VIROLOGY 225, 235–239 (1996)
ARTICLE NO. 0594

SHORT COMMUNICATION

EBERHARD HILDT,*,[1] GESINE SAHER,* VOLKER BRUSS,† and PETER HANS HOFSCHNEIDER*

*Department of Virus Research, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany; and
†Department of Medical Microbiology, University of Göttingen, Kreuzbergring 57, D-37075 Göttingen, Germany

Received April 30, 1996; accepted August 29, 1996

It has been shown that a C-terminally truncated form of the middle-sized hepatitis B virus (HBV) surface protein (MHBs^t) functions as a transcriptional activator. This function is dependent on the cytosolic orientation of the N-terminal PreS2 domain of MHBs^t, but in the case of wild-type MHBs, the PreS2 domain is cotranslationally translocated into the ER lumen. Recent reports demonstrated that the PreS2 domain of the large HBV surface protein (LHBs) initially remains on the cytosolic side of the ER membrane after translation. Therefore, the question arose as to whether the LHBs protein exhibits the same transcriptional activator function as MHBs^t. We show that LHBs, like MHBs^t, is indeed able to activate a variety of promoter elements. There is evidence for a PKC-dependent activation of AP-1 and NF-κB by LHBs. Downstream of the PKC the functionality of c-Raf-1 kinase is a prerequisite for LHBs-dependent activation of AP-1 and NF-κB since inhibition of c-Raf-1 kinase abolishes LHBs-dependent transcriptional activation of AP-1 and NF-κB.

The gene for the HBV surface antigen consists of a single ORF divided into three coding regions, preS1, preS2, and S, each starting with an in-frame ATG codon. The corresponding protein domains are therefore referred to as PreS1, PreS2, and S. By translational initiation at the first AUG the large hepatitis surface antigen (LHBs) encompassing PreS1, PreS2, and S is synthesized; by initiation at the second AUG the middle surface protein (MHBs, encompassing PreS2 + S), and by initiation at the third, the small surface protein (SHBs, encompassing S) is synthesized. All three HBV surface proteins are integral membrane proteins. MHBs occurs in a mono- or biglycosylated form. Glycan residues are linked at aa 4 of the PreS2 region and at aa 146 of the S region. LHBs and SHBs are monoglycosylated; the glycan residue is linked to aa 146 of the S region. Surprisingly, LHBs is monoglycosylated although it harbors a second glycosylation site at aa 4 of the PreS2 region. Recent work, however, revealed that in the case of LHBs the PreS1–PreS2 region is not cotranslationally translocated across the ER membrane and therefore is oriented toward the cytoplasm (*1–3*). This orientation makes the glycosylation site at asn 4 of the PreS2 region inaccessible to the glycosyltransferases, which are localized in the lumen of the ER. Only in a fraction of the LHBs chains does the PreS1–PreS2 region seem to be posttranslationally translocated across the membrane in the secretory pathway during viral assembly. In the viral particle the Pres1–PreS2 domain of a fraction of LHBs is found on the viral surface.

Whereas MHBs and SHBs are secreted as subviral particles, the secretion of LHBs requires coexpression of the MHBs and SHBs. A strong overproduction of LHBs with respect to MHBs and SHBs causes the intracellular retention of all proteins (*4–6*). In the case of 3′ terminal truncations of the preS2/S gene — at least the coding region for the transmembrane region 3 has to be deleted — the resulting MHBs derivative gains a transcriptional activator function (*7–9*). In contrast to full-length MHBs, the transcriptional activators are not secreted and not glycosylated (*10*).

In a recent report (*11*) it was demonstrated that the PreS2 region is sufficient for generation of the transcriptional activator function. It was shown that the reason for the functional difference between the structural protein MHBs and the transcriptional MHBs^t activators is a difference in the localization of the amino-terminal PreS2 region. In the case of MHBs this domain is oriented toward the lumen of the ER (*10, 12*), whereas in the case of functional MHBs^t activators this region faces the cytoplasm (*11*). This orientation apparently makes an interaction with cytosolic binding partners possible. A direct interaction with PKC was shown to be essential for triggering the MHBs^t-dependent transcriptional activation (Hildt *et al.,* submitted for publication).

