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The hepatitis B virus X gene potentiates c-*myc*-induced liver oncogenesis in transgenic mice

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The hepatitis B virus X protein (HBx) is thought to be implicated in the development of hepatocellular carcinoma, but its exact function remains controversial. Transgenic mice from PEX7 and AX16 lineages that express HBx in the liver under control of different viral regulatory elements develop no liver pathology (Billet *et al.*, 1995). We have crossed these two mouse lineages with WHV/c-*myc* oncomice in which liver-specific expression of c-*myc* driven by woodchuck hepatitis virus (WHV) regulatory sequences causes liver cancer in all animals. The average tumor latency was shortened by 2 to 3 months in bitransgenic animals from all populations compared with simple c-*myc* transgenic littermates. At preneoplastic stages, adult bitransgenic mice showed four to fivefold enhanced expression of the c-*myc* transgene, increased hepatocyte proliferation and more extensive liver lesions compared with simple WHV/c-*myc* transgenics. Thus in this model, HBx alone has no direct pathological effect but it is shown to accelerate tumor development induced by c-*myc*. The data presented here firmly establish the oncogenic potential of HBx, apparently acting as a tumor promoter. This model offers unique opportunities to investigate the mechanisms by which HBx trans-activates the expression of target genes and deregulates the hepatocyte growth control *in vivo*.

Keywords: hepatitis B virus; hepatocellular carcinoma; transactivation; oncogene; apoptosis

Introduction

Hepatitis B virus (HBV) is a major causative agent of acute and chronic hepatitis in humans worldwide. Despite overwhelming epidemiological evidence linking persistent HBV infection and the development of hepatocellular carcinoma (HCC) (Beasley *et al.*, 1981), the mechanisms by which the virus contributes to liver cell transformation remain elusive. Inspection of cloned HBV genomes revealed four overlapping reading frames conserved among different genotypes, that encode virion structural proteins (the surface and core antigens) and proteins implicated in viral replication (the DNA polymerase and the X transactivator protein, HBx). None of these viral gene products has proved to behave as a dominant oncogene, and it is generally agreed that HBV can neither directly nor acutely transform liver cells.

Studies of HBV-related animal viruses have strengthened the link between HBV and HCC and shed some light on the mechanisms operating in malignant conversion of infected hepatocytes (reviewed in Buendia, 1992). The woodchuck and ground squirrel hepatitis viruses have been associated with hepatocarcinogenesis in the rodent hosts (Marion *et al.*, 1986; Summers *et al.*, 1978). In contrast, the oncogenic potential of avian hepadnaviruses, that are devoid of an X open reading frame, has not been conclusively established (Duflot *et al.*, 1995). This led to suggest that X gene products from mammalian hepadnaviruses might play a role in liver carcinogenesis. During the last decade, seemingly contradictory data have accumulated on HBx function in natural infections. At least, two salient features of the hepadnavirus X gene products deserve consideration. On one hand, the requirement for a functional X gene in the establishment of viral infection in woodchucks has been firmly established (Chen *et al.*, 1993; Zoulim *et al.*, 1994), although the exact role of the X protein in viral replication is still unknown. On the other hand, HBx transcriptional trans-activation properties have been demonstrated on a variety of viral and cellular regulatory elements (Aufiero *et al.*, 1990; Spandau *et al.*, 1988; Twu *et al.*, 1987). Noticeably, trans-acting activity of HBx was rather weak (<10-fold activation) in most experimental systems used, and it has not been demonstrated so far in the context of the whole viral genome. HBx does not directly bind DNA and may stimulate gene expression by interacting with transcriptional factors or with elements of the basal transcription machinery (Cheong *et al.*, 1995; Haviv *et al.*, 1995; Maguire *et al.*, 1991; Qadri *et al.*, 1995; Unger *et al.*, 1990; Williams *et al.*, 1995). Other studies support an indirect effect of HBx by activating signalling pathways mediated by the Ras-Raf-MAPK cascade or by protein kinase C, a function consistent with the predominant cytoplasmic location of HBx *in vivo* and in most experimental systems (Benn *et al.*, 1994; Kekulé *et al.*, 1993; Natoli *et al.*, 1994).

Arguments favoring a significant role of HBx in liver cancer were provided by studies of HBV-related tumors and in experimental models. Integrated HBV sequences in tumor DNA are frequently interrupted between the viral direct repeats DR1 and DR2 (Nagaya *et al.*, 1987), generating 3′ truncated versions of the X gene that retain trans-activating capacity (Koshy *et al.*, 1991; Takada *et al.*, 1990; Wei *et al.*, 1995; Wollersheim *et al.*, 1988). Moreover, in HCC patients negative for the HBV surface antigen,

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accumulation of viral RNAs containing X but not surface or core sequences was evidenced by polymerase chain reaction (PCR) (Paterlini *et al*., 1995). This suggests that the integrated X gene may be important for maintaining the tumor phenotype at early stages of carcinogenesis. It has been reported that HBx binds the tumor suppressor p53 protein and inactivates its functions (Feitelson *et al*., 1993; Truant *et al*., 1995; Wang *et al*., 1994). Interaction of HBx with XAP-1, a cellular DNA repair protein homologous to the monkey u.v.-damaged DNA-binding protein, might affect cell ability to repair mutated DNA sequences and allow the accumulation of genetic changes (Lee *et al*., 1995). Another potential target of HBx was identified with XAPC7, a novel proteasome subunit; this interaction may be functionally relevant to the pleiotropic action of HBx (Huang *et al*., 1996). However, evidence for a direct oncogenic effect of HBx *in vivo* is scarce. HBx expression can transform rodent hepatocytes immortalized with the SV40 large T antigen (Höhne *et al*., 1990). In a transgenic mouse line generated in the outbred CD1 background, high level expression of HBx in the liver induced the progressive appearance of altered hepatocyte foci, proliferative adenomatous nodules, and malignant carcinomas that killed most male animals before 15 months of age (Kim *et al*., 1991). In contrast, a second mouse lineage with lower HBx expression developed liver tumors at the same rate as normal CD-1 mice (Koike *et al*., 1994), and other HBx transgenic lines generated in different mouse strains developed no obvious hepatic pathology, although the HBV X gene was expressed in liver cells and the HBx protein could be detected in some cases (Balsano *et al*., 1993; Billet *et al*., 1995; Lee *et al*., 1990; Perfumo *et al*., 1992; Yen, 1996). Similarly, the related X protein of WHV (WHx) had no apparent oncogenic potential by itself when expressed in transgenic mouse liver; it was shown to enhance the occurrence of preneoplastic lesions in mice treated with diethylnitrosamine (Dandri *et al*., 1996). These data suggest that the hepadnavirus X protein has no acutely transforming activity, but its overexpression in the susceptible CD-1 mouse strain might cooperate with activated cellular oncogene(s) in multistep hepatic transformation. Moreover, recent studies of the expression of the WHx protein in chronically infected woodchuck livers and in HCCs suggest that it may play a role at an early stage of the oncogenic process, but is not required to maintain a malignant phenotype (Dandri *et al*., 1996).

In this report, we have analysed the oncogenic potential of HBx by crossing transgenic mice from PEX7 and AX16 lineages that develop no pathology with WHV/c-*myc* transgenic mice highly predisposed to liver cancer. We find significant acceleration of the tumorigenic process, associated with upregulation of c-*myc* and increased hepatocyte turnover in the livers of bitransgenic animals.

