Abstract
The cycles of cell death and compensatory regeneration that occur during chronic hepatitis B virus infection are central to viral pathogenesis and are a risk factor for the development of liver cancer. The HBV genome encodes one regulatory protein, HBx, which is required for virus replication, although its precise role in replication and pathogenesis is unclear. Because HBx can induce the G0-G1 transition in cultured cells, the purpose of this study was to examine the effect of HBx during liver regeneration. Transgenic mice expressing HBx (ATX) and their wild-type (WT) littermates were used in the partial hepatectomy (PH) model for compensatory regeneration. Liver tissues collected from ATX and WT mice at varying sacrifice time points after PH were examined for markers of cell cycle progression. When compared with WT liver tissues, ATX livers had evidence of premature cell cycle entry as assessed by several variables (BrdUrd incorporation, proliferating cell nuclear antigen and mitotic indices, and reduced steady-state p21 protein levels). However, HBx did not affect apoptosis, glycogen storage, or PH-induced steatosis. Together, these results show that HBx expression can induce cell cycle progression within the regenerating liver. Our data are consistent with a model in which HBx expression contributes to liver disease and cancer formation by affecting early steps in liver regeneration.

Introduction
Chronic infection with hepatitis B virus (HBV) affects over 350 million people worldwide and is a major risk factor for hepatocellular carcinoma (HCC; refs. 1, 2). The HBV genome encodes one regulatory protein, HBx, which is required for virus replication in vivo (3–5). Although the precise role of HBx in virus replication and pathogenesis is unclear, HBx likely has multiple functions in these processes. Studies in transfected cells revealed that HBx can transactivate viral and cellular promoters (6, 7), can activate cytoplasmic signaling pathways (8, 9), can be either proapoptotic or antiapoptotic (reviewed in ref. 10), can inhibit cellular DNA repair (11), and can behave as a tumor promoter in transgenic mice (12, 13; reviewed in ref. 14). Cycles of immune-mediated cell death and compensatory regeneration that accompany chronic HBV infection are thought to contribute to the development of liver disease (15), but the role of HBx in this process is unknown. Previous studies have shown that in immortalized cells in culture, HBx induces cell cycle progression to overcome a serum-induced G0 block (16) and causes accelerated transit through cell cycle checkpoints (17; reviewed in ref. 18). If HBx is similarly able to alter cell cycle progression during compensatory regeneration, it could be contributing to viral pathogenesis over the decades of chronic hepatitis.

The purpose of our study was to examine the effect of HBx in vivo during the early steps of liver regeneration. We used transgenic mice expressing HBx (ATX) and their wild-type (WT) littermates in the partial hepatectomy (PH) model for compensatory regeneration. In this model, surgical removal of 70% of the liver leads to carefully regulated signaling events causing remaining hepatocytes to undergo synchronized growth and cell division (reviewed in refs. 19–21). Based on studies of HBx in cell culture (16, 17), we hypothesized that HBx expression would interfere with the regulation of early steps in liver regeneration. This function of HBx could contribute to the development of liver disease, particularly if it occurred in the context of exposure to environmental carcino gens.

Materials and Methods

Transgenic mice. Animal procedures were done in accordance with federal regulations for the care and treatment of laboratory animals. Transgenic mice expressing HBx under the control of the liver-specific α-1 antitrypsin regulatory region (ATX mice; ref. 22) and their nontransgenic (WT) littermates were used in this study. Genotypes were determined by PCR analysis of high molecular weight tail DNA using X-specific primers as described (23) and confirmed by immunoprecipitation (IP) and Western blot as described below.

PH. All PH surgeries were performed in the morning hours after a 4-h fast using 10–12-wk-old ATX and WT mice as previously described (24). Animal body weight was determined at both the time of PH and at sacrifice, and livers were removed at PH and remnant liver masses were weighed. Representative portions of liver tissues were either flash-frozen in 2-methylbutane, or fixed in neutral buffered formalin and paraffin-embedded for histologic analysis.

