

# Detection of the human hepatitis B virus X-protein in transgenic mice after radioactive labelling at a newly introduced phosphorylation site

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Besides the three essential genes encoding the envelope, core and polymerase proteins, all mammalian hepadnaviruses examined to date contain a fourth gene which is referred to as the *x*-gene. This gene is believed to encode a transcriptional transactivator which positively regulates viral gene expression. Attempts to detect X-protein *in vivo* or in tissue culture lead to varying results. Whereas some groups could detect a protein of the expected size, other groups did not. To establish optimal conditions for the isolation of the human hepatitis B virus X-protein, we introduced a recognition site for protein kinase A into the *x*-gene. Upon phosphorylation with radioactive ATP, this modified X-protein can be detected with very high specificity and sensitivity. Tissue culture experiments showed that X-protein expressed from a cytomegalovirus-driven plasmid is not soluble in non-ionic detergent but rather has to be extracted from the cell pellet by boiling with SDS at a slightly alkaline pH. This method was then used to examine the organs of several transgenic mouse lines which expressed the modified *x*-gene under control of the authentic promoter. The data show that expression of the *x*-gene and subsequent biosynthesis of the X-protein is not tissue-specific but rather can occur in most organs.

## Introduction

Sequencing of the genomes of the three mammalian hepadnaviruses characterized to date, the human, woodchuck and ground squirrel hepatitis B viruses, revealed four open reading frames (Summers, 1981). By biochemical and genetic analyses three of them could be assigned to the essential viral proteins, namely the surface proteins, the nucleocapsid protein and the polymerase (Ganem & Varmus, 1987). However, the relevance of the fourth open reading frame, which due to its unknown function was designated as the *x*-gene, is still unclear. Numerous *in vitro* studies have shown that the *x*-gene product can transactivate not only viral but also many cellular promoters (reviewed by Rossner, 1992). It is also required for the establishment of a woodchuck hepatitis B virus infection *in vivo* (Chen *et al.*, 1993; Zoulim *et al.*, 1994), but is not essential for the production of virus particles in tissue culture (Yaginuma *et al.*, 1987; Blum *et al.*, 1992).

Due to the transactivating activity of the X-protein, great efforts were undertaken to test whether this viral gene product could be involved in the induction of liver carcinoma, a complication which is frequently seen in patients who are chronically infected with the human hepatitis B virus (HBV). In particular, several transgenic mouse lines were established which expressed the HBV X-protein under authentic or foreign promoter control. One problem which was encountered during these studies was the fact that it proved quite difficult to detect the X-protein in organs of the animals although, as could be proven by the detection of the corresponding mRNA, the transgene was well expressed (Perfumo *et al.*, 1992). Until today, the following efforts to detect the X-protein *in vivo* have been described.

Billet *et al.* (1995) generated transgenic mice under authentic promoter/enhancer control (lines PEX) as well as under the hepatospecific antithrombin III gene regulatory region (lines AX). Balsano *et al.* (1994) found a 16.5 kDa band in Western blot analysis in liver tissue of 8-day-old transgenic mice of line AX16, whereas Billet *et al.* (1995) only found a positive Northern blot signal. However, a positive Western blot signal was shown in the liver of a 10-day-old mouse of line PEX7 (Terradillos *et al.*, 1997).

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Likewise, Lee *et al.* (1990) detected X-protein in the liver of 4-week-old X-transgenic mice using the human  $\alpha$ -1-antitrypsin regulatory region. By immunohistochemistry only weak staining could be noted; in older mice the X-protein was undetectable. In another study a similar Western blot analysis shows a continual X-protein expression level in animals up to 9 months old (Slagle *et al.*, 1996).

Likewise, Kim *et al.* (1991) show expression of the *x*-gene under its own regulatory elements in a transgenic mouse only by Northern blot analysis. An 18 kDa band in SDS-PAGE could be found in a primary culture of hepatocytes labelled with [<sup>35</sup>S]methionine (Koike *et al.*, 1994).

In a different study detection of X-protein by Western blotting succeeded after preparation of a whole tissue homogenate resuspended in Western blotting sample buffer (Moriya *et al.*, 1996).

The aim of this study was to establish optimal conditions for the isolation of X-protein from tissue culture cells as well as from organ material. To facilitate this analysis we used a modified X-protein which could be radioactively labelled with high efficiency and specificity. Our data show that most, if not all, of the X-protein expressed from a cytomegalovirus (CMV)-driven plasmid is not soluble in aqueous buffer containing a non-ionic detergent but rather has to be extracted from the insoluble fraction by boiling with a slightly alkaline SDS buffer. With this method we were able to detect the X-protein in transgenic mice expressing this gene under authentic promoter control.

## Methods

**■ DNA constructs.** For generation of a plasmid expressing the *x*-gene under authentic promoter control (pHBV-X) plasmid p24.6/29 (Reifenberg *et al.*, 1997; Fig. 1a) containing the enhancer I, the *x*-promoter, the *x*-gene, the enhancer II, the basal core promoter and the *preC/core* gene (HBV 2104–709), cloned as a PCR fragment with newly introduced *Hind*III and *Pst*I sites, and a repetition of the poly(A) signal (*Bam*HI–*Sac*I fragment, HBV 2682–87) was digested by *Bgl*II and religated, removing the major part of the *core* gene (HBV 84–523). The 1941 bp *Hind*III–*Sac*I fragment had previously been successfully used for pronuclear injection, generating the lines HBxI and HBxIV (Reifenberg *et al.*, 1997).