Results

HBV X gene expression in PEX7 and AX16 mice

Transgenic mice expressing HBX mRNA in the liver (designated PEX7 and AX16) have been described (Balsano *et al*., 1993; Billet *et al*., 1995). Neither of these transgenic lines have developed any pathology over two years of observation. A schematic representation of the PEX7 and AX16 transgenes and the RNAs produced from multiple transgene copies inserted in tandem array is given in Figure 1a. Abundant X-specific mRNAs of 0.8 and 1.8 kb were detected in the livers of PEX7 and AX16 mice during the perinatal period by Northern hybridization of total liver RNA with a HBX probe (Figure 1b). In a previous report, transcription was shown to initiate preferentially at the X promoter in PEX7 mice, and curiously, at the HBV

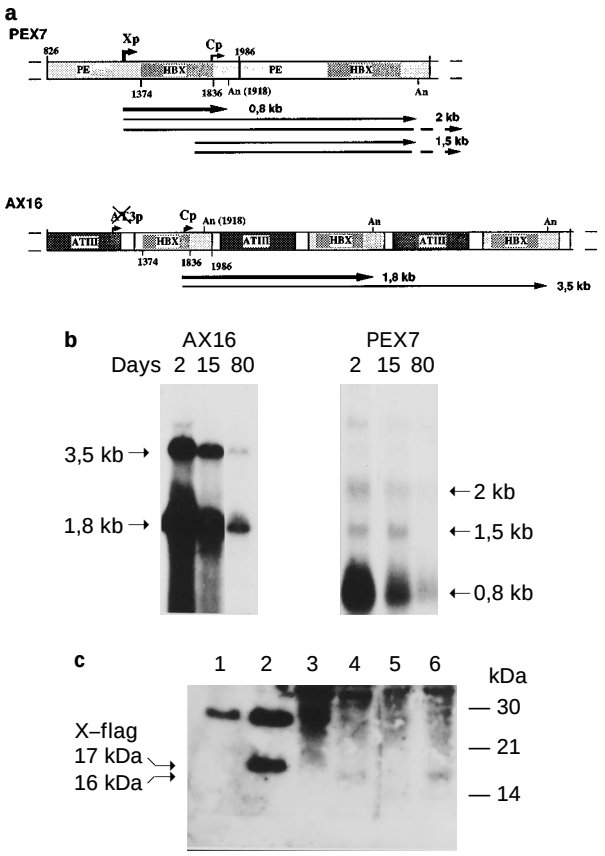

Figure 1 (**a**) Structure of the PEX7 and AX16 transgenes in HBX mice. Plasmid constructs are as described in Billet *et al*., 1995 and in Materials and methods. PE, promoter/enhancer of the HBV X gene; HBX, HBV X open reading frame; ATIII, antithrombin III regulatory region; Xp, promoter of the HBV X gene; Cp, promoter of the HBV core gene; AT3p, antithrombin III promoter; An, HBV polyadenylation site. Nucleotide positions on the HBV genome are numbered according to (Galibert *et al*., 1979). Transcripts arising from multiple copies of the transgenes integrated in tandem array are depicted under the corresponding DNA region (Billet *et al*., 1995). (**b**) Northern analysis of HBX transgene expression during post-natal liver development. Twenty μg of total RNA isolated from AX16 and PEX7 livers at different ages as indicated were separated on agarose gels, transferred onto nylon membranes and hybridized with a X-specific probe. Loading of equivalent amounts of RNA was controlled by ethidium bromide staining of ribosomal RNA. (**c**) Western blot showing weak expression of the HBx protein in 10-day-old transgenic livers. Western blotting was performed on total cell extracts prepared from CCL13 cells (lane 1), CCL13 cells transiently transfected with a HBX/flag fusion gene driven by the CMV promoter (lane 2), and from the livers of 10-day-old PEX7 transgenic mice (lanes 4 and 6) and nontransgenic littermates (lanes 3 and 5) as described in Materials and methods. The blot was probed with a polyclonal anti-HBx antibody and revealed using alkaline phosphatase-conjugated secondary antibodies and Western-star chemiluminescence reagents

core gene promoter in AX16 mice, in which the ATIII promoter remained silent (Billet *et al*., 1995). Minor RNA species of larger sizes resulted from leaky transcription termination at the HBV polyA signal. In mouse livers from both lineages, the levels of X mRNAs gradually declined as the animals aged, reaching very low levels at the adult stage (Figure 1b). By Western blotting of total liver cell proteins, a polypeptide of 16 kDa was detected at low levels in 10-day-old PEX7 and AX16 mouse livers, but not in adult transgenic mice nor in nontransgenic controls, using a polyclonal antiserum raised against the HBx antigen (Kay *et al*., 1991), as illustrated for PEX7 mice in Figure 1c. A normal rabbit serum revealed no signal at the corresponding position (data not shown). For comparison, high level expression of HBx/flag fusion protein was observed in CCL13 cells transiently transfected with a CMV/HBX/flag vector (C Transy, unpublished) (Figure 1c, lane 2).

*Liver tumors develop more rapidly in WHV/c-*myc *mice when crossed with HBX transgenics*

In other published lineages that similarly develop no spontaneous malignancy, HBx was shown to increase cellular susceptibility to chemical hepatocarcinogens (Slagle *et al*., 1996 and M Tripodi, unpublished results), suggesting that HBx may act as a co-factor in liver oncogenesis. This led us to investigate whether HBx could cooperate with a dominant oncogene in hepatocarcinogenesis. Recently, we generated transgenic mice carrying the c-*myc* gene and nearby integrated WHV sequences isolated from a woodchuck HCC (Etiemble *et al*., 1994). In two different lineages (93-7 and 93-10), liver tumors developed almost invariably, despite temporally limited expression of the woodchuck c-*myc* transgene in the neonatal liver. Previously noted differences in tumor kinetics between the 93-7 and 93-10 lineages (average 8 months *versus* 12 months) have been attributed to higher copy number and additional rearrangements of the transgene in the former.

Transgenic mice from the AX16 and PEX7 lineages were crossed with 93-10 WHV/c-*myc* mice, and PEX7 mice were crossed with 93-7 mice. Although the parental lineages were maintained mostly through backcrosses with C57BL/6 inbred mice, slightly divergent genetic backgrounds (see Materials and methods) might interfere with individual susceptibility to cancer development. We therefore set up crossing experiments between heterozygous carriers of HBx and c-*myc* transgenes from three different generations to produce offspring either heterozygous for one or two transgenes, or nontransgenic. All animals were examined by weekly palpation and sacrificed when presenting clinical signs of liver tumors. As shown in Figure 2, HCCs were detected earlier in bitransgenic animals carrying either PEX7 or AX16 transgenes than in simple WHV/c-*myc* littermates. All bitransgenic animals developed large HCCs within less than 60 weeks. Fifty percent of PEX7 × 93-10 and AX16 × 93-10 bitransgenic animals developed liver tumors by 38 weeks of age (T_{50}) (compared with simple 93-10 mice in which $T_{50} = 48$ and 52 weeks). Similarly, the T_{50} of PEX7 × 93-7 bitransgenics was 33 weeks, while that of simple 93-7 mice was 41 weeks (Figure 2c). Thus, HCC formation was accelerated to a similar extent (by 8 to 14 weeks) in WHV/c-*myc* animals carrying the PEX7 and AX16 transgenes. These data were found to be statistically significant ($P < 0.05$) in each bitransgenic group by using the Mann–Whitney's non-parametric test.

