinary protein (MUP)-WHx transgene used to obtain WHx transgenic mice was constructed by inserting an *Alu*I fragment from the WHV genome (nucleotides 1503 to 1996) (18), which contained the entire WHx ORF, into an *Xba*I-digested, blunt-ended MUP-SV40 vector, which has been previously reported (48). The resultant MUP-WHx-SV40 vector contained the 5′ MUP promoter (2.5 kb) (22), the WHx ORF, and the 3′ SV40 small-T intron along with the poly(A) sites. A 3.9-kb transgene was excised from the vector by digestion with *Pst*I and *Eco*RI. Microinjection of the purified transgene into C57/BL6 × CBA F_1 fertilized eggs and production of founder animals were done by the transgenic mouse facility of the Chanin Cancer Research Center, AECOM. Four WHx founders transmitted the transgene to their progeny, in which the transgene was expressed in the liver (data not shown), and these were bred with C57/BL6 and developed into lines. Animals from each line (>25 of each) were maintained for 1.5 to 2 years before sacrifice and autopsy for liver or other tumors. One line, Mup-WHx-4, was bred to homozygosity, and these animals and those of a nontransgenic line with a similar genetic background were used in liver carcinogenesis experiments. Tail DNAs were genotyped by Southern blotting for the presence of the MUP-WHx transgene.

Carcinogen treatment and experimental design. Diethylnitrosamine (DEN) from Sigma was resuspended in sterile saline and administrated to 12-day-old male transgenic and nontransgenic mice as a single intraperitoneal injection of 2 μg/g of body weight. Mice were housed for 6 to 10 months, and their livers were examined upon sacrifice for the presence of observable lesions. Liver tissues were cut into random sections, and one part of the material was snap-frozen in a dry-ice–isopentane bath and stored in liquid nitrogen for DNA and protein analyses, and another part was fixed in 10% neutral buffered formalin for 24 h, embedded, and sectioned. Eight DEN-treated Mup-WHx-4 transgenic mice and 12 DEN-treated nontransgenic control mice were analyzed at the age of 6 months. Three random tissue sections per animal were stained with hematoxylin and eosin and analyzed for the presence of foci of abnormal growth by using criteria previously established for precancerous lesions in rats treated with chemical carcinogens (2, 58). We measured the sizes of the AHF with a micrometer in the eyepiece of a microscope, and the smallest group of cells scored as a focus had a minimum diameter of 0.1 mm. The focal lesions seen on liver sections were approximately circular, and that is consistent with observations made by us and others (6) that liver foci are generally spherical.

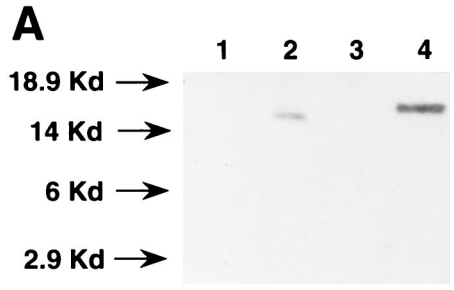
To determine the total area of the tissue sections, we photocopied (10-fold magnification) the original sections on paper sheets of known weight and cut out the images. By weighing such images, we obtained an estimate of the total area of the tissue sections examined. This was used as the denominator for our calculation of percent liver occupied by foci. For mathematical estimation of the number of foci per unit of liver volume, we used the quantitative stereologic analysis method developed and described by Campbell et al. (6) and Pugh et al. (44).

RESULTS

WHx protein is present in persistently infected woodchuck liver. To assay for the presence of WHx protein, we assembled a panel of woodchuck liver tissues (Table 1). The panel consisted of liver specimens from (i) six uninfected woodchucks (negative for all WHV markers), (ii) five woodchucks which had recovered from acute infection (anti-WHs^+/anti-WHc^+), and (iii) five woodchucks with persistent infection and no apparent HCC ($WHsAg^+$/anti-WHc^+). Histological analysis of liver specimens from the persistently infected animals revealed either chronic active hepatitis or chronic persistent hepatitis (Table 1). WHV replication in the livers of all of the animals was confirmed by Southern blot analysis as previously reported (17). None of the liver specimens from uninfected and recovered animals contained any detectable WHV DNA. Replication forms were detected as a band of open circular WHV DNA of 3.3 kb plus lower-molecular-weight replicative intermediates in all of the persistently infected liver tissues (Table 1).

