C-terminal-truncated hepatitis B virus X protein enhances the development of diethylnitrosamine-induced hepatocellular carcinogenesis

Ivan Quetier,1,2,3 † Nicolas Brezillon,1,2,3 † Julien Revaud,1,2,3 James Ahodantin,1,2,3 Lucie DaSilva,1,2,3 Patrick Soussan1,2,3,4,5 and Dina Kremsdorf1,2,3

Correspondence
Dina Kremsdorf
dina.kremsdorf@inserm.fr

1Inserm, U845, Team ‘Pathogenèse des hépatites virales B et immunothérapie’, Paris, France
2Université Paris Descartes, Sorbonne Paris-Cité, Faculté de Médecine Necker, Paris, France
3Institut Pasteur, Département de Virologie, Paris, France
4Service de Virologie, Hôpital Tenon, Paris, France
5Université Pierre et Marie Curie, Paris, France

Hepatitis B virus X protein (HBx) is involved in the development of hepatocellular carcinoma (HCC). The HBx sequence is a preferential site of integration into the human genome, leading to the formation of C-terminal-truncated HBx proteins (Ct-HBx). We previously reported that Ct-HBx proteins were able to potentiate cell transformation in vitro. Our present goal was to compare the ability of Ct-HBx and full-length HBx (FL-HBx) proteins to develop or enhance HCC in transgenic mice. In the absence of treatment, neither Ct-HBx- nor FL-HBx-transgenic mice developed HCC. In young mice treated with diethylnitrosamine (DEN) at 8 months of age, a significantly higher incidence and number of liver lesions were observed in Ct-HBx mice than in FL-HBx and control mice. The earlier development of tumours in Ct-HBx-transgenic mice was associated with increased liver inflammation. At 10 months, macroscopic and microscopic analyses showed that, statistically, FL-HBx mice developed more liver lesions with a larger surface area than control mice. Furthermore, during DEN-induced initiation of HCC, Ct-HBx- and FL-HBx-transgenic mice showed higher expression of IL-6, TNF-α and IL-1β transcripts, activation of STAT3, ERK and JNK proteins and an increase in cell apoptosis. In conclusion, in DEN-treated transgenic mice, the expression of Ct-HBx protein causes a more rapid onset of HCC than does FL-HBx protein. HBV genome integration leading to the expression of a truncated form of HBx protein may therefore facilitate HCC development in chronically infected patients.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC), accounting for more than 50% of cases worldwide (Kew, 2011). The direct mechanisms whereby HBV causes malignant transformation remain elusive. Nevertheless, much of the available evidence supports a pathogenic role of the viral HBx protein. HBx is a small protein of 17 kDa, which is highly conserved among all mammalian hepadnaviruses and is essential to the enhancement of HBV replication (Assrir et al., 2010). HBx protein does not bind directly to DNA but, rather, acts on cellular promoters via protein–protein interactions and displays pleiotropic effects on different pathways involved in intracellular signalling, transcriptional activation that modulates cell responses to genotoxic stress, protein degradation, apoptosis and cell division, which may be responsible for the potential transforming activities of HBx (for a review, see Kew, 2011). HBx is expressed at low levels during acute and chronic hepatitis and induces a humoral and cellular immune response (Malmassari et al., 2007; Wei et al., 2010). The HBx gene is maintained and transcribed in most integrated subviral DNA, and several studies have demonstrated the expression of HBx mRNA and/or protein in human HCC in the absence of any HBV replication (Toh et al., 2013). Whether HBx directly contributes to the development of HBV-associated HCC or functions as a co-factor in HCC development continues

†These authors contributed equally to this work.
to be debated. Indeed, despite in vivo data showing that HBx-transgenic mice may directly develop HCC, it is more likely that HBx expression participates in its development by sensitizing transgenic mice to chemical carcinogens or by cooperating with cellular oncogenes (for a review, see Brechot et al., 2010).

