Hepatitis B Virus X Protein Acts as a Tumor Promoter in Development of Diethylnitrosamine-Induced Preneoplastic Lesions

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Chronic infection with hepatitis B virus (HBV) is one of the major etiological factors in the development of human hepatocellular carcinoma. Transgenic mice that express the HBV X protein (HBx) have previously been shown to be more sensitive to the effects of hepatocarcinogens. Although the mechanism for this cofactor role remains unknown, the ability of HBx to inhibit DNA repair and to influence cell cycle progression suggests two possible pathways. To investigate these possibilities in vivo, we treated double-transgenic mice that both express HBx (ATX mice) and possess a bacteriophage lambda transgene with the hepatocarcinogen diethylnitrosamine (DEN). Histological examination of liver tissue confirmed that DEN-treated ATX mice developed approximately twice as many focal lesions of basophilic hepatocytes as treated wild-type littermates. Treatment of mice with DEN resulted in a six- to eightfold increase in the mutation frequency (MF), as measured by a functional analysis of the lambda transgene. HBx expression was confirmed by immunoprecipitation and Western blotting and was associated with a modest 23% increase in the MF. Importantly, the extent of hepatocellular proliferation in 14-day-old mice, as measured by the detection of proliferating cell nuclear antigen and by the incorporation of 5-bromo-2′-deoxyuridine, was determined to be approximately twofold higher in ATX livers than in wild-type livers. These results are consistent with a model in which HBx expression contributes to the development of DEN-mediated carcinogenesis by promoting the proliferation of altered hepatocytes rather than by directly interfering with the repair of DNA lesions.

Liver cancer is the fourth-leading cause of cancer mortality worldwide and results in more than 400,000 deaths annually (41). One of the primary risk factors for the development of hepatocellular carcinoma (HCC) is chronic infection with hepatitis B virus (HBV) (1, 53). For individuals chronically infected with HBV, concurrent exposure to dietary aflatoxin increases the probability of developing HCC by at least threefold (57). The synergistic contributions of these two factors to the development of HCC has led to speculation that chronic HBV infection predisposes an individual to the detrimental effects of hepatocarcinogens.

Several studies using transgenic mouse lines provide experimental evidence to support the hypothesis that chronic HBV infection acts synergistically with environmental carcinogens. The increased sensitivity to aflatoxin B1 of mice that overexpress the HBV surface antigen (18) is believed to be due in part to elevated levels of cytochrome P450 isoenzymes (27) that metabolize aflatoxin B1 into a mutagenic epoxide. Transgenic mice that express the HBV (54) or woodchuck hepatitis virus (13) X proteins (HBx or WHx, respectively) are also more sensitive to the effects of the hepatocarcinogen diethylnitrosamine (DEN). However, the levels of carcinogen-metabolizing enzymes do not appear to be elevated in mice expressing HBx (12). Therefore, the mechanism by which HBx influences the development of liver cancer in mice following exposure to environmental carcinogens remains to be elucidated.

Several lines of evidence suggest that HBx could influence the development of liver cancer by promoting the survival and growth of transformed hepatocytes. HBx is a 17-kDa regulatory protein necessary for the establishment of hepatitis B virus infection in woodchucks and, presumably, in all mammals (11, 63). Detectable in both the cytoplasm and the nucleus of infected cells (14, 40), HBx interacts with numerous cellular proteins (reviewed in reference 16) and is capable of transactivating cellular and viral genes (reviewed in reference 9) and activating protein kinase signaling cascades (3, 14, 24, 28, 34, 39). Transactivation of cellular genes by HBx (8, 50, 55) and the induction of one or more signaling pathways (3, 14, 24, 28, 34, 39) may lead to changes in cell cycle progression and/or regulation. Indeed, HBx is reported to induce cell cycle progression in Chang liver cells (4) and in quiescent skin fibroblasts (30). By affecting cell cycle regulation, HBx could facilitate the survival and proliferation of hepatocytes that were altered after exposure to a mutagenic agent.

