Virus replication and virion export in X-deficient hepatitis B virus transgenic mice

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The function of the X protein (pX) in the replication cycle of mammalian hepadnaviruses is enigmatic. Using tissue culture experiments it has been shown that the X gene product is not central to hepatitis B virus (HBV) replication and virion export. However, at present it is still unclear whether this also applies to the in vivo situation. Using a terminally redundant X-deficient HBV DNA construct, transgenic mice were established that exhibited high-level expression of the viral core protein in liver and kidneys. Importantly, replicative DNA intermediates and mature viral genomes could be detected in the liver and serum of these mice, respectively. These findings indicate that, in the in vivo model of transgenic mice, the HBV X (HBx) gene product is not required for HBV replication and virion secretion.

Introduction

Hepadnaviruses represent a small group of enveloped DNA viruses characterized by a strong species specificity and liver tropism. The most prominent members of this genus are human hepatitis B virus (HBV), woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and duck hepatitis B virus (DHBV). The genomes of all hepadnaviruses comprise three open reading frames (ORFs) encoding the core, envelope and polymerase proteins. However, all mammalian hepadnaviruses known today possess an additional ORF, which is referred to as X, since the function of its gene product, protein X (pX), is unknown. As was shown in the WHV (Dandri et al., 1996) and HBV (Levrero et al., 1991; Pfaff et al., 1987; Su et al., 1998) systems, pX is expressed at least during certain stages of hepadnaviral infection. The strict conservation of the X ORF in the genomes of the mammalian hepadnaviruses strongly suggests an important role of pX in the viral life-cycle. However, to date it can only be speculated what role this protein plays.

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Using tissue culture experiments it has been demonstrated that pX represents a moderate transcriptional transactivator of the native viral promoters (Colgrove et al., 1989; Spandau & Lee, 1988; Zahm et al., 1988). However, in contrast to retroviral transactivators, which exert their function specifically on the viral LTR elements, pX was found to act on a broad multitude of cellular genes (for reviews see Murakami, 1999; Rossner, 1992). Furthermore, since X-defective hepadnaviral genomes remained replication-competent upon transfection into differentiated hepatoma cell lines (Blum et al., 1992; Koike et al., 1989; Yaginuma et al., 1987), the activity of the HBV promoters could not strictly depend on the coexpression of pX. On the other hand, studies performed in the WHV system have suggested that an intact X gene is required for the successful establishment of hepadnaviral infection in vivo (Chen et al., 1993; Zoulim et al., 1994).

Recently, we have generated several lineages of transgenic mice carrying the HBV core gene either alone or in cis arrangement with the X gene (Reifenberg et al., 1997, 1999). By comparing the quantity of hepatic core gene expression between the X-intact and the X-deficient mice, we have demonstrated that the X gene product transactivates the expression of HBV core transcript in vivo. However, expression
of pX was not mandatory for synthesis of core mRNA in the livers of transgenic mice.

Here we have established a lineage of transgenic mice expressing all HBV gene products except for the X gene product. These transgenic animals enabled us to address the question whether the X protein is required for virus replication and virion export in vivo. We found that in this transgenic mouse model, pX is not essential for HBV replication and virus secretion.

Methods

■ Transgenic mice. All animal experiments were performed in accordance with the German regulations for the use of animals in biomedical research. Pronuclear injections were performed into F2 (C57BL/6 × DBA/2) (F2) embryos according to standard procedures (Hogan et al., 1994). Transgenic lines were established by backcrossing the transgenic founder individuals to the C57BL/6 (B6) inbred strain. Hemizygous transgenic mice were identified from their non-transgenic littermates by PCR amplification of transgene-specific sequences from leukocyte DNA using the HBV specific primers 5’ CAG TGG AAT TCC ACA ACC 3’ and 5’ AGA AAA ACC CCG CCT GTA 3’. All animals were maintained under strict specific pathogen-free conditions at the Animal Research Unit of the University of Ulm. All analyses presented in this report were performed with hemizygous animals of the first or second backcross generation.

■ Northern blot analysis. For Northern blotting, snap-frozen tissue samples were pulverized in liquid nitrogen with a micro-dismembrator (Braun Biotech International) and total RNA was prepared using the RNeasy Mini Kit (Qiagen). The samples were treated with DNase (Gibco BRL) and the purified RNA was separated on a formaldehyde–agarose gel, blotted onto nylon membranes and hybridized with an HBV-specific 32P-labelled probe.

