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Hepatitis B virus transactivator MHBs^t: activation of NF-κB, selective inhibition by antioxidants and integral membrane localization

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C-terminal truncation of the middle surface antigen from hepatitis B virus (MHBs) gives rise to a novel trans-activating protein, called MHBs^t. In this study we show that MHBs^t like the HBx protein of HBV, can cause nuclear appearance of NF-κB DNA binding activity and induce various κB-controlled reporter genes. While an inhibitor of protein kinase C could not block gene induction by MHBs^t, the antioxidants *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) could potently suppress transactivation at mM and μM concentrations, respectively. Also, κB-dependent gene induction by the transactivator HBx was blocked. The effects were selective because PDTC did not interfere with MHBs^t and HBx-induced activation of the c-*fos* promoter/enhancer, nor with the basal activity of several other reporter genes lacking functional NF-κB binding motifs. Our data suggest that induction of a prooxidant state is crucial for the activation of NF-κB by MHBs^t and HBx and might be related to the hepatocarcinogenic potential of the viral proteins. MHBs^t had a subcellular localization unusual for a viral transactivator: it appeared to be an integral membrane protein of the endoplasmic reticulum.

Key words: HBV/MHBs^t/HBx/NF-κB/antioxidants/protein kinase C

Introduction

Epidemiological studies have shown that patients who are seropositive for hepatitis B virus surface (HBs) antigen have an ~100-fold higher risk of developing hepatocellular carcinomas (HCC) than HBs negative controls (for review see Beasley, 1988). Because integrated HBV DNA is found in the majority of HCCs, it was speculated that disruption of genes by integrated HBV DNA or an overexpression of cellular sequences by viral promoter or enhancer insertion is important for carcinogenesis. However, this seems to be a rare mechanism since only two cases have been reported where HBV DNA was integrated within or close to a cellular gene (Dejean *et al.*, 1986; Wang *et al.*, 1990). The finding that the HBx protein can potently stimulate expression of a variety of cellular and viral genes provided an alternative mechanism for HBV-associated liver carcinogenesis (Twu and Schloemer, 1987; Spandau and Lee, 1988; Zahm *et al.*, 1988). Support for a role of HBx in the development of HCCs came from the recent findings that expression of HBx causes malignant transformation of NIH3T3 cells (Shirakata *et al.*, 1989) and gives rise to tumours in transgenic mice (Kim *et al.*, 1991). The underlying mechanism is, however, poorly understood. Because HBx has no intrinsic DNA binding activity (Siddiqui *et al.*, 1987; Wu *et al.*, 1990), it is believed to transactivate genes by recruiting host transcription factors. HBx was indeed shown to transactivate genes via *cis*-acting elements binding the inducible transcription factors NF-κB (Siddiqui *et al.*, 1989; Twu *et al.*, 1989; Mahe *et al.*, 1991), AP-1 and AP-2 (Seto *et al.*, 1990), CREB/ATF (Maguire *et al.*, 1991) and C/EBP (Mahe *et al.*, 1991). This indicates that the viral protein has pleiotropic activity as found, for instance, for the transactivator Tax from HTLV-I (Smith and Greene, 1991). Many inducible transcription factors, such as AP-1 or NF-κB/Rel, are composed of DNA binding subunits encoded by proto-oncogenes (for a recent review see Karin, 1991). This suggests that viral transactivators might perturb growth regulation by disrupting the tight control generally imposed on inducible transcription factors.

A well characterized transcription factor induced by HBx is NF-κB (for reviews see Baeuerle, 1991; Blank and Israel, 1992). The nuclear form of NF-κB contains two DNA binding subunits, p50 and p65. The subunits share a novel DNA binding and dimerization motif which is also present in the product of the c-*rel* proto-oncogene (for reviews see Blank and Israel, 1992; Nolan and Baltimore, 1992). In most cells, the p50–p65 heterodimer is kept inactive by association with an inhibitory subunit, called IκB (Baeuerle and Baltimore, 1988; for review see Schmitz *et al.*, 1991). IκB inhibits DNA binding and suppresses uptake of NF-κB into the nucleus. It is assumed that phosphorylation events trigger the release of IκB and thereby cause activation of the factor (Ghosh and Baltimore, 1990; Kerr *et al.*, 1991; Link *et al.*, 1992). *Cis*-acting elements binding NF-κB are functional in many inducible genes encoding cytokines, cell surface receptors, acute phase response proteins and viruses, such as HIV-1 and CMV. Extracellular stimuli that activate NF-κB and subsequently induce gene expression via κB elements include the cytokines TNF-α and IL-1, double-stranded RNA, the viral transactivators Tax and HBx, bacterial lipopolysaccharide, T cell mitogens, UV-A (for references see Baeuerle, 1991) and hydrogen peroxide (Schreck *et al.*, 1991). Because various antioxidants can potently suppress the activation of NF-κB by different stimuli, it is assumed that reactive oxygen intermediates (ROI) serve as a common messenger in the activation process (Schreck *et al.*, 1991, 1992a; Schreck and Baeuerle, 1991). The most potent inhibitor of NF-κB activation that has been characterized so far is the antioxidant pyrrolidine dithio-

carbamate (PDTC). Less than 100 μM PDTC could interfere with the release of IκB in response to all agents tested so far.

Recently, a novel transactivating function was found to be encoded by the preS/S region of the HBV genome (Caselmann *et al.*, 1990; Kekulé *et al.*, 1990). It corresponds to C-terminally truncated forms of the middle (MHBs) and large surface antigens (LHBs). Full-length surface antigens cannot detectably stimulate transcription indicating that the hydrophobic C-terminal sequence interferes with the transactivating potential of the proteins. Truncated preS/S sequences isolated from HBV-induced hepatomas displayed transactivating activity towards chloramphenicol acetyl transferase (CAT) reporter genes controlled by SV40 enhancer-early promoter elements and the c-*myc* P2 promoter (Caselmann *et al.*, 1990; Kekulé *et al.*, 1990). *Cis*-acting elements transactivated by the encoded proteins were, however, not yet defined and it is not known which host transcription factors are recruited by truncated HBs proteins. The C-terminally truncated form of middle HBs characterized in this study will be referred to as MHBs^t.

In this study, we have identified NF-κB as one of the target transcription factors for MHBs^t and investigated the involved signalling mechanism. Transient expression of MHBs^t activated the DNA binding and nuclear appearance of NF-κB and induced the expression of various CAT reporter genes controlled by κB elements. MHBs^t could also induce CAT reporter constructs with promoter and enhancer sequences lacking κB elements showing that the viral protein has pleiotropic activity. The κB-dependent gene induction by MHBs^t appeared to be independent of protein kinase C (PKC) because the kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) could not interfere with CAT induction by the viral protein. Only the antioxidants PDTC and NAC could selectively suppress the κB-dependent transactivation by MHBs^t and HBx. We discuss a possible involvement of ROIs in the activation of NF-κB by MHBs^t and HBx, and in the hepatocarcinogenic potential of the viral proteins. Evidence is presented that MHBs^t is an integral membrane protein of the endoplasmic reticulum (ER).

Results

A transactivating fragment from the preS2/S sequence of the wildtype HBV *ayw* genome was inserted into the expression vector pRc/CMV controlled by the cytomegalovirus (CMV) immediate-early enhancer (see Figure 1A). The preS2/S^t sequence in pCMVS486 corresponds to an N-terminal portion of the middle surface antigen truncated at nucleotide 486. The polypeptide encoded by this sequence will be referred to as 'truncated' MHBs or MHBs^t. Upon *in vitro* translation, a [^{35}S]methionine-labelled polypeptide of ~18 kDa was detected when the sense construct was used as template (Figure 1B, lane 1). This is in accordance with