Since LHBs shares with MHBs^t activators a cytoplasmic orientation of the PreS2 domain, we investigated whether LHBs displays a transcriptional activator function like MHBs^t. HepG2 cells (1.0×10^6 cells) were cotransfected

[1] To whom correspondence and reprint requests should be addressed.

0042-6822/96 $18.00

with 1.5 μg of the LHBs expression plasmid pSVLM$^-$S$^-$ (*13*) and the reporter constructs pSV2-CAT (0.5 μg), p3xAP-1-CAT (1 μg), or p2xNF-κB-CAT (1.5 μg). The vector pSVLM$^-$S$^-$ directs the production of the LHBs protein in the absence of MHBs and SHBs and allows a characterization of LHBs-dependent transcriptional activation without the influence of other HBV proteins. Upon cotransfection with the expression vector pSVLM$^-$S$^-$ and each of the reporter constructs, a seven- to ninefold induction of the CAT reporter gene was observed. This induction is comparable to the transcriptional activation following transfection with the expression plasmid pSVMHBs^{t76}, which encodes a MHBs^t protein truncated at aa 76 (*10*). As a negative control cotransfection with an equal amount of pUC 19 was performed (Fig. 1a). In all transfection experiments transfection efficiencies were standardized with a luciferase reporter construct containing the luciferase gene under control of the nonstimulatable minimal promoter pTK/-37luc.

Various minimal promoters (2xNF-κB or 3xAP-1) or the SV40 early promoter were activated by the production of LHBs. This indicates that the structure protein LHBs is a pleiotropic transcriptional activator.

The question thus arose as to whether L proteins, in the presence of MHBs and SHBs, also serve as transcriptional activators. Therefore, HepG2 cells were cotransfected with the reporter constructs described above and expression vector pSVLHBs (*1*), leading to the production of LHBs, MHBs, and SHBs. Compared to the cotransfection of cells with pSVLM-S-, a slightly reduced, but still six- to sevenfold transcriptional activation was observed (Fig. 1b). The slight reduction might reflect a reduced synthesis of LHBs.

The MHBs^t-dependent transcriptional activation was shown to be mediated by PKC-dependent activation of the c-Raf-1/MAP2-kinase signal transduction pathway (Hildt *et al.,* submitted). To study whether the LHBs-dependent transcriptional activation is also triggered by the same signaling pathway, the cotransfection experiments described were first performed in the presence of the PKC inhibitor H7. The LHBs-dependent activation of the reporter constructs p3xAP-1-CAT or p2xNF-κB-CAT was completely abolished by the presence of 0.1 m*M* H7 in the culture fluid (Fig. 2a). Inhibition of the MHBs^{t76}-dependent activation of these reporter constructs as demonstrated earlier (Hildt *et al.,* submitted for publication) was also observed. Transfection of cells with the cloning vector pUC19 served as the negative control. Since H7 displays no absolute PKC specificity, the PKC was depleted by continuous TPA stimulation (100 ng/ml) 30 hr prior to transfection. Under these conditions the LHBs-dependent as well as the MHBs^{t76}-dependent activation of the NF-κB- (7-fold of the control) and the AP-1-driven (9-fold of the control) reporter constructs was abolished (1.3- to 1.5-fold of the control). These results suggest that the LHBs-dependent transcriptional activation could be mediated by an activation of the PKC (Fig. 2a).