■ Southern blot analysis. Liver or kidney tissue samples (20 mg) were transferred into 500 µl of the ATL tissue lysis buffer from the QIAamp DNA Mini Kit (Qiagen) supplemented with 1 mg/ml proteinase K and incubated at 56 °C for 2 h. After centrifugation through a QIAshredder spin column (Qiagen), the DNA was purified by adsorption on to silica columns as recommended by the supplier. Serum DNA was obtained by using the QIAamp Blood Kit (Qiagen) as described in the manufacturer’s handbook. Nucleic acids were separated by agarose gel electrophoresis, transferred onto nylon membranes and hybridized with an HBV-specific 32P-labelled probe.

The viral titre was estimated in the sera of transgenic mice by comparing the Southern blot hybridization signal of purified serum DNA with a known quantity of cloned HBV DNA.

■ Western blot analysis. Liver or kidney tissue samples (20 mg) were disrupted in 1 × Laemmli loading buffer supplemented with 5% β-mercaptoethanol. The lysates were passed through QIAshredder spin columns and denatured by incubating at 95 °C for 5 min. After gel electrophoresis (13% polyacrylamide, 0.1% SDS) the proteins were blotted onto a PVDF transfer membrane (Schleicher & Schuell). The membrane was probed with a polyclonal rabbit antiserum directed against HBeAg (Nassal, 1992) or pX (antibodies 70606 or anti-X-Ecoli) and bound antibodies were visualized using the ECL Plus Western blotting detection system (Amersham).

■ Detection of HBeAg and HBsAg in murine serum. For detection of serum HBeAg, 30 µl of murine serum was diluted with 170 µl foetal calf serum and analysed using a commercial diagnostic assay (Abbott HBe 2 ELISA). HBsAg seropositivity was analysed by using the AUSAB assay (Abbott).

■ Sequence analysis. Liver DNA of transgenic mice was amplified using the two HBV-specific oligonucleotides 5’ TGC CAT TTG TTC AGT GGT TCG TAG GGC 3’ and 5’ CCG GCA GAT GAG GCA CAG ACG G 3’. The PCR products were sequenced using the Thermo Sequenase cycle sequencing kit (Amersham) and the 32P-labelled primer 5’ CCG GCA GAT GAG GCA CAG ACG 3’.

■ Immunohistochemistry. Tissue specimens were fixed in 4% formaldehyde solution in PBS (pH 7.2). HBV antigen-specific immunostaining was performed with paraffin sections using the avidin–biotin complex method (Hsu et al., 1981). Paraffin sections were treated with a commercial ‘target unmasking fluid’ (Dianova) in a microwave oven before antibody incubation. The sections were incubated overnight at 4 °C with a 1:4000 diluted polyclonal rabbit antiserum specific for HBeAg (Schlicht & Schaller, 1989) or for pX (antibodies 70606 or anti-X-Ecoli), both diluted 1:1000 to 1:4000. Specifically bound antibodies were detected with a biotinylated secondary antibody and subsequent incubation with phosphatase-conjugated streptavidin (BioGenex) and staining with naphthol AS-BI phosphate in combination with hexazonized new fuchsin (Merck). Endogenous avidin-binding activity was reduced by pretreatment of the sections with avidin and biotin solutions (Zymed Laboratories). Negative controls comprised naive rabbit serum and tissues from non-transgenic mice.

Results

Establishment of X-intact and X-deficient HBV-transgenic lineages

We generated two terminally redundant HBV constructs. The first of these constructs had identical structure and sequence to the transgene used previously to establish transgenic mice synthesizing high levels of wild-type HBV particles (Guidotti et al., 1995). The second transgene construct was derived from the wild-type control; however, as shown in Fig. 1, a C → T mutation was introduced within the X coding region. This mutation generates a translational stop signal at codon 8 of the X ORF and prevents the synthesis of pX (Blum et al., 1992). Importantly, this mutation does not affect the overlapping polymerase ORF. Since the DNA construct used to establish transgenic mice is terminally redundant, the mutation was introduced in both copies of the X ORF.