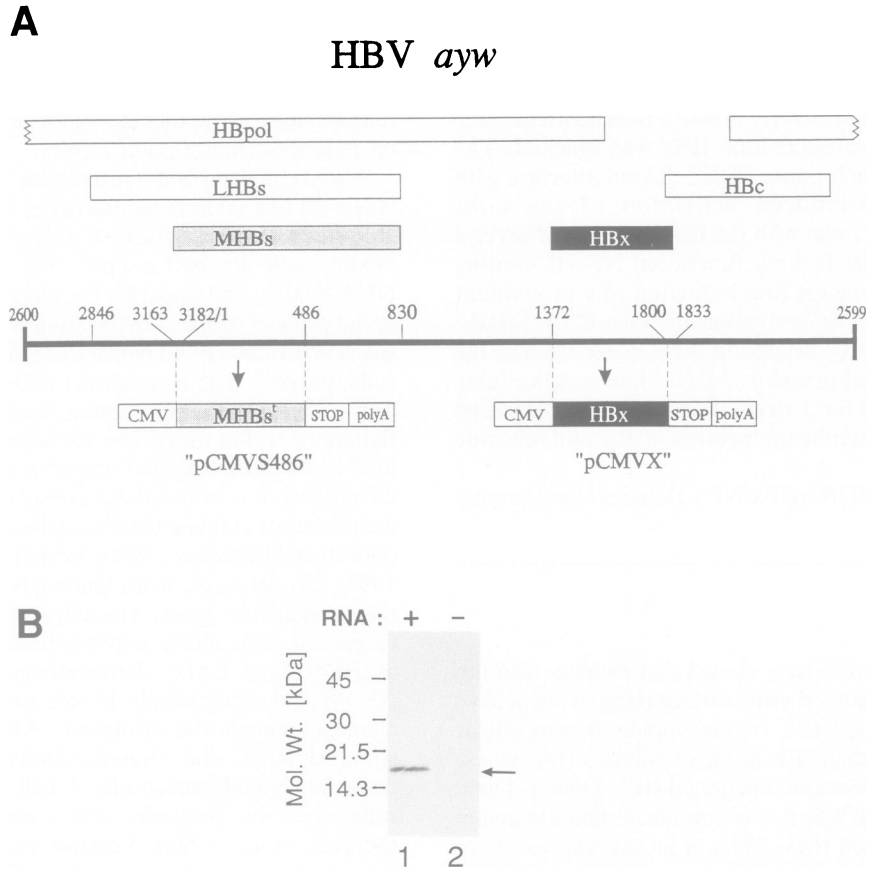


Fig. 1. Construction of expression vectors. (**A**) Genomic organization of HBV *ayw*. Coding sequences are shown as boxes over a viral sequence chart linearized after position 2599 (numbering system of Galibert *et al.*, 1979). The open reading frames for MHBs and HBx proteins are shown as grey and black boxes, respectively. Part of the expression vectors containing a C-terminally truncated form of MHBs (MHBs^t) and HBx are shown below. (**B**) *In vitro* translation reaction programmed with RNA transcribed from pCMVS486 (lane 1) and without added RNA (lane 2). An autoradiograph from a 15% SDS−polyacrylamide gel is shown. The arrow indicates the position of [^{35}S]methionine labelled MHBs^t. Molecular size standards were ovalbumin (46 kDa), carboanhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

the calculated molecular mass of 18.4 kDa (167 amino acids). An antisense construct gave no [^{35}S]methionine-labelled band (data not shown). A sequence from HBV DNA encoding the HBx protein was cloned into pRc/CMVstop (Figure 1A) and was found to give rise to a protein of 17 kDa upon *in vitro* translation (data not shown).

MHBs^t can transactivate gene expression via κB elements

We have tested whether transiently expressed MHBs^t can transactivate in HeLa cells a variety of CAT reporter constructs that harbour various copy numbers or mutated forms of the well characterized NF-κB binding motif 5′-GGGACTTTCC-3′. This motif is bound with high affinity by the p50−p65 heterodimer (Urban *et al.*, 1991) but barely by p65−cRel (Hansen *et al.*, 1992). A construct containing a repeat of six NF-κB binding motifs upstream of the SV40 minimal promoter (6xκB/SV1CAT) showed a 13.5-fold induction of CAT activity upon expression of MHBs^t in the sense orientation (Figure 2A, lane 2). Transfection of the antisense construct (lane 3) or the control plasmid pRc/CMVstop (lane 1) had no detectable effect. A minimal promoter from the SV40 genome was not detectably induced by MHBs^t (Figure 2A, lanes 4−6).

Similar results were obtained with a set of CAT reporter constructs containing a minimal promoter sequence from the mouse c-*fos* gene. Cotransfection of the MHBs^t expression vector in sense or antisense orientation could not significantly transactivate the basal c-*fos* promoter construct (Figure 2A, lanes 13−15). A 19-fold induction of CAT activity was observed with a construct into which two minimal NF-κB binding motifs were inserted as oligonucleotides (construct J16; Pierce *et al.*, 1988; Lenardo *et al.*, 1989) (Figure 2A, lane 8). With the MHBs^t antisense expression vector, only a 4-fold induction was obtained (lane 9). To provide further evidence for a κB-specific gene activation by MHBs^t, we tested a construct homologous to J16 which contains slightly mutated NF-κB binding sites that do not allow significant binding of the factor in electrophoretic mobility shift assays (EMSA; Pierce *et al.*, 1988; Lenardo *et al.*, 1989). With this mutant construct, the gene induction by MHBs^t was abolished (Figure 2A, lane 11).

Because HBV has a tropism for liver cells, we also tested κB-dependent transactivation by MHBs^t in liver-derived

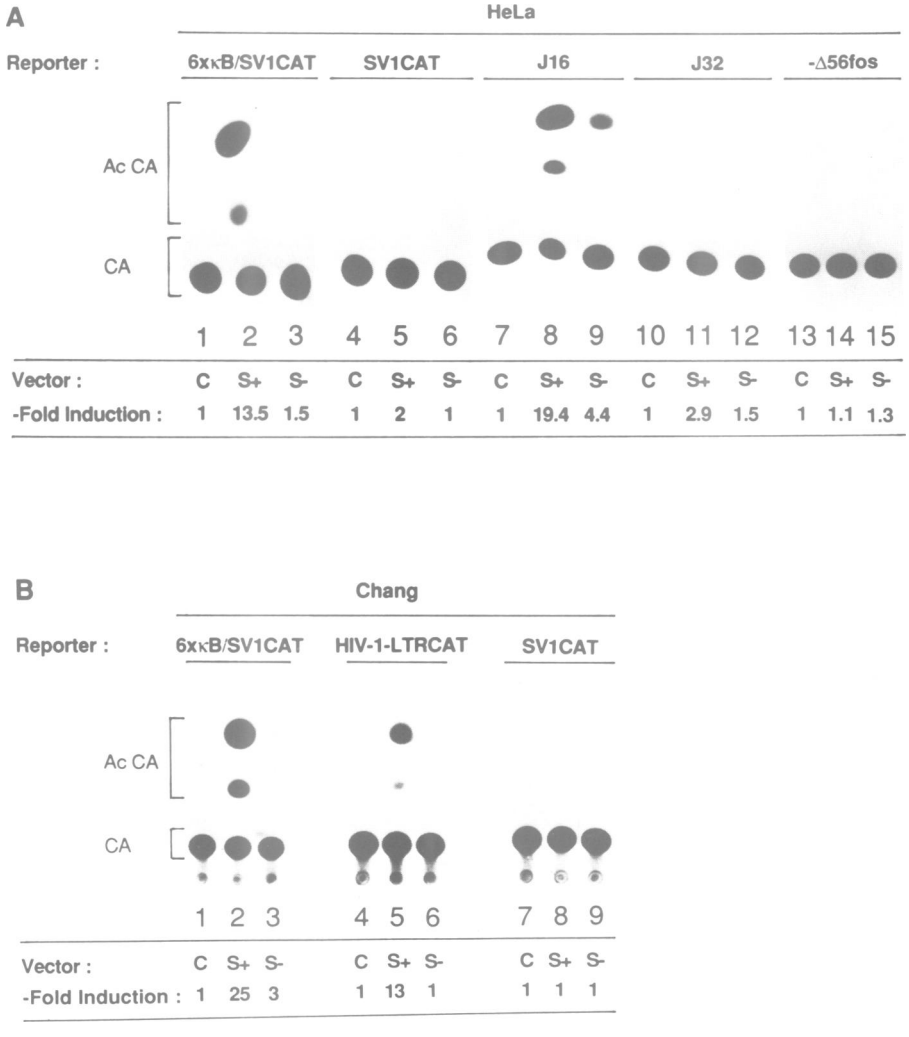


Fig. 2. The effect of MHBs^t on κB-controlled CAT reporter gene activity. (**A**) In HeLa cells. (**B**) In liver-derived Chang cells. CAT reporter constructs were cotransfected with an empty pRc/CMVstop vector (C) or with CMV-controlled expression vectors with the MHBs^t sequence in sense (S+) or antisense (S−) orientation (see Figure 1A). Plasmids and transfection conditions are described in detail in Materials and methods. Only the CAT reporter constructs 6xκB/SV1CAT, J16 and HIV-1-LTRCAT contain functional NF-κB binding sites. Autoradiographs from thin layer plates are shown. AcCA, acetylated [^{14}C]chloramphenicol; CA, [^{14}C]chloramphenicol.