Importantly, the population of WHV/c-*myc* mice that did not carry an HBX transgene developed tumors at the same rate as the parental population (Etiemble *et al*., 1994). Therefore, breeding with PEX7 or AX16 mice did not modify the susceptibility of WHV/c-*myc* mice to HCC development, which clearly implicates the HBX transgenes in the observed acceleration of tumor formation. Whether earlier detection of advanced stage

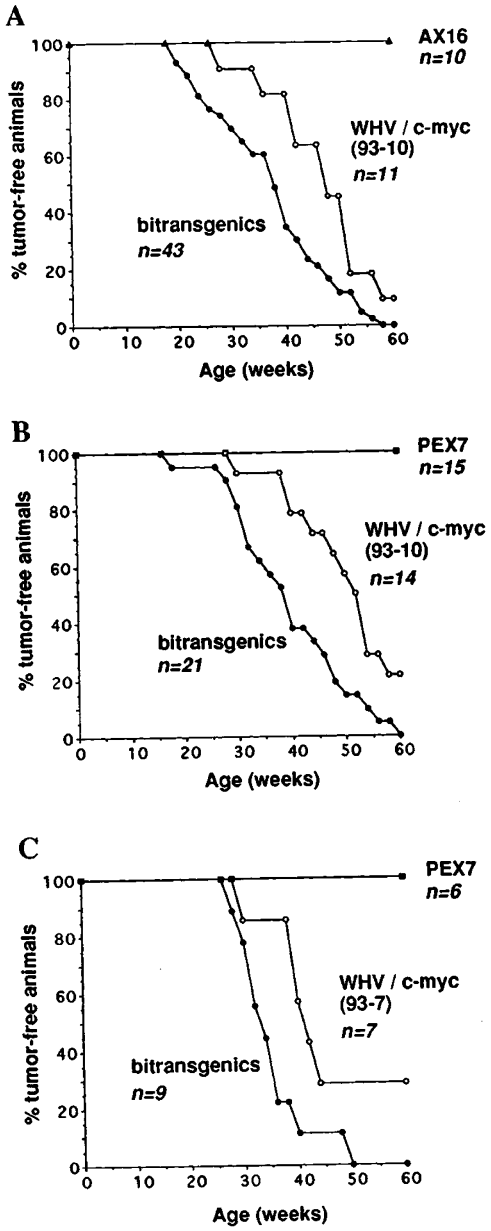

Figure 2 Kinetics of tumor occurrence in HBX × WHV/c-*myc* bitransgenic mice. The percentage of tumor-free animals as a function of age is shown in (**a**) for mice resulting from crossings between AX16 and WHV/c-*myc* 93-10 mice, in (**b**) for PEX7 × WHV/c-*myc* 93-10 mice, and in (**c**) for PEX7 × WHV/c-*myc* 93-7 mice. Animals were sacrificed when they became terminally ill and post-mortem examination was carried out. *n* is the number of animals in each population

tumors in dual-positive animals reflected earlier tumor onset or increased proliferation of neoplastic cells was not determined. Liver tumors, predominantly well differentiated HCCs of the trabecular type, were indistinguishable from those previously described in the parental WHV/c-*myc* lineages (Etiemble *et al*., 1994), and none of the animals examined showed other neoplasms. Moreover, Southern blot analysis of liver and tumor DNAs from simple and double-transgenic animals did not reveal any gross rearrangement of the WHV/c-*myc* transgene (data not shown). Finally, consistent with the largely predominant C57BL/6 background of all lineages, none of the nontransgenic animals analysed in the present study ($n=35$) developed any type of cancer over 2 years of observation.

*Activation of WHV/c-*myc *transgene expression in adult bitransgenic mice*

In WHV/c-*myc* mice, liver-specific expression of the transgene gives rise to two different transcripts: a normal 2.5 kb c-*myc* mRNA driven by the c-*myc* P1 promoter, and a 1.8 kb WHV transcript initiated at a viral preS2/S promoter (Wei *et al*., 1996) (Figure 3a). Expression of the WHV/c-*myc* transgene was assessed in PEX7 × 93-10 double transgenic and in 93-10 simple transgenic littermates at different ages by Northern hybridization of total liver RNA with a woodchuck c-*myc* exon 1 probe, used under stringent hybridization conditions, and with WHV DNA. As shown in Figure 3b, high steady-state levels of woodchuck c-*myc* and WHV mRNAs were detected during the first month of birth in WHV/c-*myc* transgenics. These mRNAs were produced in a strictly co-regulated fashion suggesting a predominant control of viral regulatory sequences. The specificity of the woodchuck c-*myc* exon 1 probe was confirmed by hybridization of the blots with the corresponding murine c-*myc* exon 1 probe, which detected very low levels of endogenous c-*myc* mRNAs (data not shown). HBx expression had no detectable effect on WHV/c-*myc* transgene expression in the neonatal liver (Figure 3b). At older ages, transgene expression was strongly down-regulated in simple WHV/c-*myc* transgenics as described previously (Etiemble *et al*., 1994). In contrast, bigenic animals at 60 and 80 days of age still expressed WHV and c-*myc* transcripts at detectable levels (Figure 3b and c). Quantitative analysis of the hybridization signals indicated that both c-*myc* and WHV-specific mRNAs were elevated by four to fivefold in adult bigenic mice compared with simple WHV/c-*myc* littermates. Significantly, these data were reproducibly observed when 80-day-old animal pairs from six different litters were analysed in parallel (Figure 3c). At this age, X mRNAs were produced at low levels in PEX7 and AX16 mice (see Figure 1b) as well as in double-transgenic animals (data not shown).

Increased cell proliferation and morphological alterations in bitransgenic mouse livers at preneoplastic stages

To better define the effects of HBx expression at preneoplastic stages, the rates of hepatocyte proliferation and apoptosis were assessed in simple WHV/c-*myc* and double transgenic mouse livers at different ages. We

