JOURNAL OF VIROLOGY, Dec. 1990, p. 5939–5947
0022-538X/90/125939-09$02.00/0
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Vol. 64, No. 12

Hepatitis B Virus Transactivator X Protein Is Not Tumorigenic in Transgenic Mice

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Received 30 July 1990/Accepted 4 September 1990

The hepatitis B virus X protein acts as a transcriptional transactivator in vitro. To elucidate possible biological effects of X protein on liver cells in vivo, we generated four lines of transgenic mice carrying the X gene open reading frame under the control of the human α-1-antitrypsin regulatory region. The plasmid construct used to introduce the transgene was shown to encode a 16-kDa X protein with transactivating capability. The expression of X protein was detectable in liver tissue of transgenic animals of three of the lines by immunoblot analysis; levels of expression were highest in the first month after birth of the animals. Over 80 animals from the expressing lines were examined histologically. Most transgenic mice, some of which were observed for up to 2 years, remained normal. However, a few transgenic animals developed mild focal hepatitis, nuclear pleomorphism, focal necrosis, and/or nodular hyperplasia in the liver. Increased mitotic activity of hepatocytes also was observed. We conclude that, at the level of expression achieved in these transgenic mice, the hepatitis B virus transcriptional transactivator X protein alone does not appear to mediate the development of serious liver damage or hepatocellular carcinomas.

Hepatitis B virus (HBV) is a small, enveloped virus with a partially double-stranded DNA genome. It can cause acute and chronic hepatitis and is a factor in the genesis of hepatocellular carcinoma. Analyses of cloned HBV genomes have revealed at least four open reading frames (ORFs), designated S, C, P, and X (20, 47). The X ORF lies between nucleotides 1376 and 1840 (subtype adw_2) and can encode a 16-kDa protein (30, 41). Although the X ORF is conserved among different HBV subtypes, its role in viral replication is not known.

Twu and Schloemer (49) showed that the chloramphenicol acetyltransferase (CAT) gene, under the control of the regulatory element of the human β-interferon gene, was transactivated in the presence of a plasmid containing the HBV X gene. The putative X protein was then demonstrated by Spandau and Lee (46) to transactivate the HBV enhancer as well as heterologous viral regulatory elements. Subsequently, several studies (3, 15, 37, 46, 48, 54) confirmed a transactivational function by the X protein on a variety of viral and cellular genes in vitro. The transcriptional regulatory activity of the X protein has been extended to include class III genes (3) and is expressed efficiently whether from an integrated HBV genome or in productive infection of permissive cells (3, 15). The pattern of transactivation is cell type specific but not species specific (38). The underlying mechanism of the observed X-gene-mediated transactivation remains obscure, although interactions of X protein with cellular transcriptional factors, probably including AP-2 and TFIIIC, have been suggested (3, 15, 36, 50).

Seroepidemiological studies have shown that anti-X antibody is detectable in a small percentage of infected individuals, most commonly those suffering from chronic active hepatitis or primary hepatocellular carcinoma (25, 26, 28–30, 32, 34). Recently, the detection of X protein by immunohistochemical means in tissues of patients with chronic hepatitis and hepatocellular carcinoma (10, 24, 55) and in sera of HBV-infected patients (19) was reported, although the identity of the detected reactivity was not established in some cases (24, 55). It remains unclear whether the expression of X protein plays any functional role in the pathogenesis of hepatitis, hepatocarcinogenesis, or other liver diseases. It is of interest that the X protein reportedly induces growth changes and tumorigenic transformation of mouse NIH 3T3 cells (40).

Transgenic mouse systems have been widely employed as tools to analyze the biological effects of gene expression under physiological conditions that cannot be reproduced in cell cultures (16, 22). The lack of susceptible tissue cultures for HBV propagation, as well as the narrow host range of the virus, emphasize the value of the transgenic animals for elucidating HBV molecular biology. Previous studies have addressed the developmental regulation (4, 7, 17, 18, 33), structural effects (11), and molecular pathogenesis of HBV S (12, 13) and C (2) genes in transgenic mice. In this study, we generated several lines of transgenic mice carrying the HBV X ORF under the control of the regulatory region of the human α-1-antitrypsin (AAT) gene, and the synthesis of X protein in the livers of transgenic animals was demonstrated. Potential pathological effects of X protein in vivo were then analyzed. The results indicate that X protein alone promotes very limited pathology in the livers of the transgenic mice.

MATERIALS AND METHODS

Plasmid constructions. The construction of the X-gene-expressing plasmid pGem-1-ATX is depicted in Fig. 1A. The 1.2-kb 5′-flanking sequence (nucleotides [nt] −1200 to +46) of the AAT gene cloned in pUC18 (39) was digested with *Bam*HI, trimmed with mung bean nuclease, and subcloned into the *Sma*I site of the pGem-1 vector. The X-gene-containing *Sph*I-*Bgl*II fragment (nt 1234 to 1986) of HBV (subtype adw_2) was subcloned into the *Hin*cII site of the