Chang cells. A strong induction of CAT activity from the SV40 promoter under control of six κB elements was seen (Figure 2B, lane 2). The minimal SV40 promoter was not induced (lane 8). Also another κB-controlled promoter/enhancer, the HIV-1-LTR (Nabel and Baltimore, 1987), could confer in Chang cells a 13-fold induction of CAT activity upon transient expression of MHBs^t (Figure 2B, lane 5). With both constructs, the MHBs^t antisense vector had no or only a small transactivating effect (lanes 3 and 6). MHBs^t could also transactivate a CAT reporter construct controlled by the IL-6 promoter/enhancer (data not shown), which is also known to be induced by NF-κB (Lieberman and Baltimore, 1990; Shimizu *et al.*, 1990). Taken together, these results show that MHBs^t is a potent transactivator of genes controlled by *cis*-acting elements which bind the inducible host transcription factor NF-κB.

As shown below (Figure 8), MHBs^t can also induce expression of a CAT reporter gene under control of the c-*fos* promoter/enhancer. This promoter/enhancer is not known to contain functional NF-κB binding sites but is thought to be induced via proteins of the CREB transcription factor family (Sassone-Corsi *et al.*, 1988) and serum response factor (Treisman, 1986). We have further observed that MHBs^t can enhance the expression of CAT reporter genes under control of the HTLV-I LTR and the promoter of the c-Ha-*ras* genes (data not shown). It thus appears that MHBs^t is a pleiotropic viral transactivator.

In order to understand how MHBs^t can transactivate κB-controlled reporter genes, we investigated whether expression of MHBs^t leads to an increase in nuclear DNA binding activity of NF-κB. A ^{32}P-labelled oligonucleotide encompassing the two κB motifs from the HIV-1 LTR was used in EMSAs. In nuclear extracts from uninduced HeLa cells, a low constitutive κB binding activity was found (Figure 3A, lanes 1 and 2). Transient expression of MHBs^t could increase the κB binding activity in nuclear extracts from HeLa and COS7 cells (Figure 3A, lane 3; Figure 3B, lane 1). The novel complex had a mobility in the native gel that was indistinguishable from the complexes activated upon transient expression of the HBx protein (lane 4) and by a phorbol 12-myristate 13-acetate (PMA) treatment of cells (lane 5). The PMA-induced complex in HeLa cells was previously shown to contain NF-κB (Baeuerle and Baltimore, 1988; Mauxion and Sen, 1990). The κB-specific binding of the MHBs^t- and HBX-induced complexes was evident from the strong inhibitory effect of a 100-fold molar excess of unlabelled HIV-1 probe (Figure 3B, lane 2). A faster migrating complex was not affected. Expression of MHBs^t in HeLa cells had only a small influence on activities binding to a labelled SP1 oligonucleotide probe (Figure 3C, lane 3). These results suggest that MHBs^t can transactivate κB-controlled genes by inducing the nuclear appearance of the transcription factor NF-κB.

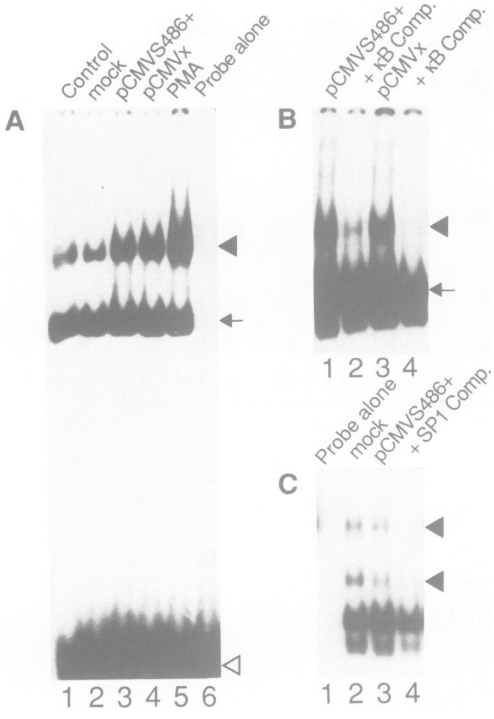
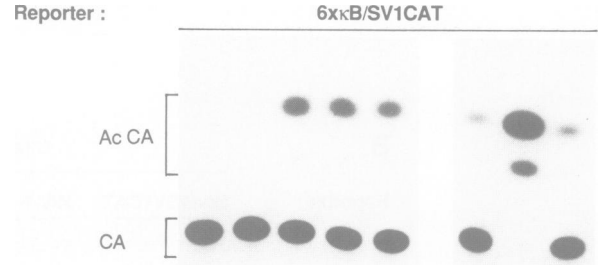


Fig. 3. The effect of MHBs^t and HBx expression on the DNA binding activity of NF-κB in HeLa and COS7 cells. (**A**) Nuclear extracts from HeLa cells transfected with the indicated plasmids and control cells were analysed by electrophoretic mobility shift assays with a ^{32}P-labelled probe encompassing the two NF-κB binding sites from the HIV-1-LTR. A fluorograph of a native gel is shown. (**B**) Competition experiment. 100-fold molar excess of unlabelled HIV probe was added to the binding reactions with nuclear extracts from COS7 cells (lanes 2 and 4). A section of the fluorograph is shown. (**C**) The effect of MHBs^t expression on the binding activity of Sp1 in COS7 cells. A ^{32}P-labelled oligonucleotide with a binding site for the constitutive factor Sp1 was used. 100-fold molar excess of unlabelled Sp1 oligonucleotide was added to the reaction in lane 4. A section of a fluorograph is shown. Filled arrowheads indicate the positions of specific complexes, the small arrows the positions of non-specific complexes. The open arrowhead indicates the position of uncomplexed DNA probe.

Mobilization of NF-κB by MHBs^t appears to be independent of protein kinase C

There are currently two concepts of how the cytoplasmic NF-κB−IκB complex is activated (reviewed in Schreck and Baeuerle, 1991). One suggests a direct phosphorylation of IκB by PKC, which would explain why phorbol esters can



	1	2	3	4	5	6	7	8
Vector /: Activator	-	C	S+	S+	S+	-	PMA	PMA
H7 [μM] :	-	-	-	100	150	-	-	100
-Fold Induction :	1	1	26	25	24	1	35	2.5

Fig. 4. The effect of the kinase inhibitor H7 on MHBs^t- and PMA-induced gene expression. For details of illustration, see legend to Figure 2. HeLa cells transfected with the κB-dependent CAT reporter gene 6xκB/SV1CAT were activated either by expression of MHBs^t (lanes 3−5) or 100 ng/ml PMA (lanes 7 and 8) in the absence or presence of the indicated concentrations of H7. H7 was added 12 h after transfection, a point in time after which there was not yet gene induction by MHBs^t and cells were harvested 24−26 h later. PMA was added 3 h prior to harvesting.

induce the activation of NF-$\varkappa$B (Ghosh and Baltimore, 1990). Based on the suppressing effect of antioxidants and the activation of NF-$\varkappa$B by H_2O_2- induced oxidative stress, the other proposes an involvement of ROI (Schreck *et al.*, 1991). The latter model does not exclude phosphorylation events but would argue against a direct action of PKC on I$\varkappa$B.

In order to test whether PKC is required for the activation of NF-$\varkappa$B by MHBs^t transactivation experiments were performed in the presence of the PKC inhibitor H7. At concentrations of 100 and 150 μM, H7 could not detectably interfere with transactivation of the 6x$\varkappa$B/SV1CAT reporter construct by MHBs^t (Figure 4, compare lanes 3–5). However, the same treatment could almost completely inhibit the induction of the reporter construct after treatment of HeLa cells with 100 ng/ml PMA (lane 8). This shows that no H7-sensitive isotypes of PKC or other H7-sensitive kinases were involved in the MHBs^t-induced activation of the $\varkappa$B-controlled CAT reporter gene.

