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The hepatitis B virus X protein activates nuclear factor of activated T cells (NF-AT) by a cyclosporin A-sensitive pathway

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The X gene product of the human hepatitis B virus (HBx) is a transcriptional activator of various viral and cellular genes. We recently have determined that the production of tumor necrosis factor-α (TNF-α) by HBV-infected hepatocytes is transcriptionally up-regulated by HBx, involving nuclear factor of activated T cells (NF-AT)-dependent activation of the TNF-α gene promoter. Here we show that HBx activates NF-AT by a cyclosporin A-sensitive mechanism involving dephosphorylation and nuclear translocation of the transcription factor. Luciferase gene expression assays demonstrated that HBx transactivates transcription through NF-AT-binding sites and activates a Gal4–NF-AT chimeric protein. DNA–protein interaction assays revealed that HBx induces the formation of NF-AT-containing DNA-binding complexes. Immunofluorescence analysis demonstrated that HBx induces the nuclear translocation of NF-AT, which can be blocked by the immunosuppressive drug cyclosporin A. Furthermore, immunoblot analysis showed that the HBx-induced activation and translocation of NF-AT are associated with its dephosphorylation. Thus, HBx may play a relevant role in the intrahepatic inflammatory processes by inducing locally the expression of cytokines that are regulated by NF-AT.

Keywords: cyclosporin A/HBx/hepatitis B virus/NF-AT/transcription

Introduction

The hepatitis B virus (HBV) is a hepatotrophic virus composed of a partially double-stranded circular DNA genome that causes acute and chronic hepatic injury. Persistent HBV infection is strongly associated with the development of hepatocellular carcinoma (Ganem and Varmus, 1987). Four genes, S/preS, C/preC, P and X, are encoded by the viral genome (Tiollais *et al*., 1985). The X gene encodes a 17 kDa protein, termed HBx, that has been shown to function as a transcriptional transactivator of a variety of viral and cellular promoter/enhancer elements (reviewed in Yen, 1996).

HBx does not bind directly to DNA, but it is able to transactivate transcription through multiple *cis*-acting elements including AP-1, AP-2, ATF/CREB, NF-κB, C/EBP and Egr-1-binding sites (Maguire *et al*., 1991; Kekulé *et al*., 1993; Yoo *et al*., 1996). However, the exact mechanism of transactivation still remains unresolved. It has been determined that HBx interacts in the nucleus with components of the basal transcription machinery, including RPB5, a subunit of all three mammalian RNA polymerases, and several transcription factors (Maguire *et al*., 1991; Cheong *et al*., 1995; Qadri *et al*., 1995; Haviv *et al*., 1996, 1998; Lin *et al*., 1997). Thus, HBx may exert its effect by mimicking the cellular coactivator function (Haviv *et al*., 1996). Another proposed mechanism for HBx activity involves the activation of signal transduction pathways such as the Ras/Raf/ERK and MEKK-1/JNK cascades, leading to induction of AP-1, NF-κB and probably other transcription factors (Benn and Schneider, 1994; Natoli *et al*., 1994b; Doria *et al*., 1995; Benn *et al*., 1996; Su and Schneider, 1996; Klein and Schneider, 1997). Whether protein kinase C (PKC) is involved in the signal transduction pathways activated by HBx is less clear (Cross *et al*., 1993; Kekulé *et al*., 1993; Benn and Schneider, 1994; Murakami *et al*., 1994; Natoli *et al*., 1994a; Chirillo *et al*., 1996). HBx has been found to be distributed in the cytoplasm, but also to some extent in the nucleus of transfected cells (Doria *et al*., 1995; Haviv *et al*., 1998). Thus, HBx may have a dual function, one related to its cytoplasmic localization, that can mediate the activation of signal transduction pathways, and another nuclear, that may account for the interaction with transcription factors and components of the transcription apparatus to enhance the binding or activity of these proteins (Doria *et al*., 1995).

Although there is emerging evidence of the involvement of HBx in hepatocarcinogenesis (Koike, 1995), very little is known about the role that this viral protein plays in the intrahepatic inflammatory processes. In this context, we previously have reported the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) by hepatocytes from patients chronically infected by HBV. We also demonstrated that transient or stable transfection of the hepatoma cell line HepG2 with either the whole HBV genome or HBx expression vectors resulted in TNF-α production (González-Amaro *et al*., 1994). In addition, it has been reported that the gene encoding human interleukin 8 (IL-8) is also transactivated by HBx (Mahé *et al*., 1991). We have shown recently that HBx-induced TNF-α production by hepatocytes is regulated at the transcriptional level involving the activation of nuclear factor of activated T cells (NF-AT) (Lara-Pezzi *et al*., 1998).

The expression of TNF-α, IL-8 and other cytokine-encoding genes is regulated in a co-ordinate manner by the transcription factor NF-AT in cells of the immune system (Okamoto *et al*., 1994; Tsai *et al*., 1996; Rao *et al*.,

1997). Therefore, NF-AT is required for initiating and controlling effective immune and inflammatory responses. NF-AT is a family of transcription factors that includes at least four structurally related proteins; NF-AT1 (previously named NF-ATp), NF-ATc, NF-AT3 and NF-AT4. Multiple isoforms and species-specific variants of these proteins have also been identified. Although NF-AT proteins are not expressed exclusively by cells of the immune system (reviewed in Rao *et al*., 1997), little information is available regarding the expression and function of NF-AT-related proteins outside the immune system (Rao, 1994; Rao *et al*., 1997).

The activity of NF-AT proteins is tightly regulated by the calcium/calmodulin-dependent phosphatase calcineurin (Sigal and Dumont, 1992; Jain *et al*., 1993; Crabtree and Clipstone, 1994; Cantrell, 1996; Loh *et al*., 1996), a primary target for inhibition by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (Schreiber and Crabtree, 1992; Shaw *et al*., 1995). Calcineurin controls the translocation of NF-AT proteins from the cytoplasm to the nucleus of activated cells by interacting with an N-terminal regulatory domain conserved in all the members of the NF-AT family (Luo *et al*., 1996b). NF-AT proteins are able to bind cooperatively with transcription factors of the AP-1 family to form composite NF-AT:AP-1 sites (Jain *et al*., 1993), which are found in the regulatory elements of many genes that are transcribed inducibly by cells of the immune system (Boise *et al*., 1993; Cockeril *et al*., 1995; Jain *et al*., 1995; Rooney *et al*., 1995).

In this report, we demonstrate that HBx transactivates transcription through NF-AT-binding sites in liver-derived Chang (CHL) cells, and that a chimeric Gal4–NF-AT protein, containing the N-terminal transactivation domain of NF-AT1 fused to the Gal4 DNA-binding domain (DBD) is activated by HBx. We also show that HBx induces, in CHL cells, NF-AT-containing protein complexes that bind independently of AP-1 to an NF-AT site of the murine IL-4 promoter. Furthermore, we demonstrate that HBx triggers dephosphorylation and nuclear translocation of NF-AT by a CsA-sensitive mechanism.

Results

Transactivation of NF-AT-dependent transcription by HBx

To analyze whether HBx was able to transactivate transcription through NF-AT-binding sites, CHL cells were transiently co-transfected with the HBx expression vector pSV-X along with a luciferase reporter plasmid driven by three tandem copies of the distal NF-AT site of the IL-2 enhancer (pNF-AT-Luc), which has been reported to bind NF-AT cooperatively with transcription factors of the AP-1 family (Boise *et al*., 1993; Northrop *et al*., 1993; Jain *et al*., 1995). As shown in Figure 1A, the expression of HBx induced the NF-AT-dependent transcription 4- to 6-fold. Moreover, in the presence of either the mitogen phorbol 12-myristate 13-acetate (PMA) or the calcium ionophore A23187, HBx further induced (11- to 14-fold) NF-AT transcriptional activity, leading to luciferase expression levels similar to those obtained by co-stimulation with PMA plus the calcium ionophore, which provides a full stimulus for NF-AT-dependent transcription (Rao *et al*., 1997). In contrast, PMA or calcium ionophore alone exerted a weak effect on the induction of the transcriptional activity of the NF-AT enhancer, thus suggesting that HBx may compensate both the mitogenic signals induced by PMA and the calcium signals induced by calcium ionophore in the activation of NF-AT sites. Strikingly, HBx further increased the induction of the NF-AT enhancer by co-stimulation with PMA plus calcium ionophore (Figure 1B). A dominant-negative version of NF-AT, which lacks the DNA-binding domain and the C-terminal transactivation domain (Northrop *et al*., 1994), was able to prevent the induction by HBx, either alone or in the presence of PMA and/or calcium ionophore (Figure 1A and 1B). In addition, CsA, a pharmacological inhibitor of the phosphatase calcineurin that controls NF-AT activation, also blocked the induction by HBx as well as the synergistic induction by HBx and PMA plus calcium ionophore (Figure 1C), suggesting that activation of NF-AT proteins was mediating the induction of the NF-AT enhancer element by HBx.