To obtain high expression of the *x*-gene in tissue culture, the *x*-gene was cloned downstream of the immediate early 1-promoter/enhancer of human cytomegalovirus (HCMV-P/E; Fig. 1b). The *x*-gene together with its poly(A) signal (2654–84) was amplified from plasmid pMH3/3097, which contains an overlength HBV genome (Junker *et al.*, 1987). The fragment thus generated contained a *Hind*III site at the 5' end, which was introduced by the upstream primer, and a *Sac*I site at the 3' end, which originated from the plasmid. This fragment was cloned into the vector pUC19 (Gibco BRL). HCMV-P/E was amplified by PCR from plasmid pcDNAIamp (Invitrogen), introducing a *Pst*I site at the 5' end and a *Hind*III site at the 3' end, and cloned upstream of the construct described above, generating the plasmid pCMV-X.

For generation of a plasmid expressing a modified X-protein with a recognition site for protein kinase A (pCMV-X-PKA, Fig. 1c), the *x*-gene was amplified from plasmid pMH3/3097 using the primers X-PKA+, 5' CCC GGA TCC ACC ATG GCT GCT AGG CTG TGC 3', and X-

PKA–, 5' ACG GAT ATC TTA CAA CGA TAG TCT CCG GGC AGA GGT GAA AAA GTT 3'. The upstream primer introduces a *Bam*HI site (underlined) immediately 5' from the *x*-gene ATG (shown in bold face), whereas the downstream primer provides the PKA recognition site (shown in bold face) as well as a new stop codon and an *Eco*RV site (underlined). This fragment was inserted into pcDNAIamp (Invitrogen) containing the HCMV-P/E and the simian virus 40 poly(A) signal.

For expression of the newly modified gene *x*-PKA under the authentic promoter control (pHBV-X-PKA, Fig. 1c), the *x*-PKA fragment was isolated by digestion with *Nco*I/*Eco*RV and recloned into plasmid pHBV-X, replacing the *x*-gene with the newly modified *x*-PKA gene. The *Hind*III–*Sac*I fragment was used for pronuclear injection, generating the lines X-PKA I, III–VII.

**■ Generation of transgenic mice.** All animal experiments were performed in accordance with the German regulations for the use of animals in biomedical research. Transgenic mice were produced according to standard procedures (Hogan *et al.*, 1994). The transgenic lines expressing the wild-type *x*-gene under authentic promoter control (HBxI and -IV) have been described (Reifenberg *et al.*, 1997). For generation of transgenic lines expressing the modified X-protein, the *Hind*III–*Sac*I fragment from plasmid pHBV-X-PKA was microinjected into one pronucleus of (C57BL/6J × CBA/Ca) F2 fertilized eggs. Transgene integration was detected by PCR analysis.

**■ Transient expression of X-protein in tissue culture.** HuH-7 cells, a human hepatoma cell line, were grown in six-well tissue culture plates (9 cm<sup>2</sup> per well) to 80% confluency in 2 ml Dulbecco's minimal essential medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin G, 0.1 mg/ml streptomycin sulfate. Cells were rinsed once with serum-free medium. Two  $\mu$ g plasmid DNA was diluted into 100  $\mu$ l serum-free medium; 8  $\mu$ l lipofectamine (Gibco BRL) was also diluted into 100  $\mu$ l serum-free medium. The two solutions were combined, mixed gently and incubated at room temperature for 30 min. After adding 800  $\mu$ l serum-free medium the DNA-liposome complexes were overlaid onto the cells. After 6 h of incubation at 37 °C, 1 ml medium containing 20% foetal calf serum was added and the cells were grown for another 42 h, replacing the medium once.

**■ Lysis of tissue culture cells.** One ml 1% Triton lysis buffer [1% Triton X-100, 100  $\mu$ M PMSF, 0.1% sodium azide in TNE (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA)] was added to each well. Alternatively, an acidic or alkaline Triton lysis was performed (Fig. 2): 1% Triton X-100 in acidic lysis buffer (C/PNE pH 4.5), containing 2 mM citrate-phosphate buffer (pH 4.5), 130 mM NaCl, 1 mM EDTA, or 1% Triton X-100 in alkaline lysis buffer containing TNE (pH 8.8).

The cells lysed by 1% Triton lysis buffer were collected with a cell-scraper and non-soluble material was pelleted by centrifugation (10 min, 14 000 r.p.m.). The cleared supernatant was directly used for immunoprecipitation (Triton lysate), the pellet was further treated with SDS- $\beta$ -mercaptoethanol (ME) lysis (see below). Proteins contained in the pellets were dissolved by boiling in 50  $\mu$ l 2% SDS-ME lysis buffer (2% SDS, 2% ME in TNE pH 8.0). Again, acidic or alkaline lysis buffers were used alternatively (Fig. 2), containing either C/PNE pH 4.5 or TNE pH 8.8, 2% SDS, 2% ME. The samples were subsequently pulled through 23G and 27G needles and diluted to 950  $\mu$ l 1% Triton X-100 in the corresponding buffers (TNE pH 8.0, C/PNE pH 4.5, TNE pH 8.8). Again, the samples were cleared by centrifugation prior to immunoprecipitation.

**■ Lysis of mouse tissue samples.** Organs were frozen in liquid nitrogen and pulverized in a micro-dismembrator (Braun Biotech). One hundred mg (ovaries 30 mg) of frozen tissue powder was lysed in 2 ml 1% Triton lysis buffer (pH 8.0). After centrifugation the Triton X-100 soluble fraction was dispelled, the insoluble fraction was homogenized by