The transgenic DNA constructs were injected into F2 hybrid zygotes and transgenic lineages were established by mating transgenic founder animals positive for HBeAg and HBsAg to C57BL/6 (B6) mice. We succeeded in generating a lineage of X-positive HBV transgenic mice (designation 1.3HBV-3), which exhibited virus replication in the liver and secreted wild-type virions into the serum (data not shown). This result is in line with a previous report (Guidotti et al., 1995). Importantly, we also succeeded in establishing an X-deficient HBV-transgenic lineage (designation 1.3HBV-Xstop-3) stably expressing the HBV seromarkers HBeAg and HBsAg.

Hepadnaviral gene expression

For analysis of hepadnaviral gene expression, the organs of X-deficient 1.3HBV-Xstop-3 transgenic mice were investigated
HBV replication in X-deficient mice

Fig. 1. DNA constructs used for the generation of X-intact and X-deficient 1.3HBV transgenic mice. The X-intact 1.3HBV and the X-deficient 1.3HBV-Xstop constructs used for transgenesis represented more than full-length HBV genomes of the same basic architecture. Since all HBV genes utilize the unique transcriptional termination signal \( [p(A)] \), both constructs were terminated downstream of this signal at position 84. In accordance with a previous report (Guidotti et al., 1995), the 5'-terminal redundancy of both constructs initiated at position 2348 and thus comprised the enhancer I (Enh-I) and enhancer II (Enh-II) elements. The Enh-I and Enh-II elements colocalize with the putative X promoter region and the X ORF, respectively. Thus, both transgene constructs comprised two X genes. The X-intact 1.3HBV wild-type construct carried the authentic ayw HBV sequence. In contrast, in the X-deficient 1.3HBV-Xstop construct a C\textsuperscript{T} mutation was introduced at codon 8 (nucleotide position 2677) of both X ORFs. Introduction of this mutation was shown previously (Blum et al., 1992) to prevent X-gene expression. Importantly, the mutation is silent in the overlapping polymerase ORF. All nucleotide positions are relative to the HBc-ATG with the A being nucleotide 1. Hepadnaviral ORFs are depicted as boxes. With respect to the extensive overlap of the hepadnaviral ORFs, the architecture of the transgene constructs is demonstrated in three levels (upper level: X ORF; medium level: core (pre-C/C) and surface (pre-S1/pre-S2/S) ORFs; lower level: polymerase (Pol) ORF). Translational start codons are indicated by the black dots. The start region of the X ORFs are indicated by the black arrows.

Fig. 2. Expression of core- and surface-specific transcripts in X-negative 1.3HBV-Xstop transgenic livers. Hepadnaviral gene transcription of an X-negative 1.3HBV-Xstop liver (lane 2) was analysed by Northern blotting. Total liver RNA of a non-transgenic B6 mouse (lane 1) was loaded as a negative control. Note that the X-negative transgenic mouse expresses core- and surface-specific transcripts of characteristic sizes.

Fig. 3. HBcAg expression in organs of X-positive 1.3HBV- and X-negative 1.3HBV-Xstop-transgenic mice. Hepatic core protein expression (HBcAg) of X-positive 1.3HBV-3 and X-negative 1.3HBV-Xstop-3 mice was analysed by staining Western blots of liver and kidney proteins with a polyclonal rabbit antibody recognizing the core gene products (Schlicht & Schaller, 1989). Note, that a comparable hepatic HBcAg expression level could be observed in the livers of the X-positive (lane 4) and X-negative (lane 2) transgenic mice. Likewise, renal core protein expression was adequate between X-positive 1.3HBV-3 (data not shown) and X-negative 1.3HBV-Xstop-3 (lane 3) animals (B6, non-transgenic control). Transgenic mice (Fig. 3, lane 2) by Western blotting was comparable to that of the X-positive control (Fig. 3, lane 4). Comparable amounts of HBcAg could also be detected in the kidneys of the X-positive and X-negative HBV transgenic mice (Fig. 3). To investigate the cellular distribution pattern of core protein expression, paraffin sections of formaldehyde-fixed liver and kidney specimens of X-intact and X-deficient mice were immunostained with an HBcAg-specific polyclonal antibody. As depicted in Fig. 4, the HBcAg staining pattern was similar in the livers of the X-intact (Fig. 4A) and X-deficient (Fig. 4B) mice. In both types of transgenic animals, HBcAg-positive hepatocytes were predominantly located in the centrolobular liver regions and only a few labelled cells were detectable in the periphery of the liver lobules. A considerable
but inter-individually varying proportion (maximally 50%) of labelled hepatocytes also exhibited a positive cytoplasmic immunostaining as well as the nuclear immunoreactivity.