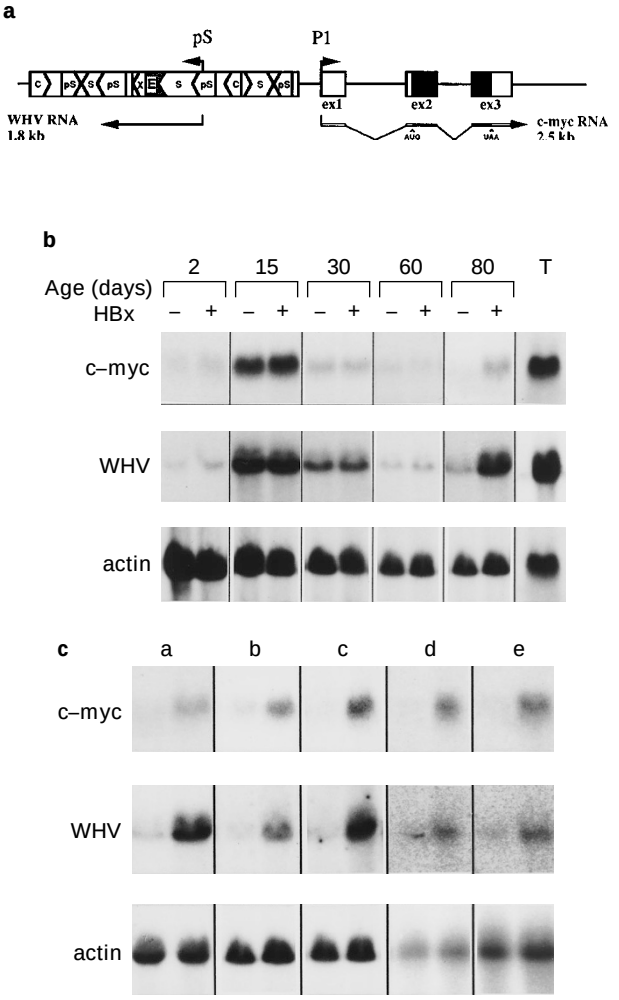

Figure 3 (**a**) Schematic structure of the WHV/c-*myc* transgene. The genes of the rearranged viral insert are indicated (C, core antigen gene; S, major surface antigen gene; pS, preS region). X denotes a 65 bp long fragment of the X gene, and viral enhancer I sequences are designated E. (**b**) Northern blot analysis of total RNA from livers of WHV/c-*myc* 93-10 mice (lanes −) and from PEX7 × 93-10 mice (lanes +) at the indicated ages. Simple and bitransgenic littermate pairs were analysed in parallel. T, liver tumor RNA from a 93-10 mouse. Blots were probed successively with woodchuck c-*myc* exon 1, total WHV genome DNA and a mouse actin cDNA. (**c**) Activation of the WHV/c-*myc* transgene expression in adult bitransgenic mice. Northern analysis was performed on total liver RNA from paired simple and bitransgenic littermates from six different litters (denoted a–e) at 80 days of age

determined the proliferation index by counting BrdU-labeled S-phase hepatocytes, and the rate of cells undergoing cell death by *in situ* DNA nick end-labeling (Gavrieli *et al*., 1992). As shown in Figure 4a, proliferation of liver cells was twofold higher in 10 day-old WHV/c-*myc* transgenics than in nontransgenic littermates (mean values: $20.2\pm5.5\%$ *vs* $11.3\pm1.7\%$, $P<0.05$), and reached approximately the same value ($18.2\pm4.5\%$, $P<0.01$) in PEX7 × 93-10 bigenic animals. As the animals aged, hepatocyte proliferation decreased progressively in all populations but remained significantly higher in simple c-*myc* and bitransgenic animals than in nontransgenic controls. At 80 days, liver cell proliferation was three times higher in WHV/c-*myc* mice than in controls (mean values: $1.7\pm0.7\%$ *vs* $0.5\pm0.1\%$, $P<0.05$) (Figure 4d). The rate of BrdU-positive cells was further increased by twofold ($2.9\pm1.2\%$) in PEX7 × 93-

10 bitransgenics (Figure 4a and e). These differences were found to be statistically significant. Thus, the effects of combined expression of c-*myc* and HBx in adult mouse liver resulted in sixfold activation of hepatocyte proliferation in bitransgenic animals.

Investigation of cells undergoing cell death by the TUNEL method showed a slight tendency toward elevated rates of hepatocyte apoptosis in simple WHV/c-*myc* transgenics compared with nontransgenic controls, particularly during the neonatal period (mean values at 10 days: $0.35 \pm 0.05\%$ *vs* $0.21 \pm 0.07\%$, $P < 0.05$) (Figure 4b). Only fluorescent hepatocytes

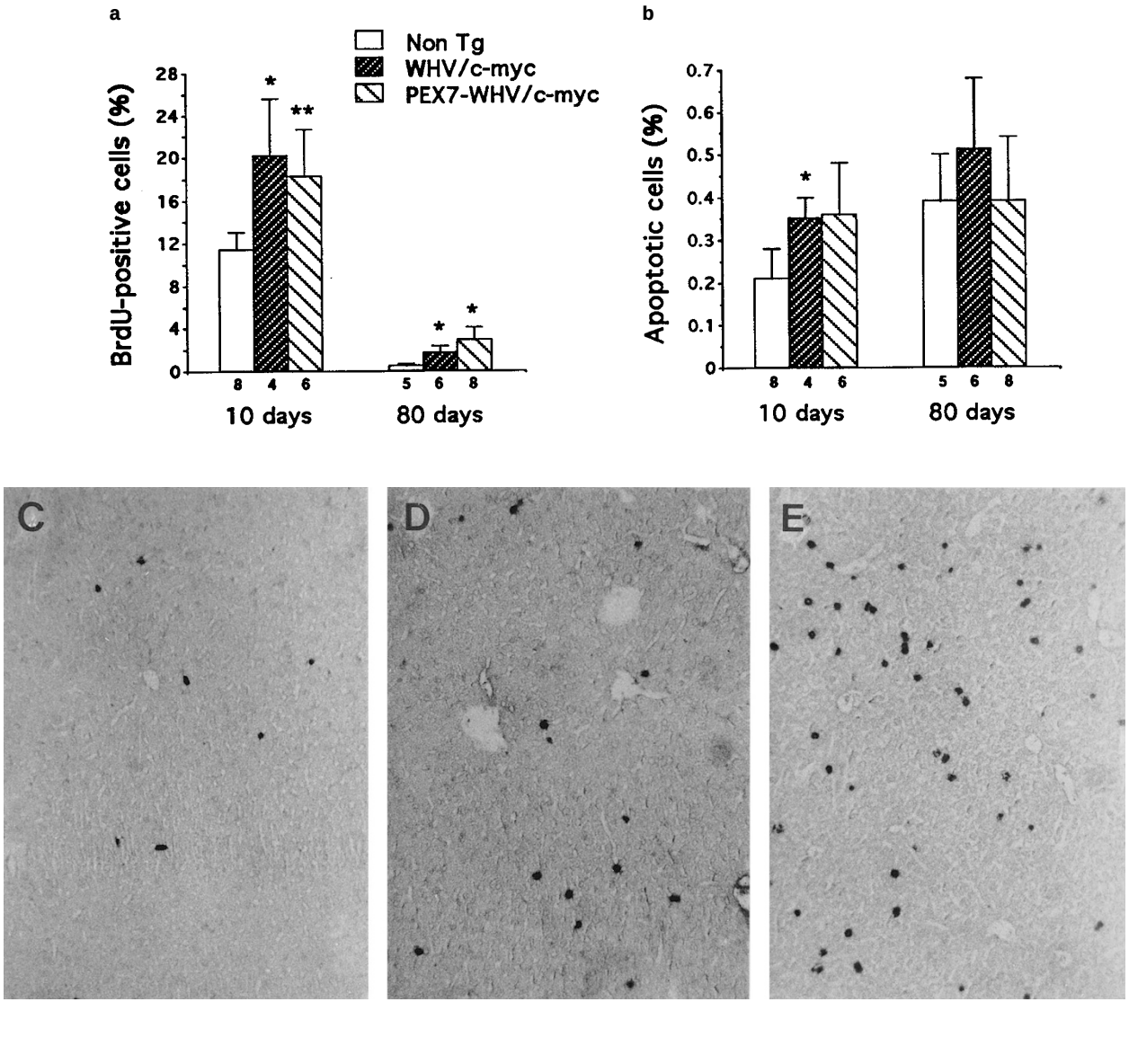

Figure 4 Temporal changes of hepatocyte turnover in WHV/c-*myc* 93-10 mice and PEX7 × 93-10 transgenic mice. (**a**) S-phase labeling index, (**b**) percentage of apoptotic cells detected by *in situ* DNA nick end-labeling and microscopic examination of 2000 hepatocytes. Error bars represent SE of the mean. Numbers at the base of each bar represent the number of mice examined in each group. Asterisks denote significant difference between transgenic and nontransgenic populations. ($^{*}P < 0.05$, $^{**}P < 0.01$). Immunohistochemical detection of S-phase hepatocytes in (**c**): a nontransgenic mouse, (**d**): a WHV/c-*myc* 93-10 transgenic mouse, and (**e**): a PEX7 × 93-10 bitransgenic mouse at 80 days of age. BrdU-positive nuclei are black. Non-labeled nuclei were counterstained with hematoxylin (× 100). (**f**) Representative nuclear staining in an apoptotic hepatocyte by *in situ* DNA nick end labeling. Visualization of the cellular morphology was obtained by superimposing u.v. and light pictures (× 1000)