To detect WHx protein in persistently infected liver, we developed an immunoprecipitation-Western blot protocol which utilized a rabbit anti-WHx serum for detection of WHx protein in immunoprecipitates by using the ECL chemiluminescence detection system (see Materials and Methods). With this protocol, we specifically immunoprecipitated a protein of 15.5 kDa from extracts of persistently infected woodchuck liver (Fig. 1A, lane 2). The WHx antiserum did not precipitate a 15.5-kDa protein from uninfected woodchuck liver (Fig. 1A, lane 3), and normal rabbit serum also did not immunoprecipitate a 15.5-kDa protein from the same persistently infected liver (Fig. 1A, lane 1). The WHx antiserum recognized a recombinant WHx protein which was run in parallel with the unknown samples (Fig. 1A, lane 4). The recombinant protein was slightly larger than the native WHx because of the presence of a short stretch of additional amino acids in the recombinant WHx protein.

Western blots of immunoprecipitates from our panel of woodchuck livers revealed that WHx protein was present in 100% of the persistently infected tissues in our panel and in some of the tumors (Fig. 1B). Interestingly, we did not detect any additional smaller WHx-related proteins in any of the samples from our panel of woodchuck livers and tumors.



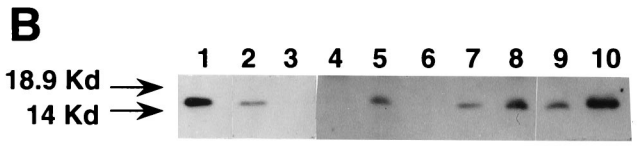


FIG. 1. Detection of WHx proteins in woodchuck liver tissues with an immunoprecipitation-Western blot-enhanced chemiluminescence detection protocol. (A) Lysates from a WHV-positive liver (woodchuck CW796) (lanes 1 and 2) or a WHV-negative liver (woodchuck CW873) (lane 3) were subjected to immunoprecipitation with either WHx antiserum (lanes 2 and 3) or normal rabbit serum (lane 1); this was followed by Western blotting of immunoprecipitated proteins and enhanced chemiluminescence detection with WHx antiserum. Lane 4 contained 2 ng of recWHx that was loaded directly onto the gel as a positive control. (B) Representative survey of WHx protein in lysates from WHV-positive livers (lanes 1, 3, 5, 7, and 9; woodchucks J40, CW796, CW819, and CW825) that was immunoprecipitated with WHx antiserum (lanes 1, 5, 7, and 9) or rabbit normal serum (lane 3), of WHx protein immunoprecipitated from an uninfected liver (woodchuck CW806; lane 4) with WHx antiserum, or of WHx protein immunoprecipitated from lysates from HCCs (woodchucks J40, CW796, and CW819; lanes 2, 6, and 8) with WHx antiserum. Lane 10 is a positive control, in which 2 ng of recombinant WHV X protein was loaded directly onto the gel. Kd, kilodaltons.

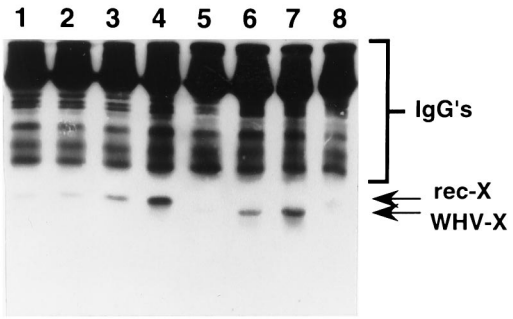


FIG. 2. Analysis of the abundance of WHx polypeptides in primary woodchuck hepatocytes from a WHV carrier woodchuck as determined by parallel immunoprecipitation of recWHx with endogenous WHx proteins. Lanes: 1 to 4, immunoprecipitation of recWHx from WC-3 cell lysates in which either 1, 2, 4, or 8 ng of recWHx protein was added, respectively (10^6 WC-3 cells were used per lysate); 5 to 7, immunoprecipitation of endogenous WHx from lysates prepared from 1×10^6, 2×10^6, or 4×10^6 WHV-positive primary hepatocytes; 8, immunoprecipitation of a lysate prepared from 4×10^6 WHV-positive hepatocytes with normal rabbit serum; 1 to 7, immunoprecipitation conducted with an excess of WHx antiserum. The nonspecific upper bands represent immunoglobulins G precipitated by Sepharose beads.

Therefore, translation products initiated in the internal translation initiation sites of the WHx ORF or otherwise truncated WHx proteins appear not to be present in persistently infected woodchuck liver.

The level of WHx varied approximately fivefold in samples of persistently infected liver (Fig. 1B, lanes 1, 5, 7, and 9). Immunoprecipitation from lysates of WHV-positive liver with normal rabbit serum consistently produced no bands on our Western blots (Fig. 1B, lane 3), and the WHx antisera did not precipitate a 15.5-kDa protein from uninfected woodchuck liver lysates (Fig. 2B, lane 4).