Antioxidants suppress $\varkappa$B-dependent gene induction by MHBs^t and HBx

A possible involvement of ROI in the activation of NF-$\varkappa$B and $\varkappa$B-controlled reporter genes by MHBs^t was tested by the use of the antioxidants NAC and PDTC. Both NAC and PDTC were shown to be selective inhibitors of $\varkappa$B-controlled gene activation and mobilization of NF-$\varkappa$B (Staal *et al.*, 1990; Schreck *et al.*, 1991, 1992a). Although chemically distinct, both agents are thought to counteract the production of oxygen radicals in intact cells.

NAC and PDTC had a small inhibiting effect on the activity of the $\varkappa$B reporter in uninduced cells (data not shown). This is also observed in L cells which have enhanced constitutive NF-$\varkappa$B levels, but not in Jurkat T cells (Schreck

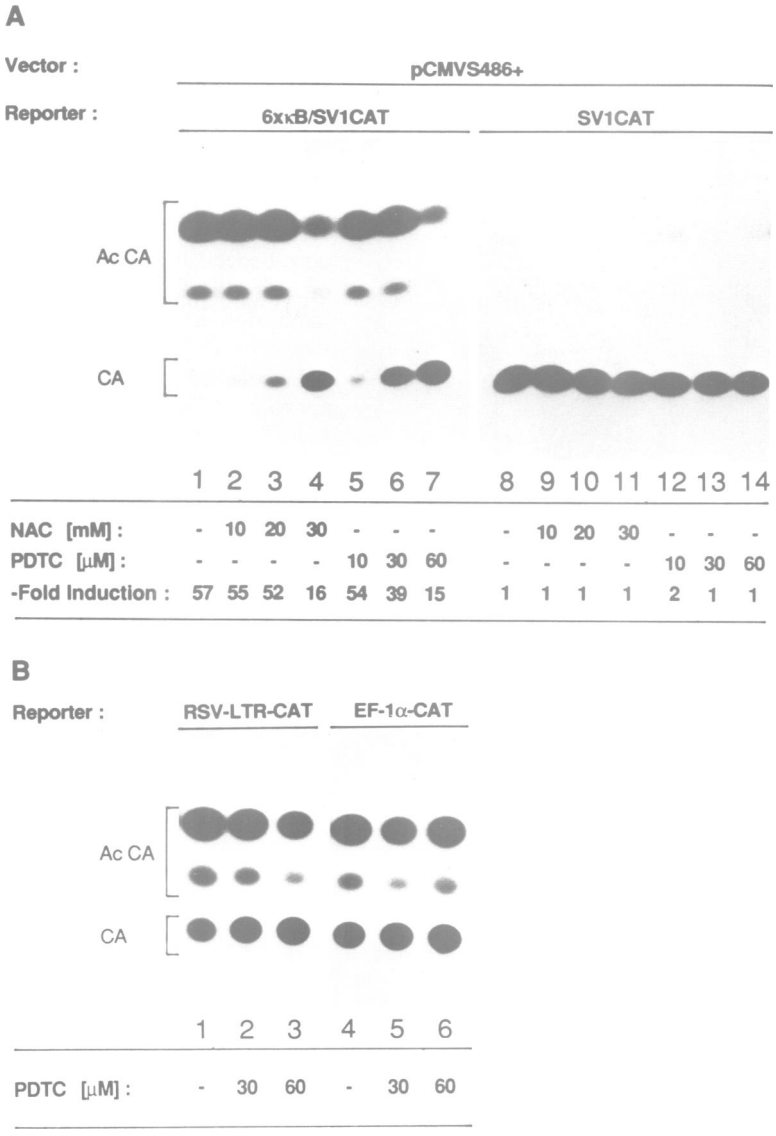


Fig. 5. The effect of NAC and PDTC on gene induction by MHBs^t. For details of illustration see legend to Figure 2. (**A**) The effect of the two antioxidants on MHBs^t-induced $\varkappa$B-specific gene expression. HeLa cells transfected with $\varkappa$B-dependent (lanes 1–7) and basal reporter CAT constructs (lanes 8–14) were treated with the indicated amounts of NAC and PDTC for 12–14 h. All cells received 0.5 μg of the MHBs^t expression vector pCMC486+. (**B**) The effect of PDTC on non-$\varkappa$B-specific gene expression. HeLa cells were transfected with CAT reporter constructs controlled by the Rous sarcoma virus long terminal repeat (RSV-LTR) and the promoter of the elongation factor 1α (EF-1α; Mizushima and Nagata, 1990), and cells were treated for 12–14 h with the indicated concentrations of PDTC.

et al., 1992a). If 30 mM NAC was present in the culture medium, a 70% suppression of the induction of CAT activity by MHBs^t was observed (Figure 5A, lane 4), while 20 mM or less NAC had no effect (lanes 2 and 3). A similar dose response of NAC was observed when the transactivation of a HIV-1 LTR-controlled reporter gene was tested in Jurkat T cells stimulated with TNF or PMA plus lectin (Staal *et al.*, 1990). PDTC could also strongly interfere with the activity of MHBs^t but at a several hundred-fold lower concentration than NAC (Figure 5A, lanes 6 and 7). 60 μM PDTC was sufficient to yield a similar reduction in CAT activity as seen with 30 mM NAC. The dose response of PDTC is also in accordance with recent findings (Schreck *et al.*, 1992a). 30–90 μM PDTC was found to increasingly inhibit transactivation of various κB-controlled CAT reporter constructs in Jurkat T cells stimulated with PMA plus lectin.

The basal activity of a reporter gene without κB motifs was not affected by PDTC (Figure 5A, lanes 8–14). Also, two other constructs with promoter and enhancer sequences which do not contain functional NF-κB binding sites showed virtually no change in CAT activity when cells were treated with 30 or 60 μM PDTC (Figure 5B). One construct was under control of the Rous sarcoma virus LTR (lanes 1–3) and the other regulated by the human elongation factor 1α promoter (lanes 4–6). This shows a selective effect of PDTC on κB-controlled gene induction (see also Schreck *et al.*, 1992a).

Using EMSA, we tested whether PDTC can interfere with MHBs^t-induced mobilization of NF-κB in HeLa cells. In concordance with the CAT activity (Figure 5A), the induction of the κB-specific complex by MHBs^t was not detectably impaired in the presence of 30 μM PDTC (Figure 6, lane 4) but was strongly suppressed with 60 μM of the antioxidant (lane 5). In the same extracts, nuclear activities binding to a ^{32}P-labelled octamer DNA probe was unaffected by the treatment (Figure 6, lane 10). It was previously shown that PDTC does not simply block the DNA binding activity of NF-κB in the assay system but seems to interfere with a reaction required in intact cells for release of IκB from the cytoplasmic form of NF-κB (Schreck *et al.*, 1992a).

A recent report suggested that also the NF-κB dependent gene expression induced by the HBx protein was independent of PKC activity (Lucito and Schneider, 1992). This prompted us to test whether the induction of the 6xκB/ SV1CAT reporter construct seen upon expression of the HBx protein, is also suppressed by the antioxidant PDTC. As shown in Figure 7 (lane 4), 60 μM PDTC could almost completely block induction of CAT activity by HBx. The effect was dose-dependent (compare lanes 2–4). We consistently observed that the basal SV40 promoter construct was slightly induced in the presence of 10 and 30 μM PDTC (Figure 7, lanes 6 and 7).

In conclusion, both transactivating proteins from HBV appeared to mobilize the transcription factor NF-κB and induce κB-controlled gene expression depending on a prooxidant state of the cell. In that respect, MHBs^t and HBx proteins are not different from the Tax protein of HTLV-I (Schreck *et al.*, 1992b) and from all other inducers of NF-κ tested so far (listed in Schreck and Baeuerle, 1991).