A second general mechanism by which HBx might increase the sensitivity of transgenic mice to the effects of hepatocarcinogens involves its ability to inhibit DNA repair. Studies performed in transiently transfected cell cultures and in primary transgenic mouse hepatocytes revealed that HBx expression leads to a significant inhibition (25 to 60%) of global nucleotide excision repair (NER) following exposure to either UV light or aflatoxin B1 (2, 20, 23, 44). Although mapping studies suggest that HBx-mediated inhibition of NER correlates with its ability to interact with the NER component.
DDB1 (32, 52), HBx has also been shown to interact with at least two other proteins or protein complexes that are also directly involved in DNA repair: p53 (17, 60) and TFIH (46). These results suggest that HBx expression could cause an accumulation of DNA mutations in vivo by compromising the repair ability of cells.

The purpose of the present study was to identify the cofactor role of HBx in transgenic mice exposed to DEN. Double-transgenic mice that express HBx (ATX mice) and possess an integrated lambda transgene, allowing measurement of mutation frequency (MF) (29), were used to measure the impact of HBx on repair of DEN-induced DNA damage. Although DEN-treated ATX mice developed 70% more expansile, basal-like, focal lesions than DEN-treated wild-type mice, there was not a significant increase in the accumulation of DNA mutations. However, livers of ATX mice had significantly increased rates of hepatocellular proliferation as measured by immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and by 5-bromo-2′-deoxyuridine (BrdU) incorporation. In humans, HBx has been shown to interact with at least two other proteins or protein complexes that are also directly involved in DNA repair: DDB1 (32, 52), and DDB2 (33, 54).

**MATERIALS AND METHODS**

**Transgenic mice.** Transgenic mice harboring the X gene (nucleotides 1376 to 1840 of subtype adw 2) under the control of the human a1-antitrypsin inhibitor regulatory region (ATX mice) (33, 54) were maintained by the breeding of hemizygous ATX males (ICR × B6C3) with wild-type females (ICR). Hemizygous ATX female (ICR × B6C3) were then mated with homozygous α males (C57Bl/6 Big Blue) (29) obtained from Stratagene Corporation, and the female F1 progeny were used for this study.

The genotypes of F1 progeny were determined by Southern blot hybridization. High-molecular-weight (HMW) DNA was purified from transgenic mouse tail samples using the Wizard genomic DNA purification kit (Promega). DNA was digested with BamHI, resolved on a 1% agarose gel, and transferred to nylon membrane (Boehringer Mannheim). X-gene-specific probe DNA was prepared by PCR amplification of HBV plasmid DNA using the primer set 5′-ATGG CTGCTAGCGGTCTAC-3′ and 5′-CTACAAAGAGATGATTAGGCAGATTTGCGAGA-3′. Probe DNA for the cII gene was amplified from 3 μg of homozygous Big Blue mouse DNA using the cII-specific primer set 5′-ACCACATCTAGTGGTAT GCA-3′ and 5′-GTCTAATGCTCTGGAGAGGAGG-3′. DNA was radiola-beled with [32P]dCTP (3,000 Ci/mmol) (ICN) using the Rediprime II random priming labeling kit (Amersham/Pharmacia). Standard conditions for hybridization and X-ray film exposures were used (51).

**Carcinogen treatment.** At 12 days old, male mice were given a single injection (intraperitoneal) of DEN (Sigma) at 2 μg of body weight as previously described (54). At appropriate time points (14, 30, 90, and 240 days old), mice were sacrificed, and portions of three liver lobes were fixed in 10% neutral buffered formalin for 16 h and then stored in 70% ethanol. Tissues were paraffin embed-ded, and coded hematoxylin-and-eosin-stained sections were submitted for his-tological analysis by M.J.F. Remaining tissue was frozen in liquid nitrogen and stored for later experiments.

**Quantification of mouse liver tissue foci.** The size and number of basophilic, nodular foci were determined by microscopic examination of hematoxylin-and-eosin-stained liver tissue sections. These values were used in conjunction with the overall area of the tissue section examined to calculate the number of foci per cubic volume of liver tissue according to the method of Pugh et al. (45).