Quantitation of WHx. To obtain a quantitative estimate of the number of WHx molecules in woodchuck hepatocytes, we first isolated primary hepatocytes from a WHV carrier woodchuck. The hepatocytes were 95% viable after initial isolation and retained 75% viability after freezing and thawing.

To quantitate WHx levels per hepatocyte, we conducted parallel immunoprecipitation assays in which we either (i) added known amounts of recWHx to cell lysates or (ii) immunoprecipitated endogenous WHx from extracts prepared from 1×10^6, 2×10^6, or 4×10^6 WHV-positive hepatocytes. To provide the immunoprecipitation standard, we added 1, 2, 4, or 8 ng of recWHx to extracts prepared from 10^6 uninfected woodchuck liver epithelial cells (WC-3 cells) and conducted immunoprecipitation in parallel with extracts prepared from the above-mentioned numbers of primary woodchuck hepatocytes from a WHV carrier. The Western blot from these immunoprecipitation assays revealed that the amount of WHx precipitated from 10^6 WHV-infected hepatocytes was equivalent to the amount of recWHx immunoprecipitated from extracts to which 1 to 2 ng of recWHx had been added (10^6 WC-3 cells were used for each control extract). This is equivalent to approximately 40,000 to 80,000 molecules of WHx per woodchuck hepatocyte (assuming 3.9×10^{10} molecules of WHx per ng of protein).

Addition of larger amounts of WHx antisera did not increase the immunoprecipitation signal, suggesting that antibody was not limiting in our assays. An average level of WHx protein was detected in extracts from the liver of the woodchuck used to obtain the primary hepatocytes, compared with the other chronically infected livers in our panel. Therefore, our data provide an average estimate of the level of WHx in persistently infected livers in the absence of detectable HCC. The presence of WHx in HCCs and peritumor liver is discussed later in this report.

Subcellular localization of WHx. To begin to determine the subcellular distribution of WHx in primary hepatocytes, we fractionated hepatocytes into crude cytoplasmic and nuclear fractions. Analysis of these fractions revealed the great majority of WHx protein in the cytoplasmic fractions (compare lanes 3 and 5 of Fig. 3A). In a second set of experiments, we used nuclei which had been purified by sucrose gradient centrifugation. Such nuclei have been previously shown to preserve WHV covalently closed circular DNA in the nucleus (data not shown), and intactness of nuclei was also monitored by microscopy. Immunoprecipitation of nuclear lysates from this preparation, followed by Western blot analysis, failed to detect any WHx in the nuclear extracts (Fig. 3B, lane 7), although the presence of WHx in these same cells was confirmed by immunoprecipitating, in parallel, the cytoplasmic fraction (Fig. 3B, lane 5). Therefore, purification of woodchuck hepatocyte nuclei eliminated any detectable WHx protein signal. Our immunoprecipitation experiments with known amounts of recombinant WHx showed that 0.2 ng (8×10^9 molecules) was the minimum amount of WHx detectable in our assay. Since we isolated and analyzed nuclei from 10^7 primary hepatocytes per assay, WHx could have been present in fewer than 800 molecules per nucleus and we would not have been able to detect it. Therefore, we cannot formally exclude the possibility of the complete absence of WHx from nuclei.

WHx is present in HCCs which are permissive for WHV replication but undetectable in HCCs which lack WHV replication. To study the distribution of WHx protein in HCCs, we compiled a panel of tissues which consisted of the peritumor liver and HCC tissues from woodchucks with single and mul-

