The PreS2 activator MHBs\(^\d\) of hepatitis B virus activates c-raf-1/Erk2 signaling in transgenic mice

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The large hepatitis B virus (HBV) surface protein (LHBs) and C-terminally truncated middle size surface proteins (MHBs\(^\d\)) form the family of the PreS2 activator proteins of HBV. Their transcriptional activator function is based on the cytoplasmic orientation of the PreS2 domain. MHBs\(^\d\) activators are paradigmatic for this class of activators. Here we report that MHBs\(^\d\) is protein kinase C (PKC)-dependently phosphorylated at Ser28. The integrity of the phosphorylation site is essential for the activator function. MHBs\(^\d\) triggers PKC-dependent activation of c-Raf-1/Erk2 signaling that is a prerequisite for MHBs\(^\d\)-dependent activation of AP-1 and NF-κB. To analyze the pathophysiological relevance of these data in vivo, transgenic mice were established that produce the PreS2 activator MHBs\(^\d\) specifically in the liver. In these mice, a permanent PreS2-dependent specific activation of c-Raf-1/Erk2 signaling was observed, resulting in an increased hepatocyte proliferation rate. In transgenics older than 15 months, an increased incidence of liver tumors occurs. These data suggest that PreS2 activators LHBs and MHBs\(^\d\) exert a tumor promoter-like function by activation of key enzymes of proliferation control.

**Keywords:** hepatitis B virus/hepatocellular carcinoma/signal transduction/surface proteins/transgenics

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Introduction

In addition to causing acute and chronic hepatitis, hepatitis B virus (HBV) is considered to be a major etiological factor in the development of human hepatocellular carcinoma (HCC). Almost all HBV-associated HCCs studied so far harbor chromosomally integrated HBV DNA (Beasley et al., 1981). HBV DNA also does not integrate at specific sites of the host genome. Thus a common cis-effect onto flanking cellular genes can be excluded as a general mechanism of HBV-associated carcinogenesis.

However, integrated HBV DNA can encode two types of transcriptional activators: the already well studied HBx (for a recent review see Murakami, 1999) and the PreS2 activators LHBs (large hepatitis B virus surface protein) and MHBs\(^\d\) (C-terminally truncated middle size surface proteins) (Kekulé et al., 1990; Hildt et al., 1996a).

The sequence encoding the PreS2 activators is localized in the HBV surface gene. The surface gene consists of a single open reading frame divided into three coding regions: preS1, PreS2 and S, each starting with an in-frame ATG codon. Through alternate translational initiation at each of the three AUG codons, a large (LHBs; PreS1 + PreS2 + S), a middle size (MHBs; PreS2 + S) and a small (SHBs; S) envelope glycoprotein can be synthesized. The activator function of the surface protein requires the cytoplasmic orientation of the PreS2 domain (the minimal functional unit) that occurs in the case of MHBs\(^\d\) (Hildt et al., 1995) and in a fraction of LHBs (Bruss et al., 1994). In contrast, full-length MHBs displays no transcriptional activator function: here the PreS2 domain directs into the lumen of the endoplasmic reticulum (ER). The generation of functional MHBs\(^\d\) from MHBs requires deletion of the 3’ end of the preS2/S gene encoding at least the last 70 amino acids corresponding to the hydrophobic region III of the S domain during the integration process (Lauer et al., 1992). Deletions of 3’ sequences of the preS2/S gene generating functional MHBs\(^\d\) activators were found in one-third of the integrates investigated so far (Schlüter et al., 1994; Zhong et al., 1999). This fact and the presence of complete preS/S genes in some other systematically studied integrates indicate the biological significance of the PreS2 activators.

Previous reports have described that transgenic mice that specifically overproduce LHBs in the liver develop liver tumors (Chisari et al., 1989). Up to now, intracellular retention of LHBs, resulting in a situation analogous to storage disease, and subsequent permanent inflammatory processes were considered to cause the tumors in these transgenics. The physiological situation of these mice is closely related to that of HBV carriers, who strongly overproduce HBsAg. In the livers of these patients, development of ground glass hepatocytes can be observed. In light of the recent observations that LHBs functions as a transcriptional activator (Hildt et al., 1996a), the question arose of whether this activator function could confer LHBs-dependent development of HCC.