The functionality of c-Raf-1 kinase is essential for triggering the activation of AP-1 and NF-κB in MHBs^t-producing cells (Hildt *et al.,* submitted for publication). To investigate whether the LHBs-dependent transcriptional activation of AP-1 and NF-κB is also mediated by an activation of c-Raf-1 kinase, 1.0×10^6 HepG2-cells were transfected with 1.5 μg of the expression plasmid pSVLM$^-$S$^-$, the reporter constructs p3xAP-1-CAT (1 μg) or p2xNF-κB-CAT (1.5 μg), respectively, and 1.5 μg of the plasmid pHCR13.1 (*14*), which encodes a transdominant negative mutant of c-Raf-1 kinase. Coexpression of the transdominant negative mutant caused a complete loss of the LHBs-dependent activation of AP-1 (1.7-fold) or NF-κB (1.4-fold), respectively (Fig. 2b). The MHBs^{t76}-dependent activation of these transcription factors, which was used as a positive control, was also completely abolished. In the control experiments the plasmid pHCR13.1 was replaced by an equal amount (1.5 μg) of the cloning vector pMNC. Under these conditions the already described (Fig. 1) activation of both transcription factors by LHBs or MHBs^{t76} was observed. To demonstrate that the inhibition of the LHBs-dependent activation of AP-1 or NF-κB is not due to nonspecific toxicity caused by the construct pHCR13.1, cells were cotransfected with pHCR13.1 and the reporter plasmid p2xNF-κB-CAT. The cells were stimulated by the addition of H_2O_2 to a final concentration of 500 μ*M.* The H_2O_2-dependent activation of NF-κB was not affected by the coexpression of the transdominant negative mutant of c-Raf-1 compared to control-transfected cells (data not shown). These experiments clearly demonstrate that the LHBs-dependent transcriptional activation is mediated by c-Raf-1 kinase. However, elevation of the endogenous c-Raf-1-kinase level by transfection of the cells with the c-Raf-1 kinase expression plasmid pMNC-c-raf instead of pHCR13.1 did not cause a significant increase in the LHBs- or MHBs^{t76}-dependent activation of the reporter constructs. It can be concluded that the endogenous level of c-Raf-1 kinase is not limiting for triggering LHBs-dependent activation of AP-1 or NF-κB.

Altogether, in this study we have demonstrated that LHBs is indeed a functional activator. Furthermore, we found that the mechanism of LHBs-dependent transcriptional activation is mediated by PKC-dependent activation of the c-Raf/MAP-kinase signal transduction pathway, which is also used in the case of MHBs^t-dependent transcriptional activation. Identical targets, i.e., AP-1 or NF-κB, are activated, and in both cases the PKC seems to be essential for triggering the transcriptional activation as well as c-Raf-1 kinase. Therefore, we conclude that the transcriptional activator function of LHBs is generated by the cytoplasmic orientation of its PreS2 region.

The observed transcriptional activation appears not to be caused by the ER retention of LHBs resulting in generation of an increased level of intracellular radicals. Cotransfection experiments in the presence of 30 m*M*