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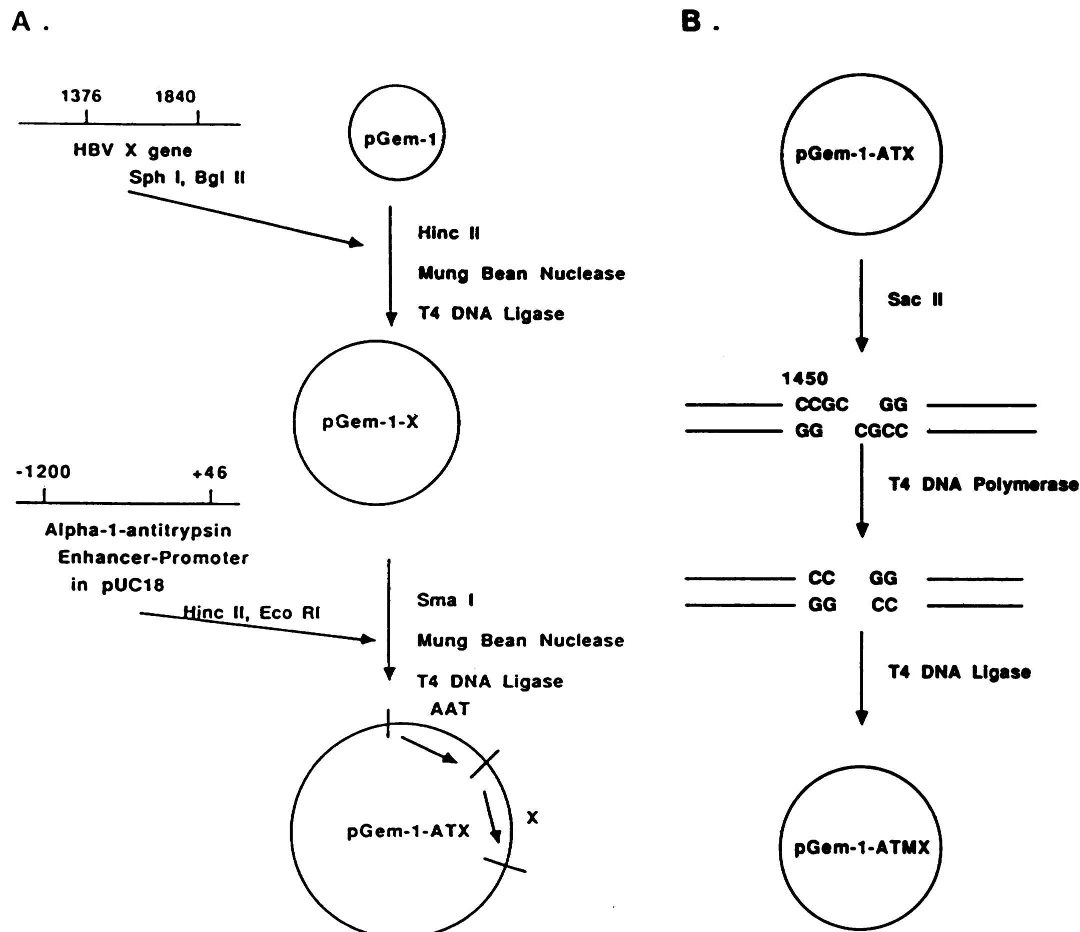


FIG. 1. (A) Construction of the HBV X-gene-expressing plasmid pGem-1-ATX. This plasmid was cleaved with *Eco*RI and *Hin*dIII to generate a 2.0-kb ATX gene fragment for microinjection. (B) Construction of a frameshift mutation (pGem-1-ATMX) in the X gene in pGem-1-ATX.

same pGem-1 vector from the plasmid pHBV-adw2-T (42). The mutated X-gene-expressing plasmid pGem-1-ATMX was constructed by digesting pGem-1-ATX with *Sac*II, which cuts the plasmid once in the X gene (nt 1452). The digested DNA was treated with T4 DNA polymerase to remove the 5′ overhangs and was religated with T4 DNA ligase (Fig. 1B). The indicator plasmid pA10HBeCAT was constructed by inserting the HBV *Spe*I-*Sph*I fragment (nt 681 to 1234) that contained the enhancer sequence into the *Bgl*II site of the enhancerless pA10CAT vector (39). The orientation of each subcloned insert was determined by restriction endonuclease mapping. The enzymes used above were purchased from New England BioLabs, Inc., Beverly, Mass.

In vitro transcription and translation. For in vitro transcription, 2 μg of plasmid DNA was incubated in a 100-μl reaction mixture containing transcription buffer (40 mM Tris hydrochloride [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol, 100 U of RNasin, 0.5 mM of nucleotide triphosphates, and 40 U of T7 RNA polymerase for 1 h at 37°C. The synthesized mRNA was used for in vitro translation. One microliter of RNA solution was incubated with 20 μl of nuclease-treated rabbit reticulocyte lysate, 20 U of RNasin, and 10 μCi of $Tran^{35}S$ label (ICN Biochemical Inc., Irvine, Calif.) at 30°C for 1 h. The synthesized protein product was detected by immunoprecipitation and gel electrophoresis. Rabbit reticulocyte lysate was prepared by the method of Clemens (14).