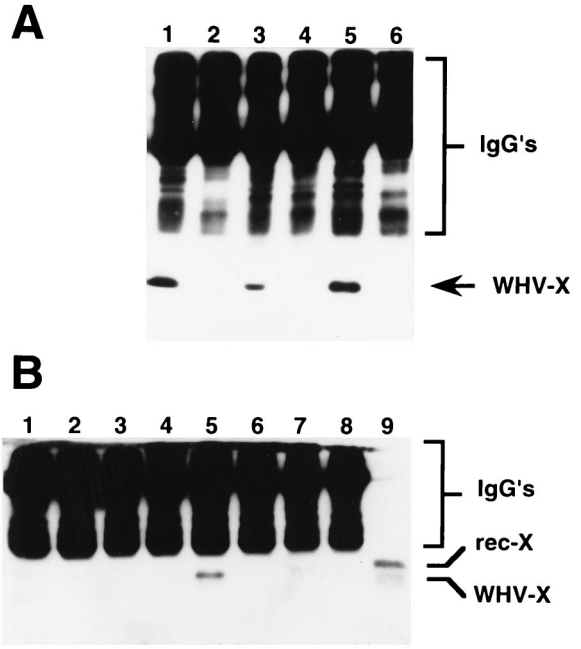


FIG. 3. Subcellular distribution of WHx in nuclear and cytoplasmic fractions of WHV-positive hepatocytes. Analysis was done with both crude nuclear extracts (A) and purified nuclei (B). (A) Total cell extracts from primary hepatocytes (lanes 1 and 2), crude nuclear extracts (lanes 3 and 4), and cytoplasmic fractions (lanes 5 and 6) were prepared as described in Materials and Methods and immunoprecipitated with WHx antiserum (lanes 1, 3, and 5) or normal rabbit serum (lanes 2, 4, and 6). (B) Extracts from cytoplasmic fractions and nuclei purified by sucrose centrifugation were immunoprecipitated with WHx antiserum (lanes 1, 3, 5, and 7) or normal rabbit serum (lanes 2, 4, 6, and 8). Lanes: 1 to 4, immunoprecipitation of cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) fractions from WHV-negative WC-3 cells as negative controls; 5 to 8, immunoprecipitation of cytoplasmic (lanes 5 and 6) and nuclear (lanes 7 and 8) fractions from WHV-positive primary hepatocytes. Lane 9 contained 2 ng of recWHx protein that was loaded onto the gel as a positive control. IgG, immunoglobulin G.

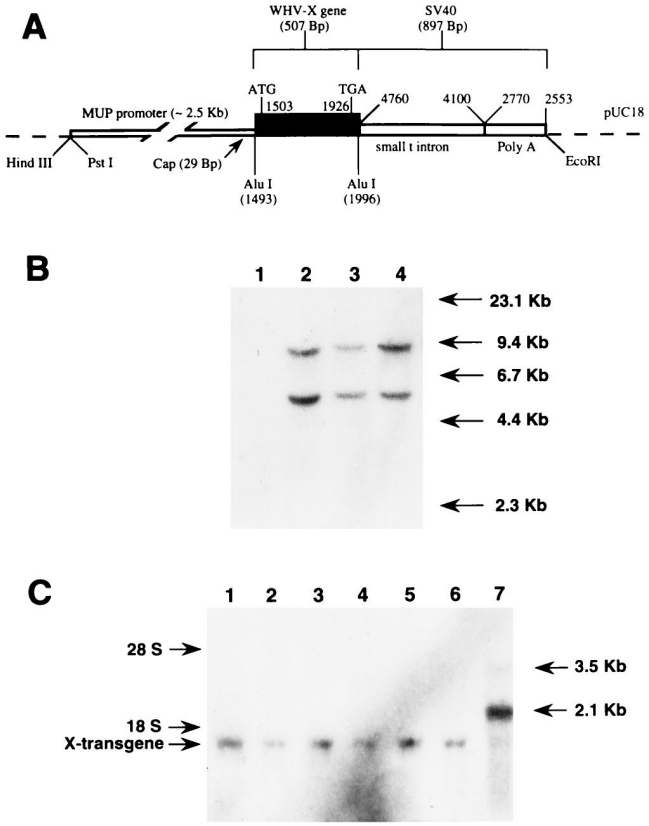


FIG. 4. Structure of the WHx transgene and measurement of its expression in Mup-WHx-4 transgenic mice. (A) Diagram of the Mup-WHx-4 transgene. The nucleotide numbers above the WHV sequences are those of Galibert et al. (18), in which the *Eco*RI site is nucleotide 1. The nucleotide numbers above the SV40 sequences refer to those in the established SV40 map, and the MUP promoter used was that reported earlier by Held et al. (22). The plasmid vector was pUC18. (B) Southern blot analysis of genomic DNA (*Hin*dIII digested) from Mup-WHx-4 transgenic mice hybridized with ^{32}P-labeled WHV DNA. Lanes: 1, genomic DNA from a nontransgenic littermate. 2 and 3, genomic tail DNA and liver DNA, respectively, from a Mup-WHx-4 mouse 1 month old; 4, liver DNA from a 6-month-old Mup-WHx-4 mouse. At least one copy of the intact transgene was integrated at two different sites of the mouse genome. These transgenes cosegregated in progeny mice. (C) Northern blot analysis of transgene expression in the livers of Mup-WHx-4 transgenic mice (20 μg of total liver RNA per lane). Transgene expression in the livers of male and female homozygous mice at the ages of 1 (lanes 1 and 2), 3 (lanes 2 and 4), and 6 (lanes 5 and 6) months. Odd-numbered lanes, males; even-numbered lanes, females. Lane 7 contained 20 μg of total liver RNA from persistently infected woodchuck CW824 as a positive control. The predominant WHV RNAs in lane 7 migrated to positions expected of the WHV pregenome (3.5 kb) and surface antigen transcript (2.1 kb).