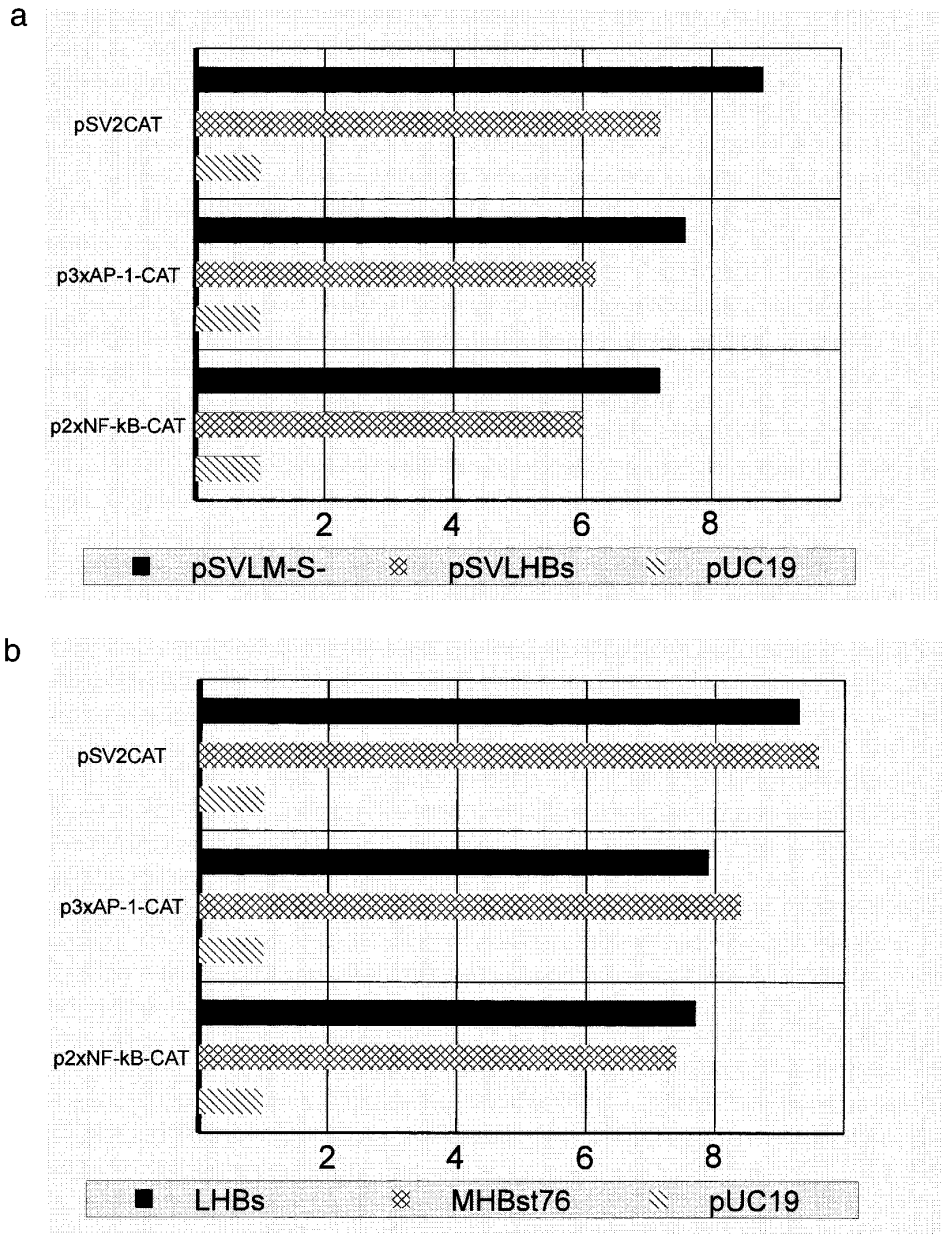

FIG. 1. LHBs has a transcriptional activator function. (a) CAT assay after cotransfection of HepG2 cells with the reporter plasmid pSV2-CAT, p3xAP-1-CAT, or p2xNF-κB-CAT and the expression plasmid pSVLM-S- (encoding for LHBs), pSVMHBs^{t76} (encoding for MHBs^{t76}), or pUC19 as the negative control. Fold inductions are the mean values from four independent transfection experiments, calculated as the values for the vector control. (b) CAT assay after cotransfection of HepG2 cells with the reporter plasmid pSV2-CAT, p3xAP-1-CAT, or p2xNF-κB-CAT and the expression plasmid pSVLHBs (encoding for LHBs, MHBs, and SHBs), pSVMHBs^{t76}, or pUC19 as the negative control. Fold inductions are the mean values from four independent transfection experiments, calculated as the values for the vector control.

N-acetyl-L-cysteine demonstrated that under these conditions no significant influence on the transcriptional activator function could be observed (data not shown).

Continuous overproduction of LHBs results *in vivo* in the generation of ground glass hepatocytes in transgenic mice (*5*), which are characterized by large amounts of intracellular LHBs. In a transgenic mouse model the production of HCC was observed under these conditions (*5*). The development of HCC was considered so far to be caused by a permanent inflammatory process under these conditions. Since overproduction of LHBs results in the activation of several kinases (PKC, c-Raf-1 kinase) triggering cellular proliferation control, it is possible that continuous activation of these enzymes might additionally contribute to the development of a hepatocellular carcinoma. According to a multifactorial model of carcinogenesis, the continuous expression of LHBs might have a tumor promoter function.