MHBs\(^\d\) proteins are paradigmatic for the family of PreS2 activators. However, in contrast to LHBs, the MHBs\(^\d\) activators are produced only in very small amounts even if the expression is driven by strong promoters. This permits an analysis of signal transduction pathways in the absence of effects based on overexpression and intracellular accumulation of LHBs. In this study, we used, in particular, MHBs\(^{\text{76}}\) (truncated at amino acid 76) corresponding to the MHBs\(^\d\) activator encoded by the integrate of the human hepatoma cell line huH4 (Kekulé et al., 1990). MHBs\(^{\text{76}}\) belongs to the ER-localized subtype of MHBs\(^\d\) activators (Hildt et al., 1993). To exclude unequivocally any effects such as ER-overload based on the ER membrane association of MHBs\(^{\text{76}}\), MHBs\(^{\text{463}}\) that belongs to the non-membrane associated cytosolic-
fraction of MHBs proteins (Hildt et al., 1996b). To analyze this in more detail, highly purified hexahistidine (His$_6$)-tagged MHBs$^{76}$ derived from S9 cells was subjected to two-dimensional gel electrophoresis. The two-dimensional gel shows that S9 cell-derived His$_6$-MHBs$^{76}$ consists of at least three differently charged populations (Figure 1A). The identity of these three spots with His$_6$-MHBs$^{76}$ was confirmed by western blot analysis (Figure 1B). To investigate whether the more negatively charged spots represent phosphorylated His$_6$-MHBs$^{76}$, acid phosphatase treatment was carried out. Subsequent western blot analysis of the two-dimensional separation showed that the two more negatively charged spots disappeared, reflecting their phosphatase sensitivity (Figure 1C). To prove unequivocally that phosphorylation of MHBs takes place in a liver cell system in vivo, labeling of His$_6$-MHBs$^{76}$- or His$_6$-MHBs-producing HepG2 cells with $[^32]$Porthophosphate was performed. The autoradiograph of the affinity-precipitated proteins shows that both the ER-localized MHBs$^{76}$ (Figure 1D, lane 2) and the cytoplasmic MHBs$^{63}$ (Figure 1D, lane 4) are phosphoproteins (Figure 1D). In the case of full-length MHBs, no specifically phosphorylated forms were detectable (Figure 1D, lane 7). Further mutants analyzed in this experiment are described below. These results demonstrate that MHBs activator proteins, in contrast to MHBs, are phosphorylated.

**The PreS2 activator MHBs is phosphorylated at Ser27/28**

Western blot analysis using a phosphoserine- and phosphotyrosine-specific antibody demonstrated the presence of phosphoserine in MHBs (data not shown), but provided no evidence for tyrosine phosphorylation.

To study phosphorylation of various MHBs proteins in more detail, an in vitro system was used. Purified *Escherichia coli*-derived proteins were incubated with isolated cytoplasm and [$\gamma$-32P]ATP. Analysis was performed by SDS–PAGE followed by autoradiography. His$_6$-MHBs$^{76}$ characterized by a mol. wt of ~6.5 kDa (Figure 2A, lanes 1 and 3) is phosphorylated efficiently under these conditions. In the case of the negative control (Figure 2A, lane 4), His$_6$-MHBs$^{76}$ was replaced by an unrelated His$_6$-tagged protein (His$_6$-grb2). To exclude any autophosphorylation activity of His$_6$-MHBs$^{76}$, the cytoplasm was replaced by homogenization buffer. The autoradiograph indicates that His$_6$-MHBs$^{76}$ possesses no autophosphorylation activity (Figure 2A, lane 2) and excludes phosphorylation by contaminating bacterial kinases. A series of C- or N-terminal deletion mutants His$_6$-MHBs$^{63}$, His$_6$-MHBs$^{52}$, His$_6$-MHBs$^{51}$-76 (starts at amino acid 11 of the PreS2 region, truncated at amino acid 76) and His$_6$-MHBs$^{31}$-76, and a structural mutant MHBs$^{52,}$ (the amphipathic $\alpha$-helix between amino acids 41 and 52 was converted to a $\beta$-sheet conformation; Hildt et al., 1995) were purified from a eubacterial expression system and used for mapping the phosphorylation site. The autoradiograph (Figure 2B) shows that neither deletion of the hydrophobic transmembrane region 1 (MHBs$^{63}$, Figure 2B), further C-terminal deletion up to amino acid 52 (MHBs$^{52}$, Figure 2B) nor mutation of the amphipathic $\alpha$-helix (MHBs$^{52}$, Figure 2B) abolishes the phosphorylation of MHBs$^{76}$. Even N-terminal deletions in the case of

**Results**

**The PreS2 activator MHBs is a phosphoprotein**

In a recent report, mass spectrometry of MHBs$^{76}$ provided preliminary evidence for the existence of a phosphorylated localized subtype was used as an appropriate control. Elsewhere, MHBs$^{63}$ and MHBs$^{76}$ have identical functions (Hildt et al., 1995). The molecular basis of MHBs-dependent transcriptional activation, however, remained enigmatic.
Fig. 2. MHBS1 is phosphorylated between amino acids 27 and 31.
(A) Left: autoradiograph of eubacteria-derived MHBS1, incubated with cytoplasm derived from CCL13 cells and [γ-32P]ATP (lane 1); as negative control, MHBS1 was incubated in the absence of lysate, which was replaced by an equal volume of homogenization buffer (lane 2). The molecular weight marker is given on the right. Right: autoradiograph of eubacteria-derived MHBS1, incubated with cytoplasm derived from CCL13 cells and [γ-32P]ATP (lane 3); as negative control, MHBS1 was replaced by HistGly2 (lane 4).
(B) Autoradiograph of in vitro phosphorylated MHBS1 fragments (MHBS1-52, MHBS1-78, MHBS1-111-78, MHBS1-111-52; here the amphiphatic α-helix between amino acids 41 and 52 was replaced by a β-sheet conformation) isolated from the eubacterial expression system. The experiments were performed in duplicate. In the case of MHBS1-111-78, only one-fifth of the amount of MHBS1 employed in the other reactions was used. (C) Autoradiograph of in vivo phosphorylated and Ni-NTA-agarose-precipitated MHBS1-specific proteins: lane 1, MHBS1-mutA; lane 2, MHBS1-mutB; lane 3, MHBS1-mutC; lane 4, MHBS1-mutD; lane 5, wild-type MHBS1; lane 6, control transfected cells. (D) CAT assay after co-transfection of HepG2 cells with reporter plasmid p3xAP-1-CAT and expression plasmid pKSVMHBs1/2, encoding the wild-type, or plasmids pKSVMHBS1/2mutA, pKSVMHBS1/2mutB, pKSVMHBS1/2mutC, pKSVMHBS1/2mutD, or cloning vector pKSV10 as negative control. Fold inductions are mean values from three independent transfection experiments, calculated as the ratio of the induced values to the vector control. The standard deviation is shown in the diagram.