Transfection and CAT assay. HepG2 human hepatoma cells (1) were seeded at a density of 2×10^5 cells per 60-mm-diameter dish 24 h before transfection. Ten micrograms of pGem-1-ATX or pGem-1 plasmid DNA was mixed with 2 μg of pSV_2CAT (21) for transfection by using the calcium phosphate coprecipitation method. At 4 h posttransfection, cells were shocked with 15% glycerol for 3 min. Cells were harvested at 48 h posttransfection and sonicated in 0.25 M Tris hydrochloride, pH 7.5, and the clarified cell lysate was assayed for CAT activity as described by Gorman et al. (21). Briefly, 1/10 of the cell lysate from each dish was incubated with 20 μl of 8 mM acetyl coenzyme A (Sigma Chemical Co., St. Louis, Mo.) and 0.4 μCi of $[^{14}C]$chloramphenicol (Dupont, NEN Research Products, Boston, Mass.) at 37° for 1 h. $[^{14}C]$chloramphenicol and its acetylated products were resolved by thin-layer chromatography. The product spots were scraped and counted.

Generation of transgenic mice. The ATX fused gene was isolated from pGem-1-ATX by digestion with *Eco*RI and *Hin*dIII and was used for microinjection as described by Brinster et al. (5). Fertilized eggs for microinjection were obtained from ICR females mated with $B6C3F_1$ males.

Screening of transgenic mice. The transgene from founder animals and progeny was analyzed by Southern blot analysis

of tail DNA (35, 45). DNA was digested with *Pst*I, electrophoresed through a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled ATX DNA probe. After being washed under stringent conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], 68°C), the filters were exposed to XAR-5 film for autoradiography.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE). Mouse liver tissue was homogenized in 10 volumes of RIPA buffer (50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 1% Trasylol) by using a Polytron homogenizer. Cell extracts were clarified by centrifugation prior to immunoprecipitation. Mixtures containing in vitro-translated products were diluted 20-fold with RIPA buffer before analysis. Lysates were incubated with polyclonal anti-X antiserum (Lee and Butel, unpublished data) overnight at 4°C, and then immune complexes were adsorbed by using a 10% Formalin-fixed *Staphylococcus aureus* (strain Cowan I) preparation. The precipitate was washed three times with RIPA buffer and suspended in disruption buffer (50 mM Tris hydrochloride [pH 6.8], 4% SDS, 4% β-mercaptoethanol, 1% glycerol, 1 mg of bromophenol blue per ml). After heating at 100°C for 3 min, the supernatant was applied to a 14% SDS-polyacrylamide gel and electrophoresed (23, 27, 35). The gel either was fixed, dried, and autoradiographed or was processed for immunoblot analysis.

Immunoblot analysis. After SDS-PAGE, proteins were transferred to nitrocellulose filters. The filters were blocked with 0.1% Nonidet P-40 and 0.25% gelatin and reacted with anti-X antiserum, and bound immunoglobulins were detected with ^{125}I-labeled protein A (0.5 μCi per lane) as described previously (44). The filters were washed and autoradiographed.

Histological procedures and cytohistochemical staining. Tissues were fixed in 10% buffered Formalin and processed on a Miles VIP automatic tissue processor on a 4-h cycle at 13°C. The tissue was then embedded in Tissue Prep. Sections (4 μm thick) were stained with hematoxylin and eosin (35).

RNA preparation and dot blot analysis. The procedure for RNA preparation from mouse liver was adopted from Chirgwin et al. (9). Briefly, liver tissue was disrupted in homogenization buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosine, and 0.1 M β-mercaptoethanol). The homogenate was centrifuged at 5,000 × *g* for 10 min. The supernatant was layered on a cushion of 6.2 M CsCl (in 0.1 M EDTA) solution for ultracentrifugation at 30,000 rpm for 16 h by using an SW41 rotor. The RNA pellet was suspended in buffer (10 mM Tris hydrochloride [pH 7.6], 2 mM EDTA, 0.2 M NaCl, and 0.5% SDS), extracted with chloroform-butanol (4:1), and precipitated twice with ethanol. The RNA pellet was dissolved in water and used for dot blot analysis.

Suspended RNA samples (20 μg) were denatured by heating in buffer [50% formamide, 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0)] at 60°C for 10 min, quick chilled, and then applied to 10× SSC-pretreated nitrocellulose filters under vacuum. The filters were dried, prehybridized, and then hybridized with ^{32}P-labeled DNA probes. Hybridized filters were washed under conditions of moderate stringency (2× SSC and 0.1% SDS at 25°C followed by 0.2× SSC and 0.1% SDS at 55°C) before autoradiography.

RESULTS

Construction of an expression plasmid encoding a functional HBV X gene. AAT is the major protease inhibitor in plasma. It is synthesized primarily in hepatocytes but also in specific cell types of several nonhepatic tissues (8). Previous studies have shown that the human AAT gene controlled by its own enhancer-promoter is expressed at high levels in the livers of transgenic mice (43). In an effort to maximize the expression of the HBV X gene in the livers of transgenic animals, we subcloned the human AAT gene regulatory region (nt −1200 to +46) in front of the X ORF, as described in Materials and Methods (Fig. 1A). This plasmid construct, pGem-1-ATX, contained a T7 promoter in front of the hybrid ATX gene. To confirm that the X gene reading frame remained intact, we examined this construct by in vitro transcription and translation. The reaction mixture was immunoprecipitated with anti-X antiserum, and the precipitated proteins were resolved by SDS-PAGE. A 16-kDa protein was identified (Fig. 2A, lane 2). No comparable protein was detected in the reaction mixture when the control pGem-1 plasmid was added as the template (Fig. 2A, lane 1). This result established that the X ORF was intact after subcloning.