LHBs is essential for the assembly of viral particles. On the basis of our results, we propose that a second function

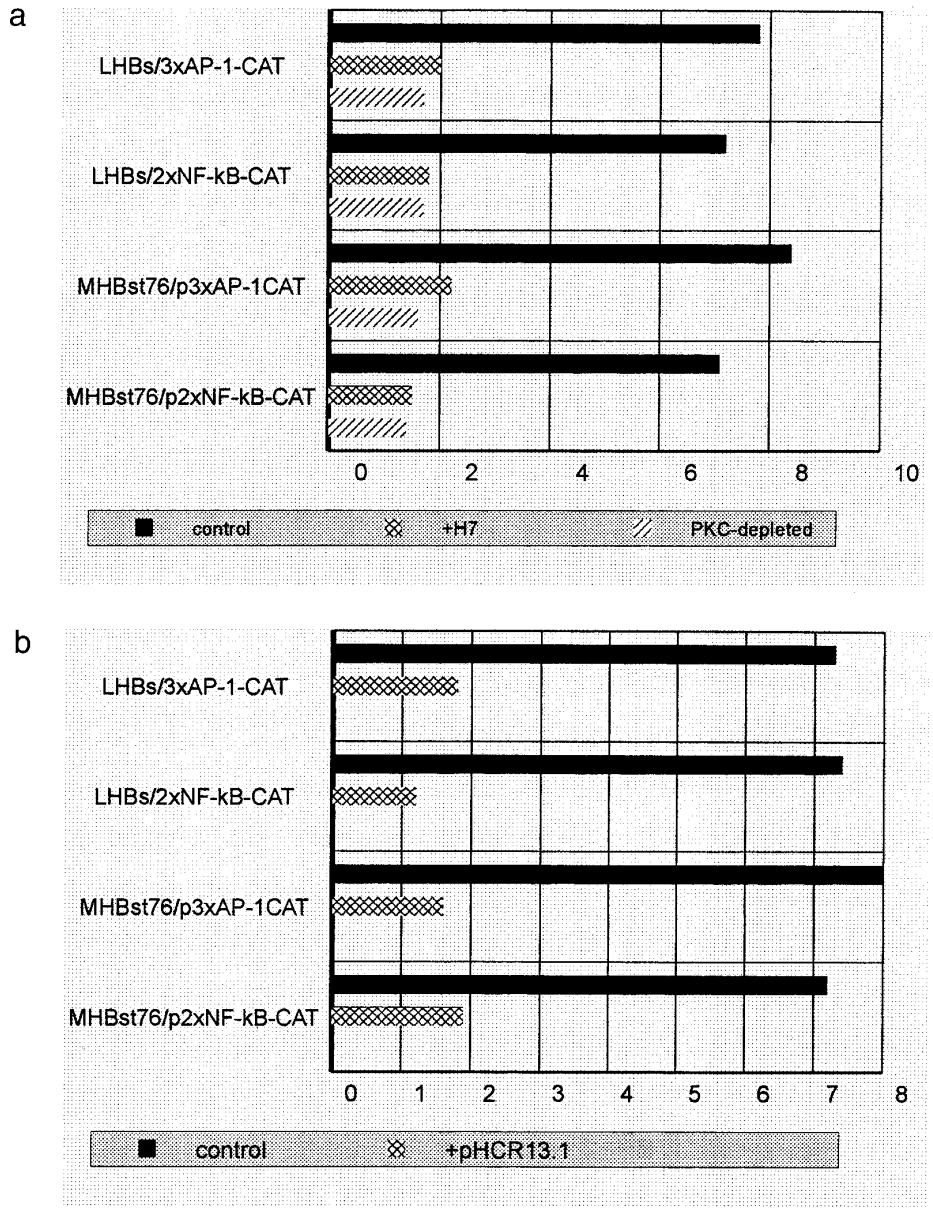

FIG. 2. Transcriptional activator function of LHBs depends on PKC and c-Raf-1 kinase. CAT assay after cotransfection of HepG2 cells with pSVLM-S- and the reporter constructs p3xAP-1-CAT or p2xNF-κB-CAT. Cotransfection of the cells with pSVMHBs^{t76} served as a positive control, cotransfection with pUC19 as a negative control. Fold inductions are the mean values from four independent transfection experiments, calculated as the values for the vector control. (a) Cells were grown in the presence of 0.1 m*M* H7 in the culture fluid to inhibit PKC, or PKC was depleted by exposure of the cells to 200 ng/ml TPA 30 hr prior to transfection. (b) Cells were cotransfected with pHCR13.1, which encodes a transdominant negative mutant of c-Raf-1 kinase, pSVL M^-S^-, or pSVMHBs^{t76} and the reporter constructs p2xNF-κB-CAT or p3xAP-1-CAT. In the control experiments cells were cotransfected with the cloning vector pMNC instead of pSVL M^-S^- or pSVMHBs^{t76}.

of LHBs is to support viral replication by a transcriptional activation of the viral promoter elements. This appears to be parallel to HBx, whose function does not appear to be a prerequisite for HBV replication. However, the presence of HBx appears to support the viral replication (*15*) by a transcriptional activation of viral and/or cellular promoters. In particular, immediately upon infection of hepatocytes, when HBx is not yet available, LHBs may replace HBx functions. Later on during the infection LHBs and HBx functions may overlap. This could be one explanation of why HBx, at least *in vitro,* seems to be dispensable for HBV replication. It should also be noted that the reported findings link the transcriptional activator function of LHBs to the increased risk of HBs carriers of developing HCC, since these carriers are also LHBs-positive.