MHBS1 and MHBS3 did not result in a loss of MHBS1 phosphorylation (Figure 2B). From these data, it can be concluded that MHBS1 is phosphorylated between amino acids 23 and 41. The corresponding peptide harbors two S/T clusters, one between amino acids 27 and 31 (PreS2aa23-34: PAGGSSSSTVNP) and the other between amino acids 37 and 40 (PreS2aa34-41: PVLMTAS). To identify the phosphorylation site more precisely, two mutants were generated. In the case of mutant I, Ser27, Ser28, Ser29 and Tyr31 were replaced by alanine (MHBS1/2mutA; MHBS3mutB); in the case of mutant II, Tyr37, Tyr38 and Ser40 were replaced by alanine (MHBS1/2mutC; MHBS3mutC).

In vivo labeling experiments with [32P]orthophosphate of HepG2 cells producing MHBS1mutAII demonstrated that mutation of the S/T cluster at amino acids 27–31 abolished the phosphorylation of MHBS1mutAII (Figure 1D, lane 3) or MHBS3mutC (Figure 1D, lane 5), whereas mutation of the S/T cluster at amino acids 37–40 had no influence on the phosphorylation of MHBS1mutAII (Figure 1D, lane 6) or MHBS3mutC (Figure 1D, lane 8). Western blot analysis revealed that MHBS3mutC and

Fig. 3. Functionality of PKC is essential for phosphorylation of MHBS1.
(A) Competitive inhibition of in vitro phosphorylation of MHBS1 using increasing concentrations (2 × 10^-4 M, lane 2, to 2 × 10^-5 M, lane 5) of a PKC-specific peptide. In lanes 4 and 5, the lower band represents the phosphorylated peptide. Lane 1 represents the phosphorylation of MHBS1 in the absence of the peptide. (B) In vivo labeling using [32P]orthophosphate of HepG2 cells producing His6-MHBS1 (lanes 1 and 2) grown in the absence (lane 2) or presence of the PKC inhibitor Go6976 (lane 1). His6-MHBS1 was enriched by Ni-NTA precipitation.

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MHBs\textsuperscript{676mutII} were produced in similar amounts as the respective wild-type proteins (data not shown).

To identify the exact phosphorylation site, a series of mutants were generated in which the following residues were replace by alanine: Ser27 in MHBs\textsuperscript{676mutI}, Ser28 in MHBs\textsuperscript{675mutII}, Ser29 in MHBs\textsuperscript{672mutIc}, and Tyr31 in MHBs\textsuperscript{671mutId}. \textit{In vivo} phosphorylation experiments using these mutants (Figure 2C) revealed that mutation of Ser28 to alanine abolishes the phosphorylation, indicating that the phosphorylation of MHBs\textsuperscript{i} takes place at Ser28.

**Destruction of the phosphorylation site abolishes activator function of MHBs\textsuperscript{i}**

The PreS2 activators are known to activate transcription factor AP-1 (Hildt \textit{et al.}, 1995). To investigate whether the integrity of the identified phosphorylation site is a prerequisite for the functionality of the MHBs\textsuperscript{i} activators, reporter gene assays were performed. HepG2 cells were co-transfected with the expression vectors pKSVMBHs\textsuperscript{676} (positive control), pKSVMBHs\textsuperscript{676mutI} or pKSVMBHs\textsuperscript{676mutII} and with the reporter construct p3xAP-1-CAT (Lauer \textit{et al.}, 1992). The vector pKSV10 served as negative control. Destruction of the phosphorylation site in the case of MHBs\textsuperscript{676mutI} abolishes the activator function of MHBs\textsuperscript{676} (Figure 2D) as compared with cells transfected with pKSVMBHs\textsuperscript{676} (positive control) or pKSVMBHs\textsuperscript{676mutII}; here, an \textapprox{}10-fold activation of the reporter gene was observed (Figure 2D). Co-transfection of pKSVMBHs\textsuperscript{676mutID28} (Ser28 was replaced by aspartate to mimic a constitutive phosphorylation) failed to induce activation of the reporter gene.

These data demonstrate that the integrity of the identified phosphorylation site is a prerequisite for the functionality of the MHBs\textsuperscript{i} activators.

**PKC phosphorylates MHBs\textsuperscript{i}**

The identified phosphorylation site showed a weak homology to protein kinase C (PKC)-dependent phosphorylated sequences. To investigate a possible implication of PKC isoforms in phosphorylation of MHBs\textsuperscript{i} proteins, \textit{in vitro} phosphorylations were performed in the presence of a PKC-specific substrate peptide. The autoradiograph (Figure 3A) shows that increasing concentrations of the peptide compete phosphorylation of His\textsubscript{677}MBHs\textsuperscript{663}. This indicates that MHBs\textsuperscript{i} is PKC-dependently phosphorylated.