BrdUrd incorporation and detection. Mice were either injected with 0.1 mg BrdUrd per gram of body weight 2 h before sacrifice, or given 1 mg/mL BrdUrd in their drinking water for 60 h before sacrifice. Fixed liver sections were stained with anti-BrdUrd antibody (Dako; 1:100) using the ARK kit (Dako). Liver BrdUrd labeling index was calculated for each animal in this study as the percent of BrdUrd-positive cells in 10 random fields each containing ~300 hepatocytes.

Histologic analyses and immunohistochemistry. Paraffin-embedded liver sections were stained with H&E for histologic analysis and for quantitation of mitotic cells. Mitotic cells were observed in high-powered fields (~400) and included those with condensed chromosomes, and those in late prophase, metaphase, or anaphase. Mitotic index was calculated as the percentage of mitotic cells in 10 random fields each containing ~100 hepatocytes for 3 ATX and 3 WT livers at each of 6 sacrifice time points. To examine cellular glycogen content, deparaffinized liver sections were treated with 0.5% periodic acid and stained with Schiff’s reagent (PAS). To measure apoptosis, liver sections were stained by terminal deoxynucleotidyl
transfection dUTP nick end labeling assay (TUNEL) using the Apop Tag kit (Millipore) according to the manufacturer's protocol. TUNEL-positive cells were quantitated in 10 random fields each containing ~300 hepatocytes for 3 ATX and 3 WT livers at each of 4 sacrifice time points. To optimize detection of proliferating cell nuclear antigen (PCNA), liver sections were treated with heat-induced epitope retrieval (HIER; Sigma). After blocking steps, tissues were incubated with primary anti-PCNA (FITC-261; Santa Cruz Biotechnology; 1:5,000), secondary antibody (goat anti-rabbit; Vector; 1:200), and Avidin-biotin complex (ABC; Vector). PCNA labeling index was calculated in livers 0 and 24 h after PH by quantitating positive cells in 10 random fields each containing ~300 hepatocytes for 3 ATX and 3 WT livers. Using the HIER system, a basal level of PCNA was detected in 0 h samples and this was subtracted from each sample data point during analysis as previously described (25).

For the identification of neutral lipids, frozen tissue sections were stained with Oil Red O (ORO) dye. Severity of steatosis in liver sections was scored using the method of Brunt and colleagues (26) for 3 ATX and 3 WT mice at 0 and 28 h after PH. Briefly, coded ORO-stained liver sections from each animal were scored for the percentage of hepatocytes that contained ORO-positive cytoplasmic vacuoles in 10 random fields per animal as described previously (23).

Detection of HBx and p21 by IP/Western blot and Western blot. Liver extracts from 2 to 3 animals of each genotype per sacrifice time point were prepared by homogenizing frozen liver tissue in extraction buffer containing 1% NP-40 and 1% aprotinin as described (13). HBx was detected by IP/Western blot as described (13). Western blot detection of p21 and tubulin control was by primary antibodies anti-p21 (sc-6246; Santa Cruz; 1:500) and anti-tubulin (sc-9104; Santa Cruz; 1:1,000). For all Western blots, bound antibody was detected and quantitated as previously described (5). For each sacrifice time point examined, the normalized WT p21 value was set to 1.00 and compared with the normalized ATX p21 value.

Reverse transcription real-time PCR. RNA was extracted from 10-mg liver tissue using the RNAqueous-4PCR kit (Applied Biosystems), and 2 µg RNA were reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR analysis was done using ~1 µg cDNA, Taqman Universal PCR Master Mix, and primer/probe sets for Serpina1a (MM0274877_g1, p21 (MM01303209_s1), 18S rRNA (4319413E), or HBx primers (5'-CCGGTCCGTGTGCACTTC-3', 5'-AGGATCT-GATGGGCGTTCAC-3') and probe (5'-6FAM ACGTCTGACGGTATGGCA-GGAC-3') all from Applied Biosystems. Values for Serpina1a, p21, and HBx were normalized to 18S rRNA values. Copy number was calculated using the method of Brunt and colleagues (26) for 3 ATX and 3 WT livers at each of 4 sacrifice time points. To optimize reaction efficiencies, the Y-intercept was assumed to be 38 and the slope assumed to be -3.3 as described by Applied Biosystems.