REFERENCES

1. Bruss, V., Lu, X., Thomssen, R., and Gerlich, W., *EMBO J.* 13, 2273–2279 (1994).

2. Ostapchuk, P., Hearing, P., and Ganem, D., *EMBO J.* 13, 1048–1057 (1994).
3. Prange, R., and Streek, G., *EMBO J.* 14, 247–256 (1995).
4. Chisari, F. V., Filippi, P., McLachlan, A., Milich, D. R., Riggs, M., Lee, S., Palmiter, R. D., Pinkert, C. A., and Brinster, R. L., *J. Virol.* 60, 880–887 (1986).
5. Chisari, F. V., Filippi, P., Buras, J., McLachlan, A., Popper, H., Pinkert, C. A., Palmiter, R., and Brinster, R. L., *Proc. Natl. Acad. Sci. USA* 84, 6909–6913 (1987).
6. Kuroki, K., Russnak, R., and Ganem, D., *Mol. Cell. Biol.* 9, 4459–4466 (1989).
7. Caselmann, W. H., Meyer, M., Kekulé, A. S., Lauer, U., Hofschneider, P. H., and Koshy, R., *Proc. Natl. Acad. Sci. USA* 87, 2970–2974 (1990).
8. Kekulé, A. S., Lauer, U., Meyer, M., Caselmann, W. H., Hofschneider, P. H., and Koshy, R., *Nature* 343, 457–461 (1990).
9. Lauer, U., Weiß, L., Hofschneider, P. H., and Kekulé, A. S., *J. Virol.* 66, 5284–5289 (1992).
10. Hildt, E., Urban, S., Lauer, U., Hofschneider, P. H., and Kekulé, A. S., *Oncogene* 8, 3359–3367 (1993).
11. Hildt, E., Urban, S., and Hofschneider, P. H., *Oncogene* 11, 2055–2066 (1995).
12. Eble, B. E., Lingappa, D., and Ganem, D., *J. Virol.* 64, 1414–1422 (1990).
13. Bruss, V., and Vieluf, K., *J. Virol.* 69, 6652–6657 (1995).
14. Kölch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U., *Nature* 364, 249–252 (1993).
15. Nakatake, H., Chisaka, O., Yamamoto, S., Matsubara, K., and Koshy, R., *Virology* 195, 305–314 (1993).