To analyze the relevance of classical PKC isoforms for phosphorylation of MHBs\textsuperscript{i}, their activity was blocked using the cPKC-specific inhibitor Goe6976. \textit{In vivo} phosphorylation experiments under these conditions revealed that phosphorylation of MHBs\textsuperscript{i} depends on the functionality of cPKC isoforms (Figure 3B).
PKC is activated in MHBsΔ-producing cells

Next, we analyzed whether MHBsΔ affects the activity of PKC. Activation of the classic isoforms of PKC such as the liver-specific PKCα/β isoforms is associated with a translocation of PKC from the cytoplasm to the cell membrane. Immunofluorescence microscopy of a clonal mixture of HepG2 cells stably transfected with the expression plasmid pCMVHBSΔ revealed in these cells a translocation of PKCα/β from the cytoplasm to the cell membrane. In the case of control-transfected HepG2 cells, PKCα/β was found in the cytoplasm (Figure 4A and B).

For a quantification of PKC activation, the ratio of membrane-bound to cytosolic PKC activity was determined in MHBsΔ or MHBsΔmutI-producing CCL13 cells. pKSV10-transfected cells served as negative control. An ~5-fold activation of PKC was measured in the case of MHBsΔ-producing cells as compared with the negative control (Figure 4C). The phosphorylation-deficient mutant MHBsΔmutI did not activate PKC.

MHBsΔ proteins induce activation of the transcription factors AP-1 and NF-κB (Lauer et al., 1992; Meyer et al., 1992). To study the relevance of PKC activation for MHBsΔ-dependent induction of AP-1 and NF-κB, the PKC inhibitor Go6976 was employed. A complete loss of the MHBsΔ-dependent activation of the AP-1- or NF-κB-dependent reporter gene was observed (Figure 4D). These data indicate that (i) MHBsΔ triggers activation of PKC and (ii) the functionality of PKC is essential for MHBsΔ-dependent activation of AP-1 and NF-κB.

MHBsΔ induces PKC-dependent activation of c-Raf-1 and Erk2

The activation of PKC can be mediated by the c-Raf-1/Erk2 signal transduction pathway (Caroll and May, 1994; Marais et al., 1998). In the case of MHBsΔ-producing cells, an ~4-fold increase of c-Raf-1 activity was found (Figure 5A) as compared with the c-Raf-1 activity in control-transfected cells. As a positive control, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) stimulation resulted in an ~9-fold activation.

In addition, the phosphorylation-deficient mutant MHBsΔmutI, which fails to activate PKC, also caused no activation of c-Raf-1. Activation of neither B-Raf nor A-Raf could be observed in MHBsΔ-producing cells (data not shown). To confirm that the observed MHBsΔ-dependent activation of c-Raf-1 is triggered through PKC, Go6976 was used to inhibit cPKC. Here, determination of c-Raf-1 activity revealed a complete loss of MHBsΔ-dependent activation of c-Raf-1, whereas the epidermal growth factor (EGF)-dependent activation of c-Raf-1 was not significantly affected by inhibition of PKC (data not shown).

In the case of MHBsΔ-producing cells, Erk2 activity was increased ~2.5-fold as compared with the lysate of control-transfected cells. Again, the phosphorylation-deficient mutant MHBsΔmutI causes no activation of Erk2. TPA-stimulated cells served as a positive control. Here, an ~6-fold activation was determined (Figure 5B).

In a recent report (Marais et al., 1998), it was revealed that in contrast to the receptor tyrosine kinase-mediated activation, the PKC-dependent activation of c-Raf-1 is not inhibited by a transdominant-negative mutant of Ras (pRasN17). Reporter gene assays revealed that MHBsΔ-dependent activation of AP-1 or NF-κB is not affected by the co-expression of RasN17 (Figure 6). Moreover, expression of transdominant-negative Ras failed to influence the MHBsΔ-dependent activation of c-Raf-1 or Erk2 (data not shown). The functionality of pRasN17 was demonstrated by the inhibition of HBx-dependent activation of AP-1 and NF-κB (data not shown). These results show that expression of the MHBsΔ activator results in PKC-dependent activation of c-Raf-1 and Erk2.

MHBsΔ fails to promote activation of AP-1 and NF-κB in cells producing a transdominant-negative mutant of c-Raf-1

In the next set of experiments, we investigated whether activation of c-Raf-1 is a prerequisite for MHBsΔ-dependent activation of AP-1 and NF-κB. For this
purpose, reporter gene assays were performed and endogenous c-Raf-1 was inhibited by co-expression of a transdominant-negative mutant (pHCR13.1; Kölch et al., 1991) in MHBs<sup>76</sup>-producing HepG2 cells. Transdominant-negative c-Raf-1 abolished MHBs<sup>76</sup>-dependent activation of AP-1 or NF-κB (Figure 6). These results indicate that the integrity of c-Raf-1 is essential for triggering the activation of AP-1 and NF-κB in MHBs<sup>76</sup>-producing cells.

**Liver-specific expression of the transgene encoding MHBs<sup>76</sup>**

To study the pathophysiological relevance of the detected PKC-controlled signaling pathway, two lines of transgenic mice were established that specifically produce the PreS2 activator MHBs<sup>76</sup> in the liver. To direct the expression of the transgene to the liver, the transgene was placed under the control of the albumin promoter. Due to the expected very low expression level of the transgene, the β-globin intron was inserted to increase the expression. To permit an efficient enrichment of MHBs<sup>76</sup> from tissue lysates, MHBs<sup>76</sup> was produced as a His<sub>6</sub>-tagged fusion protein. For stabilization of the mRNA, a poly(A) site was introduced at the 3’ end. A schematic representation of the transgene is given in Figure 7A.