To determine if pGem-1-ATX encoded a functional product, we analyzed its transacting ability on the basis of CAT assays. HepG2 cells were cotransfected with the pGem-1-ATX and the indicator pSV_2CAT plasmids, and the CAT activity in the cell lysate was analyzed 48 h after transfection. CAT activity was elevated 10- to 15-fold in lysates from cells cotransfected with pGem-1-ATX (Fig. 2B). Similar results were obtained when pSV_2CAT was replaced with indicator plasmids containing either the CAT gene or the β-galactosidase gene under the control of the HBV enhancer or the Rous sarcoma virus long terminal repeat region (data not shown). These experiments suggested that the pGem-1-ATX construct could direct the synthesis of an X protein capable of functioning as a transactivator when the plasmid was transfected into HepG2 cells.

Two types of control experiments established that the observed transactivation was mediated by X protein per se. First, the possibility that transactivation was due to the nonspecific binding of putative transcriptional repressors by the plasmid sequences, thereby indirectly increasing the expression of the indicator CAT plasmid, was ruled out. A related plasmid, pGem-1-ATIX, in which the regulatory region of the AAT gene was in reverse orientation, was constructed. The AAT gene regulatory region contains *cis*-acting sequences that function in an orientation-dependent manner (39). In cells cotransfected with a plasmid containing an inverted AAT sequence (pGem-1-ATIX) and an indicator plasmid containing the HBV enhancer (pA10HBeCAT), CAT activity increased only slightly (1.5-fold) compared with that in the control cells (Table 1, experiment 1). In contrast, CAT activity was about 11-fold more than the control in the presence of pGem-1-ATX carrying the correct orientation of AAT sequences. This result showed that inversion of the regulatory region directing the expression of the X gene markedly reduced the transactivation displayed by the X gene, indicating that expression of the X gene was essential for transactivation of the HBV enhancer.

Finally, a mutagenesis approach was taken to prove that the protein product of the X gene was the authentic transactivator. A frameshift mutation was introduced into the X gene in pGem-1-ATX, as described in Materials and Methods. The mutated plasmid pGem-1-ATMX has a 2-bp deletion at nt 1452 compared with the original pGem-1-ATX

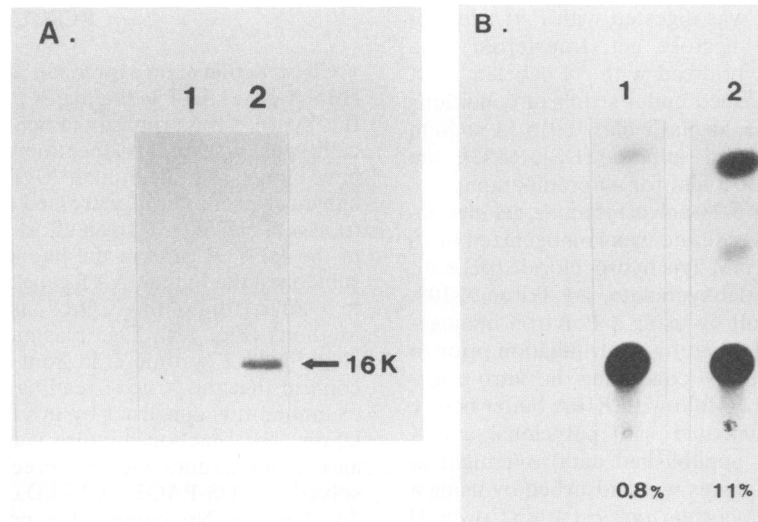


FIG. 2. (A) Expression of HBV X protein in vitro. Control pGem-1 (lane 1) or pGem-1-ATX plasmid (lane 2) DNA was transcribed and translated in vitro as described in Materials and Methods, and reaction mixtures were immunoprecipitated with anti-X antiserum. The arrow marks X protein (16 kDa) synthesized in lysates primed with the pGem-1-ATX plasmid. (B) Transactivation by the X protein encoded by pGem-1-ATX. HepG2 cells were transfected with indicator pSV_2CAT and pGem-1 plasmid DNAs (lane 1) or with pSV_2CAT and pGem-1-ATX DNAs (lane 2). CAT activity was determined in cell lysates 48 h after transfection. The percentage of acetylation is given at the bottom of each lane.

plasmid (Fig. 1B), resulting in an altered reading frame. This presumed frameshift mutation in pGem-1-ATMX was confirmed by in vitro translation. Only the parental pGem-1-ATX encoded a 16-kDa protein that could be immunoprecipitated by anti-X antiserum (data not shown). The mutated pGem-1-ATMX plasmid was then tested in cotransfection assays. CAT activity increased slightly in its presence (2.6-fold) compared with the negative control plasmid pGem-1. The positive control plasmid pGem-1-ATX mediated a 13.6-fold enhancement of CAT activity (Table 1, experiment 2). These results indicated that a frameshift mutation in the X gene markedly diminished its transactivating activity, supporting previous reports that the X-gene product is a trans-acting protein.