showing simultaneous nuclear staining and apoptotic morphology were counted, as shown in Figure 4f. The percentage of apoptotic cells was not further increased ($0.36 \pm 0.12\%$) in the livers of PEX7 × 93-10 bigenic neonates. At the adult stage, no significant variation was detected in our assays in the rate of apoptotic hepatocytes among the different groups (0.4 to 0.5%, Figure 4b). We also compared the liver/body weight ratios of WHV/c-*myc* mice and PEX7 × WHV/c-*myc* bitransgenics at different ages. A reproducible increase

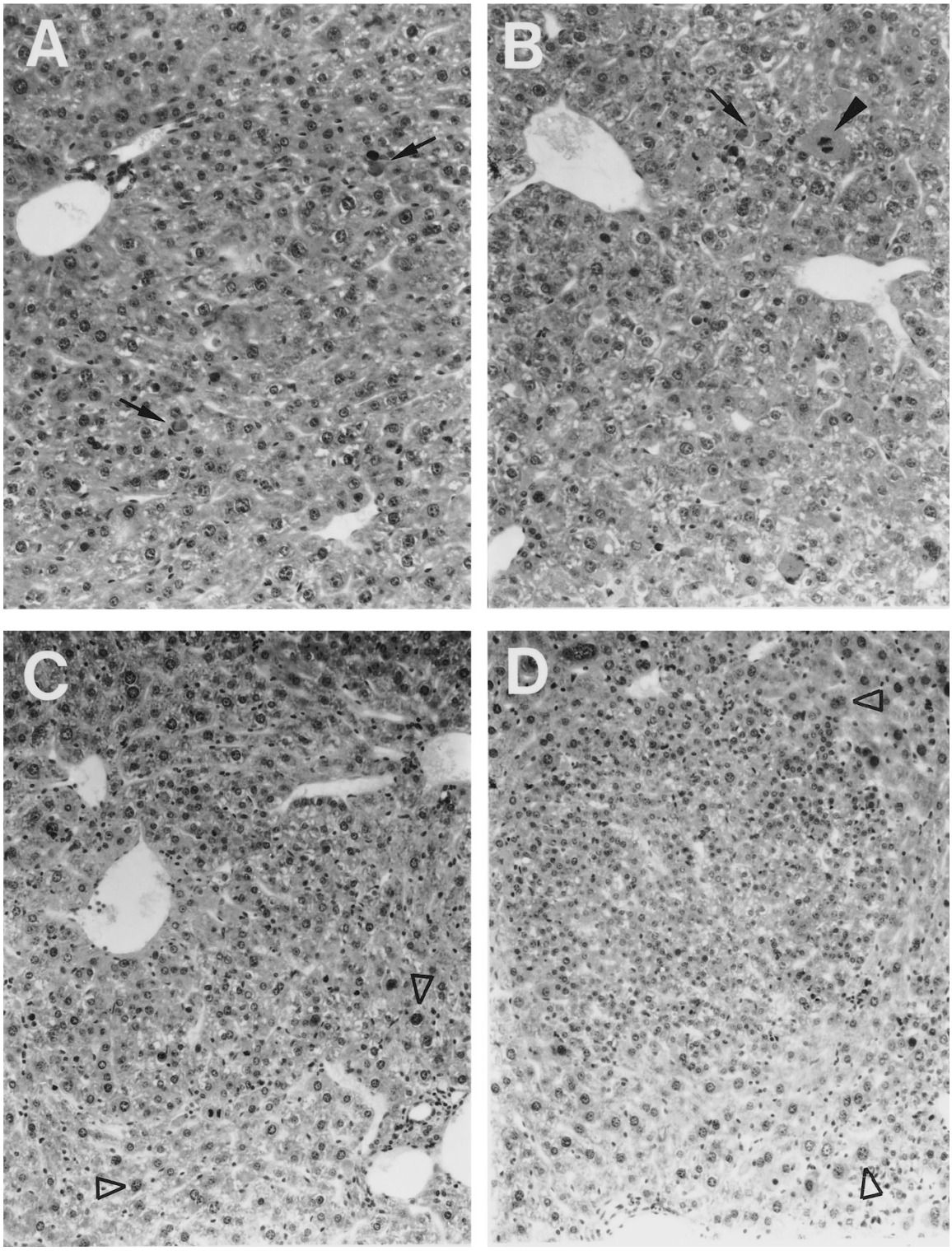

Figure 5 Histopathology of WHV/c-*myc* and PEX7 × WHV/c-*myc* transgenic mouse liver. (**a**) Liver from a 30 day-old WHV/c-*myc* mouse (lineage 93-10) showing normal liver architecture. Note the uniform size and arrangement of hepatocytes and the presence of apoptotic bodies (arrows). H&E, ×100. (**b**) Liver from a 30 day-old PEX7 × WHV/c-*myc* mouse. Numerous mitotic figures (arrowheads) and apoptotic bodies (arrows) are present. (**c**) Liver from a 80 day-old WHV/c-*myc* mouse. Note the disarrayed hepatocytes of variable size. Huge dysplastic cells are marked by an open triangle. Few mitosis and apoptotic cells are present. (**d**) Liver from a 80 day-old PEX × WHV/c-*myc* mouse, showing disrupted hepatic architecture and enhanced rate of mitotic and apoptotic figures. A hyperplastic nodule is present in the center, causing mild compression of surrounding dysplastic hepatocytes

(about 20%) in the liver/body weight ratio of transgenic compared with nontransgenic animals was seen in both cases at all ages tested (data not shown). This suggests that proliferative pathways are predominant over apoptotic pathways in these livers.

Histological examination of liver sections from WHV/c-*myc* monotransgenic and X/*myc* bitransgenic animals revealed no gross abnormality in liver architecture before 30 days, although a marked increase in mitotic figures and apoptotic bodies was noted (Figure 5a and b). At older ages, significant differences were observed in the extent and severity of liver lesions between simple and double transgenic mice. At 80 days, hyperplastic nodules could be seen in double transgenic mice whereas only anisocaryosis, anisocytosis, mitosis and apoptotic bodies were seen in WHV/c-*myc* animals (Figure 5c and d). A comparison of the lesions per unit cross-sectional liver area revealed that they increased more rapidly in double transgenic mice. This increase could result from enhanced initiation of foci or the more rapid growth of these foci, making their identification easier.