The expression of the transgene was analyzed by immunofluorescence microscopy of liver sections derived from transgenic animals and the respective wild-type littermates stained with a PreS2-specific antiserum. An inhomogeneous, specific staining of the cytoplasm can be observed, while the nucleus remains unstained, reflecting the ER localization (Hildt et al., 1993) of MHBs<sup>76</sup> (Figure 7B).

To verify that (i) the observed staining is specific for authentic MHBs<sup>76</sup> and (ii) the expression is liver specific, lysates derived from the liver or kidney of transgenic animals were analyzed by western blotting using the PreS2-specific antibodies HBV-25-19 or F124 (Neurath et al., 1987; Mimms et al., 1990). Under these conditions, the MHBs<sup>76</sup> protein was not detectable (data not shown). Therefore, the lysates were loaded on metal-chelating columns to enrich the His<sub>6</sub>-tagged MHBs<sup>76</sup>-specific proteins. The eluate was analyzed by western blotting using a PreS2-specific antiserum [monoclonal antibody (mAb) HBV25-19].

The western blot shows that MHBs<sup>76</sup> was only detectable in the lysate derived from the liver of the transgenic animals (Figure 7C/I), confirming the liver specificity of the transgene expression. The liver-derived MHBs<sup>76</sup> was also analyzed for phosphorylation using a phosphoserine-specific antiserum. The western blot (Figure 7C/II) confirms that MHBs<sup>76</sup> is a phosphoprotein.

Determination of JNK2 activity and of the hsp72 level revealed that production of MHBs<sup>76</sup>, in contrast to LHBs, does not result in unspecific activation of signal

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**Fig. 7.** Liver-specific expression of the transgene encoding MHBs<sup>76</sup>. (A) Schematic structure of the transgene encoding MHBs<sup>76</sup>. To direct the expression to the liver, the albumin promoter that was taken from the plasmid pAlb-PSX (Chisari et al., 1985) was used. To increase the expression level, the β-globin intron, derived from plasmid p5Wglob (Werner et al., 1993), was inserted. The HBV-specific fragment nt3174–3221 was fused to the sequence coding for an N-terminal His<sub>6</sub> tag. The poly(A) site was taken from SV40, subcloned in plasmid pAK2 (Lauer et al., 1992). The HBV-specific fragment nt3174–3221 codes for MHBs<sup>76</sup>. (B) Immunofluorescence microscopy of liver sections derived from 5-month-old female MHBs<sup>76</sup>-producing mice (line 2) (tg). Sections derived from sex-matched wild-type littermates served as control (wt). The PreS2-specific mAb F124 (Neurath et al., 1987) was used for detection of MHBs<sup>76</sup>. The staining was performed using a Cy3-conjugated donkey-derived secondary antibody. The micrographs were taken at a 400× magnification. (C) I: Western blot analysis of the eluates from Ni-loaded Hitrap chelating columns. The columns were loaded with lysates derived from the liver or kidney of MHBs<sup>76</sup>-producing transgenic mice or their respective littermates (5-month-old, female mice, line I). Elution was performed by a gradient of increasing imidazole concentration. The PreS2-specific mAb HBV 25-19 (Mimms et al., 1990) was used for detection of MHBs<sup>76</sup>. II: Western blot analysis, using a phosphoserine-specific antiserum (Sigma), of the eluates from Ni-loaded Hitrap chelating columns. The columns were loaded with lysates derived from the liver of wild-type mice (lanes 1 and 3) or MHBs<sup>76</sup>-producing transgenic mice (5-month-old, female mice, line I, lane 2; line II, lane 4). The western blot shows that the mouse-derived MHBs<sup>76</sup> is a phosphoprotein.
transduction (see Supplementary data available at *The EMBO Journal* Online).

A comparable amount of MHBs<sup>76</sup> was observed in the transgenic lines I and II. Here, the expression level seems to be sex independent (data not shown). In the case of lines I and II, no significant reduction of transgene expression was detectable over the investigated time period of 12 months.

![Figure 8: MHBs<sup>76</sup> triggers in vivo specific activation of the c-Raf-1/Erk2 signal transduction pathway resulting in an increased proliferation rate of the hepatocytes.](image-url)