Statistical analysis. The Student’s t test was used to compare ATX versus WT mice for the following: liver mass, BrdUrd, mitosis and PCNA labeling indices, reverse transcription real-time PCR (RT-qPCR), and TUNEL, using the Microsoft Excel software package, and significance assigned for P value of <0.05. Error bars are reported as SE or SD, as indicated in figure legends. Mann-Whitney rank sum test for significance (for ORO) was done using the SigmaStat software package.

Results

ATX transgenic mice and PH. ATX mice express HBx under the control of the liver-specific α-1-antitrypsin (A1AT) regulatory region (22). HBx expression continues throughout the life span of these mice at levels similar to that observed during chronic infection, as discussed previously (13). There is no observable pathology in the livers of untreated ATX or WT mice. However, ATX mice develop more tumors (versus WT littermates) when exposed to carcinogenic insults such as diethylnitrosamine (13, 27) and hepatitis C virus (23).

In cell culture, HBx expression has been shown to induce G0-G1 progression (16, 17). To address whether HBx might similarly affect cell cycle progression in vivo, we performed 70% PH on ATX mice and their WT littermates. Seventy-seven mice were included in the initial study (Supplementary Table S1), and total body weight did not differ between 37 ATX and 40 WT mice at the time of surgery, indicating that HBx expression did not affect animal size (Fig. 1A). Equivalent amounts of liver tissue were removed from animals of each genotype and represented ~70% of the total mass as predicted from body weight (Fig. 1B). Finally, HBx protein expression was confirmed by IP/Western blot using livers collected at 0 hour and in regenerating livers (Fig. 1C). These data show that any differences observed during regeneration will be due to the presence of HBx rather than to issues related to the surgery or animal physiology.

Altered DNA synthesis after PH in ATX mice. After PH, the liver progresses through a well-described series of steps from priming, which prepares hepatocytes to move from G0 to G1 phase of the cell cycle, to DNA synthesis, and finally to cell division (reviewed in refs. 19–21). In cell culture, HBx induces the G0-G1 transition (16, 17), so we examined the effect of HBx on the early stages of liver regeneration. To measure DNA synthesis, mice were injected with BrdUrd 2 hours before each of 8 sacrifice time points. BrdUrd incorporation was detected by antibody staining of fixed liver tissue and was observed as dark brown nuclei (Fig. 2A). Calculation of the BrdUrd labeling index in WT livers revealed an increasing percentage of BrdUrd-positive cells beginning at

Figure 1. Characteristics of mice used in the present study. A, body mass of mice (40 WT, 37 ATX) was determined before PH. B, liver mass removed during surgery was measured for the same WT and ATX mice. Columns, mean; bars, SD. NS, not significant as determined by Student’s t test. C, HBx was detected by IP/Western blot analysis of liver extracts as described in Materials and Methods. Top, representative results from two WT and two ATX livers removed at surgery. Bottom, representative results from 2 WT and 2 ATX livers harvested 40 hours post-PH.
Evidence of early DNA synthesis in ATX livers. BrdUrd that incorporated into newly synthesized DNA was detected by IHC staining. A, representative liver sections from 0, 28, and 40 h after PH (×200). B, mean BrdUrd labeling indices from three to seven mice per genotype per time point (see Supplementary Table S1). Points, mean; bars, SE; *, time points at which P value is <0.05, determined using the Student’s t test.

32 hours post-PH with a synchronous peak in DNA synthesis 40 hours after PH (Fig. 2B). This result is in agreement with previously published data for nontransgenic mice (28, 29). In contrast, the BrdUrd labeling index was more complex in ATX livers. First, we observed a population of ATX hepatocytes that entered S phase earlier than WT hepatocytes (28 hours; P < 0.02: Fig. 2B). In addition, a second peak in BrdUrd incorporation in ATX livers occurred between 40 and 44 hours, corresponding with the single 40-hour peak observed in WT livers (Fig. 2B). These results show that HBx expression alters early steps in liver regeneration.