Natural mutants of HBx have been described in the liver and serum of patients with different clinical diseases and may play a role in the development of HBV-related HCC (lavarone et al., 2003; Ma et al., 2008; Sirma et al., 1999; Toh et al., 2013; Tu et al., 2001). These include hot-spot mutations in HBx at aa 130 and 131, which have been associated with the development of HCC (lavarone et al., 2003; Kuang et al., 2004; Lee et al., 2011). Furthermore, the random integration of HBV DNA into the host genome frequently leads to 3'-end-deleted sequences of the HBx gene and the observation of chimeric transcripts which often carry deletions at the 3'-end of HBx gene, resulting in C-terminal-truncated HBx protein (lavarone et al., 2003; Jiang et al., 2012; Ma et al., 2008; Sirma et al., 1999; Sung et al., 2012; Sze et al., 2013; Toh et al., 2013; Tu et al., 2001). Interestingly, a recent study with human HCC samples reports an association between the presence of C-terminal truncation of HBx and venous invasion (Sza et al., 2013). However, most of the data in favour of the contribution of C-truncated HBx proteins in tumorigenesis are from in vitro studies. We previously reported that HBx proteins with deletion of amino acids at the C-terminal region have altered capacity to control cell proliferation and transcriptional activity but were able to potentiate ras and myc cell transformation (Sirma et al., 1999; Tu et al., 2001). Other in vitro data have shown that these HBx mutants retain the ability to bind to p53 and to block p53-mediated apoptosis (Huo et al., 2001). The C-terminal region of HBx is dispensable for HBx-specific localization in mitochondria, which is involved in activating several cytosolic signal transduction pathways (Shirakata & Koike, 2003). Although the extent of deletion of the 3'-end of the hbx gene varies, the integrity of the DDB1-binding domain (aa 76–125) probably protects HBx truncated protein from proteasome-mediated degradation (Bergametti et al., 2002; Lin-Marq et al., 2001). By contrast, C-terminal-truncated HBx proteins showed specific mechanisms involved in regulation of cell cycle and apoptosis (Bock et al., 2008; Jiang et al., 2010; Liu et al., 2008b).

These in vitro results suggest that the HBx protein with C-terminal deletions may be implicated in the development of HCC through distinct biological functions than the full length HBx protein. Therefore, to better define the involvement of naturally occurring HBx protein mutants in the evolution of liver disease to HCC, we decided to perform an in vivo comparison of the carcinogenic or co-carcinogenic capacities of C-terminal-truncated HBx (Ct-HBx) and non-truncated HBx (FL-HBx) proteins obtained from tumour and non-tumour biopsies from the same patient.

RESULTS

Characterization of HBx-transgenic strains

We previously reported that, in vitro, HBx proteins with natural deletion at the C-terminal region had altered capacity to control cell proliferation and transcriptional activity but were able to potentiate ras and myc cell transformation and that a deletion of 16 aa was enough to observe these effects (Sirma et al., 1999; Tu et al., 2001). Thus, to generate HBx-transgenic mice we used a couple of tumour/non-tumour sequences (patient 1) differing only by a deletion of 18 aa of HBx and the addition of three amino acids from the cellular genome in the tumoural region (Fig. 1b) (Sirma et al., 1999; Tu et al., 2001).

Transgenic mice expressing FL-HBx (pX-HBx-1336 and pX-HBx-1368) or Ct-HBx (pX-HBx-1280) under HBV regulatory elements were generated (Fig. 1a). HBx transcripts were quantified using reverse transcription and quantitative PCR (RT-qPCR) and the highest expression of transcripts was observed in pX-HBx-1336 transgenic mice (Fig. 1c). Assuming an identical level of expression in each hepatocyte, the HBx RNA copy number was estimated to be 8 to 25 per hepatocyte for the 1336 lineage, 0.2 to 0.5 per hepatocyte for the 1368 lineage, and 0.07 to 0.2 per hepatocyte for the 1280 lineage. In accordance with the level of viral transcript expression, the HBx protein was detected in the two FL-HBx-transgenic mouse liver tissues but barely detected in Ct-HBx-transgenic mice (Fig. 1d). Finally, different organs in addition to the liver were investigated for the expression of HBx mRNA; a significant expression of HBx transcript was only observed in kidney by RT-qPCR (Quétier et al., 2013).

Susceptibility of HBx-transgenic mice to DEN-induced hepatocarcinogenesis

At 18 months of age, no direct carcinogenesis was observed in Ct-HBx- or FL-HBx-transgenic mice (data not shown).