**Immunoprecipitation and Western blot verification of HBx expression.** Liver extracts were prepared by homogenizing tissue in extraction buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% NP-40). The protein concentration of each liver extract was determined using the DC Protein assay system (Bio-Rad Laboratories) according to the manufacturer's directions. Subsequent immunopre-cipitation and Western blot analysis, to verify expression of HBx, were performed using 2 mg of total liver protein and have been described previously (54). Briefly, following electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gels, separated proteins were transferred to nitrocellulose filters. The presence or absence of HBx was verified using rabbit anti-HBx polyclonal serum, an avidin-biotin detection kit (Vector Laboratories), and enhanced chemiluminescence (Amersham/Pharmacia). Enhanced chemiluminescence (Amersham/Pharmacia) according to the manufacturer's directions. PCNA-positive hepatocytes located within altered hepatic foci were excluded from quantitation. For Western blot analysis, liver tissue was homogenized in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA), and extracts were clarified by incubation with formalin-fixed, heat-inactivated Staphylococcus aureus. Following electrophoresis on sodium dodecyl sulfate–12% polyacrylamide gels, separated proteins were transferred to Immobilon-P (Amersham/Pharmacia) filters. Immunolabeled PCNA was subsequently visualized using a primary anti-PCNA monoclonal antibody (PC-10; Santa Cruz Biotechnology). Sections were heated in 10 mM sodium citrate (36) and incubated for 5 min in 3,3′-diaminobenzidine according to the manufacturer's directions. PCNA-positive hepatocytes located within altered hepatic foci were excluded from quantitation. For Western blot analysis, liver tissue was homogenized in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA), and extracts were clarified by incubation with formalin-fixed, heat-inactivated Staphylococcus aureus. Following electrophoresis on sodium dodecyl sulfate–12% polyacrylamide gels, separated proteins were transferred to Immobilon-P (Amersham/Pharmacia) filters. Immunolabeled PCNA was subsequently visualized using a primary anti-PCNA monoclonal antibody (PC-10) followed by an avidin-biotin detection kit (Vector Laboratories) and enhanced chemiluminescence (Amersham/Pharmacia) according to the manufacturer's directions.

**RESULTS**

**Double-transgenic mice.** Double-transgenic mice used in this study possess both an X transgene and multiple copies of an integrated bacteriophage (λ) reporter gene. For the purposes of clarity, single-transgenic mice harboring only λ are referred to as wild-type mice, while double-transgenic mice harboring both λ and X transgenes are referred to as ATX mice. Mice were generated as described in Materials and Methods and genotyped by Southern blot hybridization (data not shown). Recent studies have established that under normal conditions (i.e., no exogenous DNA damage), the expression of HBx in these double-transgenic mice has no apparent deleterious effects and does not influence the accumulation of spontaneous mutations (35). The expression of HBx was con-
confirmed for all ATX mice used in this study by immunoprecipitation (IP) and Western blot hybridization (Fig. 1). For this system, increasing the amount of antibody used for IP does not result in a noticeable increase in HBx, indicating the IP is likely being performed in an excess of antibody. It should be noted that the carefully optimized IP step is required prior to Western blot detection of HBx in ATX livers. Indeed, the 2 mg of ATX liver extract required for detection of HBx in the present study is very similar to the amount of total protein needed to obtain a similar HBx signal from 5 x 10^6 pSVX-transfected HepG2 cells (2) or from 4 x 10^6 chronically woodchuck hepatitis virus-infected woodchuck hepatocytes (13). Together, these results demonstrate that HBx is expressed throughout the course of this study and at levels comparable to those observed in chronically infected liver tissue.