Generation and screening of transgenic mice. Having validated that the pGem-1-ATX construct encoded an authentic X protein that possessed transactivating capability, we proceeded to generate transgenic mice. Linear ATX DNA cleaved from the pGem-1-ATX plasmid was microinjected into one-cell mouse embryos that were transferred to pseudopregnant foster mice. Progeny that were born were screened by Southern blot hybridization for integration of the ATX gene. Two DNA fragments 0.9 and 1.1 kb in size were expected in transgenic animals when mouse tail DNA was digested with *Pst*I and probed with ATX DNA (Fig. 3A). Of 25 founder mice, 4 (16%) mice were found to bear the ATX gene (animal numbers 1648, 1655, 1714, and 1715). The transgene copy numbers ranged from 5 to 15 in these

TABLE 1. Transactivation function of the X protein encoded by an HBV X-gene-expressing plasmid[a]

Expt	Plasmid: Expression	Plasmid: Indicator	CAT activity (% acetylation)	Fold enhancement
1	pGem-1	pA10HBeCAT	0.9	
	pGem-1-ATX	pA10HBeCAT	10.0	11.1
	pGem-1-ATIX[b]	pA10HBeCAT	1.4	1.5
2	pGem-1	pA10HBeCAT	0.9	
	pGem-1-ATX	pA10HBeCAT	12.2	13.6
	pGem-1-ATMX[c]	pA10HBeCAT	2.4	2.6

[a] HepG2 cells were cotransfected with 2.5 μg of indicator plasmid and 10 μg of expression plasmid. CAT activity was determined as described in Materials and Methods.

[b] pGem-1-ATIX contains the AAT *cis*-acting sequences in inverted orientation.

[c] pGem-1-ATMX contains a frameshift mutation in the X gene coding region at nt 1452.

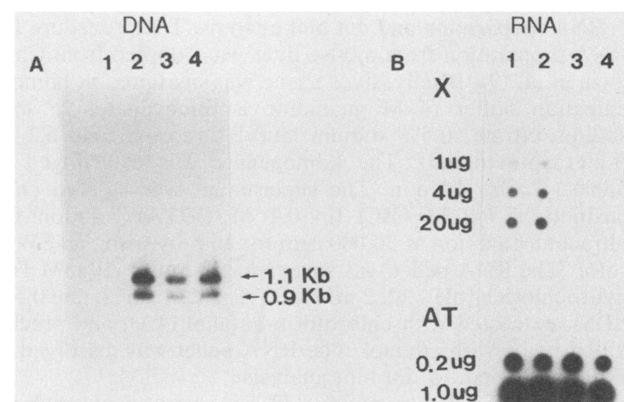


FIG. 3. (A) Southern analysis of ATX transgenic mice. Mouse tail DNA samples were digested with *Pst*I, resolved by gel electrophoresis, transferred to nitrocellulose, and probed with ^{32}P-labeled ATX DNA. Lane 1, Nontransgenic littermate; lanes 2 to 4, transgenic mice. The positions of the expected 1.1- and 0.9-kb DNA fragments in transgenic mice are marked. (B) RNA dot blot analysis of ATX transgenic mice. Mouse liver RNA samples from F_1 generation transgenic mice of line 1655 (lanes 1 and 2) or from nontransgenic littermates (lanes 3 and 4) were applied to a nitrocellulose filter and probed with ^{32}P-labeled X or AAT gene DNA.

TABLE 2. Features of ATX transgenic mice

Founder animal	Features			
	Sex	No. of ATX gene copies[a]	X mRNA expression[b]	X protein expression[b]
1648	Male	5–10	–	ND
1655	Female	10–15	+	++
1714	Male	5–10	+	+
1715	Male	5–10	+	+

[a] ATX gene copy numbers were estimated by Southern blot analysis.
[b] X mRNA expression was determined by an RNA dot blot assay, and X protein synthesis was measured by an immunoblot method. ND, Not done.

founder animals (Table 2). Lines were successfully established from all four founder mice.

The transcription of the transgene in members of the four transgenic lines was determined by RNA dot blot analysis. Mouse liver samples from the F_1 generations were extracted with guanidine isothiocyanate, and the RNA was isolated by CsCl gradient centrifugation. Equivalent amounts of RNA were applied to filters and were tested with X gene or AAT gene probes. HBV X gene mRNA transcripts were detectable in the extracts of livers from three of the four lines (lines 1655, 1714, and 1715), albeit at low levels (about 1%) relative to the amount of mouse endogenous AAT mRNA (Fig. 3B). The level of expression of X gene mRNA was similar to that observed for other foreign genes controlled by the human AAT enhancer-promoter in transgenic mice (35).

Synthesis of X protein in livers of transgenic mice. The expression of X protein in liver tissues of the transgenic mice was determined by immunoblot analysis. The amount of X protein expressed varied among different lines of transgenic mice, but the pattern of expression was similar (Table 2). Animals of line 1655 consistently contained higher levels of X protein than did those from the other two lines positive for X gene mRNA. Maximal X protein accumulation occurred in the first month after birth and then dropped, with the protein becoming undetectable by the immunoblot method by 2 months of age (Fig. 4). Immunohistochemical staining reactions of sections of liver tissue from transgenic animals were weak but revealed that the X protein was localized predominantly in the cytoplasm of hepatocytes, especially in cells surrounding the portal vein (data not shown). Livers from animals of line 1655 were perfused with collagenase to collect hepatocytes, which were plated in culture and labeled with [^{35}S]methionine. Immunoprecipitation of extracts of the labeled liver cells with anti-X antiserum confirmed the production of the 16-kDa X protein in the transgenic mice.