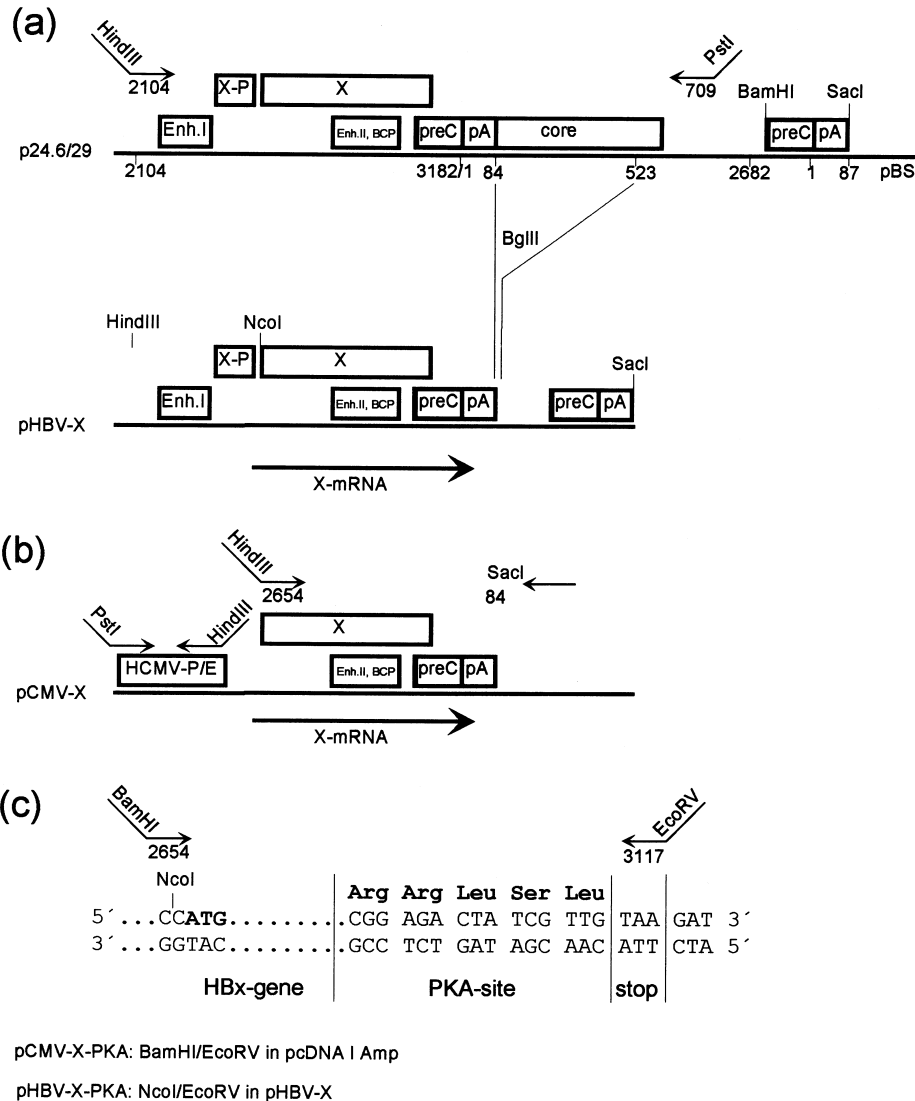


Fig. 1. HBV-X and CMV-X constructs used for the generation of transgenic mice and transfection of tissue culture cells. (a) The plasmid pHBV-X was obtained by *Bgl*III digestion of p24.6/29, deleting the major part of the *core* gene. It served for expression of the *x*-gene in tissue culture as well as in transgenic mice (Reifenberg *et al.*, 1997). (b) In pCMV-X the immediate-early 1 promoter/enhancer of the human cytomegalovirus (HCMV-P/E) was used instead of the *x*-promoter (X-P)/enhancer I (Enh.I) to obtain a high expression of the *x*-gene in tissue culture cells. (c) Five nucleotide triplets encoding a PKA-recognition site (PKA-site) were added to the 3' terminus of the *x*-gene by PCR mutagenesis, yielding the plasmids pCMV-X-PKA (used for transient transfection of tissue culture) and pHBV-X-PKA (used for generation of transgenic mouse founders X-PKA I-VII).

subsequent pulling through 20G and 23G needles in 750  $\mu$ l 2% SDS–ME lysis buffer (pH 8.8), boiled, centrifuged and the supernatant was dissolved 1:20 in 1% Triton lysis buffer (pH 8.8). To reduce the background, this solution was cleared again by centrifugation at 20000 *g* before immunoprecipitation.

■ **Isolation of X-protein by immunoprecipitation.** Fifteen  $\mu$ l of the anti-X specific polyclonal rabbit antiserum 70646 (raised against a recombinant HBx–cellular fusion protein expressed in *Escherichia coli*) was adsorbed to 70  $\mu$ l pre-swollen protein A–Sepharose CL-4B (Pharmacia) in 300  $\mu$ l PBS for 30 min and then added to organ or tissue culture lysates. Immunoprecipitation was carried out overnight at 4  $^{\circ}$ C.

■ **Radioactive labelling of X–PKA protein with PKA and [ $\gamma$ - $^{32}$ P]ATP.** The immunoprecipitates were washed three times with PBS

buffer and twice with PKA buffer (20 mM Tris–HCl, pH 7.0, 100 mM NaCl, 10 mM MgCl<sub>2</sub>). After adding 30  $\mu$ l PKA buffer supplemented with 1 mM dithiothreitol (DTT), 20  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP and 4 U PKA (Sigma) reconstituted in deionized water containing 6 mg/ml DTT, samples were incubated three times for 15 min at 30  $^{\circ}$ C, adding 4 U of the enzyme each time.