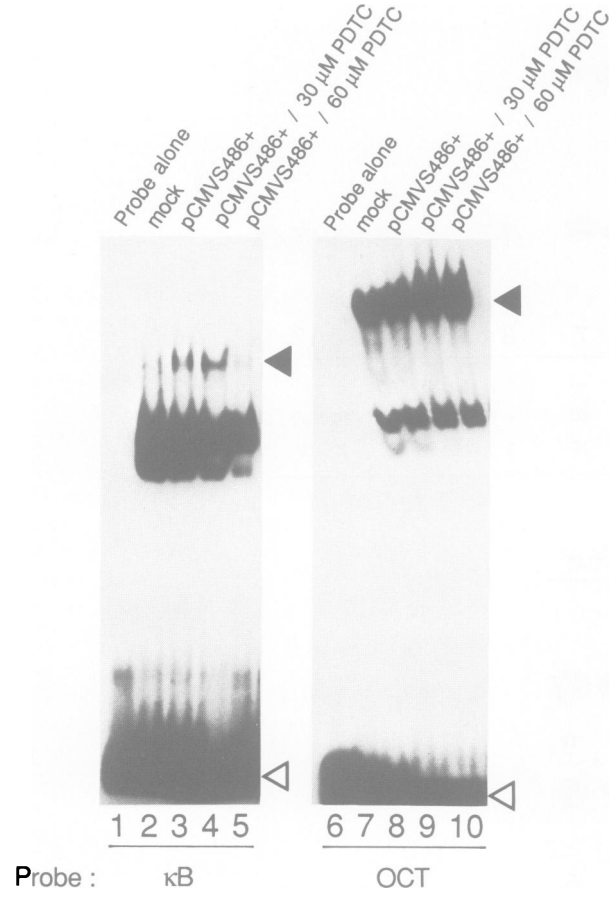


Fig. 6. The effect of PDTC on the induction of NF-κB DNA binding activity in COS7 cells. Nuclear extracts from cells treated as indicated were analysed by EMSA with ^{32}P-labelled HIV-1-LTR (lanes 1–5; see legend to Figure 3) and octamer probes (lanes 6–10). PDTC treatment of transfected cells was for 8–12 h. Fluorogaphs of native gels are shown. The filled arrowheads indicate the positions of specific complexes, the open arrowheads the positions of uncomplexed DNA probes.

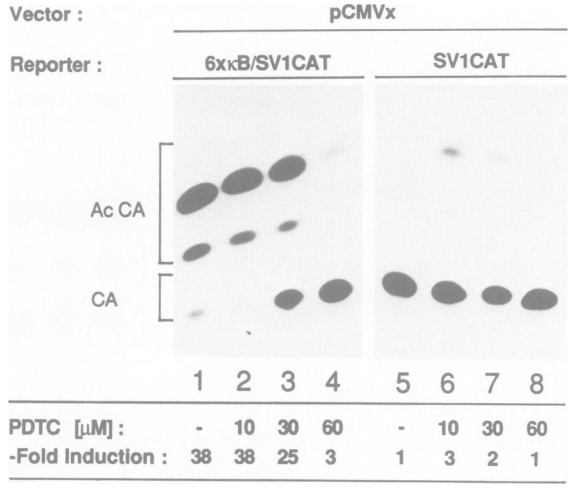


Fig. 7. The effect of PDTC on HBx-induced κB-specific gene expression. For details of illustrations, see legend to Figure 2. HeLa cells transfected with κB-dependent (lanes 1–4) and basal CAT reporter constructs (lanes 5–8) were treated with the indicated concentrations of PDTC for 12–14 h. All cells received 0.2 μg of the HBx expression vector pCMVX.

PDTC does not impair other activities of the MHBs^t and HBx proteins

The inhibiting effect of PDTC on transactivation by MHBs^t and HBx could result from an inactivation of the viral proteins or from their impaired biosynthesis. We tested these possibilities by investigating the effect of PDTC on the induction by MHBs^t and HBx of a c-*fos* promoter/enhancer controlled reporter gene. This element is not known to be under control of $\varkappa$B elements but was nevertheless induced upon expression of MHBs^t and HBx (Figure 8). The pRc/CMVstop vector had no effect. In the presence of 30 and 60 μM PDTC, the induction of CAT activity seen upon transient expression of MHBs^t and HBx was virtually unaffected (Figure 8). This shows that the antioxidant PDTC selectively interfered with $\varkappa$B-dependent transactivation by MHBs^t and HBx, and influenced neither biosynthesis nor other transactivating activities of these pleiotropic viral proteins.

MHBs^t, a membrane-integrated viral transactivator

Transcription factors activated by MHBs^t are localized in the cytoplasm or the nucleus. MHBs, the precursor for MHBs^t, is, however, an *N*-glycosylated integral membrane protein (see Neurath and Kent, 1988; Figure 10). We therefore asked whether the gain in transactivating function upon C-terminal truncation of MHBs coincides with an altered subcellular localization of the protein.

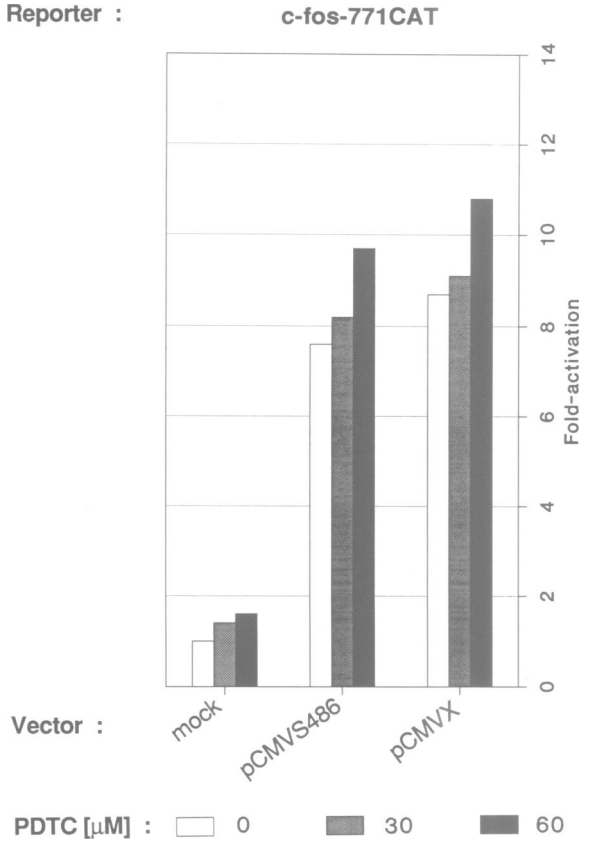


Fig. 8. The effect of PDTC on induction of the c-*fos* promoter/enhancer by MHBs^t and HBx. HeLa cells transfected with a CAT reporter construct controlled by upstream sequences of the human c-*fos* promoter/enhancer (from position 41 to −771) were treated with the indicated concentrations of PDTC for 12−14 h. Cells were cotransfected with 0.5 μg MHBs^t or 0.2 μg HBx expression plasmids and the amount of CAT protein determined by CAT ELISA.

MHBs and MHBs^t were expressed in HeLa cells by a vaccinia virus/T7 polymerase system (see Materials and methods). Total cell extracts, a high speed supernatant and particulate fraction were analysed by Western blotting with the monoclonal antibody F-124 specific for the N-terminal half of the preS2 domain (Budkowska *et al.*, 1986). Cells infected with T7 recombinant vaccinia virus alone did not give a specific signal (Figure 9a, lane 1). In cells expressing MHBs^t under control of T7 polymerase, two immunoreactive bands were observed (Figure 9a, lane 5). The more abundant, and faster migrating band had an apparent molecular size of 18.5 kDa (Figure 9a, lane 2) and comigrated with MHBs^t translated *in vitro* in the absence of microsomes (see Figure 1B; data not shown). A minor band of 22 kDa had the same size as a form of MHBs^t obtained upon *in vitro* translation in the presence of microsomes (data not shown). The non-glycosylated 18.5 kDa form and the apparently *N*-glycosylated 22 kDa form both partitioned completely into the particulate fraction (Figure 9a, lane 7). No significant amounts of the proteins were detected in the high speed supernatant (lane 6).

To distinguish whether MHBs^t is a peripheral or membrane-integrated protein, the particulate fraction was treated with 0.1 M Na_2CO_3 at pH 11.5 (Fujiki *et al.*, 1982). This treatment could not detectably release MHBs^t into the supernatant (Figure 9A, compare lanes 8 and 9) suggesting that the protein was firmly associated with membranes. The insolubility at high pH also indicated that the protein was not present as protein aggregate without membranes.