HBx-associated increase in DEN-induced liver lesions. Previous studies with DEN-treated transgenic mice revealed that expression of HBx (and WHx) leads to a statistically significant increase in the development of expansive nodules of basophilic hepatocytes and that these focal lesions reliably predict the development of hepatocellular carcinoma (13, 54). To confirm the cofactor effect of HBx in the double-transgenic mice used in this study, male ATX and wild-type mice were treated at 12 days old with a single dose of DEN. All mice appeared normal at the time of sacrifice. However, histological examination of liver tissue sections revealed the development of expansive nodules of basophilic hepatocytes in both ATX and wild-type mice by 240 days old (Fig. 2A). The incidence of foci per cubic volume of liver tissue was increased by approximately 70% in ATX mice relative to that in wild-type mice (Fig. 2B) (P < 0.012). In addition to the clusters of small, basophilic hepatocytes observed in all foci, some altered hepatocytes also possessed globular eosinophilic inclusions. These inclusions were found in both ATX (four out of seven) and wild-type (two out of nine) animals. All other histological abnormalities cited (e.g., mild anisocytosis, isolated foci of inflammation, and decreased glycogen content) appeared in a random distribution and were consistent with observations reported for untreated mice of the same lineage and age (data not shown) (35). These observations establish that the double-transgenic mice (ATX\lambda)

used for this study are more sensitive than wild-type littermates (\lambda) to the carcinogenic effects of DEN.

Effect of HBx on in vivo MF. Previous studies have established that HBx can inhibit the ability of cells to repair damaged DNA (2, 20, 23, 44). A similar inhibitory effect of HBx in vivo should lead to an increase in the accumulation of DNA mutations in DEN-treated mice. To test this hypothesis, we measured the relative MF in liver tissue samples obtained from DEN-treated ATX and wild-type mice (Table 1). Compared to the MF reported for untreated mice (35), exposure to DEN
resulted in a six- to eight-fold increase in the MF, confirming that this assay reliably detects changes in mutation accumulation. A slight elevation in the mean MF for ATX mice was found at each time point (30, 90, and 240 days old); however, this increase was not statistically significant (all $P$ values were $>0.21$). A comparison of the mean MF among all ATX ($n = 15$) and wild-type ($n = 13$) mice revealed a 23% increase in the MF that was also not significant ($P = 0.1$). These results demonstrate that the expression of HBx in vivo does not lead to major alterations in the spectrum of DEN-induced DNA mutations.

**Determination of DNA mutation spectrum.** While HBx expression did not result in a measurable increase in the MF, it was conceivable that it could cause a change in the spectrum of mutations induced by DEN by inhibiting the repair of only a certain subset of DNA lesions. To examine this possibility, mutant phage derived from DEN-treated ATX and wild-type mice were picked at random, and the $cII$ genes were sequenced ($n = 63$) to establish the mutation spectrum (Table 2). Compared to the mutation spectrum reported for untreated mice (49), DEN treatment resulted in a relatively large increase in transition and transversion events at A and T base pairs, a finding consistent with the long half-life and mutagenicity of $O^4$-ethyldeoxythymine ($O^4$-EtT) moieties formed by DEN ($t_{1/2} = 11$ days) (48). In addition, there was an approximately twofold increase in the incidence of transitions from G or C to A or T base pairs in ATX relative to wild-type animals. These results establish that HBx expression does not lead to major alterations in the spectrum of DEN-induced DNA mutations.

**HBx-induced hepatocyte proliferation.** Since previous studies have shown that HBx may stimulate cell cycle progression and proliferation in cell culture (4, 30), we hypothesized that a similar effect of HBx in vivo might explain the 70% increase in the incidence of DEN-induced preneoplastic lesions in ATX mice. To determine the possible effect of HBx on hepatocellular proliferation, livers of ATX and wild-type animals were examined for the expression of PCNA, a marker of cellular proliferation (25). A significant increase in the percentage of