■ **SDS–PAGE and detection of X–PKA protein by autoradiography.** After four washing steps with PBS, the precipitated proteins were dissolved by boiling the immunocomplexes for 5 min in 40  $\mu$ l of reducing protein sample buffer (200 mM Tris–HCl pH 8.8, 0.5 M sucrose, 5 mM EDTA, 0.1% bromophenol blue; adding 3% SDS, 2% ME shortly prior to use) and separated on a 15% discontinuous SDS–polyacrylamide gel, with a constant current of 20 mA/cm<sup>2</sup>, using prestained protein molecular mass standards (Gibco BRL) as marker



Fig. 2. Solubility of the modified protein X-PKA. Cells grown in a 9 cm<sup>2</sup> well and transiently transfected with the plasmid pCMV-X-PKA were lysed using different detergents and pH values in order to determine the appropriate conditions for detecting X-PKA protein. After immunoprecipitation with the X-specific polyclonal rabbit antiserum 70646 X-PKA protein was radiolabelled with PKA and [ $\gamma$ -<sup>32</sup>P]ATP and analysed by SDS-PAGE and subsequent autoradiography. X-PKA protein was not soluble in 1% Triton lysis buffer, regardless of the applied pH (pCMV-X-PKA transfections in lanes 1–3, control transfections in lanes 4–6). After boiling the Triton-insoluble pellet in 2% SDS, 2% ME (pCMV-X-PKA transfections in lanes 8–10, control transfections in lanes 11–13), X-PKA protein was soluble in alkaline buffers (lanes 9, TNE at pH 8.0; 10, TNE at pH 8.8), but not in acidic buffer (lane 8, citrate-phosphate buffer at pH 4.5).

proteins. Subsequently the gel was dried and exposed to a film. Corresponding to the calculated molecular mass a specific band should be expected at 17 kDa.

■ **Western blotting.** The proteins separated in the SDS-polyacrylamide gel were transferred to a nitrocellulose membrane (Schleicher & Schuell) in transfer buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol; overnight transfer, 60 V, 4 °C) according to the method of Towbin *et al.* (1979). The membrane was briefly washed in PBS and unspecific protein binding sites were saturated with PBS-1% BSA-0.1% sodium azide over 6 h. The polyclonal anti-X antiserum 70646 was added to a dilution of 1:2000 and incubated overnight at room temperature. After three washing steps (PBS, PBS-0.1% Triton X-100, PBS) the specifically bound antibodies were detected by protein A-alkaline phosphatase conjugate (15 U) in PBS-1% BSA-0.1% sodium azide for 3 h. After another three washing steps the membrane was incubated twice for 15 min in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and briefly rinsed in 100 mM Tris-HCl (pH 9.2), 100 mM NaCl, 50 mM MgCl<sub>2</sub>. Ninety  $\mu$ l NBT (nitro blue tetrazolium) solution (75 mg/ml NBT, 70% dimethylformamide) and 70  $\mu$ l BCIP (5-bromo-4-chloro-3-indolyl phosphate) solution (50 mg/ml BCIP in dimethylformamide) were added to 20 ml 100 mM Tris-HCl (pH 9.2), 100 mM NaCl, 50 mM MgCl<sub>2</sub> for the colour reaction (stopped with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

## Results

### The X-protein is not soluble in aqueous buffer containing a non-ionic detergent

Although there are several reports describing the detection of the X-protein *in vivo*, we as well as other groups have been unsuccessful in repeating these data. In particular, we had been unable to detect the X-protein in transgenic mice by Western

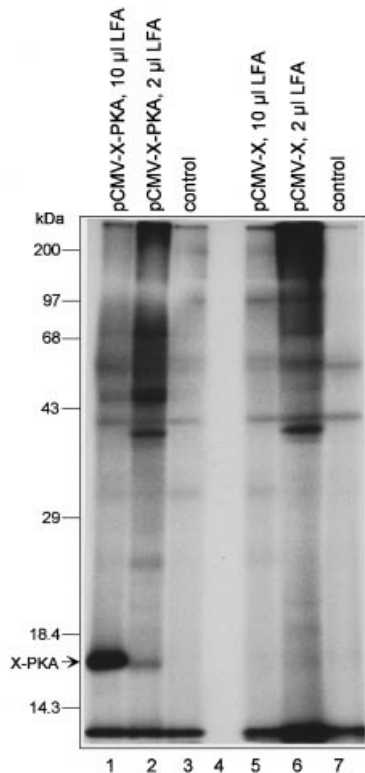
blotting although, as could be proved by Northern blotting, the transgene was expressed. In principle, this negative result could be explained by weak expression, an instability of the protein, technical problems or a combination of these possibilities.

In order to examine these points we decided to produce a construct expressing a slightly modified X-protein which can be detected with high sensitivity. To this end, a short sequence of 15 nucleotides was attached to the 3' end of the *x*-gene by PCR mutagenesis and the gene thus modified was cloned downstream of the HCMV major immediate-early promoter/enhancer (pCMV-X-PKA; Fig. 1). The five amino acids encoded by the extra sequence represent a specific recognition site for PKA. Since the PKA phosphorylation using [ $\gamma$ -<sup>32</sup>P]ATP is both efficient and specific, it allows the detection of even trace amounts of a protein. This method has previously been used for the detection of the HBV polymerase in a vaccinia virus system (Bartenschlager *et al.*, 1991).

In the first experiment, HuH-7 cells, transiently transfected with pCMV-X-PKA, were lysed with buffer containing 1% Triton X-100 and subjected to immunoprecipitation with an X-specific antiserum. The immunoprecipitated proteins were incubated with PKA and [ $\gamma$ -<sup>32</sup>P]ATP and then analysed by SDS-PAGE and subsequent autoradiography. Surprisingly, no specific band could be detected in the samples obtained from the cells transfected with pCMV-X-PKA in comparison to untransfected cells (data not shown).

In the next experiment we therefore decided to analyse not only the soluble but also the non-soluble fraction of the cells and also to test the effects of different pH values. As is shown in Fig. 2, again no specific protein band could be detected in the cell lysates, regardless of whether an acidic or an alkaline lysis buffer was used (Fig. 2, lanes 1–3). However, by boiling the cell pellets with a slightly alkaline buffer containing 2% SDS and 2% ME a protein with the expected size of about 17 kDa could be extracted from the transfected cells which was specifically phosphorylated by PKA (Fig. 2, lanes 9 and 10). Interestingly, this band was not observed if an acidic extraction buffer was used (lane 8).