Thus, the DEN-induced liver tumour model was used to explore whether the Ct-HBx protein was able, like the FL-HBx protein, to participate in development of hepatocarcinogenesis (Lee et al., 2004; Madden et al., 2001). To exert its carcinogenic effect, DEN needs to be bioactivated in the liver by several P450 isoforms, including CYP2E1, resulting in DNA adducts formed through an alkylation mechanism (Verna et al., 1996). The resulting O'-alkylguanine leads to GC–AT transitions, which are believed to be largely responsible for DEN-induced carcinogenesis. Exposure to DEN is also associated with hepatocellular accumulation of reactive oxygen species, which may enhance DEN-induced hepatocarcinogenesis (Santos et al., 2012). Livers of Ct-HBx- and FL-HBx-transgenic mice, and genetically matched WT treated mice were removed from post-mortem mice at 8, 10 or 12 months. Macroscopically visible lesions on the liver surface, representing both pre-neoplastic focal lesions and benign or malignant neoplastic lesions were counted and microscopic analyses were
performed (Figs 2 and 3). At 8 months, the incidence and the number of macroscopic liver lesions were significantly higher in Ct-HBx-transgenic mice than in FL-HBx-transgenic and control mice (Fig. 2b, e). Similarly, the microscopic evaluation of haematoxylin and eosin (H&E)-stained slides showed a higher incidence of tumours in Ct-HBx-transgenic mice than in FL-HBx-transgenic and control mice (Fig. 2b). However, probably because of the small size and number of observed lesions per slide, no statistical difference in tumour area was observed between the three groups of mice (Fig. 2e). The microscopic evaluation of H&E-stained slides showed essentially preneoplastic foci and areas with inflammatory cells (Fig. 3a–c). At 10 months, the incidence of macroscopic liver lesions was similar in the WT and FL-HBx-transgenic mice (Fig. 2c). However, the number of macroscopic lesions was statistically higher in FL-HBx-transgenic mice than in WT mice (Fig. 2f). Similarly, histological assessment of the liver tumours showed a significantly higher tumour area in FL-HBx-transgenic mice than in WT mice (Fig. 2f). Histopathological examination revealed that most of the tumour nodules seen in the livers were of a neoplastic type (Fig. 3d–f). In 12-month-old FL-HBx- and Ct-HBx-transgenic animals, macroscopic and microscopic examinations did not evidence differences (Fig. 2d, g). Histopathological examination of the livers revealed enlarged cancerous tissue in a high proportion of animals, corresponding to well-differentiated HCC or multifocal nodules of poorly differentiated HCC (Fig. 3g–i).

In order to evaluate the liver damage in DEN-treated mice, blind scores for steatosis, necrosis and inflammatory cell

**Fig. 1.** Characterization of FL-HBx- and Ct-HBx-transgenic mouse strains. (a) Scheme of the transgene (numbers correspond to nucleic acid position on HBV sequence) and FL-HBx and Ct-HBx proteins (shaded boxes correspond to three amino acids belonging to the human genome). (b) Amino acid sequences of FL-HBx and Ct-HBx proteins compared with the prototype of adr serotype. Underlined amino acids correspond to cellular sequence. (c) HBx mRNA expression in the liver of pX-HBx-1336, pX-HBx-1368 and pX-HBx-1280 mice at 1, 2, 4 and 6 months of age (n=3). Error bars, ± the standard deviation. (d) Western blot analysis of FL-HBx and Ct-HBx expression at the indicated times. The positive controls were FL-HBx-Myc-His-tag or Ct-HBx-Myc-His-tag vectors transfected into Huh7 cells. The negative control was WT mice.
infiltration were determined (Fig. 4). In 8-month-old Ct-HBx-transgenic mice, but not in FL-HBx-transgenic mice and control mice, significant increase in inflammation was observed (Fig. 4d). Indeed, scattered small inflammatory cells in the lobule, associated or not with portal inflammation, were observed in all of the Ct-HBx-transgenic mice (data not shown). In 10-month-old FL-HBx-transgenic and control mice, no differences were seen with respect to hepatic damage (Fig. 4e).

Overall, in this model of DEN-induced carcinogenesis, our findings are in favour of a co-carcinogenic role for FL-HBx and Ct-HBx proteins, the effect of the latter occurring earlier. Furthermore, the earlier development of tumours in Ct-HBx-transgenic mice was associated with increased liver inflammation.

Fig. 2. Increased susceptibility of Ct-HBx- and FL-HBx-transgenic mice to DEN-induced hepatocarcinogenesis. (a) Representative photographs of livers from male WT mice, 8, 10 and 12 months after DEN treatment. Green arrows indicate lesions. Lesions on the liver surface were pre-neoplastic focal lesions and benign or malignant neoplastic lesions. (b, c, d) The incidence of macroscopic lesions and the number of microscopic tumours were determined in WT, FL-HBx and Ct-HBx livers at 8 (b), 10 (c) and 12 months (d) after DEN treatment. (e, f, g) Determination of the number of macroscopic lesions (left panels) and tumour area (right panels) in WT, FL-HBx and Ct-HBx livers at 8 (e), 10 (f) and 12 months (g) after DEN treatment. Filled circle, WT; filled square, FL-HBx; filled triangle, Ct-HBx. * or † or ‡ or § Significant Student’s t-test analysis. For FL-HBx-transgenic mice, black circles correspond to the pX-HBX-1368 strain and red circles to the pX-HBX-1336 strain. nb, number.