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**TABLE 1. Determination of the MF in DEN-treated transgenic mice**

| Age (days) | Genotype | Animal ID$^a$ | Total no. of PFU ($10^5$)$^b$ | No. of mutant PFU$^c$ | MF ($10^{-4}$) | Mean MF ± SD ($10^{-4}$) |
|-----------|----------|---------------|-------------------------------|----------------------|----------------|--------------------------|
| 30        | ATX      | 2101          | 4.72                          | 121                  | 2.57           | 2.01 ± 0.67             |
|           |          | 2108          | 2.12                          | 55                   | 2.59           |                          |
|           |          | 2133          | 4.56                          | 102                  | 2.24           |                          |
|           |          | 2134          | 6.52                          | 102                  | 1.57           |                          |
|           |          | 2136          | 3.39                          | 36                   | 1.06           |                          |
|           | WT$^d$   | 2084          | 3.13                          | 76                   | 2.43           | 1.83 ± 0.38             |
|           |          | 2085          | 4.12                          | 69                   | 1.67           |                          |
|           |          | 2096          | 4.20                          | 65                   | 1.55           |                          |
|           |          | 2097          | 5.85                          | 116                  | 1.98           |                          |
|           |          | 2098          | 4.78                          | 72                   | 1.51           |                          |
| 90        | ATX      | 2055          | 3.58                          | 139                  | 3.88           | 2.39 ± 1.33             |
|           |          | 2058          | 2.95                          | 58                   | 1.97           |                          |
|           |          | 2128          | 3.02                          | 40                   | 1.32           |                          |
|           | WT       | 2052          | 3.87                          | 82                   | 2.12           | 1.79 ± 0.70             |
|           |          | 2056          | 3.13                          | 71                   | 2.27           |                          |
|           |          | 2127          | 4.23                          | 42                   | 0.99           |                          |
| 240       | ATX      | 2059          | 2.54                          | 87                   | 3.43           | 2.49 ± 0.75             |
|           |          | 2061          | 2.80                          | 70                   | 2.50           |                          |
|           |          | 2062          | 2.77                          | 95                   | 3.43           |                          |
|           |          | 2070          | 2.07                          | 47                   | 2.27           |                          |
|           |          | 2082          | 4.01                          | 104                  | 2.59           |                          |
|           |          | 2137          | 5.94                          | 95                   | 1.60           |                          |
|           |          | 2139          | 3.87                          | 63                   | 1.63           |                          |
|           | WT       | 2060          | 3.58                          | 94                   | 2.62           | 1.99 ± 0.46             |
|           |          | 2063          | 5.28                          | 104                  | 1.97           |                          |
|           |          | 2065          | 2.57                          | 39                   | 1.52           |                          |
|           |          | 2067          | 2.49                          | 56                   | 2.25           |                          |
|           |          | 2132          | 3.87                          | 61                   | 1.58           |                          |
|           | WT (untreated)$^e$ | | | | 0.306 ± 0.28 | |

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$^a$ ID, identification.

$^b$ Calculated from dilutions incubated at 37°C.

$^c$ Isolated mutants replated and incubated at 24°C to verify phenotype.

$^d$ WT, wild type.

$^e$ MF value reported for untreated WT mice at 240 days old by Madden et al. (35).
PCNA-positive hepatocytes was observed in 14-day-old ATX mice versus results for wild-type mice (Fig. 3A) (all P values were <0.004). This difference was apparent for both DEN-treated and untreated mice and was confirmed independently by the determination of steady-state levels of PCNA in mouse liver tissue sections by Western blot analysis (Fig. 3B). Hepatocellular proliferation had diminished considerably by 30 and 240 days of age, with no measurable effect of HBx observed (data not shown). These results indicate that the contribution of HBx-induced cellular proliferation is likely limited to the early stages of DEN-induced carcinogenesis.

To establish that the increased levels of PCNA found in ATX mouse livers correlated with an increase in hepatocellular replication, the incorporation of BrdU into hepatocellular DNA was measured in 14-day-old mice. Significantly more BrdU-positive hepatocytes were detected in DEN-treated ATX than in treated wild-type mouse liver tissue sections (Fig. 3C and D) (P = 0.03). Together, these data clearly demonstrate that under certain conditions, HBx expression can promote hepatocellular proliferation in vivo.