**Fig. 8.** MHBs<sup>76</sup> triggers in vivo specific activation of the c-Raf-1/Erk2 signal transduction pathway resulting in an increased proliferation rate of the hepatocytes. (A) Immunocomplex assay of c-Raf-1 activity in liver-derived lysates of MHBs<sup>76</sup>-producing mice (lanes 1 and 2), of LHBs-producing transgenic mice, or of the corresponding littermates as negative control. In the case of the lysates derived from LHBs- or MHBs<sup>76</sup>-producing mice, the autoradiograph shows a significant activation of c-Raf-1 as compared with the control. The activation factors (f.a.) are mean values of three independent experiments. Both f.a. and standard deviation (s.d.) are shown at the bottom of the figure. (B) Immunocomplex assay of Erk2 activity in liver-derived lysates of MHBs<sup>76</sup>-producing mice (lanes 1 and 2), of LHBs-producing transgenic mice or of the corresponding littermates as negative control. In the case of the lysates derived from LHBs- or MHBs<sup>76</sup>-producing mice, the autoradiograph shows a significant activation of Erk2 as compared with the control. The f.a. are mean values of three independent experiments. Both f.a. and s.d. are shown at the bottom of the figure. (C) Western blot analysis of lysates derived from the liver of the two lines of MHBs<sup>76</sup>-producing transgenic mice (lanes 2 and 3) and of the corresponding littermates (lane 1) using a PCNA-specific antiserum. The western blot shows that in the case of the lysates derived from the MHBs<sup>76</sup>-producing transgenics, an elevated amount of PCNA as compared with the control was detectable. The induction factors (f.i.) are mean values of three independent experiments. Both f.i. and s.d. are shown at the bottom of the figure. (D) Immunofluorescence microscopy of liver cryosections derived from a male 6-month-old MHBs<sup>76</sup>-producing mouse (a and b). A sex- and age-matched wild-type littermate served as control. For detection of PCNA (a and c), a PCNA-specific monoclonal antibody (Santa Cruz) was used and was visualized using a Cy3-conjugated anti-mouse secondary antiserum (Dianova). The nuclei were visualized by DAPI staining (b and d).
These results demonstrate that in the two investigated transgenic mice lines, detectable, comparable, liver-specific expression of the transgene occurred.

Specific activation of the c-Raf-1/Erk2 signal and increased proliferation rate in the liver of transgenic mice producing the PreS2 activator MHBs^76^.

The results described above demonstrate that this system is suitable to perform an in vivo analysis of signal transduction pathways specifically activated by the PreS2 activator. The activity of c-Raf-1 and Erk2 was determined by immunocomplex assays in liver-derived lysates from MHBs^76^- or LHBs-producing mice or the respective controls. The autoradiographs (Figure 8A and B) show a strong phosphorylation of MEK or MBP, respectively, in the case of lysates derived from LHBs- or MHBs^76^-producing mice as compared with the control. These results confirm that the PreS2 activator is able to trigger a significant specific activation of the c-Raf-1/Erk2 signaling pathway in vivo that is not subjected to depletion. Activation of c-Raf-1/Erk2 signaling can result in increased cellular proliferation. The proliferating cell nuclear antigen (PCNA) is a well established marker of cellular proliferation. Western blotting of liver-derived lysates (Figure 8C) as well as immunofluorescence microscopy (Figure 8D) of cryosections derived from 6-month-old transgenics using a PCNA-specific antiserum revealed an increased amount of PCNA in the case of the transgensics as compared with their wild-type littermates. These results indicate that the PreS2 activator MHBs^76^ indeed causes an increased cellular proliferation in vivo.

Increased incidence of liver tumors in transgenic mice producing the PreS2 activator MHBs^76^.

The results described above indicate that the PreS2 activator MHBs^76^ is able to trigger the activation of a tumor promoter pathway in vivo. However, according to the literature, it is well established that the HBV genome does not harbor a classic oncogene. To analyze whether expression of the transgene coding for the PreS2 activator MHBs^76^ confers a transforming potential, we performed histological analysis of liver tissue. The analysis was performed over a time period of 18 months. Transgenics younger than 1 year old did not develop tumors. However, in the case of transgenics killed at the age of between 15 and 18 months, a significant number of mice with liver tumors was found (Figure 9). From 23 investigated male mice, 16 mice (69%) displayed liver tumors, while in the case of 17 killed female mice, a liver tumor was observed in 11 animals (65%). There were 2–8 tumors per animal, with a size between 2 and 6 mm. Moreover, the histological analysis revealed the existence of hyperplastic noduli. In the case of the corresponding wild-type controls, two of 14 male mice developed a tumor (14%), as did one of 11 female mice (9%). Immunohistochemistry of six tumors derived from male mice and five from female mice revealed that MHBs^1^ was detectable in each of these tumors (data not shown). These results are summarized in

Table 1. Tumor development in wild-type and transgenic mice killed after 15 months

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Sex</th>
<th>Tumor development</th>
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<tr>
<td>Transgenic</td>
<td>23</td>
<td>male</td>
<td>16</td>
</tr>
<tr>
<td>Transgenic</td>
<td>7</td>
<td>female</td>
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<td>11</td>
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Table I. In contrast to the LHBs transgenic mice, there is no significant difference in the percentage of tumor development between male and female mice and the transgene is expressed in the tumor cells.

These results indicate that the continuous activation of the signaling cascade promotes the development of liver tumors in an age-dependent process.

Discussion

MHBs or LHBs-encoding sequences are found in many integrates subcloned from HBV-associated HCCs (Schlüter et al., 1994; Zhong et al., 1999). To evaluate the putative relevance of PreS2 activator function for the process of HBV-associated HCC development, a detailed in vitro and in vivo analysis of PreS2 activator function is of major biological significance.

Based on the in vitro and in vivo experiments, we propose that in the first step PreS2 interferes with PKC, resulting in activation of PKC and phosphorylation of PreS2.

The PreS2-dependent activation of PKC requires the structural integrity of the phosphorylation site. Conversion of the essential serine residues 27, 28, 29 to alanine abolishes the activator function, as does mutation of Ser28 to aspartate, mimicking phosphoserine. From this, it can be concluded that the conversion from the unphosphorylated to the phosphorylated form is not essential for the activator function. However, the proper interaction of the PreS2 domain with PKC is crucial to trigger activation of PKC. Co-expression of RasN17 does not affect the PreS2-dependent activation of c-Raf-1. This is in accordance with the described model of PKC-dependent activation of c-Raf-1 (Marais et al., 1998).