Effect of HBx expression on DEN-induced initiation of carcinogenesis

The next step was to determine whether FL-HBx- and Ct-HBx-transgenic mice were more sensitive to DEN-induced acute hepatic injury. In adult mice, DEN acts only as an initiator of liver injury. This is possibly associated with the lesser capacity of hepatocytes to proliferate in adult liver. Indeed, hepatocyte proliferation is needed for complete DEN-induced carcinogenesis (Maeda et al., 2005). Thus, cell death and compensatory proliferation were investigated in 2-month-old mice injected with a high dose of DEN (100 mg (kg body mass))⁻¹).

To quantify cell death, TUNEL assays were performed on liver sections 48 h after the DEN injection. Ct-HBx- and
FL-HBx-transgenic mice displayed a significantly larger labelled area than WT mice (Fig. 5a). The highest rate of hepatocyte apoptosis (with a twofold increase) was observed in Ct-HBx-transgenic mice (Fig. 5b). At the same time, compensatory cell proliferation was measured by Ki-67 labelling of nuclei (Fig. 5a,c). At this short time after DEN treatment, in agreement with previous data (Schneider et al., 2012), proliferation was essentially observed in non-parenchymal cells. Despite a higher degree of cell proliferation in Ct-HBx-transgenic mice than in either FL-HBx-transgenic or WT mice, no statistical difference was observed (Fig. 5c). As, in vitro, this specific Ct-HBx protein had lost its cytotoxic activity (Tu et al., 2001), we wanted to investigate if the observed higher increase of apoptosis in Ct-HBx-transgenic mice might be associated with a sensitization of cells to TNF-induced apoptosis. For that, we used the model of fulminant hepatitis induced by TNF-α and D-galactosamine treatment and evaluated the survival of FL-HBx- and Ct-HBx-transgenic mice livers compared with the livers of WT mice (Fig. 5d). In untreated animals, expression of transcripts and proteins was unchanged. Interestingly, activation of ERK1/2 was higher in Ct-HBx mice than in FL-HBx mice. Finally, 48 h post treatment, we did not observe major modifications in expression of mRNAs and proteins in liver of HBx-transgenic mice compared with control (data not shown). These observations suggested that the inflammatory and apoptosis responses related to DEN administration are enhanced in HBx-transgenic mice and confirmed the role of FL-HBx and Ct-HBx proteins in the modulation of hepatocyte metabolism, which could contribute to the induction of tumours following treatment with DEN.

**Activation of signalling pathways in the livers of DEN-treated mice**

In order to investigate whether Ct-HBx or FL-HBx protein modified inflammatory or oncogenic pathways in DEN-treated transgenic mice at 8 and 12 months, liver biopsies were analysed by Western blot (Fig. 6a). It should be noted that at 8 months lesions were very small, with only pre-neoplastic tumours (Fig. 2a), rendering difficult to obtain biopsies with only preneoplastic tissues. In contrast, for biopsies obtained at 12 months, most of the liver tissues contained HCC tumours (Fig. 2a). In addition, HBx mRNA expression was investigated in DEN-treated or untreated

**Fig. 3.** Representative H&E staining of liver sections from 8- and 12-month-old DEN-treated male mice. (a, b, c) Liver sections after 8 months of DEN treatment: (a) adenoma, (b) small tumour surrounded by normal liver parenchyma, (c) area with an infiltrate of inflammatory cells. (d, e, f) Liver sections 10 months after DEN treatment: (d) adenoma containing eosinophilic material at the periphery; (e) HCC tumour arranged in multiple cell layers with an infiltrate of inflammatory cells that appeared to concentrate around the portal triads; (f) necrotic area surrounded and infiltrated by inflammatory cells. (g, h, i) Twelve months after exposure to DEN, examination revealed the formation of a large adenoma (g) and well-differentiated (h) or poorly differentiated (i) HCCs.
FL-HBx- and Ct-HBx-transgenic mice at 8 and 12 months; DEN treatment did not modify HBx mRNA expression (Fig. 6c).