Histological changes in the livers of ATX transgenic mice. The livers of transgenic mice were monitored to determine if any histopathological changes could be attributed to the expression of the HBV X gene (Table 3). F_1 progeny were sacrificed periodically between the ages of 2 and 24 months; as matched controls, nontransgenic littermates were sacrificed at the same time points. All tissue specimens were processed and interpreted under code. Over 80 animals from the three X-expressing lines were analyzed.

Most of the mice appeared normal at the time of sacrifice. A few animals displayed histological abnormalities in their livers, which included mild hepatitis, nuclear pleomorphism, focal necrosis, and/or nodular hyperplasia. However, the histological alterations observed in the three lines of transgenic mice were random and did not follow a consistent pattern. As line 1655 expressed the highest level of X protein in the liver, progeny of that line (F_1 and F_2 generations) were

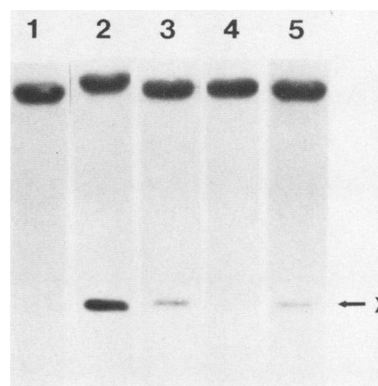


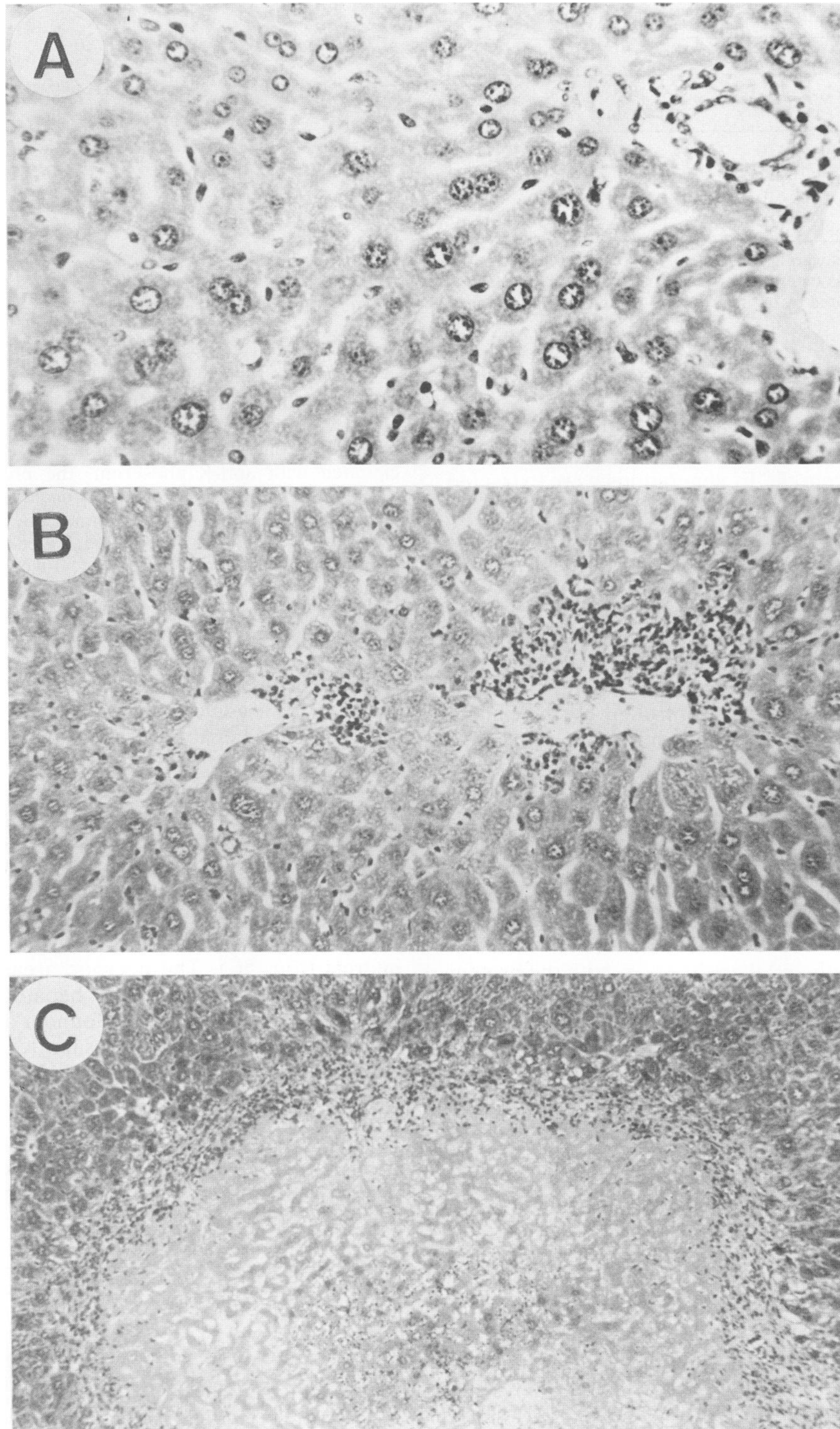
FIG. 4. Immunoblot analysis demonstrating the expression of HBV X protein in ATX transgenic mice. Liver extracts prepared from mice from various lines and at different ages were immunoprecipitated with anti-X antiserum, the proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters, and the filters were probed with anti-X antiserum. Lane 1, Nontransgenic mouse, 2 weeks old; lanes 2 to 4, line 1655, 2 weeks, 1 month, and 2 months old, respectively; lane 5, line 1714, 2 weeks old. The position of X protein is indicated on the right. The nonspecific upper band represents the heavy chain of immunoglobulin.