**Total hepatocyte division not affected by HBx.** The 2-hour BrdUrd labeling method commonly used to measure DNA synthesis after PH identifies those hepatocytes undergoing DNA synthesis during a 2-hour window between BrdUrd administration and sacrifice (28, 30–33). By summing all the hepatocytes labeled by this method, it seems that the total number of hepatocytes that have undergone DNA synthesis in ATX and WT livers by 60 hours after PH is similar. To confirm this, we performed PH and administered BrdUrd continuously for 60 hours so the total number of hepatocytes undergoing DNA synthesis could be measured. An additional nine mice (three ATX, six WT) were subjected to PH and given BrdUrd in their drinking water. Animals were sacrificed at 60 hours, BrdUrd incorporation was detected by antibody staining of fixed liver sections (Fig. 3A), and the labeling index was determined for each animal. Using this approach, equivalent percentages of BrdUrd-positive hepatocytes were detected in both ATX and WT livers (P = 0.41), indicating similar levels of DNA synthesis were achieved in both groups by 60 hours post-PH (Fig. 3B). Finally, we sought to confirm that ATX mice survived beyond 60 hours. Surgeries were performed on another 8 mice (3 ATX, 5 WT), and animals were sacrificed at 8 days after PH. Approximately 100% of the liver mass removed at surgery had regenerated by 8 days in both WT and ATX mice (Fig. 3C). This result is consistent with the 60-hour BrdUrd labeling data and indicates that, despite the effects of HBx on early regeneration (Fig. 2B), the ATX mice were able to successfully regenerate their livers.

**Possible mechanisms for early cell cycle progression in ATX hepatocytes.** Deregulated liver regeneration is hypothesized to precede the development of liver disease, so the observation of the complex pattern of DNA synthesis in ATX livers (Fig. 2B) was intriguing. There are several possible explanations for this result, and we first considered the possibility that there are two populations of ATX hepatocytes.

The idea of two populations of ATX hepatocytes leads to several testable predictions. For example, the subpopulation of ATX hepatocytes that enters S phase prematurely should also display markers of G1-S phase earlier than WT hepatocytes. This idea was tested by monitoring expression of PCNA, a commonly used marker for late G1-S phase. Previous PH studies in nontransgenic mice indicate PCNA protein expression begins to increase from baseline 24 hours post-PH, as the cells begin to progress from late G1 into S phase (25, 34, 35). Therefore, we measured the PCNA labeling index in three ATX and three WT livers. PCNA was detected in WT livers at 24 hours (Fig. 4A), indicating that some hepatocytes are in late G1-S phase. At the same time point in ATX livers, the percentage of cells expressing detectable PCNA was significantly higher than that of WT livers (P < 0.05). This result is consistent with the 2-hour BrdUrd data showing a subpopulation of ATX hepatocytes had entered S phase earlier than WT (Fig. 2B).

If ATX hepatocytes prematurely entering S phase continued through the cell cycle, we would expect to see a corresponding subpopulation of ATX hepatocytes reach mitosis sooner than those in WT livers. The possible early appearance of mitotic cells in ATX livers was examined by determining the mitotic index in fixed liver tissues at six sacrifice time points after PH (Fig. 4B). Data from 3 WT livers revealed a peak in mitosis 48 hours post-PH (Fig. 4C), as has been reported previously in PH models of nontransgenic mice (30, 36). In contrast, data from three ATX livers revealed a more complex pattern. There were significant increases in the number of mitotic cells in ATX livers at 40 and 44 hours relative to that found in WT livers at the same time points (Fig. 4C). The peak in mitotic cells in the ATX livers occurred at 44 hours, ~4 hours earlier than
We next investigated potential molecular mechanisms that might explain a premature entry into S phase. Because p21 can be regulated at the transcriptional (reviewed in ref. 40) and posttranslational levels (31, 41), we examined p21 mRNA by RT-qPCR and p21 protein by Western blot. There were no significant differences in the p21 mRNA levels in ATX and WT livers at any time point examined (Fig. 5A). In contrast, we found a trend toward lower p21 protein levels in ATX (versus WT) livers (Fig. 5B). The lack of statistical significance may be due either to variability among animals or that we are measuring an effect on p21 that occurs only in a subpopulation of hepatocytes. The generally decreased p21 levels are consistent with the observations of early increases in BrdUrd incorporation and PCNA levels in ATX (versus WT) livers. Together, these results support the observation that HBx expression leads to a premature onset of cell cycle entry during liver regeneration. These data also raise the possibility that HBx can deregulate p21 at a posttranscriptional level.