To provide additional evidence that the 17 kDa band was in fact due to the phosphorylated modified X-protein, HuH-7 cells were transfected with expression plasmids containing either the modified or the wild-type *x*-gene under the control of the HCMV-P/E (Fig. 3). For transfection, 2  $\mu$ g plasmid DNA (pCMV-X-PKA, lanes 1 and 2; pCMV-X, lanes 5 and 6) and 10  $\mu$ l (lanes 1 and 5) or 2  $\mu$ l (lanes 2 and 6) lipofectamine was added to 9 cm<sup>2</sup> cell culture wells. Cells treated with 10  $\mu$ l lipofectamine showed morphological signs of toxicity, therefore 8  $\mu$ l lipofectamine was used per each 9 cm<sup>2</sup> well in the following experiments. After 2 days of expression the cells were lysed, the soluble fraction was discarded and the cell pellet extracted with the alkaline SDS-buffer (pH 8.0) as described above. As is shown in Fig. 3, a protein of the expected molecular mass could only be detected in the cells

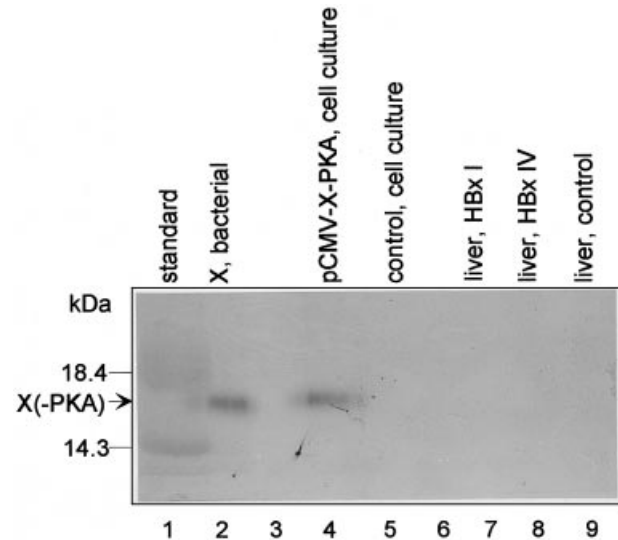


**Fig. 3.** Comparative transfection of X-PKA protein and authentic X-protein. The *x-PKA* gene (lanes 1 and 2) or the authentic *x*-gene (lanes 5 and 6) were transfected into cell cultures. Two µg plasmid DNA and 10 µl (lanes 1 and 5) or 2 µl (lanes 2 and 6) lipofectamine were used for transfection. Untransfected cells served as negative controls (lanes 3 and 7). After alkaline SDS lysis of the Triton-insoluble protein fraction, immunoprecipitation, incubation with PKA and [ $\gamma$ - $^{32}$ P]ATP, SDS-PAGE and autoradiography, a protein of the expected molecular mass could only be detected in the cells which had been transfected with the plasmid expressing the modified *x-PKA* gene. This observation suggested that the immunoprecipitated 17 kDa protein was indeed specifically labelled at the newly introduced PKA site.

which had been transfected with the plasmid expressing the modified *x*-gene and thus does in fact represent the X-PKA protein (Fig. 3, lanes 1 and 2). From these data we conclude that an alkaline buffer with a strong ionic detergent is required to extract the X-protein from the cells. This fact was probably at least one reason why our former efforts to detect the X-protein had failed and might also explain the negative results obtained by other groups.

#### Detection of the X-protein by Western blotting

As has been shown above, a special procedure had to be used to extract the X-protein from transfected cells. However, even if a suitable method for the isolation of this protein is employed, insufficient expression could still make detection of this protein impossible. To test this, X-protein was isolated from HuH-7 cells (material from four 9 cm<sup>2</sup> wells) which had been transfected with a plasmid expressing the modified X-protein under the control of the HCMV promoter/enhancer (pCMV-X-PKA) as well as from the livers of two 8-week-old



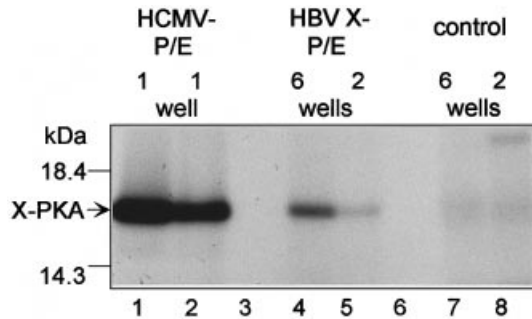
**Fig. 4.** Western blot analysis comparing liver tissue from transgenic mice to transiently transfected tissue culture cells and bacterially expressed X-protein. In transiently transfected cells or 100 mg of pulverized frozen liver tissue, the Triton-insoluble fraction was lysed with alkaline 2% SDS-ME lysis buffer, immunoprecipitated, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The X(-PKA) protein was detected by the anti-X antiserum 70646 and a colour reaction with a protein A-alkaline phosphatase conjugate. In cells transfected with the plasmid pCMV-X-PKA (material of four 9 cm<sup>2</sup> wells; lane 4) a protein only slightly heavier (five additional amino acids) than the positive control (bacterially expressed X-protein, lane 2) was detectable. The livers of two 8-week-old male X-expressing transgenic mice were negative (line HBxI in lane 7, line HBxIV in lane 8), suggesting that the Western blot was not sensitive enough for detection of X-protein *in vivo*. Untransfected cell culture (lane 5) and a liver from a non-transgenic mouse (lane 9) served as controls. Lane 1 contains a molecular mass standard, lanes 3 and 6 are clear.

transgenic mice expressing the X-protein under authentic promoter/enhancer control. After extraction of the insoluble protein fraction with an alkaline SDS buffer and subsequent immunoprecipitation the proteins thus obtained were analysed by SDS-PAGE and Western blotting. As a positive control bacterially expressed X-protein was directly loaded on the gel.