Discussion

Transgenic mice have been instrumental in demonstrating the requirement for cooperative oncogenic events in cell transformation (Adams *et al*., 1991). Liver tumors usually occur after a long latency period in mice carrying a c-*myc* transgene, making them ideal tools in crossing experiments with mice bearing potentially cooperating genes. A representative example is the synergistic action of c-*myc* and transforming growth factor α (TGFα) in hepatic oncogenesis (Murakami *et al*., 1993; Sandgren *et al*., 1993). To characterize the effects of the HBV X protein during multistage carcinogenesis, we have crossed HBX transgenic mice with WHV/c-*myc* mice and examined tumor development in liver of dual-positive animals. The data presented here demonstrate that coexpression of the HBV X gene with c-*myc* induced a more rapid development of liver tumors than did expression of c-*myc* alone, thereby reducing the host life span up to 25%. These findings clearly attribute a role to HBx in liver carcinogenesis.

The fact that HBx by itself was largely nononcogenic in PEX7 and AX16 transgenic mice confirms previous reports showing that transient or low level expression of the X gene was insufficient to induce hepatic oncogenesis in transgenic animals (Dandri *et al*., 1996; Koike *et al*., 1994; Lee *et al*., 1990; Perfumo *et al*., 1992). In our model, cooperation with a cellular oncogene was required to reveal the oncogenicity of HBx. Similarly, the transforming potential of different cellular and viral genes, like cyclin D1, Tal1 and the tax gene of HTLV1, only came to light when co-expressed with a dominant oncogene in double transgenic animals (Benvenistry *et al*., 1992; Larson *et al*., 1996; Lovec *et al*., 1994). The notion that HBx acts as a tumor promoter in liver oncogenesis was first suggested by its effects on mouse liver susceptibility to chemical hepatocarcinogens (Slagle *et al*., 1996 and M Tripodi, unpublished results). It is further supported by the present finding that combined expression of the HBX and c-*myc* genes produced a marked growth response in the entire liver together with extensive hepatic lesions at preneoplastic stages. These effects of HBx might result either from direct or indirect activation of the WHV/c-*myc* transgene expression, or from an independent pathway leading to altered hepatocyte turnover.

The coordinated increase of woodchuck c-*myc* and WHV RNA levels in adult livers from double transgenic mice may reflect a direct, trans-acting action of HBx on a common regulatory element controlling both mRNA species. In the absence of the potent WHV enhancer II in the WHV/c-*myc* transgene, one candidate element lies in the region homologous to the HBV enhancer I. This WHV domain carries a 33 bp sequence perfectly conserved between mammalian hepadnaviruses, comprising a composite binding area for transcription factors from the CREB/ATF, C/EBP, and AP1 families, and a strong X-responsive element (Faktor *et al*., 1990a,b; Unger *et al*., 1990). The strict conservation of this motif between HBV and WHV genomes suggests that HBx might be active as well as the WHV enhancer; interspecies activity of the hepadnaviral X proteins has been previously demonstrated in transient transfection assays (Colgrove *et al*., 1989; Wei *et al*., 1995). It has been shown that HBx interacts with and increases the affinity of CREB for its cognate binding site in the HBV enhancer I (Maguire *et al*., 1991; Williams *et al*., 1995). This effect presents similarities with that of the transcriptional activator tax on the long terminal repeat of HTLV1 (Kwok *et al*., 1996). It is plausible, therefore, that HBx might activate WHV regulatory sequences through a cAMP-regulated enhancer element, in turn activating expression of the WHV/c-*myc* transgene in the adult, but not in the neonatal liver. In previous studies, the transcriptional trans-activation properties of HBx *in vivo* were demonstrated in AX16 mice using an HIV LTR-βgalactosidase reporter (Balsano *et al*., 1993).

Alternatively, HBx might influence tumor development by more indirect ways, and enhanced expression of the c-*myc* transgene in bitransgenic livers might be linked to marked preneoplastic changes observed in adult animals. Seemingly contradictory effects of HBx were recently reported: activation of cell cycle progression by deregulating checkpoint controls (Benn *et al*., 1995), inhibition of apoptosis through interaction with p53 (Wang *et al*., 1995) and induction of apoptosis by activating the Ras pathway (Yen, 1996). Interestingly, some cellular genes such as c-*myc* may trigger either proliferative or apoptotic responses, depending on the cellular context (Harrington *et al*., 1994). Here we show that expression of c-*myc* in the liver of simple WHV/c-*myc* transgenics not only promotes cell proliferation, but also slightly increases the rate of hepatocyte death. It will be interesting to determine whether HBx interferes with one or the other c-*myc*-mediated pathway. Studies of the hepatocyte turnover in PEX7 and AX16 transgenic mice and of the cellular effectors of HBx action would help to clarify the mechanisms by which HBx sensitizes liver cells to malignant transformation.

The role of HBx in the development of liver cancer in HBV-infected patients is a debated question. Previous studies of the X gene function *in vitro* have shown its

ability to activate the Ras-Raf-MAP kinase cascade and to trans-activate cellular genes implicated in growth control (Avantaggiati *et al*., 1993; Balsano *et al*., 1991; Benn *et al*., 1994, 1996; Twu *et al*., 1993). In human hepatocarcinogenesis, overexpression of c-*myc* is commonly observed both at tumoral and pretumoral stages, whereas c-*myc* expression is barely detectable in the normal adult liver (Gu *et al*., 1986; Himeno *et al*., 1988; Zhang *et al*., 1990). Our evidence that co-expression of the HBx and c-*myc* transgenes accelerated HCC development in transgenic mice clearly establishes the viral transactivator as a tumor promoter and as a cooperating partner of the c-*myc* oncogene in liver cell transformation. This and other studies of transgenic models provide a basis to define the contribution of HBx in altered gene expression and deregulated cell growth during multistage carcinogenesis.

Materials and methods

Experimental animals

AX16 and PEX7 transgenic mice carry the HBV X open reading frame as described (Balsano *et al*., 1993; Billet *et al*., 1995). The chimeric 1.7 kb AX transgene contains human antithrombin III regulatory sequences (R allele of the ATIII gene) linked to the X coding sequence from HBVayw and the HBV polyadenylation signal. The PEX transgene was a 1172 bp AccI–BglII fragment of the HBVayw genome, spanning the enhancer I/X promoter, the X open reading frame, and the viral polyadenylation signal (see Figure 1a). Both lineages were produced on a C57Bl/6 × DBA2 hybrid background. WHV/c-*myc* transgenic mouse lineages 93-7 and 93-10 were described (Etiemble *et al*., 1994). Briefly, the transgene, a 14 kb DNA fragment spanning the entire c-*myc* gene and adjacent integrated WHV sequences cloned from a woodchuck HCC (see Figure 3a), was microinjected in C57Bl/6 × SJL/J hybrid embryos. Transgenic founders and progeny were backcrossed for ten or more generations with either inbred C57Bl/6 mice or transgenic littermates. Transgenic mice were identified by hybridization of tail biopsy DNA with transgene-specific probes. For X transgenics, the probe was derived from a PCR fragment spanning 420 bp of the HBVayw X open reading frame (positions 1375–1795 on the HBV map) (Galibert *et al*., 1979). For WHV/c-*myc* mice, we used a PCR fragment covering 320 bp of the WHV genome (nt 1158–1478) (Galibert *et al*., 1982). Animals were handled and euthanized according to French government guidelines (Ministère de l'Agriculture, Services Vétérinaires de la Santé et de la Production Animale).