tiple HCCs (Table 1). All of the animals in this group were WHsAg$^+$/anti-WHc$^+$. Peritumor liver samples from five such animals contained WHV replicative intermediates, and all of the liver samples were positive for the presence of WHx (Table 1). Representative results from several liver samples in this group are shown in Fig. 1.

Analysis of the HCCs from these and other animals revealed that most of the HCCs contained WHV replicative intermediates and all of the replication-positive tumors were also positive for WHx protein. Four of the HCCs did not contain WHV replicative intermediates, and these four tumors were negative for WHx protein (one example is presented in Fig. 1B, lane 6). Three of these tumors (CW824-T1, CW605-T1, and CW605-T4) contained high-molecular-weight WHV integrated DNA (data not shown). In summary, in our panel of samples, there was a 100% correlation between the presence of WHx and WHV replication, regardless of whether the tissue was chronically infected liver before the appearance of HCC, peritumor liver, or HCC permissive for viral replication. In the absence of WHV replication in HCCs, there was no detectable WHx protein.

WHx transgenic mice are more sensitive to precancerous changes induced by DEN than are control mice. The presence of WHx during persistent infection leaves open the possibility that X plays a role in hepatocarcinogenesis during the precancerous stages, while its continued presence would not be required once malignant tumors are established. To investigate the role of WHx in hepatocarcinogenesis, we developed four lines of WHx transgenic mice. The transgene used in this study is shown in Fig. 4A. This transgene utilized the murine MUP promoter (22, 48) to drive the expression of the WHx gene. Southern blot analysis of genomic DNA from Mup-WHx-4 mice revealed that the transgene was present at two loci, which cosegregated (Fig. 4B), and Northern blot analysis demonstrated that the Mup-WHx-4 transgene was expressed in the livers of both male and female mice at a moderate level throughout their lives (Fig. 4C). Recently, with our immunoprecipitation-Western blot protocol, we detected a low level of WHx in the Mup-WHx-4 transgenic mouse liver (data not shown).

A large number of animals (greater than 25 per line) from each of four WHx transgenic mouse lines were allowed to age as described in Materials and Methods. No increase in spon-