The synergism of HBx and PMA plus the calcium ionophore was not due merely to an increase in HBx expression levels, since these agents did not induce significantly the activity of the promoter/enhancer elements driving the expression of HBx within the plasmid pSV-X (data not shown). Therefore, distinct mechanisms might be mediating the synergistic activation of the NF-AT-dependent transcription by HBx. One such mechanism could be a further increase by HBx of the PMA-induced transcriptional activity of AP-1, which binds cooperatively with NF-AT to the NF-AT enhancer element. To address this issue, CHL cells were co-transfected with pSV-X vector and the reporter plasmid pAP-1-Luc, containing four AP-1-binding sites, and the transfected cells were either left untreated or stimulated with PMA or PMA plus calcium ionophore. As shown in Figure 1D, HBx further activated the AP-1-dependent transcription induced by PMA (not shown) and by PMA plus calcium ionophore. However, the activation of AP-1 transcriptional activity by HBx, either alone or in the presence of PMA or PMA plus calcium ionophore, was not blocked by CsA (Figure 1D, and data not shown). Thus, the synergistic induction of the NF-AT enhancer element by HBx and PMA plus calcium ionophore may be due, at least in part, to a stronger activation of the AP-1 component of this composite NF-AT:AP-1 site.

To substantiate further that HBx was able to induce transcription through the NF-AT enhancer element via activation of NF-AT proteins, CHL cells were co-transfected with a cDNA encoding full-length NF-ATc along with the pNF-AT-Luc reporter plasmid, either in the presence or the absence of HBx. Although NF-ATc had a weak effect on the luciferase activity, probably due to its cytoplasmic localization after transfection, when co-transfected with HBx it potently enhanced NF-AT-Luc expression in a dose-dependent manner (Figure 2A). In addition, the HBx-mediated induction in the presence of NF-ATc was blocked by the dominant-negative mutant of NF-ATc (Figure 2A). Similar functional results were obtained using clones of CHL cells stably transfected with HBx (CMX), in which NF-ATc enhanced HBx-mediated induction of the NF-AT element >6-fold (Figure 2B).

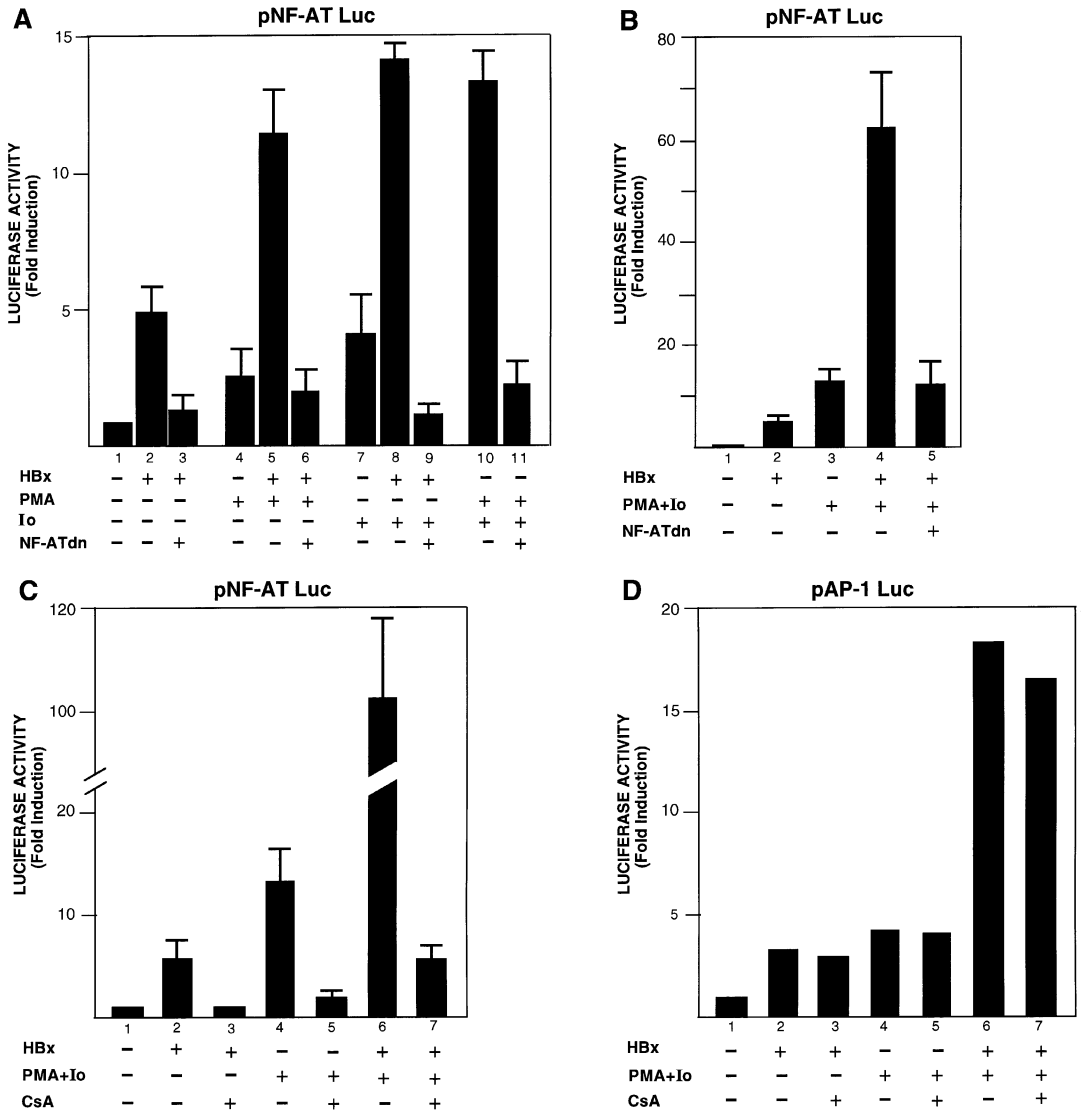

Fig. 1. HBx transactivates a multimeric NF-AT-containing plasmid. (**A**) CHL cells were co-transfected with 0.2 μg of the reporter plasmid pNF-AT-Luc along with 5 μg of either pSV-X or the control plasmid pSV-hygro. Transfected cells were either left untreated or stimulated with PMA or calcium ionophore (Io). Treatment with PMA plus calcium ionophore was used as control for full stimulus of NF-AT-dependent transcription. (**B**) HBx synergizes with PMA plus calcium ionophore in the activation of the NF-AT enhancer element. CHL cells were co-transfected as described above, and either left untreated or stimulated with PMA and calcium ionophore (PMA + Io). To demonstrate the specificity of the activation of the reporter gene expression, 2 μg of the plasmid pSH102CΔ418, which encodes a dominant-negative mutant of NF-AT, were included in the transfection experiments. (**C**) Cyclosporin A blocks the activation of the NF-AT enhancer element by HBx. CHL cells were co-transfected as described above and, where indicated, treated with CsA (200 ng/ml) and/or PMA plus calcium ionophore. (**D**) HBx synergizes with PMA plus calcium ionophore in the activation of a multimeric AP-1-containing plasmid in a CsA-independent manner. CHL cells were co-transfected with 0.2 μg of the reporter plasmid pAP-1-Luc along with 5 μg of pSV-X or pSV-hygro. Cells were either left untreated or treated with CsA and/or PMA plus calcium ionophore. The luciferase activities are represented as fold induction over the expression of pNF-AT-Luc or pAP-1-Luc in the absence of any stimuli. The values shown in (A), (B) and (C) represent the mean fold-induction (±SD) of at least three independent experiments. The values shown in (D) are representative of three experiments.