Other possible mechanisms for deregulated regeneration in ATX hepatocytes. We also considered other functions previously reported for HBx that might influence liver regeneration. These included possible effects of HBx on apoptosis (reviewed in ref. 10), glycogen storage (30), and fat accumulation (23, 30), as well as effects due to the presence of the ATX transgene. We began by investigating a possible effect of HBx on apoptosis.

Under certain conditions, HBx expression can induce and/or prevent apoptosis both in cell culture and in vivo (reviewed in ref. 10). We considered that increased cell death in the ATX livers might contribute to the complex pattern of DNA synthesis (Fig. 2B). To determine a possible effect of HBx on apoptosis, fixed liver sections were stained by TUNEL assay and TUNEL-positive cells were quantitated in ATX and WT livers (3 mice per genotype per time point) at 0, 24, 28, 32, and 36 hours after PH. Apoptotic cells were rare (fewer than 0.06%), with no genotype-specific differences in the incidence of TUNEL-positive cells (P > 0.19; Supplementary Table S2). Therefore, we concluded that apoptosis was not increased in ATX (versus WT) livers and likely does not explain the complex pattern of DNA synthesis (Fig. 2B).

The ATX mice used in this study express HBx under the control of the liver-specific A1AT regulatory region. Murine A1AT (Serpina1a) is an acute phase response (APR) protein expected to be activated in response to PH (42). We considered that the presence of the human A1AT regulatory element in the ATX transgene might alter the activation of endogenous A1AT and disrupt compensatory regeneration. To address this, RT-qPCR was used to quantitate Serpina1a levels in 3 ATX and 3 WT livers at both 0 and 36 hours after PH. There were no differences between ATX and WT livers in the levels of endogenous Serpina1a in either resting liver (0 hour; P = 0.087) or regenerating liver (36 hours; P = 0.398; Fig. 6A). We also compared the Serpina1a levels in ATX livers at 0 versus 36 hours and found no significant effect of HBx (P = 0.677), indicating that the presence of the human A1AT regulatory element did not affect endogenous Serpina1a mRNA levels during regeneration.

Although endogenous Serpina1a levels were not affected by the presence of the ATX transgene, activation of APR genes after PH might enhance HBx expression from the A1AT-driven transgene, and high levels of HBx could interfere with compensatory regeneration. A similar approach was used to evaluate whether HBx expression levels increase after PH. First, RT-qPCR analysis was used to measure HBx mRNA expression in WT and ATX livers at 0 and 36 hours after PH. HBx expression was detected exclusively in ATX livers, and there was no significant change in

the WT peak at 48 hours. When the time to progress through S and G2 phase into M phase is taken into consideration, the early onset S phase subpopulation revealed in the 2-hour BrdUrd labeling experiment and with the increased PCNA detection in ATX livers at 24 hours. The mitosis data extends those results by demonstrating the subpopulation of ATX hepatocytes that enters S phase prematurely continues through the cell cycle.