Liver examination

Mice were examined at weekly intervals and sacrificed at different ages (days 10, 20, 30 and 80 after birth) for biological and histological analyses, or at later stages when abdominal broadening provided evidence of liver tumors. Complete autopsies were performed, and gross and microscopic evaluations were done. For histological examination, liver specimens were fixed in Bouin's fluid, embedded in picolyte-paraplast, sectioned at 5 μm and stained with hematoxilin-eosin.

In vivo *labeling of S-phase hepatocytes*

5-bromo-2′-deoxyuridine (BrdU) was used for non-isotopic S-phase labeling of hepatocytes. BrdU (Sigma Chemical Co) was dissolved in sterile phosphate buffered saline (PBS) and injected intraperitoneally 2 h before sacrifice at a dose of 100 mg/kg. At sacrifice, total mouse bodies and isolated livers were weighted, and liver lobes were fixed in 4% paraformaldehyde (Flucka), then in ethanolamine 0.1 M pH 7.4 (Flucka) and embedded in paraffin. Five μm thick sections were first treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity, and then digested with 0.1% trypsin in 0.2 M TrisHCl pH 8 for 10 min at 37°C. A mouse monoclonal antibody against BrdU (Immunoglobulin G, Pharmingen, San Diego, CA) was applied at 1 μg/ml for 2 h at room temperature. The primary antibody was then detected by a sheep anti-mouse biotinylated secondary antibody (Large Volume DAKO LSAB Kit, Dako, Carpinteria, CA) according to manufacturer's instructions. Metal-enhanced 3,3′-diaminobenzidine was used as the final color-reaction product. Cell nuclei were counterstained with hematoxylin.

Detection of apoptotic hepatocytes

Liver sections fixed as described above were first treated with 20 μg/ml proteinase K for 15 min at room temperature. 3′-OH DNA termini were nick-end labeled using 1 unit of terminal deoxynucleotidyl transferase and 1.5 nmol of biotin-16-dUTP in 50 μl TdT buffer, for 1 h at 37°C ('TUNEL' method; all reagents were supplied by Boehringer Mannheim Biochemical). Slides were incubated for 15 min at room temperature in terminal buffer (300 mM NaCl, 30 mM sodium citrate, RT), rinsed in H_2O and then saturated for 10 min in 2% BSA. Samples were stained in the dark with 2% ExtrAvidin-TRITC conjugate for 40 min at room temperature (Sigma Chemical Co, France). Finally, slides were rinsed in H_2O and nuclei were stained with hematoxylin before mounting.

Quantitative morphometry

The slides were observed with a conventional light and u.v. Leitz microscope (original magnification × 400 or × 1000 under oil immersion). For S-phase quantification, 10 consecutive optic fields were counted for each liver section (about 1000 hepatocytes). For apoptotic quantification, 20 consecutive optic fields were counted. Only the fluorescent signals showing a typical morphology of apoptotic hepatocytes were selected. The S-phase labelling rate was expressed as the percentage of total hepatocytes containing BrdU-positive nuclei. The apoptotic rate was expressed as the percentage of total hepatocytes containing stained nuclei. Data were analysed statistically using the Kruskall–Wallis or Mann–Whitney's non-parametric tests. A level of 5% probability was considered statistically significant. All values are given as mean ± s.d.

DNA and RNA analysis

Frozen liver samples were crushed in liquid nitrogen and used for DNA or RNA extraction as described previously (Wei *et al*., 1992). For Southern blot analysis, total genomic DNA (20 μg) was digested with NcoI, separated on 0.8% agarose gels and transferred to nylon membranes (Hybond N^+, Amersham) in 0.4 N NaOH. For Northern blot analysis, total RNA (20 μg) was denatured with glyoxal and dimethylsulfoxyde, electrophoresed through 1% agarose gels, and transferred onto Hybond N^+ in 0.05 N NaOH. The integrity and relative amounts of RNA were checked by ethidium bromide staining of ribosomal RNAs. Hybridizations of Southern and Northern blots were carried out according to (Church *et al*., 1984), using probes labeled by random priming. The following probes were used: cloned WHV DNA (Ogston *et al*., 1982), a 1.4 kb BglII fragment specific for woodchuck c-*myc* exon 1 (Möröy *et al*., 1985), a murine c-*myc* exon 1 probe (Stanton *et al*., 1984), a PCR fragment spanning 420 bp

of the HBVayw X open reading frame (positions 1375–1795 on the HBV map) (Galibert *et al*., 1979), and a mouse actin cDNA. After hybridization with the c-*myc* probes, filters were washed under conditions of high stringency (0.1 × SSC, 0.1% SDS, 65°C for 1 h). Labeled bands were quantified directly with a PhosphoImager (Molecular Dynamics), and standardized with the actin probe. The blots were exposed to XAR-5 films (Kodak) at −80°C with intensifying screens.

Western blot analysis

Total cell extracts were prepared from AX16 and PEX7 transgenic and nontransgenic livers as previously described (Etiemble *et al*., 1994). Briefly, fresh liver tissues were homogenized in protein lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptitine and pepstatin). Cell lysates prepared by the same method from CCL13 cells were kindly provided by C Transy and S Pringent. Twenty μg of proteins were subjected to electrophoresis through 16.5% acrylamide gels, and the resolved proteins were transferred to a Hybond C extra membrane (Amersham) using the Transblot semi-dry apparatus (Biorad). The HBx protein was detected with a polyclonal anti-X serum (1 : 3000 dilution; a kind gift of A Kay) using the Western-star chemiluminescent detection system (Tropix) according to manufacturer's instructions.