This signal is mediated subsequently to the nucleus by the c-Raf-1/Erk2 signal transduction pathway. Most importantly, based on results obtained from transgenic mice, it could be clearly concluded that the PreS2-dependent activation of the c-Raf-1/Erk2 cascade is not subjected to depletion resulting in its permanent activation. In accordance with this, an increased proliferation rate of hepatocytes was indeed observed in the MHBs-transgenic producing transgenic. However, this increased proliferation rate was not reflected in a significantly enlarged liver. In many features, the PreS2 activators seem to resemble HBx. Both HBx (Su and Schneider, 1997; Pollicino et al., 1998) and the PreS2 activator MHBs (E.Hildt, unpublished results) can confer a proapoptotic potential to hepatocytes. This could compensate for the increased proliferation rate, resulting in an elevated turnover of hepatocytes. On the other hand, there are reports describing an antianapoptotic potential of HBx (Su et al., 2001).

The observed activation of c-Raf-1/Erk2 signaling in vitro and in vivo indicates that the PreS2 activators are able to activate a classic tumor promoter pathway. During the process of aging, a variety of critical DNA mutations can occur (initiation). Due to the production of the PreS2 activator and the resulting permanent activation of proliferative pathways, these cells are positively selected (promotion). The cooperation of both processes can result in tumor formation. Moreover, accumulation of mutations in these cells can also be increased by MHBs-dependent inactivation of p53 by induction of mdm2 expression and sequestration of p53 to the ER (E.Hildt, unpublished results). In the case of the LHBs-producing transgensics, critical mutations are generated with higher incidence by permanent inflammation of liver tissue due to intracellular accumulation.

In conclusion, according to the classic model of carcinogenesis (initiation and promotion), the PreS2 activators MHBs and LHBs might act as a tumor promoter through activation of key enzymes of proliferation control, i.e. PKC isofoms α/β or c-Raf-1/Erk2 as described here.

Materials and methods

Construction of expression plasmids

For construction of eukaryotic expression vectors pCMV-MHBsC-His6, the truncated preS2/S gene (ntrp3174-3221) was amplified from plasmid pTKTphbV2 (Will et al., 1985) by PCR employing primers with restriction sites for BamHI. For plasmid pCMV-MHBsC-His6p, the truncated preS2/S gene (ntrp3174-3180) was amplified. In both cases, the coding sequence for the His6 tag fused to the C-terminus of the respective MHBs protein was introduced by the reverse primer. The amplified fragments were digested with BamHI and ligated into the BamHI-restricted and dephosphorylated expression vector pCDNA.3 (Invitrogen). For construction of pCMVMHBsC-His6p, the complete preS2/S gene was amplified using primers with restriction sites for BgIII. The His6 tag was introduced by the reverse primer. Amplified fragments were digested with BgIII and ligated into BamHI-restricted and dephosphorylated expression vector pCDNA.3 (Invitrogen).

Construction of eubacterial expression vectors pQE-MHBs67, pQE-MHBsc, pQE-MHBs81, pQE-MHBs111-76, pQE-MHBs23-76 and pQE-MHBs1-52 was described in a recent report (Hildt et al., 1995). For construction of the coding sequence for the MHBs/mut1 mutants, the sequence ntrp3174-3180 was amplified. The forward primer harbored at its 5' end a BamHI restriction site, and the reverse primer a NarI restriction site. By a second PCR, fragment ntrp60-221 (MHBs67) or ntrp60-176 (MHBsc) was amplified. The forward primer introduced mutated codons, resulting in a replacement of Ser27, Ser28, Ser29 and Tyr31 by alanine; at the 5' end, the forward primer contained a NarI site, and the reverse primer a HindIII site. The NarI site introduced an inversion from A→G in the wild-type protein to G→A in the mutant. The amplified fragments were digested with NarI, ligated and inserted into the BamHI-HindIII-restricted, dephosphorylated vector pQE8. The remaining mutants were constructed in an analogous manner. The fragments were inserted into BamHI-restricted vector pCDNA.3 or into the BgIII-restricted vector pKSV10 (Pharmacia).

Cells and cell cultures

CCL13 and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. For transfection, cells were seeded at a density of 0.8 × 10⁶ cells per 60 mm plate. Stimulation of PKC was performed by addition of TPA (Sigma) to a final concentration of 100 ng/ml 30 min prior to lysis of the cells. Goe6976 was used at a final concentration of 10 nM to inhibit classic PKC isofoms.

Culture of SF9 cells (ATCC, CRL-17-11) and infection with recombinant baculovirus encoding MHBs67 was performed as described by Hildt et al. (1995).

Cell fractionation

Cell fractionation for preparation of cytoplasmic and membrane fractions was performed by differential centriugation as described earlier (Hildt et al., 1993).

Reporter gene assays

The chloramphenicol acetyltransferase (CAT) gene was used as reporter gene. The AP-1-dependent reporter plasmid p3xAP-1-CAT and the reporter plasmid p2xNF-xB-CAT (also designated p55A2) that harbors two binding sites for the transcription factor NF-xB were described in Lauer et al. (1994).