The activation of STAT3, JNK and ERK, which is frequently observed in a context of HCC, was investigated. Surprisingly, we did not observe any STAT3 activation at either 8 or 12 months in any of the mice. Activation of the stress-related JNK pathway was observed between 8 and 12 months in WT mice, and was more pronounced between 8 and 12 months in Ct-HBx- and FL-HBx-transgenic mice. Finally, the ERK pro-proliferative pathway was markedly activated in the livers of Ct-HBx- and FL-HBx-transgenic mice at 12 months of age compared with the WT mice livers. We also investigated the level of expression of the alpha fetal protein (AFP), re-expressed in the majority of HCCs. AFP was highly expressed in treated mice at 12 months, and more specifically in HBx-transgenic mice. In addition, in agreement with compensatory liver cell proliferation driving carcinogenesis in liver, proliferation cell nuclear antigen (PCNA), a marker of cell proliferation, was highly expressed in treated mice at 12 months, independently of the mouse genotype (Fig. 6a). To evaluate the impact of HBx expression in the absence of DEN treatment, we investigated the expression of ERK and PCNA in biopsies from untreated transgenic and control mice at 12 months (Fig. 6b). In contrast to ERK activation in DEN-treated Ct-HBx- and FL-HBx-transgenic mice 12 months after treatment, no difference was observed in untreated mice at this time. Interestingly, in Ct-HBx-transgenic mice at 12 months PCNA expression seemed to be increased. This difference was not observed in treated animals.

Thus, after DEN treatment, pathways involved in cell proliferation and HCC development were upregulated in livers of Ct-HBx- and FL-HBx-transgenic mice.

**DISCUSSION**

Recent studies using next-generation sequencing technologies have highlighted the fact that, following the integration
of HBV into the cellular genome, the 3’-end of the HBx gene is often deleted and this event is observed more frequently in tumour tissues (Jiang et al., 2012; Sung et al., 2012; Toh et al., 2013). Furthermore, truncated HBx human chimeric transcripts may be seen and expressed as chimeric proteins (Sirma et al., 1999; Toh et al., 2013; Tu et al., 2001). This has raised the question of a putative role for Ct-HBx proteins in the development of HCC. Our study was carried out in order to enable in vivo investigation of the oncogenic potential of Ct-HBx in comparison with FL-HBx protein.

First of all, we observed that, in the absence of any treatment, neither FL-HBx- nor Ct-HBx-transgenic mice developed HCC, as evaluated by macroscopic and microscopic analyses performed during an 18 month follow-up. The data available on the ability of FL-HBx protein to induce carcinogenesis directly are contradictory. Indeed, although few FL-HBx-transgenic mice strains might develop spontaneous HCC, there is more evidence that FL-HBx protein participates in HCC development by sensitizing the animals to carcinogen inducers. The genetic background of the mice and the level of expression or genotype of HBx protein may explain these discrepancies (Kremsdorf et al., 2006).

To evaluate, in our model, where FL-HBx and Ct-HBx were expressed under their own promoter, whether FL-HBx or Ct-HBx proteins were involved in liver carcinogenesis, DEN-induced HCC treatment was chosen because of its relevance to human HCC induction. Indeed, when injected into young animals, the gene expression patterns of HCC induced by DEN in mice were generally similar to those seen in the poorer survival group of human HCC.
(Lee et al., 2004). Our findings were in favour of an accelerated development of liver tumours in Ct-HBx- and FL-HBx-transgenic mice as compared with WT mice. Indeed, in Ct-HBx-transgenic mice, 8 months after DEN treatment, livers displayed a higher macroscopic and microscopic incidence of tumours, associated with an increase in liver inflammation, whereas in FL-HBx-transgenic mice increases in the number of tumours and in tumour area versus WT mice were only observed 10 months after DEN treatment. To our knowledge, this study provides the first indication under \textit{in vivo} conditions of the ability of the Ct-HBx mutant not only to induce HCC but to do it more rapidly than FL-HBx. This emphasizes that the HBx deletion mutant may develop distinct biological properties that contribute to liver carcinogenesis. Initial \textit{in vitro} data demonstrated the ability of the Ct-HBx protein to abrogate the growth-suppressive and apoptotic effects of FL-HBx protein (Huo et al., 2001; Sirma et al., 1999; Tu et al., 2001) and that Ct-HBx might enhance cell proliferation (Jiang et al., 2010; Liu et al., 2008b; Ma et al., 2008). The Ct-HBx protein has also been reported to modulate microRNA expression (Fu et al, 2012; Yip et al., 2011), to regulate Wnt-5a expression (Liu et al., 2008a) and to play a role in cell invasiveness by enhancing the transcription of metalloproteinase 10 (MMP10) through c-Jun (Sze et al., 2013).