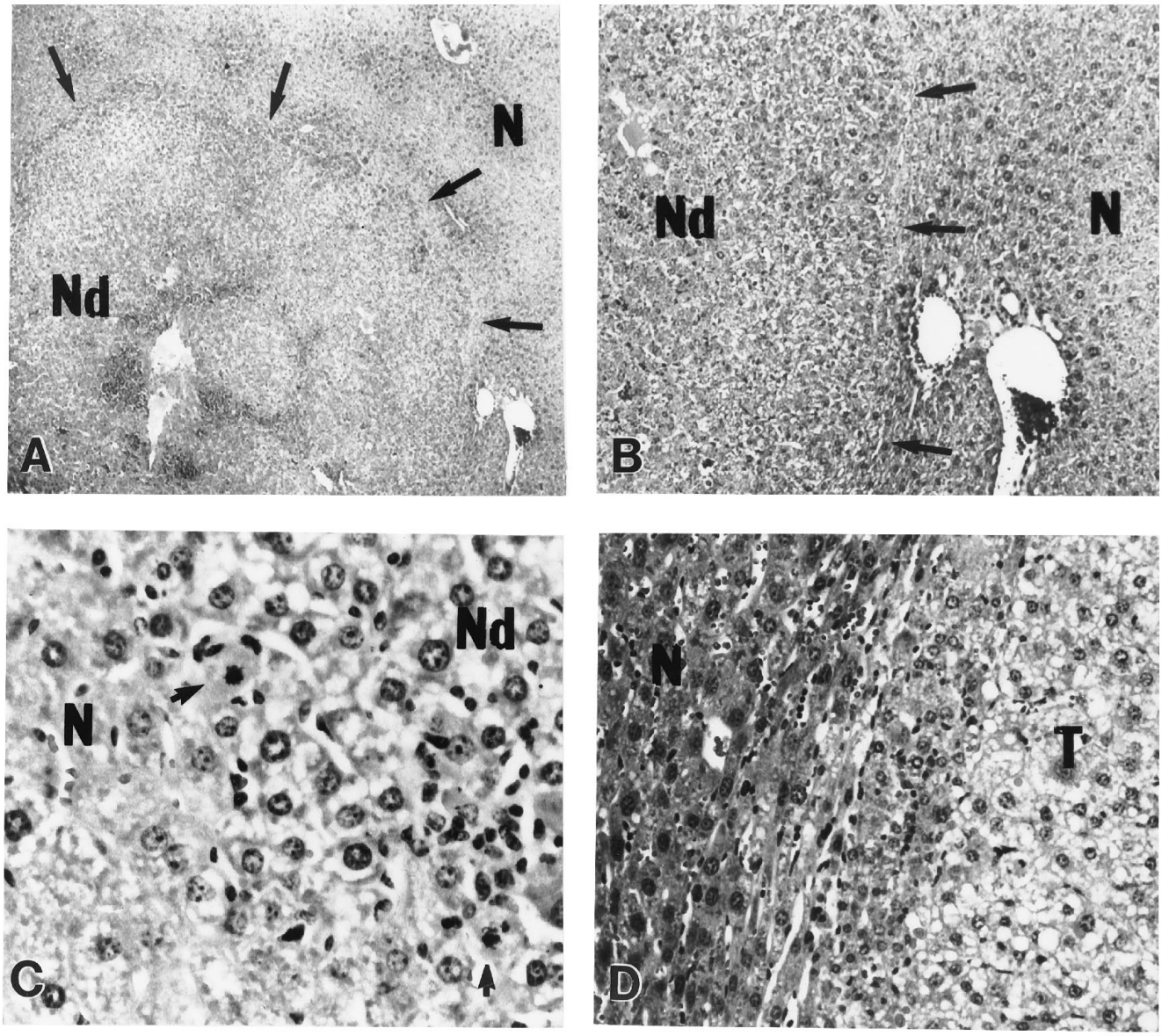


FIG. 5. Histologic analysis of liver tissues from DEN-treated Mup-WHx-4 transgenic mice sacrificed at the age of 6 months. Sections were cut from paraffin blocks and stained with hematoxylin and eosin. (A) Representative AHF observed in DEN-treated Mup-WHx-4 mice. The borders of the nodule are clearly defined (arrows), and hepatocytes inside the nodule typically show greater basophilic staining (magnification, ×15). (B) Edge of the same AHF shown in panel A (magnification, about ×38). Note that the hepatocytes in the nodule are smaller than the surrounding normal hepatocytes. (C) Hepatocytes in AHF hepatic foci (magnification, about ×150). Arrows denote mitotic figures. Nd, nodule of AHF; N, normal liver. (D) Growing edge of the single HCC observed in one 6-month-old Mup-WHx-4 transgenic DEN-treated mouse (magnification, about ×75). N, nontumorous liver; T, tumor. HCC appears to be well differentiated and trabecular with vacuolated hepatocytes.

taneous HCC or any other tumor was detected in any of the lines (data not shown). These results suggest that WHx, at the level at which it was expressed in our lines, was incapable of acting as a complete carcinogen.

To investigate whether WHx could function as a tumor promoter, we treated newborn mice of the Mup-WHx-4 line with DEN at a level (2 μg/g of mouse) which would cause only moderate formation of HCC by 10 months of age. This dose was identical to a dose used to treat HBx transgenic mice which also expressed X but did not develop HCC spontaneously (30, 55). After 6 months, we sacrificed groups of control ($n = 12$) and transgenic ($n = 8$) mice which had been treated with DEN as newborns. One of the 8 DEN-treated Mup-X-4 transgenic mice developed one small (0.6-cm) HCC at the age of 6 months (Fig. 5D), while none of the 12 control mice developed any detectable HCCs.

A blinded histologic inspection of liver sections from 6-month-old DEN-treated Mup-WHx-4 transgenic and nontransgenic control mice suggested that the extent of the precancerous lesions was greater in the Mup-WHx-4 transgenic mice than in the controls. We therefore carried out a detailed quantitation of the number and sizes of precancerous lesions, termed AHF, in the livers of transgenic and control mice. Typical AHF from Mup-WHx-4 mice are shown in Fig. 5A and B. These foci were generally similar in their phenotypes to those in control mice, except that they differed in size and number.