As is obvious from Fig. 4, a protein only slightly heavier (5 additional amino acids) than the bacterially expressed X-protein (lane 2) was detected in the transfected cells (lane 4). The fact that no such band could be observed in our previous experiments shows that the special extraction procedure described here was essential for isolation of the X-protein. However, the liver samples (lane 7 and 8) were negative, suggesting that the Western blot was not sensitive enough. Therefore, as far as our transgenic mice are considered, probably not only was the method which we had used previously not suitable but also there was not enough X-protein present.

#### Generation of transgenic mice expressing the modified X-protein

If the Western blot discussed above (Fig. 4) is compared to the autoradiographs shown in Figs 2 and 3 it is obvious that

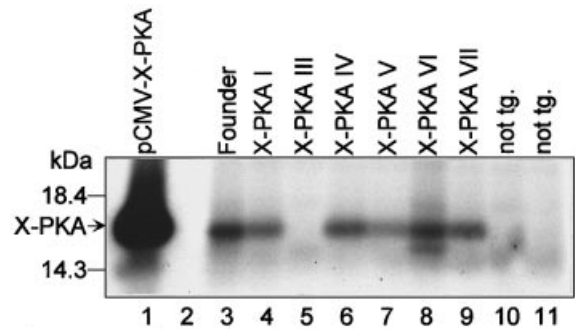


**Fig. 5.** Expression of the *x*-PKA gene driven by the HCMV-P/E or by the authentic HBV X-promoter/enhancer I and II (HBV X-P/E). The *x*-PKA gene under control of the HCMV-P/E (lanes 1 and 2, each containing the material of one 9 cm<sup>2</sup> well) as well as the authentic HBV X-P/E (lanes 4 and 5, each containing the material of either six or two wells) were transfected into cells grown in 9 cm<sup>2</sup> wells (2 µg DNA, 8 µl lipofectamine). Untransfected cells served as negative controls (lanes 7 and 8; each containing the material of six or two wells). After extraction of the Triton-insoluble protein fraction with alkaline SDS-ME buffer, immunoprecipitation, radioactive phosphorylation, SDS-PAGE and autoradiography, phosphorylated X-protein was detectable with less but still clear intensity in the cells transfected with the plasmid containing the HBV X-P/E (pHBV-X-PKA) compared to the plasmid containing the HCMV-P/E (pCMV-X-PKA). Lanes 3 and 6 are clear.

detection of the modified X-protein after phosphorylation is a much more sensitive method than detection of the authentic X-protein by Western blotting. Since we wanted to analyse X-protein biosynthesis *in vivo* under natural conditions, we therefore decided to generate a transgenic mouse expressing the modified *x*-gene under the control of the authentic *x*-gene promoter. To this end the HCMV-P/E of pCMV-X-PKA was replaced by a fragment of the HBV genome which had been shown to contain sequences controlling the expression of the *x*-gene (Shaul *et al.*, 1985; Laub & Treinin, 1987). This construct, referred to as pHBV-X-PKA, was then first used to transfect HuH-7 cells. The material of either six or two wells was pooled for immunoprecipitation. Cells transfected with pCMV-X-PKA as well as untransfected cells served as controls.

As shown in Fig. 5, phosphorylated X-protein was clearly detectable in the cells transfected with pHBV-X-PKA (lanes 4 and 5). Compared to the pCMV-X-PKA transfections (lanes 1 and 2), it can be estimated that the authentic *x*-promoter is about 40-fold (as calculated by Image Quant) less efficient. However, this appeared to be sufficient for our purposes.

For generation of transgenic mice a fragment containing the *x*-gene together with its authentic regulatory sequences was excised from pHBV-X-PKA. After microinjection of this fragment we obtained six fertile founder mice (X-PKA I, III, IV, V, VI and VII) which carried the transgene. These founders were then backcrossed to C57BL/6J mice. One of the founder mice, which had no offspring, was sacrificed and liver and kidneys analysed for X-mRNA by RT-PCR as described (Reifenberg *et al.*, 1997). Both organs were positive, showing that our construct was in fact expressed *in vivo* (data not shown).



**Fig. 6.** Detection of X-PKA protein in liver tissue of mice from different transgenic X-PKA lines. For generation of transgenic founders (X-PKA I-VII) pHBV-X-PKA was linearized and the vector was excised. Liver tissue (100 mg) of transgenic mice of the F1 generation was investigated for X-PKA protein expression applying the protein extraction protocol established in transfected cell culture (see Figs 2, 3 and 5). Five out of six transgenic lines expressed X-PKA protein in the liver (X-PKA I, IV, V, VI, VII). Lanes 3-9 show liver samples (100 mg) of transgenic animals, lanes 10 and 11 show liver samples of control animals. The liver of the sterile founder animal which had been positive in RT-PCR (lane 3) as well as pCMV-X-PKA-transfected cell culture (one-quarter of the phosphorylated immunoprecipitate of a transfected tissue culture, 1 well; lane 1) were used as positive controls. Lane 2 is clear.

#### Detection of the modified X-protein *in vivo*

One transgenic F1 offspring of each of the six remaining founders was sacrificed. Liver tissue (100 mg) was prepared and homogenized in 1% Triton lysis buffer. The soluble fraction was discarded and the pellet analysed for X-protein as described above. As shown in Fig. 6, the phosphorylated X-protein could be found in the livers of five of the six offspring animals tested as well as in the liver of the sterile founder. As a positive control one-quarter of the phosphorylated immunoprecipitate of a transfected tissue culture (1 well) was used (lane 1). Altogether, six transgenic lines could be established with the construct HBV-X-PKA, out of which five lines (X-PKA I, IV, V, VI, VII) expressed the X-PKA protein in the liver.