DEN-induced carcinogenesis promotion requires the proliferation of hepatocytes; thus in adults, where mature hepatocytes display an extremely low turnover rate, DEN administration induces only the initiation stage, without tumour development. Our experiments revealed that during the DEN-induced initiation stage, there was an increase in the sensitization of both FL-HBx- and Ct-HBx-transgenic mice to liver injury. Interestingly, a more pronounced apoptosis was observed in Ct-HBx-transgenic mice. We have evidenced that this might be linked to a higher sensitivity of Ct-HBx-transgenic mice to TNF-\(\alpha\)-induced apoptosis. These observations are in accordance with previous data demonstrating that the severity of acute hepatic injuries correlates with the level of cell proliferation and the development of liver tumours during the promotion stages (Lee et al., 2004; Maeda et al., 2005). For this reason, the increased inflammatory response and apoptosis observed in FL-HBx- and Ct-HBx-transgenic mice during the initiation phase of DEN-induced carcinogenesis might have been related to the more rapid onset and development of DEN-induced HCC, particularly in Ct-HBx-transgenic mice. We also observed, at early stages of DEN induction, an increase in cell proliferation in the livers of Ct-HBx-transgenic mice, but not in those of FL-HBx-transgenic mice, compared with WT mice. In a previous study, increased hepatocellular

**Fig. 6.** Analysis of the modulation of pathways activated during DEN-induced liver carcinogenesis in FL-HBx- and Ct-HBx-transgenic mice. Ten-day-old WT, FL-HBx and Ct-HBx mice were injected with DEN [2 \(\mu\)g (g body mass)\(^{-1}\)]. (a) Western blot analysis of STAT3, JNK and ERK activation, and PCNA and AFP expression, 8 and 12 months after treatment. (b) Western blot analysis of ERK activation and PCNA expression in 12 month untreated FL-HBx and Ct-HBx mice. Each lane represents a single mouse. (c) HBx mRNA expression in the liver of FL-HBx- (pX-HBx-1336) and Ct-HBx- (pX-HBx-1280) transgenic mice, treated or not treated with DEN, at 8 and 12 months of age.
proliferation in transgenic mice expressing a full-length HBx gene was observed 48 h after the injection of DEN into 12-day-old mice (Madden et al., 2001). The use of different experimental protocols (newborn versus adult animals) probably reflects the differences in the results obtained. However, in line with our data, in the same study, at 8 months of age, no measurable effect of HBx protein expression on hepatocyte proliferation was evidenced (Madden et al., 2001). Taken together, this indicates that the involvement of HBx protein in the induction of cell proliferation probably occurs during the early stages of DEN-induced carcinogenesis. Furthermore, the greater capacity of Ct-HBx protein to induce cell proliferation may account for the more rapid tumour development observed during the promotion stage of DEN-induced carcinogenesis.

During the initiation stage of DEN-induced carcinogenesis we observed an increased activation of mitogen-activated protein kinases (MAPKs: JNK and ERK) and STAT3 pathways in both transgenic mice compared with WT mice. DEN treatment leads to an accumulation of reactive oxygen species inducing the activation of JNK and STAT3, which are both involved in liver carcinogenesis (He et al., 2010). Two in vitro studies demonstrated the ability of FL-HBx but not CT-HBx to induce the generation of reactive oxygen species, leading to the activation of STAT3 (Jung & Kim, 2013; Waris et al., 2001). This suggests that, in our model, the increased activation of JNK and STAT3 by HBx proteins was probably independent of the accumulation of reactive oxygen species. Alternatively, JNK may be activated by diverse stimuli, including cytokines such as IL-1 and TNF-α (Seki et al., 2012). It could therefore be hypothesized that the observed increase in IL-1β and TNF-α transcripts might account for JNK activation. Furthermore, in a hepatoma cell line, it has been reported that HBx protein potentiated the phosphorylation of JNK by interacting with Jabi (Tanaka et al., 2006). Along the same line of evidence, we have observed increased expression of IL-6 in both transgenic mice. Interestingly, in addition to DEN induction of IL-6 production in Kupffer cells (Naugler et al., 2007), HBx stimulates the production of IL-6 in a MyD88-dependent manner in hepatoma cells (Xiang et al., 2011).