HBx targets the transactivation domain of NF-AT

To confirm the role of NF-AT proteins in HBx-mediated transcriptional activation of the NF-AT enhancer element, a Gal4-derived reporter system was employed, which in mammalian cells responds only to artificial activators. To carry out these studies, the chimeric vector, pGal4-NF-AT1(1–415), encoding the Gal4 DBD fused to the transactivation domain of NF-AT1, or the parental vector pRSV-Gal4-DBD (Luo *et al.*, 1996a) were co-transfected into CHL cells along with the luciferase reporter plasmid pGal4-Luc, either in the presence or the absence of the HBx expression vector pSV-X. As expected, HBx did not stimulate transcription when co-transfected with the control plasmid encoding the Gal4 DBD (Figure 3A). However, transcription of the pGal4-Luc reporter plasmid was induced up to 16-fold by HBx when co-transfected with Gal4–NF-AT1 expression vector (Figure 3A). Similarly, in CMX cells, HBx induced Gal4-Luc expression ~18-fold, when compared with control CMO cells, using three different amounts of Gal4–NF-AT1 expression vector (Figure 3B). Taken together, these results strongly indicate that HBx is able to activate NF-AT either by direct protein–protein interaction or by mimicking the signals involved in the activation of these proteins.

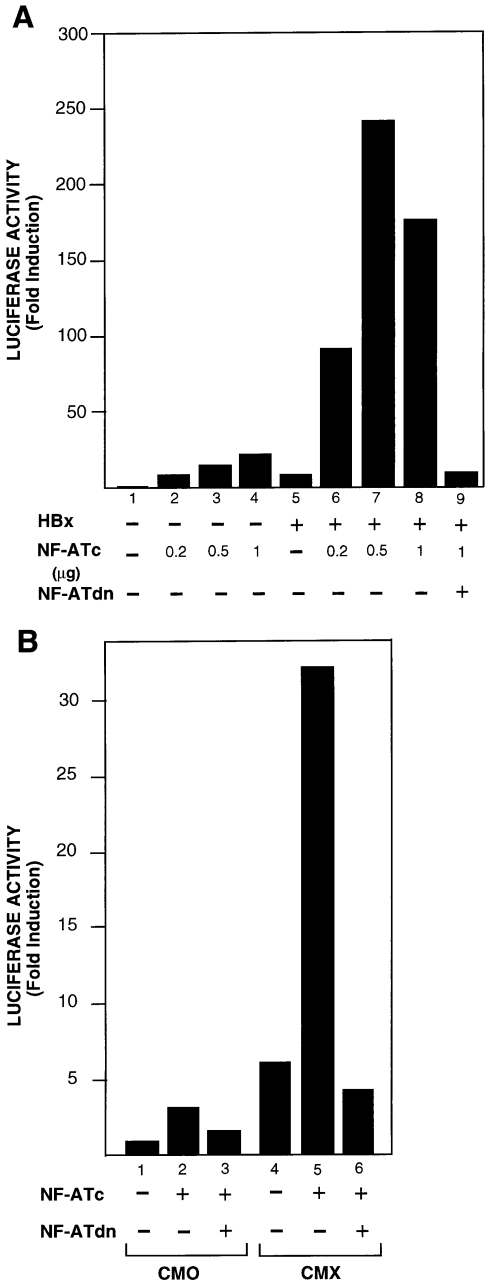

Fig. 2. HBx synergizes with transfected full-length NF-ATc-encoding cDNA. (**A**) CHL cells were co-transfected with 0.2 μg of pNF-AT-Luc, 5 μg of pSV-X or pSV-hygro, and increasing amounts of pNF-ATcwt. To keep the amount of plasmid DNA in each transfection point constant, the empty vector pBJ5 and 5 μg of the carrier plasmid pGEM7 were used. (**B**) Empty vector cells (CMO) and HBx expression cells (CMX) were co-transfected with 0.2 μg of pNF-AT-Luc, 0.2 μg of the expression plasmid pNF-ATcwt or the empty vector pBJ5. The dominant-negative NF-AT-encoding plasmid pSH102CΔ418 (2 μg) was included in the transfection experiments to demonstrate the specificity of the activation. The luciferase activities are represented as fold induction over the expression of pNF-AT-Luc in the absence of any stimuli. Results shown are representative of four experiments. Three different clones of CMO and CMX cells were employed in the experiments.

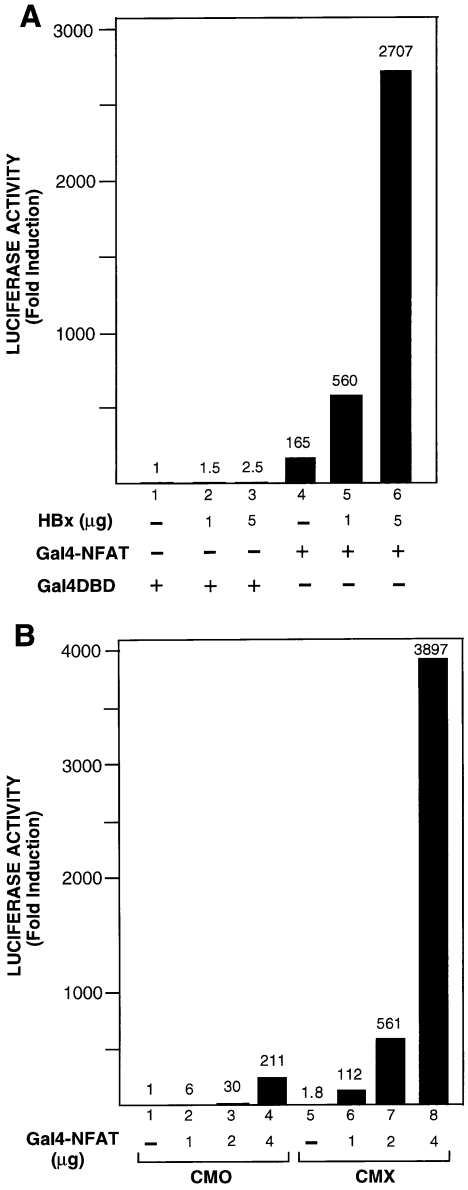

Fig. 3. HBx activates a Gal4–NF-AT1 chimeric protein containing the transactivation domain of NF-AT1. (**A**) CHL cells were co-transfected with 1 μg of the reporter plasmid pGal4-Luc along with 5 μg of either the expression vector encoding the fusion protein Gal4–NF-AT1 (1–415) or the parental empty vector pRSV-Gal4-DBD, and increasing amounts of pSV-X. To keep the total amount of DNA constant, pSV-hygro was used. (**B**) CMO and CMX cells were co-transfected with 1 μg of pGal4-Luc and increasing amounts of the expression vector pGal4-NF-AT1(1–415). To keep the amount of DNA constant, the parental vector pRSV-Gal4-DBD was used. The luciferase activities are represented as fold-induction over the values obtained in CHL cells or CMO cells co-transfected with pGal4-Luc and pRSV-Gal4-DBD, and are representative of three independent experiments. The results obtained with the stable transfectants were verified by using different clones of CMO and CMX cells.