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References

Adams JM and Cory S. (1991). *Science*, **254,** 1161–1167.
Aufiero B and Schneider RJ. (1990). *EMBO J*., **9,** 497–504.
Avantaggiati ML, Natoli G, Balsano C, Chirillo P, Artini M, Marzio ED, Collepardo D and Levrero M. (1993). *Oncogene*, **8,** 1567–1574.
Balsano C, Avantaggiati ML, Natoli G, De Marzio E, Will H, Perricaudet M and Levrero M. (1991). *Biochem. Biophys. Res. Comm*., **176,** 985–992.
Balsano C, Billet O, Bennoun M, Cavard C, Zider A, Grimber G, Natoli G, Briand P and Levrero M. (1993). *Arch. Virol*., **8,** 63–71.
Beasley RP, Lin CC, Hwang LY and Chien CS. (1981). *Lancet*, **2,** 1129–1133.
Benn J and Scheider J. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 10350–10354.
Benn J and Schneider RJ. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 11215–11219.
Benn J, Su F, Doria M and Schneider RJ. (1996). *J. Virol*., **70,** 4978–4985.
Benvenistry N, Ornitz DM, Bennet GL, Sahagan BG, Kuo A, Cardiff RD and Leder P. (1992). *Oncogene*, **7,** 2399–2405.
Billet O, Grimber G, Levrero M, Seye KA, Briand P and Joulin V. (1995). *J. Virol*., **69,** 5912–5916.
Buendia MA. (1992). *Adv. Cancer Res*., **59,** 167–226.
Chen HS, Kanako S, Girones R, Anderson RW, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, Purcell RH and Miller RH. (1993). *J. Virol*., **67,** 1218–1226.
Cheong J, Yi M, Lin Y and Murakami S. (1995). *EMBO*, **14,** 143–150.
Church G and Gilbert W. (1984). *Proc. Natl. Acad. Sci. USA*, **81,** 1991–1995.
Colgrove R, Simon G and Ganem D. (1989). *J. Virol*., **63,** 4019–4026.
Dandri M, Schirmacher P and Rogler CE. (1996). *J. Virol*., **70,** 5246–5254.
Duflot A, Mehrotra R, Yu SZ, Barraud L, Trepo C and Cova L. (1995). *Hepatology*, **21,** 1483–1491.
Etiemble J, Degott C, Renard CA, Fourel G, Shamoon B, Vitvitski-Trépo L, Hsu TY, Tiollais P, Babinet C and Buendia MA. (1994). *Oncogene*, **9,** 727–737.
Faktor O, Budlovsky S, Ben-Levy R and Shaul Y. (1990a). *J. Virol*., **64,** 1861–1863.
Faktor O and Shaul Y. (1990b). *Oncogene*, **5,** 867–872.
Feitelson MA, Zhu M, Duan LX and London WT. (1993). *Oncogene*, **8,** 1109–1117.
Galibert F, Chen TN and Mandart E. (1982). *J. Virol*., **41,** 51–65.
Galibert F, Mandart E, Fitoussi F, Tiollais P and Charnay P. (1979). *Nature*, **281,** 646–650.
Gavrieli Y, Sherman Y and Ben-Sasson SA. (1992). *J. Cell. Biol*., **119,** 493–501.
Gu JR, Hu LF, Cheng YC and Wan DF. (1986). *J. Cell. Physiol*., **4** (suppl.), 13–20.
Harrington EA, Bennett MR, Fanidi A and Evan GI. (1994). *EMBO J*., **13,** 3286–3295.
Haviv I, Vaizel D and Shaul Y. (1995). *Mol. Cell. Biol*., **15,** 1079–1085.
Himeno Y, Fukuda Y, Hatanaka M and Imura H. (1988). *Liver*, **8,** 208–212.
Höhne M, Schaefer S, Seifer M, Feitelson MA, Paul D and Gerlich WH. (1990). *EMBO J*., **9,** 1137–1145.
Huang J, Kwang J, Sun E and Liang T. (1996). *J. Virol*., **70,** 5582–5591.
Kay A, Dupont de Dichenin S, Vitvitski-Trépo L, Mandart E, Shamoon BM and Galibert F. (1991). *J. Med. Virol*., **33,** 228–235.
Kekulé AS, Lauer U, Weiss L, Luber B and Hofschneider PH. (1993). *Nature*, **361,** 742–745.
Kim CM, Koike K, Saito I, Miyamura T and Jay G. (1991). *Nature*, **351,** 317–320.
Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T and Kurokawa K. (1994). *Hepatology*, **19,** 810–819.
Koshy R and Wells J. (1991). *Adv. Appl. Biotechnol. Ser*., **13,** 159–170.
Kwok RPS, Laurance ME, Lundblad JR, Goldman PS, Shih H, Connor LM, Marriot SJ and Goodman RH. (1996). *Nature (London)*, **380,** 642–646.
Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ, Wadman I, Nottage K and Rabbitts TH. (1996). *EMBO J*., **15,** 1021–1027.
Lee T-H, Elledge SJ and Butel JS. (1995). *J. Virol*., **69,** 1107–1114.
Lee T-H, Finegold MJ, Shen RF, DeMayo JL, Woo SLC and Butel JS. (1990). *J. Virol*., **64,** 5939–5947.
Lovec H, Grzeschiczek A, Kowalski MB and Möröy T. (1994). *EMBO J*., **13,** 3487–3495.
Maguire HF, Hoeffler JP and Siddiqui A. (1991). *Science*, **252,** 842–844.

Marion PL, Van Davelaar MJ, Knight SS, Salazar FH, Garcia G, Popper H and Robinson WS. (1986). *Proc. Natl. Acad. Sci. USA*, **83,** 4543–4546.

Möröy T, Etiemble J, Trépo C, Tiollais P and Buendia MA. (1985). *EMBO J.*, **4,** 1507–1514.

Murakami H, Sanderson ND, Nagy P, Marino PA, Merlino G and Thorgeirsson SS. (1993). *Cancer Res.*, **53,** 1719–1723.

Nagaya T, Nakamura T, Tokino T, Tsurimoto T, Imai M, Mayumi T, Kamino K, Yamamura K and Matsubara K. (1987). *Genes Dev.*, **1,** 773–782.

Natoli G, Avantaggiati ML, Chirillo P, Puri PL, Ianni A, Balsano C and Levrero M. (1994). *Oncogene*, **9,** 2837–2843.

Ogston CW, Jonak GJ, Rogler CE, Astrin SM and Summers J. (1982). *Cell*, **29,** 385–394.

Paterlini P, Poussin K, Kew M, Franco D and Bréchot C. (1995). *Hepatology*, **21,** 313–321.

Perfumo S, Amicone L, Colloca S, Giorgio M, Pozzi L and Tripodi M. (1992). *J. Virol.*, **66,** 6819–6823.

Qadri I, Maguire HF and Siddiqui A. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 1003–1007.

Sandgren EP, Luetteke NC, Qiu TH, Palmiter RD, Brinster RL and Lee DC. (1993). *Mol. Cell. Biol.*, **13,** 320–330.

Slagle BL, Lee TH, Medina D, Finegold MJ and Butel JS. (1996). *Molecular Carcinogenesis*, **15,** 261–269.

Spandau DF and Lee CH. (1988). *J. Virol.*, **62,** 427–434.

Stanton LW, Fahrlander PD, Tesser PM and Marcu KB. (1984). *Nature*, **310,** 423–425.

Summers J, Smolec JM and Snyder R. (1978). *Proc. Natl. Acad. Sci. USA*, **75,** 4533–4537.

Takada S and Koike K. (1990). *Proc. Natl. Acad. Sci. USA*, **87,** 5628–5632.

Truant R, Antunovic J, Greenblatt J, Prives C and Cromlish JA. (1995). *J. Virol.*, **69,** 1951–1959.

Twu JS, Lai MY, Chen DS and Robinson WS. (1993). *Virology*, **192,** 346–350.

Twu JS and Schloemer RH. (1987). *J. Virol.*, **61,** 3448–3453.

Unger T and Shaul Y. (1990). *EMBO J.*, **9,** 1889–1895.

Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA*, **91,** 2230–2234.

Wang XW, Gibson MK, Vermeulen W, Yeh H, Forrester K, Stürzbecher HW, Hoeijmakers JHJ and Harris CC. (1995). *Cancer Res.*, **55,** 6012–6016.

Wei Y, Etiemble J, Fourel G, Vitvitski-Trépo L and Buendia MA. (1995). *J. Med. Virol.*, **45,** 82–90.

Wei Y, Etiemble J, Renard C-A, Tiollais P and Buendia MA. (1996). *J. Gen. Virol.*, **77,** 177–182.

Wei Y, Fourel G, Ponzetto A, Silvestro M, Tiollais P and Buendia MA. (1992). *J. Virol.*, **66,** 5265–5276.

Williams JS and Andrisani OM. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 3819–3823.

Wollersheim M, Debelka U and Hofschneider PH. (1988). *Oncogene*, **3,** 545–552.

Yen BTS. (1996). *J. Biomed. Sci.*, **3,** 20–30.

Zhang XK, Huang DP and Chiu JE. (1990). *Oncogene*, **5,** 909–914.

Zoulim F, Saputelli J and Seeger C. (1994). *J. Virol.*, **68,** 2026–2030.