Indirect immunofluorescence labelling of COS7 cells overexpressing MHBs^t showed a perinuclear staining with a ring-like structure around nuclei (Figure 9B, panels 1, 3 and 4). Upon higher magnification, reticular structure was seen in the cytoplasm (panels 3 and 4). The latter and the perinuclear staining could come from MHBs^t in the endoplasmic reticulum. Consistent with this idea is that MHBs^t staining colocalized with the staining of various ER marker proteins upon double immunofluorescence analysis (data not shown). We cannot exclude at present that very minor fractions of MHBs^t are present in other subcellular compartments. The weak nucleoplasmic staining was presumably not specific because non-transfected cells also gave a nucleoplasmic staining with the F-124 antibody (Figure 9B, panel 2). Using a radioimmunoassay (AUSRIA, Abott), no MHBs^t was detected in the culture medium, as was observed for a C-terminally truncated form of SHBs (Bruss and Ganem, 1991). This indicates that secretion of MHBs^t is strongly retarded in the ER.

Upon subcellular fractionation, MHBs behaved similarly to MHBs^t in that most of the protein was recovered in the particulate fraction (Figure 9a, compare lanes 3 and 4). As reported previously (Stibbe and Gerlich, 1983; for review see Neurath and Kent, 1988), the MHBs protein was present in a non-glycosylated and two glycosylated forms. The small amounts of glycosylated, soluble MHBs found in the high speed supernatant (lane 3) were presumably released from membrane vesicles. In contrast to MHBs^t, MHBs was detected in the cell culture medium by radioimmunoassay (data not shown).

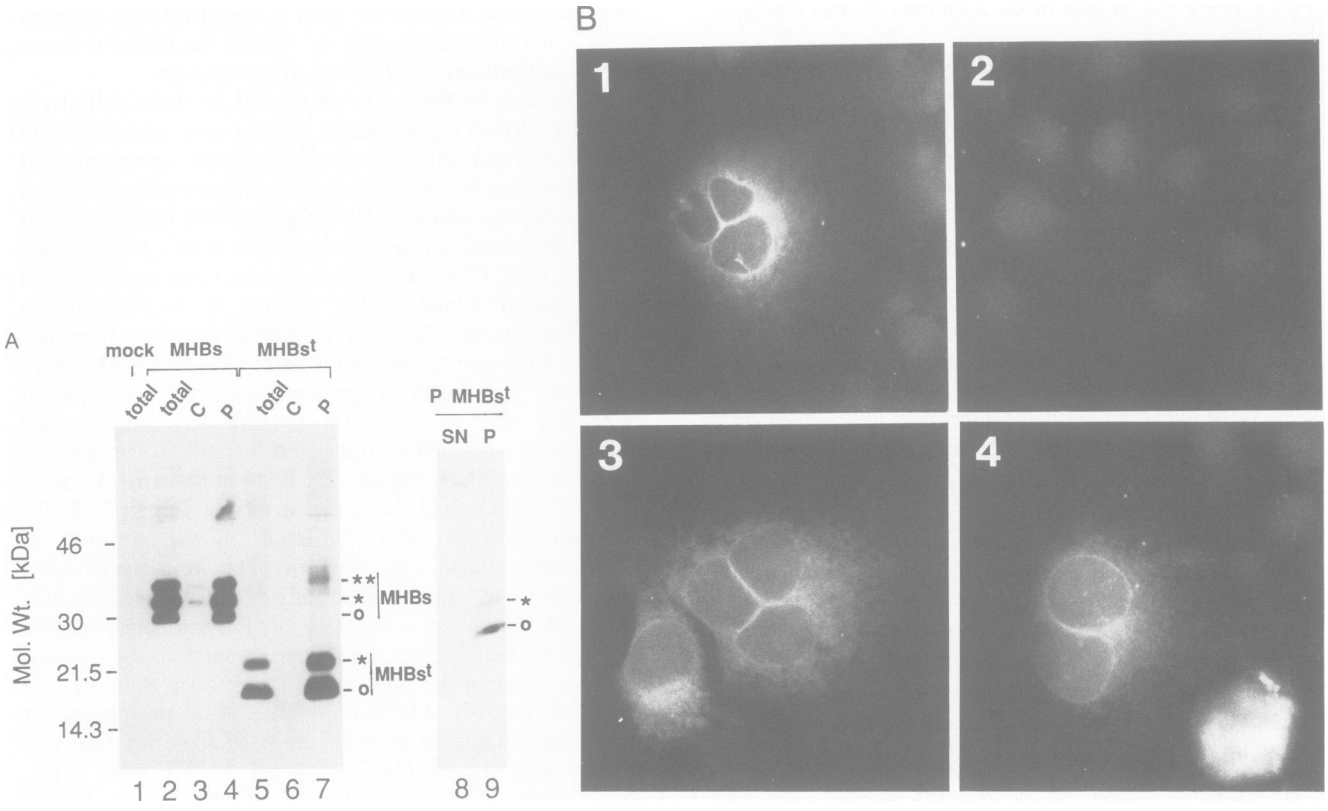


Fig. 9. Subcellular distribution of MHBs^t. (**A**) Subcellular fractionation. HeLa cells were infected with a recombinant vaccinia virus expressing T7 polymerase followed by transfection with T7 vector pTM1 alone (mock; lane 1), MHBs (pTMS486; lanes 2–4), or MHBs^t expression vectors (pTMS831; lanes 5–9) under the control of the T7 polymerase. 18 h after infection, cells were lysed in RIPA buffer (total; lanes 1, 2 and 5) or fractionated, following hypotonic cell lysis, into the cytosol (C; lanes 3 and 6) and particulate fraction (P; lanes 4 and 7). In the left panel, the particulate fraction (lane 7) was treated with 0.1 M Na_2CO_3 followed by ultracentrifugation. P, pellet; SN, supernatant. Proteins from equal cell equivalents were separated by reducing 15% SDS–PAGE and then transferred onto nitrocellulose filters. Filters were reacted with the monoclonal antibody F-124 and bound IgG visualized by a chemoluminescence detection system. A 7 s exposure of the filter is shown. For molecular size standards, see legend to Figure 1. o; non glycosylated; *, monoglycosylated; **, diglycosylated proteins. (**B**) Indirect immunofluorescence labelling of MHBs^t. COS7 cells were transiently transfected with 1.5 μg pCMV486+ (panels 1, 3 and 4) and the expressed MHBs^t protein was visualized with the monoclonal antibody F-124 and a FITC-labelled goat anti-mouse antibody at 525 nm. Panel 2, non-transfected cells. Panels 2, 3 and 4 show a 1.6-fold higher magnification than panel 1.

Discussion

HBV transactivators and pleiotropic gene induction

This study shows that the transactivator MHBs^t from HBV is a potent inducer of the transcription factor NF-κB. It also became apparent that various other promoter and enhancer sequences that are not controlled by NF-κB are induced by MHBs^t. The protein must therefore be able to activate a series of inducible transcription factors. This could happen via NF-κB as master regulator or by a simultaneous activation of factors. The latter situation is more likely since antioxidants can selectively block the activation of NF-κB without interfering with the transactivation of other regulatory elements by MHBs^t.

HBx and the transactivator Tax from HTLV-I display an activity spectrum overlapping with that of MHBs^t. Although all three proteins can transactivate reporter constructs under the control of NF-κB and AP-1 binding sites and the HTLV-I LTR (reviewed in Smith and Greene, 1991; Rossner, 1992; data not shown), they have no apparent sequence homology that would reflect the related function and specificity. Moreover, as shown in this study, it is not even required that the proteins have the same subcellular distribution. While MHBs^t is a membrane protein, HBx and Tax occur predominantly in the nucleus (Siddiqui *et al.*, 1987; Smith and Greene, 1991; Wang *et al.*, 1991). These differences could simply mean that the three proteins exert their activity in the cell by using distinct signalling systems and reaching on overlapping set of target transcription factors. Alternatively, MHBs^t, HBx and Tax might share common biophysical property that can elicit the same intracellular reaction. For instance, the expression of the viral proteins could cause a stress situation under which a common programme of gene activation is induced.

HBV transactivators and hepatocarcinogenesis: the 'radical' hypothesis

Sequences of the virus coding for HBx and C-terminally truncated LHBs and MHBs transactivators are well conserved in the HBV DNA integrated in HCC genomes (for review see Nagaya *et al.*, 1987). Indirect evidence is presented here that expression of MHBs^t and HBx transactivators causes an increase in the intracellular concentration of ROI. The evidence is based on the effect of inhibition of two well studied antioxidants, NAC and PDTC, on transactivation. Although the two substances are chemically quite distinct, they showed the same effect on the κB-dependent gene induction by MHBs^t. Similar results were obtained with Tax (Schreck *et al.*, 1992b). Future

studies may provide more direct evidence that expression of MHBs^t and HBx proteins lead to an increased production of ROI. Such studies are currently hampered by the lack of systems in which the synthesis or activity of the viral proteins is tightly controlled.