Interestingly, it has been reported that HBx protein may upregulate FoxM1 expression through the ERK/CREB pathway, and that FoxM1 overexpression indicated a poor prognosis in HBV-related HCC patients (Xia et al., 2012). Surprisingly, whereas the IL-6/STAT3 pathway is clearly upregulated during the DEN-induced initiation phase of HCC in HBx-transgenic mice, STAT3 activation was barely detectable 8 or 12 months after DEN-induced carcinogenesis in our animals.

Considering activation of MAPKs, it was reported that, in vivo, the prolonged activation of ERK by HBx expression is also accompanied by the activation of JNKs (Nijhara et al., 2001). Furthermore, it was identified that the internal region (aa 58–140) of HBx was as effective as the FL-HBx in activating MAPKs (Nijhara et al., 2001). Our observations that the ERK and JNK pathways are simultaneously activated by HBx demonstrate that activation of both signalling pathways is required to trigger a full spectrum of phenotypic traits associated with oncogenesis in HBx-expressing livers. This reinforces the idea that HBx may be involved in HBV-mediated liver carcinogenesis through pro-proliferation mechanisms. Consistent with these findings, in our study the ERK and JNK pathways in the livers of both transgenic mice, 8 and 12 months after DEN treatment, were upregulated compared with WT mice. In addition, we showed that AFP, an important biomarker of HCC, was highly expressed in treated transgenic mice at 12 months. It was demonstrated that HBx derepresses AFP expression through its interaction with p53 and that the expression of AFP receptor was a pivotal event for HBx inducing malignant transformation of liver cells (Li et al., 2013; Ogden et al., 2000).

The mechanisms underlying liver carcinogenesis involve numerous steps during which both the immune system and viral proteins may be responsible for initiating environmental and biological modifications that lead to the development of HCC. Among them, it is important to mention that abnormal activation of AFP, ERK and JNK is often observed in human HCC (Min et al., 2011; Seki et al., 2012). The HBx protein, by modulating these pathways, might contribute to the development of HCC. Our study showed that the expression of Ct-HBx protein led to a more rapid onset of HCC than did FL-HBx protein. It could be assumed that the expression of truncated forms of HBx may facilitate cell transformation by accelerating the initiation step of carcinogenesis. This suggests that the integration of HBV into the host genome in addition to cis-acting effects may exert trans-acting effects via the expression of truncated HBx proteins and thus contribute to tumorigenesis.

**METHODS**

**Transgenic mice.** Two sequences of the HBx gene isolated from either a non-tumour (FL-HBx) or tumour (Ct-HBx) region in the same patient were used to generate transgenic mice (Tu et al., 2001). HBx sequences containing the Myc-His tag were cloned downstream of the HBx promoter and enhancer 1 region (nt-832–1371) and β-globin intronic sequences (Fig. 1). Three independent lines were derived from the founders (pX-HBx-1336 and pX-HBx-1368 expressing FL-HBx, and pX-HBx-1280 expressing Ct-HBx), and expanded by back-crossing with the C57BL/6j strain (Institut Clinique de la Souris, Strasbourg, France). All the experiments were performed on heterozygous transgenic mice. The animals were treated in accordance with European Union regulations on animal care (directive 86/661/ECC).

**Animal experiments.** For the direct carcinogenic study, untreated FL-HBx, Ct-HBx and WT mice from the same litter were followed for 8, 12 and 18 months. For the co-carcinogenesis study, diethyl nitrosamine (DEN) was injected intraperitoneally at 2 μg (g body mass)⁻¹ into 10-day-old male mice. The mice were euthanized 8, 10 and 12 months after DEN injection. At the time of sacrifice, macroscopic lesions were counted on the surface of the liver.

**REFERENCES**

I. Quetier and others
order to induce acute liver damage, DEN was injected intraperitoneally at 100 µg (g body mass)$^{-1}$ into 2-month-old male FL-HBx, C57-HBx (pX-HBx-1336 strain) and WT mice. The mice were euthanized 4 or 48 h post-injection. The left lobe of the liver was fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. The remaining liver was frozen for protein or RNA analysis. For survival experiments, animals were fasted for 14 h and fulminant hepatitis was induced by intraperitoneal injection of murine TNF-α (20 µg kg$^{-1}$; AbCys) and D-galactosamine (700 mg kg$^{-1}$; Sigma). The experimental protocols were approved by the French Committee on the Ethics of Animal Experiments (MERS no. 0079303).