We calculated the total areas of sections and counted and measured all of the AHF in all of the sections (see Materials and Methods for details). There was a twofold increase in the

TABLE 2. Evaluation by quantitative stereology of the number of preneoplastic foci present in livers of 6-month-old transgenic Mup-WHx-4 and control mice, both treated with DEN[a]

Transgene status	No. of mice[b]	Total no. of focal intersections	Mean no. of focal intersections/mouse (SEM)	No. of focal intersections/cm^2	No. of foci/cm^3 of liver	Mean volume of foci (cm^3)	Volume % of foci in liver
Mup-WHx-4	8	46	5.75 (1.5)	5.07	48.84	2.547	12.44
None	12	29	2.41 (0.59)	2.63	23.46	0.862	1.97
Ratio			2.38	1.927	2.08	2.95	6.31

[a] All of the animals were treated with a single intraperitoneal injection of DEN (2 μg/g of body weight) at 13 days of age.
[b] Three random tissue sections were analyzed per animal.

number of AHF per square centimeter of liver in the Mup-WHx-4 transgenic mice compared with nontransgenic controls ($P = 0.025$; Table 2). Furthermore, these AHF were larger and occupied approximately six times more area in an average liver section.

A quantitative stereological method for the quantitation of elements in three-dimensional space from observations in two-dimensional planes (6, 44) was used to provide a more accurate estimation of the number of AHF per cubic centimeter of liver. In this analysis, the frequency of foci was also increased twofold (ratio, 2.08; Table 2) and the mean volume of foci was threefold greater in Mup-WHx-4 transgenic mice than in controls (ratio, 2.95; Table 2).

We continued the experiment to 10 months, at which time eight transgenic mice and eleven nontransgenic controls were sacrificed. The average tumor number per animal in both groups was approximately 8. However, in the Mup-WHx-4 transgenic mice it was more difficult to estimate the number of tumors accurately, since they were generally larger and grew together. Thus, we determined the tumor burden and related the total weight of the tumor tissue per animal to the weight of the whole liver. In the DEN-treated nontransgenic mice, the average tumor burden per liver was 1.57 g (40% of the whole liver mass), and in the DEN-treated Mup-WHx-4 transgenic mice, the average tumor burden per liver was 2.65 g (58.3% of the whole liver mass). Even though the average tumor burden was increased, the variability per mouse was high and the data were barely statistically significantly different ($0.1 > P > 0.5$).

Analysis of Mup-WHx-4 tumors for WHx protein did not reveal any WHx in the tumors. The reason for the absence of WHx in the HCCs is not clear. However, Northern blot analysis revealed a level of expression of the transgene in tumors slightly lower than the steady expression level observed in nonneoplastic livers from the same animals. A small reduction in the activity of the MUP promoter of the transgene could have reduced the level of WHx below the sensitivity of our assay.

In summary, our data suggest that the presence of WHx in transgenic mice promoted the development of precancerous lesions in mice at the age of 6 months. Overall, the levels of WHx present in the mice was much lower than that which normally occurs in woodchuck hepatocytes during persistent infection. Indeed, we estimated that the amount of WHx detected in our transgenic mouse livers was at least 50 times less than that present in naturally infected woodchucks. Therefore, it is necessary to generate additional WHx transgenic models to attain WHx expression equivalent to that which occurs in natural infection.

DISCUSSION

The discovery of the X gene ORFs in all of the mammalian hepadnavirus genomes and the absence of a corresponding X gene ORF in the avian hepadnaviruses have provided a continuous source of unanswered questions regarding the function of the X gene in viral replication and pathogenesis. The lack of an X gene ORF in the avian viruses, which are the least pathogenic hepadnaviruses, and the 3′ location of X genes in the mammalian hepadnavirus genomes have led to speculation that X proteins may play a role in the pathogenesis of mammalian hepadnaviruses (5, 36, 46).

Promoters and enhancers have been identified immediately upstream from the mammalian X gene ORFs (59, 62), yet specific X mRNAs are generally not detectable in RNA preparations from acutely and persistently infected livers (7). However, the presence of X antibodies in the serum of patients who recovered from acute infection or in the serum of virus carriers demonstrated that X was expressed at some point during infection (31, 40). Whether the X protein was continuously expressed in persistently infected liver was not determined in those studies.

Studies with mutant viral genomes expressed in cell culture demonstrated that X proteins were not necessary to maintain viral replication in cell culture (4). In contrast, genetic studies have established the need for a functional X ORF to establish WHV infection in woodchucks (8, 72). The first biological function of X to be described was its ability to serve as a transacting factor (63, 64). Further studies with cell cultures which overexpress X proteins have demonstrated that X proteins can affect both cytoplasmic signal transduction pathways (3, 10, 14, 15, 25, 38) and the activity of genes in the nucleus (9, 34, 35, 45, 51, 66).