There is an overwhelming number of studies supporting a role of free radicals in the initiation and progression of multistage carcinogenesis (for a recent review see Sun, 1990). Consistent with this idea, free radical scavengers and antioxidant enzymes have anticarcinogenic activity and very frequently antioxidant enzymes are found downregulated in tumour cells. Also various hepatocarcinoma cell lines exhibited very low levels of catalase and superoxide dismutases compared to normal liver cells, which were correlated with the rate of growth (Oberley *et al.*, 1978; Corrocher *et al.*, 1986; Bellisola *et al.*, 1987; Mavier *et al.*, 1988). Future studies have to investigate whether induction of oxidant stress is a prerequisite for initiation and progression of hepatocarcinogenesis by MHBs^t and HBx.

The subcellular localization of the MHBs^t protein

The subcelluar distribution, membrane topology and structural characteristics of MHBs were extensively studied (Eble *et al.*, 1987, 1990; for review see Neurath and Kent, 1988). MHBs has an internal signal peptide (amino acids 63−78), a stop transfer signal (amino acids 135−153), is *N*-glycosylated on Asn4 and Asn201, and spans the membrane by at least two hydrophobic domains (see model in Figure 10). The N-terminus is thought to be luminal and the highly hydrophobic C-terminus anchored to the luminal side of the membrane. The C-terminal truncation at position 167 giving rise to the form of MHBs^t studied here, eliminates the *N*-glycosylation site in position 201 and the hydrophobic C-terminus, but leaves both N-terminal transmembrane signals intact. This might well explain why MHBs^t was not present in a diglycosylated form and was still firmly associated with membranes. Unlike MHBs, MHBs^t was not detected in the culture medium. A similar observation was made with a form of SHBs harbouring a frame shift mutation after residue 231 (Bruss and Ganem, 1991). This protein could only be secreted if coexpressed with wild type MHBs. As indicated here by indirect immunofluorescence labelling, the apparent block in export of MHBs^t could be at the level of the rough ER: staining of nuclear envelopes, which are contiguous with the ER, and a perinuclear, reticular structure were both observed. Subcellular fractionation showed no detectable amounts of MHBs^t in the cytosol and even a treatment at pH 11.5 could not strip significant amounts of MHBs^t from membranes. MHBs^t appears to be the first viral transactivator found to be an integral membrane protein, presumably associated with the ER.

This raises two questions. First, how can an integral membrane protein activate the cytoplasmic form of NF-$\varkappa$B and various other nuclear transcription factors? Secondly, why is the parental protein inactive? Because MHBs^t still undergoes *N*-glycosylation, its membrane topology is presumably unaffected by the C-terminal truncation. Two differences that distinguish the transactivating MHBs^t from full-length MHBs are the block in secretion and a reduced *N*-glycosylation. These might, however, not be crucial for transactivation. MHBs can also stay for a relatively long time in the ER (see Gerlich *et al.*, 1989) and also part of the

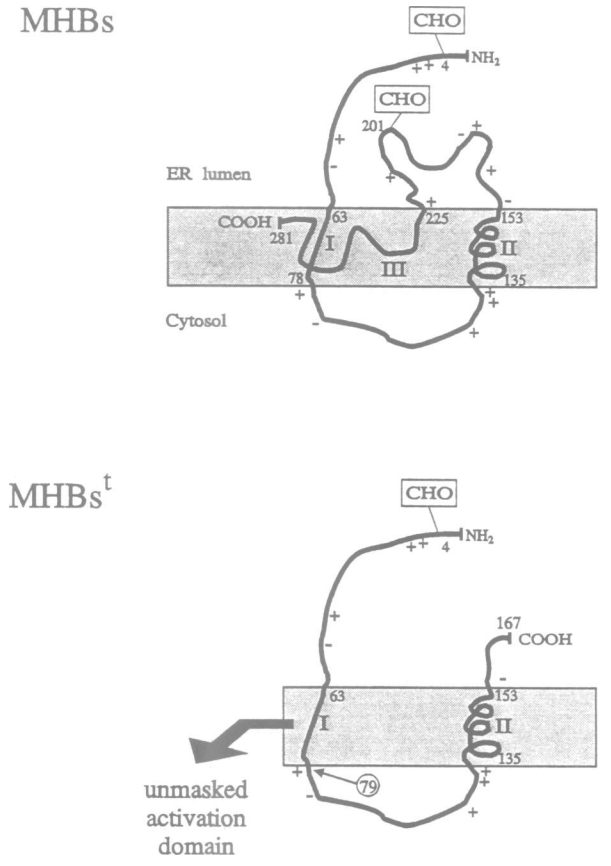


Fig. 10. Structural models of MHBs and MHBs^t. CHO, N-linked oligosaccharides on Asn4 and Asn201; +/−, charged amino acid residues; NH_2, N-terminus; COOH, C-terminus; 167, C-terminus of MHBs^t (C-terminus for MHBs is amino acid 281); 79, C-terminus of a still transactivating form of MHBs (Kekulé *et al.*, 1990). Roman numbers indicate the putative integral membrane domains of MHBs. Domains I and III are unlikely to adopt an α-helical confirmation because they are rich in proline and glycine residues. In our model, the hydrophobic C-terminal portion of MHBs physically masks the transactivating N-terminal portion within the membrane. It is also conceivable that luminal sequences are involved instead, or in addition.

MHBs is not glycosylated. The sequence exposed to the cytosol (amino acids 79−134; see Figure 10) might also not be involved in transactivation. It is present in both truncated and full-length MHBs, and its replacement by an unrelated cellular sequence does not impair transactivation (Kekulé *et al.*, 1990). In fact, a transactivating MHBs fusion protein was isolated from the HCC-derived cell line HuH-4, which encompassed only the first 79 amino acids of MHBs consisting of the luminal tail and the first transmembrane domain (see Figure 10; lower panel).

Because the C-terminal portion of MHBs exerts a repressing function on the transactivating potential of the protein, we assume that it masks a short transactivating sequence in the N-terminal portion of MHBs. This could occur via an intramolecular mechanism (as shown in Figure 10), but also other proteins could be involved. The transactivating sequence of MHBs could consist of only the first transmembrane domain, the N-terminal portion in the lumen or a combination of both sequences. From these considerations, it becomes a challenging task for future studies to understand how the transactivating activity is suppressed in MHBs and how a short membrane-attached

peptide can activate the cytoplasmic NF-κB−IκB complex and other nuclear factors in a fashion similar to that of the nuclear/cytoplasmic transactivators HBx and Tax.

Materials and methods

Cell culture

Liver-derived Chang cells (ATCC CCl13), HeLa cells (ATCC CC12) and COS-7 cells (ATCC CRL 1651) were cultured at 37°C in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum and 100 μg/ml kanamycin or 50 μg/ml gentamycin.

Constructs

The expression vector pRc/CMV containing the cytomegalovirus immediate early promoter/enhancer, the SV40 origin of replication and T7/SP6 polymerase binding sites was cleaved with *Not*I and subsequently ligated to a *Not*I-linkered double-stranded oligonucleotide carrying TAG stop codons in all three reading frames in order to generate pRc/CMVstop. For construction of the MHBs^t expression vector, *Hin*dIII linkers were added to a blunt-ended *Bsu*36I−*Bam*HI fragment from pTKTHBV2) (Will *et al.*, 1985) covering nucleotides 3163−486 of the preS/S domain of the HBV strain *ayw* (numbering system of Galibert *et al.*, 1979). The HBV sequence was inserted into *Hin*dIII-opened pRc/CMVstop. Ligation generated pCMVS486$^+$ and pCMVS486$^-$, which harbour a 3′-truncated preS2/S region of HBV in sense and antisense orientation, respectively. The expression vector pCMVX was constructed by the addition of *Hin*dIII linkers to a blunt-ended, *Nco*I−*Fsp*I fragment corresponding to nucleotides 1372−1800 of pTKHBV2 (Will *et al.*, 1985). The fragment encoding the X gene was ligated into *Hin*dIII-opened pRc/CMVstop. The correct sequence of all constructs was verified by sequencing.