In order to analyse the tissue distribution of the X-protein, mice of two different lines, one male 12-week-old mouse of line X-PKA I (Fig. 7, upper panel) and one female 21-week-old mouse of line X-PKA IV (Fig. 7, lower panel) were sacrificed to investigate the X-protein expression in 14 different tissues. One hundred mg (ovaries 30 mg) of 14 different tissues was examined for X-protein expression. As is clear from Fig. 7, X-protein could be easily detected in most organs of both animals.

#### Discussion

In this study we developed a method to detect the human HBV X-protein in liver tissue from transgenic mice. Therefore, we introduced a phosphorylation site into the X-protein which allowed us to specifically label the X-protein with [ $\gamma$ -<sup>32</sup>P]ATP and PKA.

To date, there have been extensive studies in which the transacting potential of the X-protein on autologous viral as

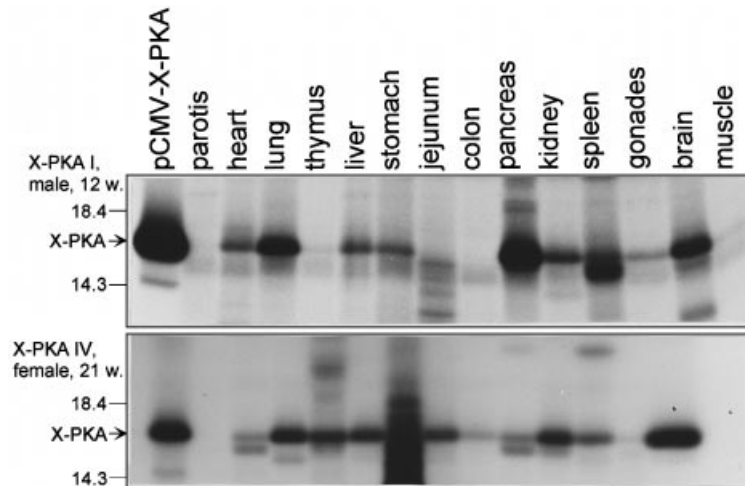


Fig. 7. Detection of X-PKA protein in 14 different tissues of two X-PKA transgenic mice of different line, age and sex. X-protein expression was investigated in organs of one male, 12-week-old mouse of line X-PKA I (upper panel) and one female, 21-week-old mouse of line X-PKA IV (lower panel). The highest expression level was found in lung, kidney and brain, and in line X-PKA I also in pancreas. Surprisingly, the liver showed only a medium expression level. Low amounts or no expression was found in parotis, heart, thymus, spleen, gonads and skeleton muscle. Overall, the female, 21-week-old mouse of line IV shows higher expression levels than the male, 12-week-old mouse of line I, as the film of the lower panel was exposed only 1 h compared to 4 h of the upper panel. Compare also the signals of the positive controls of both panels, each containing the same amount of X-PKA protein (one-quarter of a phosphorylated immunoprecipitate of a pCMV-X-PKA-transfected tissue culture well).

well as heterologous viral and cellular promoters has been examined by *in vitro* transfection assays (Rossner, 1992). However, information about the regulation and function of the X-protein *in vivo* is still limited. A major problem in investigation of the X-protein *in vivo* is the necessity of an efficient and sensitive technique for X-protein detection in tissue probes.

Studies on X-transgenic mice lack convincing data concerning the detection of the X-protein and the published data are often not consistent. Balsano *et al.* (1994) show a positive Western blot analysis using only 10 mg liver tissue of transgenic mice (line AX16), yet immunohistology in the same animals gave only a weak signal. Billet *et al.* (1995) show no protein detection, but only X-mRNA in livers of these animals.

In a transgenic mouse line which expresses the X-protein under the control of the human  $\alpha$ -1-antitrypsin regulatory region, Lee *et al.* (1990) detected X-protein in the liver only in 2–4-week-old transgenic mice in Western blot analysis, whereas Slagle *et al.* (1996) found a continuous expression level in mice of the same transgenic line from 4 weeks old up to 9 months old.

Because of the technical problems in detecting X-protein in tissue samples, Kim *et al.* (1991) as well as Koike *et al.* (1994) tried to detect X-protein in livers of transgenic mice by using a [ $^{35}$ S]methionine labelling technique either *in vivo* or *in vitro* after isolation of transgenic mouse hepatocytes with collagenase.

A recent study compared 11 mono- and polyclonal anti-X antibodies from five different laboratories (Su *et al.*, 1998). The antiserum used to detect the X-PKA protein in our study

proved to react strongly and also highly specific with X-protein not only in Western blots of recombinant X-protein but also in immunohistochemistry of human livers with HBV-associated disease. Thus, none of the antibodies tested showed any reaction with lysates of these explanted human livers in Western blot analyses.

The great variation of antibodies against X-protein in addition to different extraction procedures and detection techniques applied in previous studies of X-transgenic mice might explain in part the varying success in detection of X-protein. Likewise, the level of X-protein expression due to different promoter elements may influence the formation of aggregates and thus the solubility of X-protein.

In the study presented here we show in particular that the Western blotting technique is not sensitive enough to detect the X-protein in tissue samples in which it is expressed rather weakly under authentic promoter control.

In order to establish a method to solubilize the X-protein in tissue samples to make it accessible to immunoprecipitation a reliable detection technique was necessary. Therefore a modification was introduced into the 3' end of the *x*-gene which allowed us to radioactively label the X-protein at a newly introduced phosphorylation site. Incubation with PKA and [ $\gamma$ - $^{32}$ P]ATP enabled detection of the immunoprecipitated proteins at a very high sensitivity and specificity.

A method for the detection of proteins which are expressed at a very low level by introducing an artificial PKA site was developed by Li *et al.* (1989) for the detection of human interferon- $\alpha$ . It has also been used for the detection of HBV polymerase (Bartenschlager *et al.*, 1991). Here we show for the

first time that the radioactive labelling at a newly introduced PKA site is a valuable technique for detection of proteins in transgenic mice.