**DISCUSSION**

Previous work with humans and woodchuck X-transgenic mice has established a cancer cofactor role for the HBx (or WHx) protein (13, 54, 58). The purpose of the present study was to investigate the molecular basis of the role of the HBx cofactor in DEN-mediated carcinogenesis. Using a novel double-transgenic mouse model that allows determination of the DNA MF in vivo, we show that HBx expression is associated with a twofold increase in altered hepatic foci, a modest (23%) increase in the DNA MF, and a two- to threefold increase in hepatocellular proliferation in young mice (measured by PCNA staining and BrdU incorporation). Together, these results are consistent with the idea that HBx functions as a tumor promoter in the DEN model of liver carcinogenesis.

We originally predicted that HBx expression in the presence of exogenous DNA damage would lead to unrepaired DNA and an increased DNA MF. This hypothesis was based on the observation that HBx inhibits the repair of DNA damage in cell culture (2, 20, 23, 35, 44). Although we were able to measure an HBx-associated elevation in the MF at each time point in this study, the increase measured (23%) was very small. One interpretation of this result is that the cofactor role of HBx in this model is restricted to promoting the growth of DEN-altered hepatocytes, as was previously suggested (13). However, our data showing an HBx-associated increase in hepatocellular proliferation suggest that HBx might additionally enhance the initiation of carcinogenesis. The major DEN adduct O6-ethylguanine (O6-EtG) has a half-life of 20 h (48). HBx-induced cell division that occurs prior to repair of the O6-EtG lesions would lead to a mutation at the position of the unrepaired adducts, specifically at a guanine residue. Indeed, we are able to measure a modest increase in the MF in ATX mice at all time points and also observed an increase specifically in G (or C) to A (or T) transitions. These latter results indicate that HBx may enhance the initiation of carcinogenesis by promoting the division of hepatocytes that contain unrepaired DNA damage.

The results of the present study are consistent with current models of known tumor promoters. Analysis of the tumor promoter 12-myristate 13-acetate (TPA) has yielded results that are strikingly similar to those observed for HBx. The two- to fivefold induction of mitosis in TPA-treated keratinocytes (37) is similar to the two- to threefold induction of hepatocyte proliferation measured in ATX livers. In addition, the modest elevation in the MF in carcinogen-treated Big Blue mice additionally treated with TPA (64%) (37) is comparable to the HBx-associated 23%-increased MF found in DEN-treated mice in the present study. Finally, neither TPA nor HBx alone has any affect on the MF (35, 37); their effect is apparent only in the presence of DNA damage. Thus, the influence of HBx measured in the present study is very similar to that measured for the well-known tumor promoter TPA.

The mechanism by which HBx increases hepatocyte turnover remains unknown. This effect of HBx was measurable only in neonatal mice, when hepatocyte numbers are increasing and growth factors are abundant. It is interesting to consider that many of the growth-stimulatory factors present in neonatal liver are also induced during liver cell regeneration (19, 59). It seems likely that a similar growth-promoting effect of HBx during immune-mediated cycles of liver cell death and regeneration (reviewed in reference 53) would contribute to the pathology of chronic HBV infection in humans.

The expression of HBx in many transgenic mouse lines is not associated with any detrimental effects (6, 15, 21, 38, 42, 47). However, the ability of HBx to induce hepatocyte proliferation has been reported for a line of X-transgenic mice that are susceptible to HCC (31). Those mice also demonstrate a similar two- to threefold increase in cell proliferation. It remains unknown whether the ability of HBx to increase hepatocyte replication is responsible for the HCC in those mice. Other variables to consider when comparing those mice with the ATX mice used in the present study include genetic variations between mouse lineages, the level at which HBx is being expressed, and possible environmental cofactors unique to individual animal colonies.
A delicate balance between cellular proliferation and apoptosis is necessary for normal liver homeostasis. Indeed, a proapoptotic property of HBx has been demonstrated in several studies (26, 43, 56). However, it is difficult to reconcile an induction of apoptosis by HBx with the increase in the development of liver foci demonstrated in the present study. Rather, the prevention of apoptosis would lead to the survival of cells that contain DNA mutations. DNA-damaging agents, such as DEN, are known to induce apoptosis (62). However, we observed an HBx-associated increase in PCNA-positive cells in both the presence and the absence of DEN treatment. This result indicates that the increased rate of cellular turnover measured in ATX livers is not merely a compensatory response to increased cell death in DEN-treated ATX mice.