The vaccinia−T7 polymerase system was described earlier (Fuerst *et al.*, 1985). For directed mutagenesis, MHBs and MHBs^t sequences were cloned into the vector pGEMZ7f(+) (Promega) and a C to G transition introduced after the ATG start codon in position 3173. This generated an *Nco*I site for cloning and optimized the Kozac sequence. The respective *Nco*I−*Bam*HI insert corresponding to nucleotides 3170−486 (MHBs^t) and 3170−831 (MHBs) of HBv *ayw* were isolated and cloned into the vector pTM1 (Moss *et al.*, 1990) giving rise to pTMS486 and pTMS831, respectively.

The reporter plasmid p6xκB/SV1CAT contains three copies of a sequence from the HIV-I LTR (nucleotides −105 to −80) encompassing the two κB motifs (Hiscott *et al.*, 1987). The construct J16 contains two κB motifs (5′-GGGACTTTCC-3′) upstream of a −Δ56 mouse c-*fos* promoter and the homologous construct J32 has two copies of a mutated κB motif (5′-TTCACTTTCC-3′) instead (Pierce *et al.*, 1988; Lenardo *et al.*, 1989). CAT reporter constructs controlled by the HIV-1 LTR (nucleotides −453 to +80) and HTLV-I LTR (nucleotides −350 to +35) are described elsewhere (Nabel and Baltimore, 1987; Sodrowski *et al.*, 1984).

Transfections and CAT assays

Human Chang and Hela cells were plated one day prior to transfection at a density of 3.5 × 10^6 or 5 × 10^6 cells per 60 mm dish, respectively. 1−3 μg of reporter plasmids was cotransfected with 0.1−0.5 μg of expression vector by the calcium phosphate method (Graham and van der Eb, 1973). Cells were treated with PDTC, NAC (for details see Schreck *et al.*, 1991, 1992a) or H7 as described in the legends to figures. Cells were detached with PBS−10 mM EDTA, collected by centrifugation, resuspended in 200 μl 250 mM Tris−HCl pH 7.8, 5 mM EDTA and lysed by four cycles of freeze−thawing. Equal amounts of protein quantified by the method of Bradford (1976) were assayed for CAT activity by incubation with 75 nCi [^{14}C]chloramphenicol (Amersham International) for 90 min (Gorman *et al.*, 1982). Reaction products were analysed by ascending thin layer chromatography and the amount of acetylated and non-acetylated chloramphenicol determined by an isotope scanner (Berthold tracemaster 20). Alternatively, the amount of CAT protein was determined by a CAT ELISA system (Boehringer Mannheim). Transfections were performed in triplicates and CAT assays were performed at least twice.

Electrophoretic mobility shift assays

COS7 cells (1.5 × 10^6 cells per 100 mm dish) were transfected using DEAE dextran (450 μg/ml) with 2.5 μg of the expression vectors. HeLa cells were transfected with 5 μg of vectors using lipofection (Gibco, BRL). 40 h after transfection, PDTC was added at the indicated amounts 8−12 h prior to harvest of cells. Nuclear extracts were prepared essentially as described by Dignam *et al.* (1983) with the modification that buffer D contained 0.1% (v/v) Nonidet P-40. Binding reactions were performed with 4−8 μg of protein in 10 mM Tris−HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 8% (v/v) glyceorol, 0.1 mM dithiothreitol (DDT), 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 20 000 c.p.m. (Cerenkov) of a ^{32}P-labelled oligonucleotide in a final volume of 20 μl for 20 min on ice. Oligonucleotides were labelled using either [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) with Klenow enzyme (Gibco-BRL) or [γ-^{32}P]ATP (5000 ci/mmol. Amersham) with T4 polynucleotide kinase (Gibco-BRL). DNA−protein complexes were electrophoresed on native 4.5% polyacrylamide gels in 34 mM Tris−HCl pH 7.5, 17 mM NaCl and 0.5 mM EDTA. Gels were vacuum dried and exposed to Amersham MP films at −80°C.

The sequence of oligonucleotides were as follows (factor binding sites are underlined):

κB: 5′-AGCTTGAAGGGACTTTCCGCTGGGGACTTTCCAGT-3′
3′-ACTTCCCTGAAAGGCGACCCCTGAAAGGTCAGATC-5′

Sp1: 5′-TCGACCTGGGGCGGGGCAGG-3′
3′-GGACCCCGCCCCGTCCAGCT-5′

oct: 5′-AGCTTTGGGTAATTTGCATTTCTAAG-3′
3′-TCGAAACCCATTAAACGTAAAGATTC-5′

In vitro transcription and translation

*Not*I-linearized plasmid pCMV486$^+$ was used as template for transcription by T7 RNA polymerase. RNA synthesis was performed for 1 h at 37°C in the presence of the cap analogue m^{7}G(5′)ppp(5′) guanosine. Transcribed mRNA was purified by several rounds of phenol−chloroform extraction and subsequently used as template for translation in rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine (1000 Ci/mmol; Amersham). Canine pancreas microsomes were purchased from Promega. Approximately 10 000 TCA-precipitable c.p.m. were subjected to analysis on 15% SDS polyacrylamide gels.

Subcellular fractionation, high pH treatment and Western blotting

HeLa cells (1.2 × 10^6 cells per 60 mm dish) were infected with five MOI of the T7 recombinant vaccinia virus TF7-3 followed by transfection with 5 μg pTMS486 or pTMS831 and grown for 18 h. Cells were detached by PBS containing 10 mM EDTA and collected by centrifugation. After incubation in hypotonic buffer (10 mM HEPES−KOH pH 7.8, 5 mM KCl, 0.2 mM $MgCl_2$, 2 mM DDT, 0.5 mM PMSF and 1 μg/ml aprotinin) for 15 min, cells were lysed by 15 strokes in a glass Dounce homogenizer. The lysate was separated into cytosol and particulate fraction by centrifugation at 100 000 *g* for 1 h at 4°C. The two fractions were adjusted to equal volumes of 1 × SDS sample buffer (Laemmli, 1970) and boiled. Total cell lysates were prepared with RIPA buffer (0.15 M NaCl, 10 mM Tris−HCl pH 8.0, 0.5% (v/v) Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.1 mM PMSF and 0.1% aprotinin). Aliquots of the total lysate and fractions were subjected to reducing 15% SDS−PAGE and proteins subsequently transferred onto nitrocellulose membranes (ECL membranes; Amersham). The membranes were blocked with 5% dried milk in TBST (150 mM NaCl, 50 mM Tris−HCl, pH 7.4, 0.1% (v/v) Tween-20) for 1 h and incubated for 2−3 h with the mouse anti-preS2-specific monoclonal antibody F-124 (Budkowska *et al.*, 1986) diluted 1:400 in TBST−0.25% bovine serum albumin. After incubation for 1 h with a goat anti-mouse horse radish peroxidase-conjugated second antibody (Sigma), immunoreactivity was detected with an enhanced chemoluminescence system (ECL; Amersham International).

High pH treatment was performed by resuspending the particulate fraction in 0.1 M Na_2CO_3, pH 11.5 (Fujiki *et al.*, 1982). After 15 min on ice, the suspension was centrifuged for 90 min at 150 000 *g*. Pellet and supernatant were adjusted to the same volume of 1 × sample buffer, boiled and subjected to reducing 15% SDS−PAGE.

MHBs and MHBs^t were quantified in cell culture media by the radioimmunoassay AUSRIA-II (Abott) according to the instructions of the manufacturer.

Indirect immunofluorescence labelling

COS7 cells were transfected with 1.5 μg pCMV486$^+$ and grown on cover slips (NUNC LabTek) for 60 h. After removal of medium, cells were washed several times with PBS, fixed for 15 min in 3.7% formaldehyde dissolved in PBS−10% FCS (PBS−FCS) followed by incubation in 0.2% (v/v) Triton X-100, PBS, 10% FCS. After 1 h in PBS−FCS, fixed cells were exposed for 2 h to the preS2-specific monoclonal antibody F-124 at a 100-fold dilution in PBS−FCS. Specimens were then extensively washed with PBS and incubated for 30 min with goat anti-mouse fluoresceinisothiocyanate (FITC)-labelled second antibody (Sigma) diluted 1:40 in PBS/FCS. After four 10 min washes with PBS, cover slips were overlayed with *p*-phenylenediamine

(5 mg/ml in PBS−10% glycerol), covered with a slide and analysed by fluorescence microscopy (Leitz−Aristoplan).

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