HBx induces NF-AT DNA-binding activity

To examine whether HBx was able to induce the formation of NF-AT-containing DNA–protein complexes, electrophoretic mobility shift assays (EMSAs) were performed using a ^{32}P-labeled oligonucleotide that contained the NF-AT-binding site of the mouse IL-4 promoter, which can bind NF-AT independently of AP-1 (Rooney *et al.*, 1994, 1995), and nuclear extracts from CHL cells stably transfected with either CMX or CMO. Three major specific complexes were resolved using nuclear extracts from HBx-expressing cells that were not detectable in CMO control cells (Figure 4A). These inducible complexes resulted from specific DNA binding since their formation

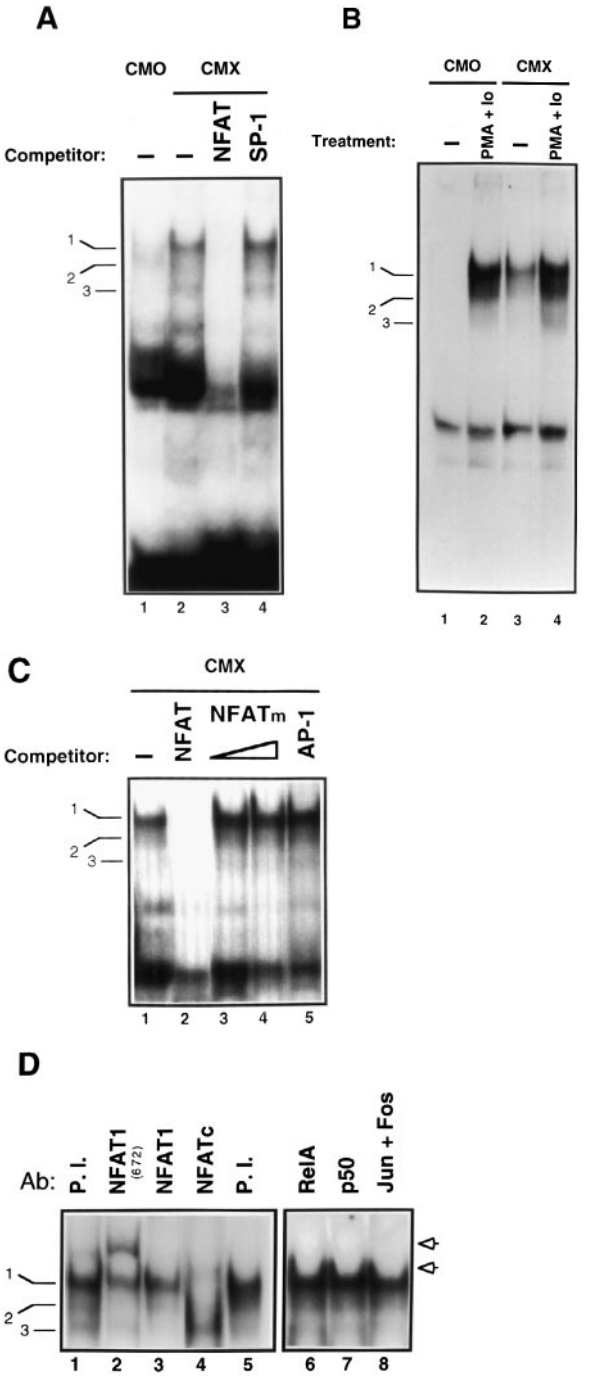

Fig. 4. HBx induces the formation of NF-AT-containing DNA-binding complexes. (**A**) A 2 μg aliquot of protein from CMO or CMX nuclear extracts was incubated with a ^{32}P-labeled probe containing the NF-AT-binding site of the murine IL-4 promoter. For competition, a 130-fold molar excess of the homologous NF-AT oligonucleotide or an oligonucleotide containing an SP-1-binding site were used. The three specific complexes formed in the presence of HBx are indicated. (**B**) CMO and CMX cells were stimulated with PMA (10 ng/ml) plus calcium ionophore (1 μM) for 16 h and the complexes formed compared with those obtained in the absence of stimuli. (**C**) The HBx-induced complexes are competed by the homologous NF-AT oligonucleotide (40-fold excess) but not by a mutated NF-AT oligonucleotide (40- and 130-fold) or an AP-1 oligonucleotide (130-fold excess). (**D**) Prior to adding the IL-4-NF-AT probe, 0.5 μl of either pre-immune antiserum (P. I.) or antibodies against different members of the NF-κB, NF-AT or AP-1 families were included in the binding reaction. Supershifted bands induced by the anti-NF-ATc monoclonal antibody and by the anti-NF-AT1 antiserum (672) are indicated by arrows.

was blocked by an excess of unlabeled IL4-NFAT oligonucleotide, but not by an unrelated oligonucleotide containing an SP-1-binding site. Three DNA–protein complexes, with electrophoretic mobility identical to those induced by HBx, were resolved using nuclear extracts from CMO cells treated with PMA plus calcium ionophore, which were used as a positive control for NF-AT activation (Figure 4B). It is noteworthy that PMA plus calcium ionophore treatment was a stronger stimulus than HBx for the formation of these complexes. In addition, HBx expression did not appear to enhance further the binding activity of these proteins induced by PMA plus calcium ionophore (Figure 4B). To characterize these HBx-induced complexes, an IL4-NFAT oligonucleotide bearing mutations in the core NF-AT-binding site was included in the competition assays. As shown in Figure 4C, two different amounts of the mutated IL4-NFAT oligonucleotide had no detectable effect on the generation of the inducible complexes, whereas similar amounts of the wild-type oligonucleotide completely abolished their formation. As an additional control, an oligonucleotide including an AP-1 consensus site failed to compete the specific bands generated with the NF-AT probe, suggesting that these complexes contained NF-AT-related proteins that bind to the oligonucleotide independently of the Fos/Jun family proteins.

To identify further the IL4-NFAT-binding factors induced by HBx, specific antibodies for NF-κB, NF-AT and Fos/Jun proteins were added to the binding reactions. As shown in Figure 4D, neither a pre-immune antiserum nor antisera against the NF-κB family members p50, Rel A and p52 (not shown) affected any of the DNA–protein complexes. In contrast, addition of a monoclonal antibody specific for NF-ATc prevented the formation of complex 1 and generated a supershifted band. An antiserum against NF-AT, which recognizes NF-AT1 and probably other members of the family (see Materials and methods), reduced complex 2 and prevented the formation of complex 3. On the other hand, an antiserum specific for NF-AT1 (672) significantly reduced complex 3, abolished the formation of complex 2 and generated a supershifted band. Taken together, these results suggest that complex 1 is composed mainly by NF-ATc, and that complexes 2 and 3 contain NF-AT1. These results also indicate that the inducible bands do not contain detectable amounts of NF-κB-related proteins, in agreement with previous observations which demonstrated that NF-κB family members bind with very low affinity to the NF-AT site of the mouse IL-4 promoter (Casolaro *et al.*, 1995). Consistent with the competition assays, the HBx-induced complexes were not affected by antibodies recognizing various members of the Fos and Jun families.

HBx triggers nuclear translocation and dephosphorylation of NF-AT

To investigate whether the activation of NF-AT-dependent transcription and NF-AT binding by HBx involved the nuclear translocation of NF-AT proteins, the cellular distribution of a transiently expressed HA-tagged NF-ATc protein (Northrop *et al.*, 1994) was analyzed by indirect immunofluorescence staining in clones of CHL cells either expressing or not expressing HBx. As summarized in Table I, ~40% of the stable CMX cells displayed a nuclear

Table I. NF-AT is translocated to the nucleus in CMX but not in CMO cells

Cell	Treatment	% Nuclear translocation[a]	n[b]
CMO	–	8.6	122
CMO/Dex	–	4.1	73
CMO	P + Io	90.1	91
CMO/Dex	P + Io	100	90
CMX	–	40.7	73
CMX/Dex	–	75.2	86
CMX/Dex	CsA	15.7	60
CMX	P + Io	90.6	85
CMX	P + Io/CsA	10.2	53
CMX/Dex	P + Io	100	52
CMX/Dex	P + Io/CsA	29.6	72

CMO and CMX cells were transfected with the HA-tagged NF-AT mutant vector pSH102CΔ418 and were stimulated or not with 1 μM dexamethasone for 16 h. Where indicated, cells were treated with 200 ng/ml CsA (2 h) or 10 ng/ml PMA plus 1 μM calcium ionophore (30 min). Cells were analyzed by immunofluorescence with an anti-HA antibody.
[a]Percentage of cells displaying total or partial nuclear translocation of HA-NF-AT are shown.
[b]Number of transfected cells analyzed at each transfection point.

pattern of staining of HA-NF-AT. Treatment of CMX cells with dexamethasone, which augmented the level of HBx expression (see Materials and methods), further increased the number of cells with nuclear HA-NF-AT up to 75% (Table I; Figure 4). In contrast, in control cells (CMO), either treated with dexamethasone or untreated, the fusion protein was found largely in the cytoplasm (Table I; Figure 5). A predominant nuclear localization of HA-NF-AT was observed in both HBx-expressing and non-expressing cells stimulated with PMA plus calcium ionophore (Table I; Figure 5). Thus, the expression of HBx appears to induce constitutive nuclear translocation of NF-AT proteins in the absence of conventional calcium-mobilizing stimuli. Interestingly, the immunosuppressor CsA prevented the nuclear translocation of NF-AT triggered by both PMA plus calcium ionophore treatment and HBx expression (Table I; Figure 5), indicating the involvement of the phosphatase calcineurin in the translocation of NF-AT induced by both stimuli. Similarly, transient co-transfection of CHL cells with the HA-NF-AT expression plasmid and the HBx expression vector pSV-X resulted in a predominant nuclear localization of HA-NF-AT, whereas the cells co-transfected with the control vector pSV-hygro displayed a cytoplasmic distribution of the fusion protein (Figure 6). Moreover, treatment of the cells transfected with the control vector with PMA plus calcium ionophore also resulted in a nuclear staining pattern of HA-NF-AT. CsA blocked the nuclear translocation induced by HBx and by PMA plus calcium ionophore (Figure 6).