studied most extensively. Mild focal lobular hepatitis was found in transgenic mice of all ages (Fig. 5B). The same patchy type of hepatitis also was observed in some nontransgenic littermates. However, weak immunohistochemical staining was noted with anti-X antiserum in the inflammatory areas of some transgenic animals. Most animals older than 4 months had mild nuclear pleomorphism of hepatocytes that was not progressive. Increased mitotic activity of hepatocytes without significant hepatitis or necrosis was noted in two transgenic animals (ages 1.5 and 14 months). The liver of one 15-month-old mouse (F_1 of line 1655) had several old infarcts (Fig. 5C). The necrotic areas were surrounded by inflammatory cells, but the hepatocytes located outside these foci were normal. The founder animal of line 1715 had a grossly nodular liver when sacrificed at 19 months (Table 3). The nodules contained hyperplastic hepatocytes focally

TABLE 3. Histopathological observations of ATX transgenic mice

Line	Generation	No. examined	Age(s) examined (mo)[a]	Observations
1655	F_0	1	Not done	Not done
	F_1	16	2–22	Mostly normal, mild hepatitis
	F_2	37	0.5–14	Some nuclear pleomorphism, increased mitoses, rare focal necrosis, infarcts
1714	F_0	1	19	Normal
	F_1	15	0.5–16	Mostly normal, mild hepatitis
1715	F_0	1	19	Hyperplastic nodule
	F_1	12	2–24	Mostly normal, mild hepatitis, fatty changes, rare focal necrosis

[a] F_1 progeny from each line were sacrificed at 2- to 3-month intervals. Nontransgenic littermates were sacrificed in parallel.



filled with fat (Fig. 5D), sometimes undergoing acidophilic necrosis. Some cells had nuclear pleomorphism (Fig. 5E), but no definitely dysplastic cells were seen. The nodule resembled an adenoma. However, progeny of the 1715 line did not develop similar hyperplastic nodules. Members of the transgenic lines did not appear to have shortened life spans relative to their nontransgenic littermates.

DISCUSSION

The aim of this study was to determine the histopathological effects of expression of the HBV transactivator X protein in mouse liver. A plasmid was constructed in which the HBV X gene was under the control of the human AAT gene regulatory region. The construct was shown to encode

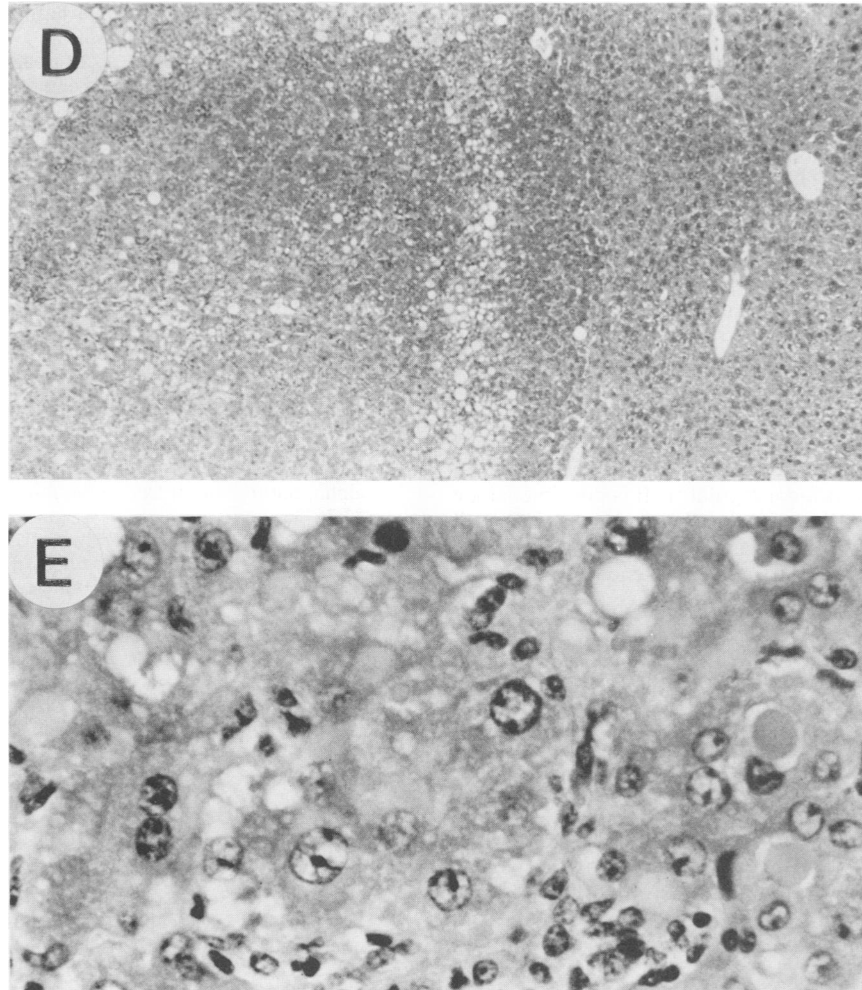


FIG. 5. Histological abnormalities in liver tissues of ATX transgenic mice. (A) Nontransgenic control mouse, 15 months old (magnification, ×400). (B) Mild portal inflammation from an F_2 mouse of line 1655, 13 months old (×200). (C) Liver infarcts from an F_1 mouse of line 1655, 15 months old (×100). (D) and (E) Hyperplastic nodule from an F_0 mouse of line 1715, 19 months old (×40 and ×640, respectively).