We next investigated potential molecular mechanisms that might explain a premature entry into S phase. One important cell cycle regulator at the G1 phase checkpoint is the cyclin-dependent kinase inhibitor p21. Liver regeneration studies in p21 knockout mice revealed an accelerated G1 and early onset of S phase evidenced by early increases in BrdUrd and PCNA after PH (31, 33). The effect of HBx on p21 has been studied in transfected cells, where HBx can repress (37), activate (38), or both repress and activate (39) p21. We considered the possibility that HBx expressed in ATX livers would deregulate p21 expression, leading to premature entry into S phase. Because p21 can be regulated at the transcriptional (reviewed in ref. 40) and posttranslational levels (31, 41), we examined p21 mRNA by RT-qPCR and p21 protein by Western blot. There were no significant differences in the p21 mRNA levels in ATX and WT livers at any time point examined (Fig. 5A). In contrast, we found a trend toward lower p21 protein levels in ATX (versus WT) livers (Fig. 5B). The lack of statistical significance may be due either to variability among animals or that we are measuring an effect on p21 that occurs only in a subpopulation of hepatocytes. The generally decreased p21 levels are consistent with the observations of early increases in BrdUrd incorporation and PCNA levels in ATX (versus WT) livers. Together, these results support the observation that HBx expression leads to a premature onset of cell cycle entry during liver regeneration. These data also raise the possibility that HBx can deregulate p21 at a posttranscriptional level.

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HBx mRNA expression in regenerating liver compared with that of resting liver ($P = 0.77$; Fig. 6B). Analysis by IP/Western showed there was no reproducible difference in HBx protein levels in the resting versus regenerating ATX livers (Fig. 6C). We conclude that HBx protein and RNA levels expressed from the ATX transgene remained constant during liver regeneration.

In response to PH, glycogen is mobilized from the remnant liver to produce enough glucose to maintain homeostasis in the body. This mobilization transiently depletes the glycogen content of liver, which is restored to normal levels at later time points during regeneration. A recent study in another lineage of HBx transgenic mice concluded that severely impaired glycogen storage contributed to inhibited regeneration after PH (30). To examine this possibility in our model, fixed liver sections collected at 0, 24, and 48 hours after PH were examined for glycogen content by treating with periodic acid and staining with Schiff’s reagent (PAS). At 0 hour, liver samples stained PAS-positive for glycogen with no obvious difference in staining patterns between ATX and WT (Supplementary Fig. S1). By 24 hours, decreased PAS-positive staining intensity in livers of both genotypes compared with that of 0-hour livers indicated that glycogen had been mobilized from the hepatocytes. At 48 hours, glycogen stores had increased to similar levels in both genotypes.

Figure 4. PCNA- and mitotic-indices in ATX (versus WT) livers. 
A, increased percentage of PCNA-positive hepatocytes in ATX (versus WT) livers. Formalin-fixed liver tissue was stained with antibodies for PCNA and the total percentage of PCNA-positive hepatocytes. Columns, mean; bars, SE; significance was determined by the Student’s $t$ test; *, $P < 0.05$. 
B, formalin-fixed liver sections from WT and ATX mice were stained by H&E. White arrows, mitotic bodies. Magnification, ×400. 
C, mean mitotic indices for three WT livers (white bars) and three ATX livers (black bars). Columns, mean; bars, SE; significance determined by the Student’s $t$ test is shown either $P \leq 0.05$ (*), or $P < 0.00001$ (**).

Figure 5. $p21$ mRNA and protein levels in ATX (versus WT) livers. 
A, levels of $p21$ mRNA were measured by RT-qPCR and normalized to 18S rRNA expression. Columns, mean of three WT livers (white bars) and three ATX livers (black bars); bars, SD. 
B, liver lysates from ATX and WT livers were examined by Western blot for $p21$ and tubulin. Each band, representing a different animal, was quantitated by densitometer scanning, and $p21$ levels were normalized to tubulin levels. For each time point, the average of normalized WT samples was set to 1.00 and compared with ATX samples.
Discussion

Chronic HBV infection is marked by cycles of cell death and regeneration, and is a risk factor for the development of HCC. In this study, the effect of HBx expression on the early steps of liver regeneration was examined using ATX and WT mice in the 70% PH model. We predicted that HBx might alter PH induced liver regeneration, given its reported ability to induce cell cycle progression in cultured cells (reviewed refs. 16–18). Our results indicate that HBx affects early steps in liver regeneration, leading to a complex pattern of DNA synthesis. Liver tissues collected at varying sacrifice time points after PH showed evidence of premature cell cycle entry as assessed by several variables (BrdUrd incorporation, PCNA and mitotic index, and reduced steady-state p21 levels). There was no effect of HBx, however, on apoptosis, glycogen storage, or PH-induced steatosis. Together, these results show that HBx expression has the ability to induce cell cycle progression within the milieu of the regenerating liver.