We further confirmed the effect of HBx on the translocation of NF-AT by transient co-transfection experiments in the T-cell line Jurkat, where the mechanism of NF-AT activation/translocation previously has been characterized (Luo *et al*., 1996b; Martínez-Martínez *et al*., 1997). As shown in Figure 7, co-expression of HBx and HA-NF-AT resulted in a clear nuclear localization of the fusion protein, whereas the control cells, transfected with the empty vector, displayed a cytoplasmic distribution of HA-NF-AT. A nuclear distribution of HA-NF-AT was also observed in both HBx-expressing cells and in control cells, when they were stimulated with PMA plus calcium ionophore. As above, CsA prevented the nuclear translocation triggered by HBx and a mitogenic stimulus (Figure 7).

The activation and translocation of NF-AT proteins by various calcium-mobilizing stimuli involve the dephosphorylation of these proteins, and the dephosphorylated forms of NF-AT migrate more rapidly than fully phosphorylated forms on SDS–PAGE (Martínez-Martínez *et al*., 1997). To examine whether HBx-mediated activation and translocation of NF-AT also resulted from its dephosphorylation, immunoblotting studies were performed in CMX and CMO cells transiently transfected with HA-tagged NF-ATc. The phosphorylation state of the fusion protein was analyzed by Western blot using an anti-HA antiserum (Figure 8). In control cells (CMO), a single HA-NF-AT protein band was detected, which corresponded to the phosphorylated form, whereas in CMX cells a significant amount of HA-NF-AT was present in its rapidly migrating dephosphorylated form. Treatment with PMA plus calcium ionophore induced the dephosphorylation of a large amount of the transfected HA-NF-AT in both CMX and CMO cells. In agreement with the translocation studies, CsA prevented the dephosphorylation induced by HBx and PMA plus calcium ionophore.

Taken together, these results indicate that the activation of NF-AT by HBx is associated, at least in part, with its dephosphorylation and translocation, and that it is mediated by a mechanism involving deregulation of calcineurin.

Discussion

Chronic infection of the liver by HBV leads to profound changes in hepatocyte physiology including the expression of various molecules that normally are not expressed, or expressed at very low levels, by uninfected hepatocytes (Chisari and Ferrari, 1995). It has been shown that the expression of many of these molecules is transcriptionally up-regulated by HBx, the transcriptional transactivator of HBV (Yen, 1996). In this context, we have demonstrated that human hepatocytes infected by HBV produce the pro-inflammatory cytokine TNF-α both *in vivo* and *in vitro* (González-Amaro *et al*., 1994). In addition, we have demonstrated that the proximal region of the TNF-α gene promoter contains target sequences for HBx transactivation (Lara-Pezzi *et al*., 1998). The expression of TNF-α is almost exclusively restricted to cells of hematopoietic origin (Vassalli, 1992), and is controlled by pre-existing transcription factors belonging to the NF-κB/Rel and NF-AT families, which ensure a rapid response to extracellular stimuli. In cells of the myeloid lineage, the inducible expression of TNF-α is regulated mainly by members of the NF-κB/Rel family (Trede *et al*., 1995; Yao *et al*., 1997), whereas in lymphocytes the expression of TNF-α is tightly controlled by NF-AT proteins (Tsai *et al*., 1996). We have shown that the HBx-mediated induction of the TNF-α gene promoter can be interfered with by a dominant-negative mutant of NF-AT, but not by the cytoplasmic inhibitor of NF-κB proteins IκBα (Lara-Pezzi *et al*., 1998), suggesting that NF-AT proteins may have a role in the expression of TNF-α by HBV-infected hepatocytes.

We have demonstrated herein that HBx is able to activate NF-AT by a CsA-sensitive mechanism involving dephosphorylation and nuclear translocation of this trans-

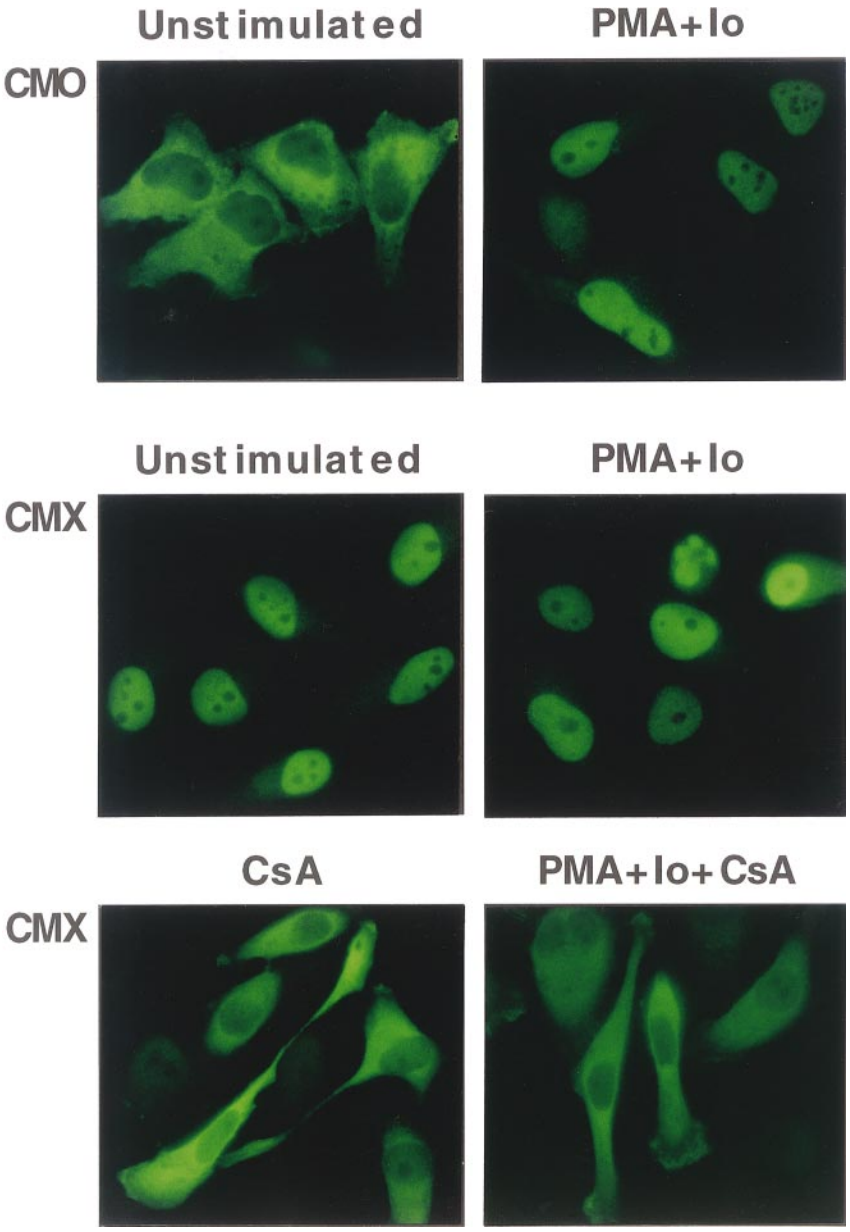

Fig. 5. NF-AT is translocated to the nucleus in CMX but not in CMO cells. Different clones of CMO and CMX cells were transfected with the plasmid pSH102CΔ418, encoding the HA-tagged NF-AT deletion mutant, and stimulated with 1 μM dexamethasone for 16 h. Where indicated, the transfected cells were treated with 200 ng/ml CsA for 2 h and/or 10 ng/ml PMA plus 1 μM calcium ionophore (PMA + Io) for 30 min. Cells were fixed and analyzed for immunofluorescence with an anti-HA antibody. Representative fields where total translocation of NF-AT took place are shown for CMX cells, either unstimulated or stimulated with PMA + Io, and for PMA + Io-treated CMO cells.