There is a need for data on the presence of X proteins in infected liver and the level and subcellular distribution of X proteins in infected hepatocytes. We detected WHx proteins in 100% of the persistently infected livers which we analyzed, although a specific X mRNA was not identified in preparations of total cellular RNA (data not shown). It is not clear whether X proteins are translated from a low-abundance (perhaps short-lived) X mRNA or whether they are translated from the WHV envelope mRNA or pregenome RNAs as a result of polymerase frame shifting or internal ribosome entry. We did not detect any X proteins other than full-length WHx in our assays. Therefore, we believe that the internal translation start sites present in the WHV X gene ORF are not utilized during persistent infection.

We have begun to study the subcellular distribution of WHx to gain insights into its possible functions. Our initial studies have localized WHx to the cytoplasmic fraction of hepatocytes. Further studies are necessary to determine whether the cytoplasmic WHx proteins are associated with cell membranes or other cytoplasmic constituents.

Independent studies carried out by ourselves and others have failed to detect WHx proteins in viral particles with several very sensitive assays (50a). Therefore, if WHx is not associated with viral core particles in the cytoplasm, the number of WHx molecules we have observed in hepatocytes could significantly affect signal transduction pathways. In that case, we

would expect WHx polypeptides to be present in the cytoplasmic membrane or in association with proteins involved in cytoplasmic signal transduction mechanisms.

Several reports have demonstrated effects of HBx proteins on the Ras signal transduction pathway (3, 10, 15, 25, 38), and our data are consistent with the possibility of such a function for WHx proteins. The cytoplasmic localization of WHx is also consistent with the possibility that it can sequester nuclear proteins in the cytoplasm, as has also been suggested (65, 67). Specific association of WHx with any cellular protein has not been demonstrated. As stated earlier, the presence of fewer than 800 molecules of WHx in the nucleus is undetectable by our assays and therefore we cannot rule out the possibility that some WHx protein does exist in the nucleus.

To investigate the role of WHx in pathogenesis and hepatocarcinogenesis, we have studied its presence in woodchuck HCCs and its effect on liver pathology in transgenic mice. The failure to detect WHx in some woodchuck HCCs indicates that high-level expression of WHx is not required to maintain the tumor phenotype. However, we cannot rule out the possible presence of lower levels of WHx (fewer than 800 molecules) in each hepatoma cell and the possibility that this amount is significant for maintenance of the tumor phenotype. This possibility is strengthened by our transgenic mouse data for WHx and those of others for HBx, which have demonstrated partial oncogenic function for X proteins (26, 28, 55).

The continued presence of WHx polypeptides in woodchuck liver tissue at late stages of persistent infection after HCC has developed, and also in HCCs which continue to replicate WHV, provides the opportunity for X to affect cellular mechanisms during the precancerous stages of persistent infection, as well as after HCC develops. We have characterized HCCs in woodchucks which are oligoclonal, as judged by their WHV integration patterns. In these tumors, one portion of the tumor contains WHV replicative intermediates and another portion contains no replicative intermediates (data not shown). In the context of those related tumors, the loss of WHx protein expression could have been a late event. Alterations of signal transduction pathways which affect cell survival versus apoptosis, or regulate the progression of hepatocytes through the cell cycle, may set the stage for further genetic changes which render the cell independent of WHx for proliferation.

Our transgenic mouse data support the growing consensus that the level of X protein expression and the genetic background of the hepatocyte are important variables which affect the biological function of X polypeptides. The level of WHx in our transgenic mouse lines was much lower than that which we have observed in WHV-infected liver, so our estimates of oncogenic functions of WHx may be minimum estimates. At the level measured in our transgenic WHx lines, transgene expression did not cause spontaneous hepatocarcinogenesis. However, when we provided a tumor initiator, in the form of a one-time dose of DEN, to the mice, there was a significant increase in the number and size of precancerous lesions in the Mup-WHx-4 transgenic line compared with those in control nontransgenic mice similarly injected with DEN. In this experimental system, DEN is a complete carcinogen; however, tumor promoters are known to increase the occurrence of precancerous changes and HCC (61). Our data are consistent with a possible tumor promoter function for WHx in Mup-WHx-4 transgenic mice, even when WHx proteins are present at very low levels.

In persistently infected woodchuck liver, tumor initiator functions may be carried out by WHV DNA integrations into *myc* protooncogenes and by point mutations induced by toxic oxygen radical release. In this context, a tumor promoter function of WHx would be expected to increase the rate of tumorigenic progression. Our approach to the detection and quantitation of WHx polypeptides in tissues with various pathophysiological conditions will allow us to further explore the above possibilities.

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