The design of the present study does not permit definitive conclusions regarding the ability of HBx to directly compromise DNA repair in vivo. At present, HBx has only been shown to inhibit the NER pathway (2, 20, 23, 44), one of several DNA repair pathways in the cell (61). DEN is a metabolically activated mutagen that ethylates nucleophilic sites in DNA, primarily the \( O^6 \) and \( O^4 \) positions of guanine and thymine, respectively (5). In addition to being repaired by the NER pathway (7), \( O^6 \)-EtG lesions are also removed via \( O^6 \)-alkylguanine DNA alkyltransferase activity in eukaryotes (5). The redundancy of repair pathways for \( O^6 \)-EtG moieties and the long half-life of \( O^4 \)-EtT lesions (11 days) may have minimized our ability to measure the impact of HBx expression on NER. We considered the possibility that HBx might inhibit the repair of

**FIG. 3.** Impact of HBx on hepatocyte proliferation. (A) Percentage of PCNA-positive hepatocytes in 14-day-old DEN-treated \( (n = 4) \) and untreated \( (n = 5) \) ATX and in DEN-treated \( (n = 5) \) and untreated \( (n = 4) \) wild-type (WT) mouse liver tissue sections. Mean values were determined by counting PCNA-positive hepatocytes in five random fields of approximately 250 cells per field. Error bars represent standard deviations. (B) Detection of PCNA in ATX and wild-type mouse liver extracts by Western blotting analysis. Liver extracts were prepared from DEN-treated 14-day-old male ATX and wild-type mouse littermates. (C) Percentage of BrdU-positive hepatocytes in 14-day-old DEN-treated ATX \( (n = 3) \) and wild-type \( (n = 4) \) mouse liver tissue sections. Mean values were determined by counting BrdU-positive hepatocytes in five random fields of approximately 250 cells per field. Error bars represent standard deviations. (D) Representative immunohistochemical staining of incorporated BrdU in DEN-treated 14-day-old ATX and wild-type mouse liver tissue sections. BrdU-positive nuclei are black, and nonlabeled nuclei are counterstained with methyl green.
a subset of DEN lesions and so investigated the DNA mutation spectrum for 63 lambda cII mutants. Those experiments revealed an increase in G (or C) to A (or T) transitions that could be explained by the HBx-induced cell division in the presence of unreplicated O'EtG (discussed above). Studies designed to measure the impact of HBx expression on the removal of lesions repaired exclusively by the NER pathway are ongoing.

In summary, a strong correlation exists between HBV status, exposure to environmental carcinogens, and the development of HCC (10, 57). In the present study, we demonstrate that the expression of HBx in vivo leads to a significant increase of DEN-induced hepatic lesions by a mechanism that does not include a large increase in the DNA MF, data consistent with a model in which HBx acts as a tumor promoter. In addition, we propose that HBx may enhance the initiation of DEN damage by inducing hepatocellular proliferation in cells that contain unrepaired DNA lesions. It is therefore possible that damage by inducing hepatocellular proliferation in cells that express HBx in vivo leads to a significant increase of HCC (10, 57). In the present study, we demonstrate that the expression of HBx in vivo leads to a significant increase of DEN-induced hepatic lesions by a mechanism that does not include a large increase in the DNA MF, data consistent with a model in which HBx acts as a tumor promoter. In addition, we propose that HBx may enhance the initiation of DEN damage by inducing hepatocellular proliferation in cells that contain unrepaired DNA lesions. It is therefore possible that expression of HBx at the time of carcinogen exposure in humans will similarly lead to enhanced carcinogenesis. Further investigation of the molecular mechanism(s) by which HBx alters the hepatocyte cycle will lead to a better understanding of the molecular basis of HBV-associated liver cancer and may reveal novel targets for intervention and treatment of HCC.

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