cription factor. Since the HBx-mediated transactivation experiments carried out in this study involved measuring reporter gene expression driven by a composite NF-AT:AP-1 site of the IL-2 enhancer, their interpretation was necessarily complex, and it might not be apparent whether NF-AT proteins or AP-1 proteins were affected. Given that both PMA and HBx can trigger the signal transduction pathways leading to activation of AP-1, it was expected that HBx would substitute effectively for PMA and synergize with calcium ionophore to stimulate the NF-AT enhancer element. In contrast, it had not been determined whether HBx was able to regulate the calcium-dependent pathways involved in the activation of the transcription factor NF-AT. In this regard, HBx appears to be able to substitute effectively for calcium ionophore and synergize with PMA to activate the pNF-AT-Luc reporter plasmid. Therefore, HBx appears to be sufficient to induce the intracellular signals necessary to activate transcription through composite NF-AT:AP-1 sites, present in many regulatory elements of cytokine-encoding genes, as demonstrated by the fact that HBx alone is able to transactivate the NF-AT enhancer element of the IL-2 gene.

In this work, we present three lines of evidence indicating that HBx is able to functionally activate NF-AT: first, the induction of the reporter plasmid pNF-AT-Luc by HBx, either alone or in synergy with PMA and/or calcium ionophore, can be disrupted by a dominant-negative mutant of NF-AT and by the immunosuppressive drug CsA; secondly, the transiently transfected NF-ATc protein potently activates the NF-AT enhancer element only in

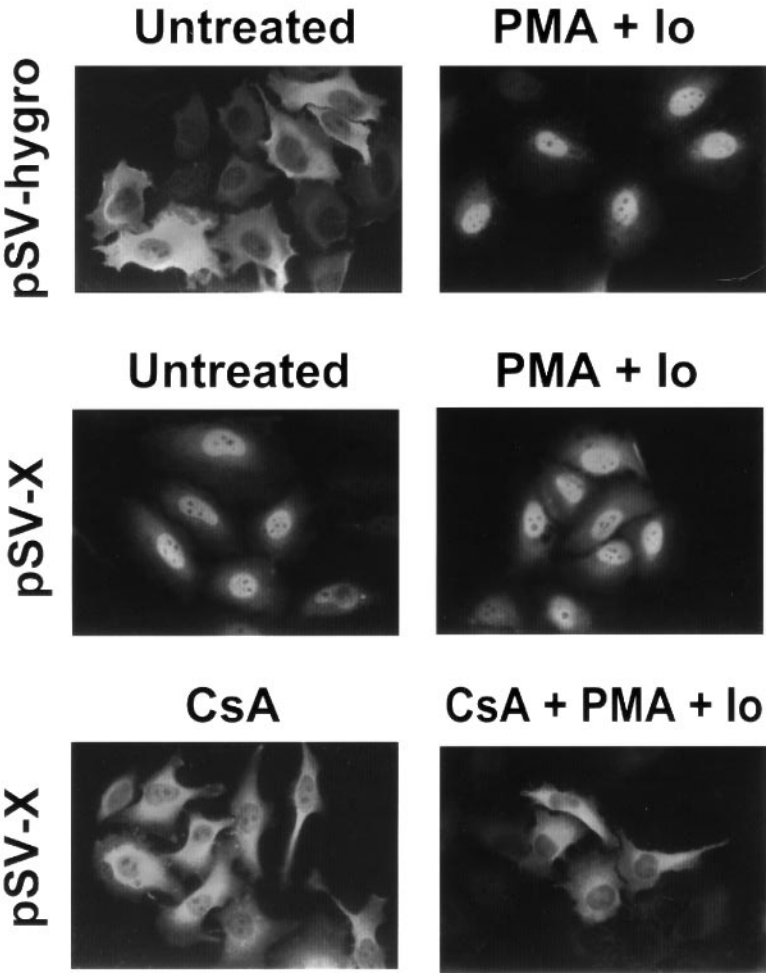

Fig. 6. NF-AT is translocated to the nucleus in CHL cells transiently transfected with the HBx expression vector. CHL cells were co-transfected with the plasmid pSH102CΔ418, encoding the HA-tagged NF-AT deletion mutant, and either the HBx-expressing vector pSV-X or the negative control pSV-hygro. The cells were either left untreated or treated with 200 ng/ml CsA for 2 h and/or 10 ng/ml PMA plus 1 μM calcium ionophore (PMA + Io) for 30 min. Cells were fixed and analyzed for immunofluorescence with an anti-HA antibody.

the presence of HBx; and thirdly, a chimeric Gal4–NF-AT protein, containing the transactivation domain of NF-AT, can mediate transcriptional activation by HBx. These functional data are supported further by the ability of HBx to trigger dephosphorylation, nuclear translocation and DNA-binding activity of NF-AT.

Mechanistically, dephosphorylation of NF-AT by the calcium/calmodulin-dependent phosphatase calcineurin appears to be the major activation pathway of NF-AT proteins in cells of the immune system (Rao *et al*., 1997). However, additional calcineurin-independent and CsA-resistant pathways of NF-AT activation have also been reported (Ghosh *et al*., 1996). In this regard, the HBx-triggered dephosphorylation and nuclear translocation of NF-AT as well as the HBx-induced transactivation of the NF-AT enhancer element are sensitive to CsA treatment, suggesting that the activation of NF-AT by HBx may be mediated, at least in part, by deregulation of calcineurin. However, a distinct mechanism that synergizes with the calcium/calcineurin pathway may also be involved in the activation of the NF-AT enhancer by HBx, as suggested by the fact that HBx is able to augment further the induction of the pNF-AT-Luc plasmid by co-stimulation with PMA plus calcium ionophore, which has been described to provide a full stimulus for NF-AT-dependent transcription in cells of the immune system (Rao *et al*., 1997). Although alternative explanations cannot be ruled out, our results indicate that the synergistic activation of the NF-AT enhancer by HBx and PMA plus calcium ionophore could be mediated, at least to some extent, through a stronger activation of the AP-1 component of this composite NF-AT:AP-1 site. It is also likely that HBx may activate the transcriptional activation domain of NF-AT, once this transcription factor has targeted its recognition sequences, by a mechanism involving direct protein–protein interaction, as has been shown for other transcription factors (Haviv *et al*., 1995, 1996; Yoo *et al*., 1996).