**Histological analysis.** From each mouse, three liver sections 4 µm thick, each separated by 200 µm, were stained with H&E and used for histological analysis. Liver sections were digitized (Hammamatsu Nanozoomer) and analysed with NDP.VIEW software. For each mouse, the three liver sections were used to determine mean tumour number and area per slide, and blind-scored (0 to 4) (two investigators) for steatosis, necrosis and inflammation using a modified scoring scale (Brandon-Warner et al., 2012). This score was representative of the mean of the three sections from each mouse.

**Cell proliferation and apoptosis.** Cell proliferation analysis was performed by immunohistochemistry using Ki-67 antibody (clone NCL-Ki67p; Novocastra). The number of positive nuclei was evaluated in 15 defined areas representing about 10% of the whole liver section and reported as the number of positive cells (mm liver$^{-2}$).

To study apoptosis, TUNEL staining was performed using the In situ Cell Death Detection kit (Roche). After digitizing the slides, TUNEL labelling was quantified using Image-J software. TUNEL brown labelling and the counter-staining were separated using threshold tools and the percentage labelled area in the whole liver section was determined. For each experiment, the mean TUNEL-positive area found in WT mice was set at 1, and the TUNEL area was expressed in relative units.

**Western blotting.** Total proteins were extracted from frozen liver tissues as previously described (Quetier et al., 2013). For HBx protein detection, a total of 200 µg liver protein and 10 µg HBx-myc-transfected HuH7 protein (positive control) was loaded onto 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with c-Myc antibody (1:100) (sc-40; Santa Cruz). For cellular protein detection, 20 µg protein extract was resolved on SDS-PAGE (10%) and transferred onto a nitrocellulose membrane, which was then incubated with the following primary antibodies (1:1000) in PBS–BSA (5%): GAPDH (Santa Cruz), PCNA (M0879; Daco), phospho-STAT3 (no. 9131), STAT3 (no. 9132), phospho-ERK1/2 (no. 9101), ERK (no. 9102), phospho-JNK (no. 9251), JNK (no. 9258) and AFP (no. 3903) (Cell Signalling). The secondary antibody was anti-rabbit antibody coupled to horseradish peroxidase, and the bands were revealed using the ECL Plus (Amersham) or ECL Prime (Invitrogen) detection systems.

**RNA quantification.** Total RNA was extracted from frozen liver tissues (Qiagen kit) and quantified using Nanodrop; 1.5 µg RNA was used for reverse transcription with random primers and SuperScript II (Invitrogen). Quantification of cDNAs was performed on a Taqman 7500 (Applied Biosystems) using either the TaqMan Master Mix (Applied Biosystems) and primers/probes specific to IL-6, TNF-α, IL-1β and TGF-β (Integrated DNA Technology), or the SybrGreen PCR Master Mix for GAPDH (forward, 5’-AGAGCCGGCATCTTTGTCTTGCA; reverse, 5’-GCGCAATTACGGCAATCGTGTC); a standard curve was included to quantify HBx mRNA as copy number (µg total RNA)$^{-1}$.

**Statistical analysis.** Statistical analyses were performed using the non-parametric Mann–Whitney U-test, Student’s t-test or χ²-test to compare the different groups. A P-value <0.05 was considered to reveal a significant difference. Comparisons of survival curves were performed using the log-rank (Manuel–Cox) test. All calculations were performed using GraphPad Prism software.

**ACKNOWLEDGEMENTS**

I.L. was supported by a grant from the French Ministry for Higher Education and Research (Ministère de l’Enseignement Supérieur et de la Recherche), N.B. and L.D. received grant support from the Bill and Melinda Gates Foundation. J.A. received grant support from the Fondation de la Recherche Médicale (FRM). The study was supported by grants from the Institut Nationale de la Santé et de la Recherche Médicale (Inserm), Association de la Recherche contre le Cancer (ARC) (5054) and Association Nationale de Recherche sur le sida et les hépatites virales (ANRS) (06202). The authors thank Professors B. Terris and J.-C. Fournet for their assistance with the histological interpretation and discussions, S. Berissi and N. Gadessaud from the U845 histological platform for tissue processing and slide coloration, and Professor F. Pagès, F. Marliot and N. Haichour for access to the HEGP immunomonitoring platform.

**REFERENCES**


MasterMix Sybr Green I (Roche) and specific HBx primers (forward, 5’-GGGCTCGTTTGGTTCGCGTTC; reverse, 5’-GGCGGAATTGCCCTGAAGTCGCGG). A standard curve was included to quantify HBx mRNA as copy number (µg total RNA)$^{-1}$.