Because the liver has many redundant pathways to facilitate regeneration (44), it is not surprising that the effects of HBx on regeneration were subtle. However, the expression of HBx in the current study caused a modest effect of premature cell cycle entry that was reproducible among animals of a given sacrifice time point. Although no single assay yielded a striking effect of HBx, it is significant that the effect of HBx in each assay consistently supported our conclusion that HBx induced premature cell cycle entry. Our conclusions are also consistent with the results from cell culture experiments indicating HBx can induce proliferation in cultured cells (16, 17).

The results of our study are in contrast to two other studies that concluded that HBx inhibited hepatocyte regeneration (30, 45). It is important to note that our analysis focused on time points within 48 hours post-PH, revealing effects of HBx that were missed in the design of those other studies (30, 45). In addition, differences in genetic background of the mice used may be important. For example, the HBx transgenic mice used in the study by Wu and colleagues (30) were on a C57BL/6 genetic background, and the WT mice in that study had steatosis that was worsened by HBx expression. It is known that steatosis alone may interfere with liver regeneration (29, 43), providing an explanation for why the mice in that study did not survive past 72 hours. In contrast, the outbred ICR background of the ATX mice showed mild PH-induced steatosis that was not worsened by HBx expression (Supplementary Fig. S2). A direct comparison of our study with the original report that HBx inhibits regeneration (45) is difficult because that study involved HBx hepatocytes transplanted into severe combined immunodeficient mice, and PH performed on 30-day-old HBx transgenic mice (compared with 10- to 12-week-old mice used in the present study). Finally, the HBx lineage studied by Wu and colleagues (30) developed HCC, whereas the ATX mice in our study do not develop spontaneous tumors (22), indicating some other underlying differences between these transgenic mouse models.

Our results suggest that HBx may act by driving a subpopulation of hepatocytes to prematurely enter the cell cycle. It is uncertain why all ATX hepatocytes do not behave in a synchronous fashion, but several possibilities may be considered. It is possible that HBx expression levels vary from cell-to-cell, and a threshold level of HBx is required to overcome a restriction point. Consistent with this idea, limited immunohistochemical (IHC) staining of ATX livers detected HBx in 26% of hepatocytes, with HBx-positive cells not restricted to a particular zone of the liver (data not shown). Another consideration is that not all hepatocytes are equal, and indeed hepatocyte function varies by location in the liver (46). Therefore, it is possible that HBx affects some hepatocytes but not others. This possibility is difficult to address experimentally, but we
do note that BrdUrd-positive hepatocytes were distributed evenly throughout the liver, similar to that of the BrdUrd-positive WT hepatocytes. Finally, it is intriguing to consider the possibility that HBx can prime G0 hepatocytes to move into G1, because ATX hepatocytes began entry into S phase (and M phase) ~4 hours earlier than hepatocytes from WT mice, consistent with the time needed to prime the liver after PH (4 hours; ref. 20).

There are several reasons why we believe the effect of HBx on cell cycle progression during liver regeneration is biologically relevant. Normal hepatocytes are typically nondividing and would have time to repair DNA adducts that form after exposure to carcinogens. However, if HBx-expressing cells are primed to enter the cell cycle, they may have less time to repair these lesions, leading to the formation of DNA mutations. This idea is supported by data showing the increased DNA mutation frequency in ATX carcinogens. However, if HBx-expressing cells are primed to enter the cell cycle prematurely, consistent with the time hepatocytes began entry into S phase (and M phase) 4 hours earlier than hepatocytes from WT mice, this would allow for the normal proliferative response during PH-induced liver regeneration (reviewed in refs. 19–21).

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Regeneration Regulatory HBx Protein during Compensatory Liver Regeneration

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