Recently, it has been shown that the *tax* gene product of the human T-cell leukemia virus (HTLV) induces IL-2 gene expression by a CsA-sensitive mechanism involving dephosphorylation and activation of NF-AT1 (Good *et al*., 1996, 1997). Moreover, it has been determined that activation of NF-ATc by the HIV transactivator Tat plays an important role on HIV gene expression and replication (Kinoshita *et al*., 1997). In contrast to these lymphotrophic viruses, HBV infects mainly hepatocytes, in which no information is available regarding the expression and function of NF-AT-related proteins. For the first time, to our knowledge, we provide herein evidence of expression

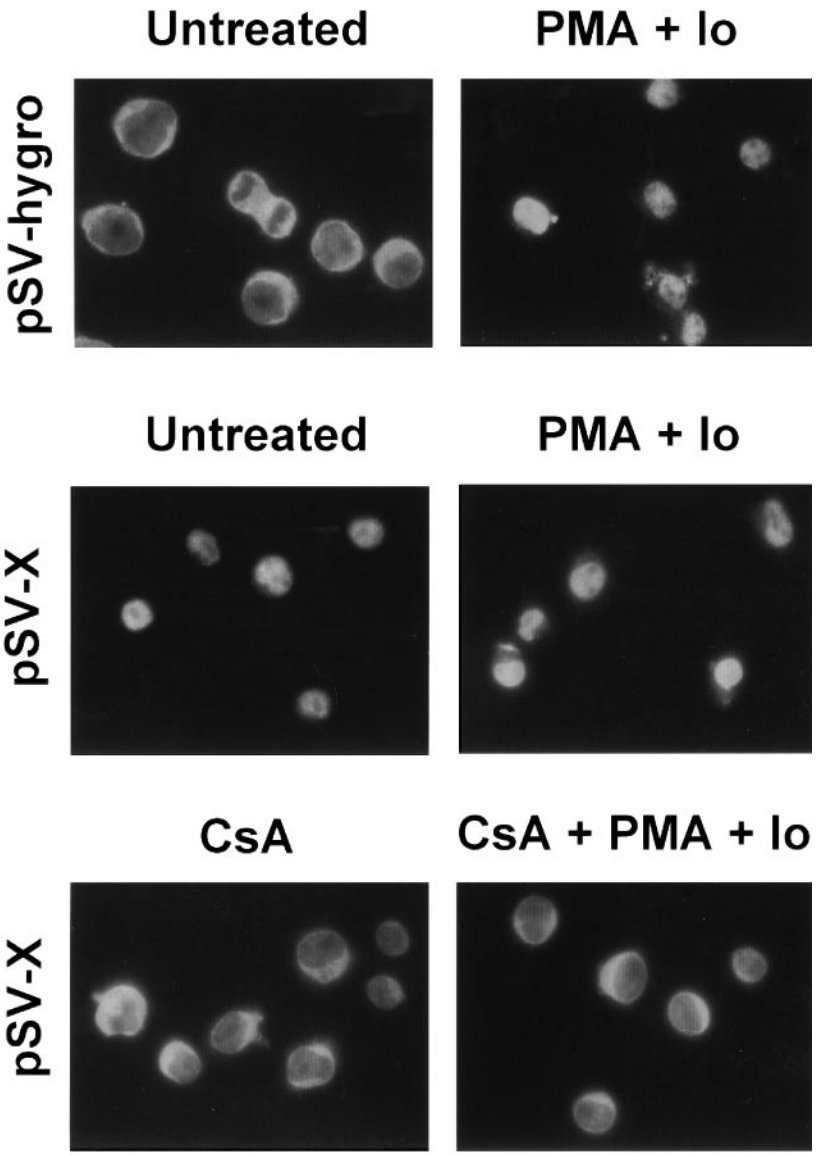

Fig. 7. HBx triggers nuclear translocation of NF-AT in the T-cell line Jurkat. Jurkat cells were transiently co-transfected with pSV-X (left column) or pSV-hygro (right column) and the plasmid pSH102CΔ418, which encodes the HA-NF-AT mutant. Where indicated, transfected cells were treated for 2 h with CsA and/or PMA plus calcium ionophore for 30 min. The cells were fixed and analyzed for immunofluorescence with an anti-HA antibody.

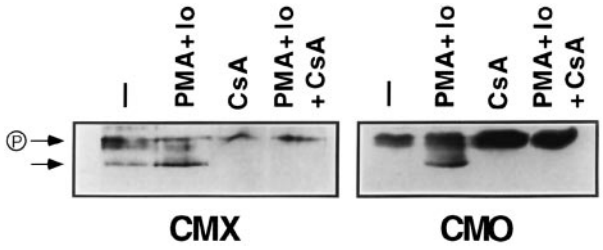

Fig. 8. HBx induces NF-AT dephosphorylation. CMO and CMX cells were transfected with 1.5 μg of the HA-tagged NF-AT mutant vector pSH102CΔ418. Cells were treated overnight with 1 μM dexamethasone and stimulated where indicated with 200 ng/ml CsA or 10 ng/ml PMA plus 1 μM calcium ionophore (PMA + Io). Total cell extracts were analyzed by Western blot using an anti-HA antibody. Dephosphorylated and phosphorylated forms of HA-NF-AT are indicated by arrows. The CMX panel displays less HA-NF-AT protein because, in this particular experiment, CMX cells were transfected less efficiently with the HA-NF-AT expression vector.

of NF-AT family members within CHL cells, and that these NF-AT proteins can be activated by HBx, as demonstrated by reporter gene and mobility shift assays. Our findings raise further questions about the expression of other NF-AT-regulated genes by HBV-infected hepatocytes. In this context, it has been determined that HBx transactivates the human IL-8 gene through a κB-like *cis*-acting element (Mahé *et al*., 1991), which recently has been shown to behave *in vivo* as a functional NF-AT site in T cells (Okamoto *et al*., 1994; Rao *et al*., 1997). The expression of other pro-inflammatory cytokines such as IL-1β, interferon-γ and IL-6 has been described to take place in the liver during acute and chronic hepatitis B, but it has not been established yet whether these cytokines are produced only by non-parenchymal cells or also by hepatocytes (Fukuda *et al*., 1995; Shindo *et al*., 1996). On the other hand, it is well known that HBV, although unable to replicate actively, can also infect cells of the immune system (Ferrari *et al*., 1992; Franco *et al*., 1992),

making it conceivable that HBx, like Tax and Tat, may also induce the expression of cytokine-encoding genes in these cells.

The liver may function as an important source of cytokine production and participates in the systemic defence mechanisms that take place after injury or infection (Andus *et al*., 1991). It is likely that the infection of hepatocytes by HBV may initiate the cytokine cascade that mediates the host immune response and inflammatory process by inducing locally the expression of several cytokines that are up-regulated by NF-AT and other HBx-inducible transcription factors. The underlying abnormality in chronic hepatitis B is an alteration of the cell-mediated immune response, which remains strong enough to cause hepatocellular necrosis but insufficient to clear the virus (Chisari and Ferrari, 1995). It recently has been reported that prolonged exposure of T lymphocytes to TNF-α, a cytokine that is highly produced in HBV-infected patients, suppresses T-cell function by attenuating T-cell receptor signaling (Cope *et al*., 1997), suggesting that this might be a mechanism of viral persistence. Chronic liver injury and the inflammatory and regenerative responses create the stimuli for DNA damage that may lead to the development of hepatocellular carcinoma. Elucidation of the molecular basis for the HBV-triggered cytokine network may yield therapeutic strategies to terminate chronic HBV infection and reduce the risk of its sequellae.

Materials and methods

Plasmid constructs

The expression vectors pSV-X and pSV-hygro, harboring the HBx open reading frame (ORF) and the bacterial hygromycin phosphotransferase gene, respectively, under the control of the SV40 early promoter/enhancer have been described elsewhere (Chirillo *et al*., 1996). The expression vectors pMMTV-X and pMMTV-CAT, containing the HBx ORF and the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the glucocorticoid-inducible promoter/enhancer of the mouse mammary tumor virus (MMTV), were a gift of Dr M.Levrero (Rome, Italy) (Chirillo *et al*., 1997). The plasmids pNFATcwt and pSH102CΔ418 (a kind gift of Dr G.Crabtree, Stanford, CA) are derivatives of pBJ5 vector and encode, respectively, the full-length protein NF-ATc cDNA and an NF-ATc deletion mutant (1–418), fused to an N-terminal HA tag, which functions as a dominant-negative mutant for all the NF-AT isoforms. pSH102CΔ418 lacks the DNA-binding domain and the C-terminal transactivation domain, but maintains its ability to interact with calcineurin through the NF-AT homology region (Northrop *et al*., 1994). The reporter construct pNFAT-Luc contains three tandem copies of the distal NF-AT-binding site of the human IL-2 enhancer fused to the minimal IL-2 promoter and was provided by Dr G.Crabtree (Durand *et al*., 1988). The plasmid pAP1-Luc that contains the minimal rat prolactin promoter and four copies of the human collagenase TRE site (Lara-Pezzi *et al*., 1998) was a gift of Dr R.A.Flavell (Howard Hughes Medical Institute, Yale University, New Haven, CT). The pGal4-Luc reporter plasmid, which carries five copies of the Gal4-binding sequence fused to the luciferase gene (kindly provided by Dr R.Perona), has been described previously (Minden *et al*., 1995). The plasmids pGal4-NFAT1(1–415) encoding the transactivation domain of NF-AT1 (amino acids 1–415) in-frame with the Gal4 DBD, and pRSV-Gal4-DBD, which includes only the DBD (amino acids 1–147), were a gift of Dr A.Rao (Boston, MA) (Luo *et al*., 1996a).