In the case of double transfections, cells were transfected with 2.5 μg of activator plasmid or 2.5 μg of pCDNA.3 as negative control, and 1 μg of p3xAP-1-CAT or 2.5 μg p2xNF-xB-CAT reporter plasmid. In the case of triple transfections, 2.5 μg of the transdominant-negative mutants of
CAT assays
Relative CAT activity was determined with 50–100 µg of protein from total cellular lysates as described (Gorman et al., 1982). Acetylated and unreacted [3H]chloramphenicol was quantified with an isotope scanner (Berthold). Fold inductions are mean values from at least three independent transfection experiments.

Indirect immunofluorescence labeling
Staining was performed as described (Hildt et al., 1995). For detection, a mixture ofPKCα/β-specific antibodies (Santa Cruz Biotechnology) diluted in phosphate-buffered saline–Tris (PBST) containing 10% bovine serum albumin (BSA) was used. Bound antibody was visualized by incubation with Cy3-conjugated anti-rabbit antibody (Dianova). A DMR fluorescence microscope (Leica) was used. In order to exclude fixation artifacts, the integrity of the fixed cells was confirmed by phase contrast microscopy of the same specimens. Sections and staining of the paraffin-embedded, formaldehyde-fixed tissue and cryosections of the liver were obtained and processed as described previously (Saher and Hildt, 1999).

In vivo phosphorylation
HepG2 cells were seeded at a density of 0.8 × 10^6 cells per 60-mm-diameter plate. The cells were transfected with 6 µg of the Hs-hMHBI expression plasmids. At 20 h after transfection, the cells were washed twice with phosphate-free DMEM (Sigma) and incubated for 2 h in this medium. Medium was removed and replaced by 1.2 ml of phosphate-free medium containing 1 µCi of [32P]orthophosphate. Cells were incubated in this medium for 2 h then washed twice with phosphate-free medium. Lysis and immunoprecipitation were performed as described (Hildt et al., 1995).

In vitro phosphorylation assays
Highly purified, renatured proteins (0.1 µg) derived from a bacterial expression system were added to 25 µl (20 µg total protein) of the cytosolic fraction of CCL13 cells. The reaction mixture contained in addition 10 µl of 10× kinase buffer (20 mM Tris–HCl pH 7.5, 20 mM β-mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2% glycerol, 110 mM NaCl), 10 µl of 100 µM ATP and 5 µl of 2 mM MgCl2 containing 10 µCi of [γ-32P]ATP. By addition of water, the reaction mixture was brought to a final volume of 100 µl. This basic assay was varied by addition of several activators or inhibitors. Competitive inhibition of the PKC activity was performed by increasing concentrations of a PKC-specific substrate peptide (AAKIQASFRGHRM). Immunocomplex assays
Cells were transfected by lipofection using 2.5 µg of pcMVMHBI's. After 12 h, cells were harvested and lysed in 20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.2 mM EDTA, 1 mM EGTA, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate, 0.25 M sucrose, 0.5 M phenylmethylsulfonyl fluoride (PMSF; Sigma), 0.15 U/ml aprotinin and 2 µg/ml leupeptin. Insoluble material was removed by centrifugation at 20 000 g at 4°C for 15 min. Protein concentrations were determined by a Bradford assay. A-Raf, B-Raf, c-Raf-1 or Erk2 were immunoprecipitated from lysates by the respective polyclonal serum (Santa Cruz Biotechnology). The subsequent procedure was described recently (Hildt and Oess, 1999). Immunocomplex assays for determination of c-Raf-1 or Erk2 kinase activity in liver tissue were performed as described recently (Saher and Hildt, 1999).

Determination of PKC activity
Cells were fractionated 6 h after the glycerol shock in the cytoplasm and membrane fractions. PKC was enriched from both fractions using ion exchange chromatography on DEAE-cellulose columns as described by Maly et al. (1989). PKC activity was determined using a commercial assay system (PKC assay system; Amersham).

Protein analysis
Proteins were separated by SDS–PAGE (Laemmli, 1970; Schägger and von Jagow, 1987). Western blotting was performed as described by Hildt and Oess (1999). The different MBHIs' fragments were detected by the PreS2-specific mAbs HBV25-19 (Mommsens, 1990) or FI24 (Neurath et al., 1987). A mouse monoclonal antiserum (Santa Cruz) was used for detection of PCNA, and phosphoserine- or phosphothreonine-specific mouse mAbs (Sigma) were used for analysis of protein phosphorylation.

Generation of transgenic mice
Animals were generated at the Laboratory Animal Research Unit of the University of Ulm and bred at the Technical University Munich under strict specific-pathogen-free conditions. For production of preS2/SNC transgenic linesages, plasmid pTG17 was linearized with NorI and EcoRV, and the HBV fragment was microinjected into F1(CBA/Ca × C57BL/6J) embryos according to standard procedures. Transgenic lines were established by backcrossing hemizygous transgenic individuals to the C57BL/6J (B6) inbred strain. Transgenic animals were distinguished from their non-transgenic littermates by PCR amplification of transgene sequences using PreS2-specific primers. In all experiments, the non-transgenic, sex- and age-matched littermates served as controls.

The LHBS-producing mice (the preS/S gene is expressed under the control of the albumin promoter) were generated by F.Chisari and co-workers and described in Chisari et al. (1985). The expression characteristics of the mice were determined with animals of the first backcross generation. For the tumor studies, we used animals of the second and third backcross.

Supplementary data
Supplementary data for this paper are available at The EMBO Journal Online.

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