an authentic X protein that possessed transactivation capability in in vitro cotransfection assays. We then generated four lines of transgenic mice carrying integrated ATX genes. Members of the independent transgenic lines were sacrificed periodically to monitor the expression of X protein and the presence of any liver abnormalities.

Of the four transgenic lines, three expressed detectable levels of X protein in the livers as determined by immunoblot analysis. Variable degrees of X protein expression occurred at different ages and in individual lines of transgenic animals. The general pattern was that the highest level of expression of X was observed during the first month after birth, after which the level of X protein dropped and became undetectable in the immunoblot assay by 2 months of age. Although the expression of mouse AAT protein in the transgenic mice remained high throughout the life span of the animals (43), the pattern of transgene expression in the ATX transgenic mice was similar to that observed in transgenic mice carrying the simian virus 40 T-antigen gene controlled by the same AAT gene regulatory region (35). In that system, liver tumors developed reproducibly in the AAT-T antigen transgenic mice after the expression of T antigen decreased markedly. It should be noted that immunoblot assays, based on extracts of tissue samples, are not highly sensitive detection methods. Further, transactivation by X protein was demonstrated to occur in transfected hepatoma cells, although the level of X protein was too low to be detected (3). These observations indicate that the level of expression of X protein achieved in these transgenic mice, although low, is probably sufficient to be functionally significant.

Several viral proteins that act as transactivators in vitro have been found to induce physiological disorders when expressed in transgenic animals. For example, transgenic mice carrying the *tat* gene of the human immunodeficiency virus or the tax_1 gene of the human T-lymphotropic virus type I develop Kaposi's sarcoma-like lesions or mesenchymal tumors (31, 51). However, none of the ATX transgenic mice had any apparent life-threatening liver changes when sacrificed over a 24-month period, although some histological abnormalities were observed. Mild focal lobular hepatitis was the most common disorder found, but similar hepatitis also was detectable in a few nontransgenic littermates. It is possible that this pathology was due to unrelated factors, such as sporadic infection by mouse hepatitis virus. Although the expression of X protein was detectable at the sites of lesions in some transgenic mice by immunohistochemical staining, it is unclear at this time whether the focal hepatitis represents an effect of X protein in liver tissue.

Other histological disorders, including liver infarcts and hyperplastic nodules, were found in very few transgenic animals, and their significance is unknown. Finally, some transgenic animals showed proliferation of liver cells without significant hepatitis, and we are currently analyzing those abnormalities for their relationship to X protein function.

HBV X protein encoded by the pGem-1-ATX plasmid was demonstrated to be a transactivator protein by cotransfection studies in cell culture. A similar function in vivo might explain the development of the histological changes observed in the transgenic mice. However, the expression levels of several cellular genes, known to be transactivated by X protein in vitro, were not up regulated at the transcriptional level in the presence of X, as compared with nontransgenic littermates (Lee and Butel, unpublished data). This apparent discrepancy between cell culture and whole animal systems suggests that the more normal physiological state in the transgenic mice may have prevented the X protein from functioning as a transcriptional regulator. It is possible, since transactivation by the X protein has been shown to be cell type specific (38), that cells growing in vitro and hepatocytes in ATX transgenic mice differ in the presence of necessary accessory regulatory factors.

The role of HBV in the development of liver cancer, one of the most common human cancers in Africa and Southeast Asia, remains an important question (20, 47). Integration into a growth-regulatory gene might be functionally important in the genesis of tumors (53). Evidence is mounting that the inactivation of one or more tumor-suppressor genes may be involved in liver carcinogenesis (6, 52; B. L. Slagle, Y.-Z. Zhou, and J. S. Butel, Cancer Res., in press). In combination with those types of events, the transactivating potential of X protein provides a theoretical mechanism that might be contributory in a subset of tumors. The retention of X gene sequences in a number of HBV integrants cloned from hepatocellular carcinomas supports that possibility (20, 47, 56). It is evident from the transgenic mouse data presented here that X protein by itself is not sufficient to function as an oncogene and to mediate liver tumorigenesis. Chisari et al. (12) have shown that overproduction of HBV large envelope polypeptide in the hepatocytes of transgenic mice causes liver cell injury and induces severe chronic hepatitis and hepatocellular carcinomas. Liver cell injury-induced regeneration over a prolonged period of time seems to generate an environment favoring the occurrence of transforming events in hepatocytes. We have experiments in progress to determine whether X gene expression predisposes mice to the development of chemical carcinogen-induced liver tumors.

ACKNOWLEDGMENTS

We thank W. S. Robinson for supplying the pHBVadw2-T plasmid.

This work was supported in part by Public Health Service research grant CA37257 from the National Cancer Institute. S.L.C.W. is an Investigator of the Howard Hughes Medical Institute.

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