There were also no significant differences detectable regarding the renal HBCAg-specific staining pattern of X-intact control mice (Fig. 4C) and X-deficient animals (Fig. 4D). In both types of transgenic kidneys, HBCAg staining was restricted to the tubular epithelium and the intensity of the nuclear staining significantly exceeded that of the cytoplasmic fraction.

**HBV replication and virion export**

To answer the question of whether pX is central to hepadnavirus replication in transgenic mice, Southern blots were prepared from hepatic and renal DNA isolated from X-deficient mice and hybridized with a $^32$P-labelled HBV-specific probe. Interestingly, we could detect HBV replicative intermediates in the livers (Fig. 5, lane 2) and kidneys (Fig. 5, lane 1) of the X-deficient mice demonstrating that these murine tissues support HBV replication even in the absence of pX. To exclude the possibility that efficient hepadnavirus replication observed in the X-deficient transgenic organs might be due to a remutation restoring the X-gene, we determined the sequence of a PCR-amplified DNA fragment generated from an X-deficient liver DNA template. These investigations confirmed the presence of the stop mutation at codon 8 in the X ORF (data not shown).

As already mentioned, the X-deficient transgenic mice expressed abundant quantities of surface proteins in their
Discussion

In the present study we have generated a transgene construct with the same basic architecture as that shown previously to drive hepadnavirus replication in transgenic mice (Guidotti et al., 1995) but lacking the X gene (Fig. 1). The mutation introduced into the X gene did not affect the overlapping polymerase ORF but had been shown previously to abolish the transactivating potential of the X gene product (Blum et al., 1992). The X-deficient construct was used to establish transgenic mice. These animals expressed the HBV core gene efficiently and exhibited similar expression characteristics to the X-intact controls (Figs 2, 3 and 4). It should be mentioned that we failed to detect the X protein in the organs of the X-positive HBV transgenic control mice by Western blotting and immunohistochemistry. However, this result is not surprising, since it has been discussed controversially whether the HBV X protein can be detected in vivo at all. There are some reports claiming successful detection of HBx in transgenic or human livers (Kim et al., 1991; Lee et al., 1990; Madden et al., 2001; Su et al., 1998) but these data could not be reproduced by others (Guidotti et al., 1996; Guo et al., 1993; Reifenberg et al., 1997). In this context it should be mentioned that the polyclonal rabbit antisera used for the present study have proved to be suited for X-protein detection in HBV-infected human livers (Su et al., 1998). However, the same sera failed to indicate X expression in the livers of a multitude of X-transgenic mice (Guidotti et al., 1996; Reifenberg et al., 1997, 1999).

Importantly, our data have demonstrated that X-negative HBV-transgenic mice are capable of HBV replication and of virion export (Fig. 5). The viral DNA detectable in the serum of the transgenic animals was of the relaxed circular species, clearly indicating that it could not have resulted from cell death and the release of intracellular core particles but originated from the export of mature enveloped viral particles. Hence, pX is not central for these two steps of the viral life-cycle in vivo, which is in accordance with previous results obtained with transfected cells (Blum et al., 1992; Koike et al., 1989; Yaginuma et al., 1987). Furthermore, it must be stated that hepadnavirus replication and virion export in the absence of X coexpression is no special feature of the transgenic lineage described in the present paper, but could also be achieved in another murine transgene model (Christa Kuhn, unpublished observations). On the other hand, two independent research groups (Chen et al., 1993; Zoulim et al., 1994) have reported that X-deficient WHV genomes injected into woodchuck livers failed to establish hepadnaviral infection, whereas injection of X-intact control DNA resulted in viraemia. These studies clearly indicate an essential role for the X protein in the natural life-cycle of WHV. In the light of our results, we assume that the failure to establish WHV infection by injecting X-deficient viral genomes is most probably not due to a replication defect of the X-deficient WHV virus particles.

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