To obtain a high expression in cell culture, the modified gene *x*-PKA was cloned downstream of the major immediate-early 1 promoter of HCMV. After transient expression of this plasmid in tissue culture cells it was proven that the X-PKA protein could be specifically immunoprecipitated (Fig. 3) and detection of the radiolabelled X-PKA protein was possible with an extremely high sensitivity. In these assays it became clear that the X-protein is not soluble in aqueous buffers (Fig. 2) but can be solubilized by boiling in 2% SDS–2% ME under slightly alkaline conditions.

In the next experiment we tried to use the technique developed in tissue culture for detection of the X-protein in liver tissue of transgenic mice (lines HBxI and -IV). As positive controls X-PKA protein expressed in tissue culture as well as bacterially expressed X-protein were used. All efforts led to negative results. Even with the improved cell lysing technique it was impossible to detect the authentic X-protein after immunoprecipitation in livers of transgenic mice by Western blotting (Fig. 4). Therefore we generated new transgenic mice which expressed the modified *x*-PKA gene with the artificial PKA site instead of the authentic *x*-gene. Thereby a substantial improvement of the detection sensitivity seemed to be possible. After transfection of cell culture cells with pCMV-X-PKA,  $10^4$  cells were sufficient to clearly detect the X-PKA protein after radioactive labelling (data not shown). This result allowed us to estimate the detection sensitivity in mouse liver: 100 mg liver tissue contains about  $10^8$  hepatocytes. So it could be expected that an expression level by a factor of  $10^4$  lower compared to pCMV-X-PKA-transfected cells should be sufficient to allow detection of the X-protein.

The ubiquitous, strong HCMV-P/E is not suitable for directing expression of the *x*-gene in transgenic mice. It can lead to embryonic death or sterility of the founder animals (unpublished data). Therefore the HCMV-P/E was exchanged for the HBV *x*-promoter and the HBV-enhancers I and II. The DNA construct used for pronuclear injection was gained by *Bgl*III digestion and religation of a construct used for generation of mice expressing the *preC/core* gene as well as the *x*-gene. These mice, described by Reifenberg *et al.* (1997), showed expression of the transgene at a level which is comparable to a normal HBV infection in humans. This construct contains both enhancer elements of HBV, the enhancer II, which is located within the *x*-gene, as well as the enhancer I, which lies about 400 bp upstream of the enhancer II (Fig. 1a; Shaul *et al.*, 1985; Tognoni *et al.*, 1985; Wang *et al.*, 1990; Yuh & Ting, 1990). By *Bgl*III digestion and religation the major part of the *core* gene was deleted, without influencing the X expression unit (Fig. 1a). The PKA site was introduced afterwards by PCR mutagenesis (Fig. 1c).

In cell culture experiments it could be proven that the new construct was suitable for expression of the X-PKA protein

and, as calculated by scanning densitometry, the relative expression level of the pHBV-X-PKA construct was a factor of 40 lower compared to the pCMV-X-PKA construct (Fig. 5).

After pronuclear injection of the HBV-X-PKA construct we obtained seven transgenic founder mice, from which six transgenic lines could be established (X-PKA I, III, IV, V, VI, VII). One male founder, which had no offspring, was sacrificed to find out if the transgene was expressed *in vivo*. The mRNA of the transgene X-PKA could be detected in liver and kidneys by RT-PCR (data not shown). Therefore the transgenic animals seemed to be suitable for the detection of the X-PKA protein *in vivo*, applying the detection technique developed in tissue culture.

In the sterile founder as well as in five out of six heterozygous offspring from the six transgenic X-PKA lines the detection of the X-PKA protein in 100 mg liver tissue by radiolabelling of the immunoprecipitated proteins was indeed successful (Fig. 6). One line, X-PKA III, expressed no X-PKA protein in the liver, which could be due to a 'silent' integration of the transgene.

To date, there are only limited studies concerning the tissue-specificity of the *x*-promoter. Billet *et al.* (1995) proved that in line PEX7, in which enhancers I and II are present, the *x*-promoter shows no tissue specificity but rather is active in all 11 tissues tested. However, the *core* promoter was liver-specific in line PEX7, in which the enhancer I is present, whereas it is active in various tissues of the SV28 and AX lines, which lack the enhancer I. These results suggested that enhancer I directs tissue specificity of the *core* promoter associated with enhancer II, but not of the *x*-promoter. These findings were consistent with Guo *et al.* (1991), who showed that the ubiquitous herpes simplex virus *thymidine kinase* promoter associated with enhancer I is active only in hepatic cell lines whereas the *x*-promoter associated with enhancer I is active in hepatic as well as in non-hepatic cell lines.

Our mice allowed us, for the first time, to investigate the tissue-specificity of the *x*-promoter by direct detection of the X-protein *in vivo*. In two out of the five transgenic lines expressing the X-PKA protein in the liver the tissue distribution of the transgene expression was investigated. This promised to yield decisive information about the tissue specificity of the *x*-promoter in a construct containing the two HBV enhancers at the authentic positions.

One male, 12-week-old mouse of line X-PKA I (Fig. 7, upper panel) and one female, 21-week-old mouse of line X-PKA IV (Fig. 7, lower panel) were sacrificed and the X-protein expression was investigated quantitatively in 14 different tissues. The highest expression level was found in lung, kidney and brain, and in line X-PKA I also in pancreas. Surprisingly, the liver showed only a medium expression level. Low amounts or no expression were found in heart, colon, gonads and skeleton muscle. Overall, the female, 21-week-old mouse of line IV showed higher expression levels than the male, 12-week-old mouse of line I. Consequently, the X-PKA protein

could be found in many different organs, irrespective of the transgenic line, the age or the sex of the animal. This finding strongly suggests that the *x*-promoter is not tissue-specific but rather is active in many different cell types.

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