Cell culture and reagents

CHL cells (ATCC CCL13) were grown at 37°C with a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamycin. CMO and CMX clones were generated by stably transfecting CHL cells with the plasmids pMMTV-CAT and pMMTV-X, respectively, which drive the expression of CAT or HBx in a glucocorticoid-inducible manner (Chirillo *et al*., 1997). Clones were selected in the presence of 0.2 mg/ml hygromycin (Boehringer Mannheim, Mannheim, Germany), and the inducible expression of HBx was analyzed by PCR and Northern blot. In order to confirm the results obtained with these transfectants, different clones of CMO and CMX cells were used in each experiment. The T-cell line Jurkat was grown in RPMI 1641 (Life Technologies LTI, Paisley, Scotland), supplemented with 10% FCS, 2 mM L-glutamine and 50 μg/ml gentamycin. Where indicated, CMO and CMX cells were treated with 1 μM of the water analog of dexamethasone D-2915 (Sigma, St Louis, MO). PMA and the calcium ionophore A23187 were obtained from Sigma. CsA was purchased from Sandoz Pharmaceuticals Co. (East Hanover, NJ).

Transfections and luciferase assays

CHL cells, at 50–70% confluence, were co-transfected with 0.2 μg of the reporter plasmids pNFAT-Luc or pAP-1-Luc, along with 5 μg of pSV-X, or the negative control pSV-hygro, using the DOSPER reagent (Boehringer Mannheim), according to the manufacturer's instructions. Where indicated, 2 μg of the dominant-negative NF-AT expression vector pSH102CΔ418 or varying amounts of the full-length NF-ATc expression vector pNF-ATcwt were added. The empty vector pBJ5 was used to keep the total amount of DNA constant. The transfected cells were either left untreated or stimulated with PMA (10 ng/ml) and/or calcium ionophore (1 μM) for 16 h prior to harvesting. Where indicated, cells were treated with CsA (200 ng/ml) for 40 h. CMO and CMX cells were co-transfected with 0.2 μg of pNF-AT-Luc, 0.2 μg of pNF-ATcwt or pBJ5 and 5 μg of the carrier plasmid pGEM-7 (Promega, Madison, WI). Where indicated, 2 μg of pSH102CΔ418 were included. For Gal4-dependent transactivation experiments, 1 μg of the pGal4-Luc reporter construct, 5 μg of pRSV-Gal4-DBD or pGal4-NFAT1(1–415) and increasing amounts of pSV-X were transfected into CHL cells using the DOSPER reagent. CMO and CMX cells were transfected with 1 μg of reporter plasmid pGal4-Luc and increasing amounts of the pGal4-NF-AT1(1–415) vector. In order to induce the expression of HBx, 1 μM dexamethasone was added to transfected CMO and CMX cells 16 h prior to harvest. The results obtained with the stable transfectants were confirmed using different clones of CMO and CMX cells. Luciferase activity was measured in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany), and normalized to transfection efficiency, which was determined by co-transfecting 0.5 μg of pRL-null and measuring Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility band shift assays

Small-scale nuclear extracts were prepared from CMO and CMX cells at 30–70% confluence either untreated or stimulated with PMA (10 ng/ml) plus calcium ionophore (1 μM) as previously described (Schreiber *et al*., 1989). Binding reactions were performed at 4°C in a volume of 18.5 μl containing 10 mM HEPES pH 7.6, 10% glycerol, 50 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 2.5 μg of poly(dI–dC), 0.75–1 ng of 3′-end-labeled probe and 2 μg of nuclear extracts. For competition, a 40- or 130-fold excess of unlabeled oligonucleotide was added to the reaction prior to the addition of the probe. The DNA–protein complexes were resolved by electrophoresis on 4% polyacrylamide gels. The nucleotide sequences of the oligonucleotides (and their complementaries) used are: 5′-gatcATAAAATTTTCCAATGTAAA-3′ (NF-AT-binding site of the murine IL-4 promoter), 5′-gatcATAAAATTTaggtATGTAAA-3′ (mutated NF-AT-binding site of the murine IL-4 promoter), 5′-gatcATTCGATCGGGGCGGGGCGAG-3′ (SP-1 consensus binding site) and 5′-gatcCGCTTGATGAGTCAGCCGGAA-3′ (AP-1-binding site).

For supershift assays, 0.5 μl of antibody was added 10 min prior to the labeled probe, using the following antibodies: anti-NFAT1 antiserum (Upstate Biotechnology, Lake Placid, NY), which recognizes NF-AT1 but its cross-reactivity with other NF-AT family members has not been tested; anti-NFAT1 antiserum 672, which was raised against the synthetic peptide NH_2-CSPPSGPAYPDDVLDYGLK (residues 53–70 of human NF-AT1) as previously described (Lyakh *et al*., 1997); anti-NFATc monoclonal antibody (Alexis Corporation, San Diego, CA); anti-Jun and anti-Fos family antisera (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-RelA and anti-p50 antisera (provided by Dr E.Muñoz, Córdoba, Spain) (Kieran *et al*., 1990).

Immunofluorescence analysis

CMO and CMX cells were transfected with 1.5 μg of pSH102CΔ418, which expresses an HA-tagged NF-ATc mutant, and 8.5 μg of an irrelevant plasmid using the DOSPER reagent. At 24 h after transfection, cells were plated on coverslips and were grown overnight in the presence

or absence of 1 μM dexamethasone. Where indicated, cells were treated for 2 h with CsA (200 ng/ml) and/or PMA (10 ng/ml) plus calcium ionophore (1 μM) for 30 min. The subcellular localization of NF-AT was analyzed by immunofluorescence with an anti-HA antibody (12CA5). The percentages of cells displaying total or partial nuclear translocation of HA-NF-AT were visually scored after counting at least 50 HA-expressing cells. The statistical analysis was performed using different clones of CMO and CMX cells.

CHL cells were co-transfected with 0.5 μg of pSH102CΔ418 and 1.5 μg of either pSV-X or the control plasmid pSV-hygro, using the FuGENE-6 liposomal reagent, following the manufacturer's instructions. Where indicated, cells were treated with CsA (200 ng/ml) for 2 h and/or with PMA (10 ng/ml) plus calcium ionophore (1 μM) for 30 min. The subcellular localizaton of HA-NF-AT was analyzed by immunofluorescence with the anti-HA antibody 12CA5.

Jurkat cells (10^7) were transiently co-transfected with 1.5 μg of pSH102CΔ418 and 3.5 μg of pSV-X, or the control plasmid pSV-hygro, using the Lipofectin reagent (Life Technologies), according to the manufacturer's recommendations. At 48 h after transfection, cells were plated on polylysine-coated coverslips and were either left untreated or treated with CsA (200 ng/ml) for 2 h and/or PMA (10 ng/ml) plus calcium ionophore (1 μM) for 30 min. HA-NF-AT localization was analyzed by immunofluorescence with the anti-HA antibody 12CA5.

Western blots

CMO and CMX cells were transfected with 1.5 μg of pSH102CΔ418 and 8.5 μg of an irrelevant plasmid, and 24 h later were stimulated overnight with 1 μM dexamethasone and, where indicated, cells were treated for 2 h with CsA (200ng/ml) and/or PMA (10 ng/ml) plus calcium ionophore (1 μM) for 30 min. After washing twice with ice-cold phosphate-buffered saline (PBS), cells were lysed on the plate with 100 μl of Laemmli buffer, boiled for 5 min and separated by electrophoresis in SDS–8% polyacrylamide gels under reducing conditions. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) that were incubated in blocking solution [5% skimmed milk in Tris-buffered saline (TBS) buffer] overnight at 4°C, washed three times in TBS-T (0.1% Tween-20 in TBS) and incubated with the anti-HA antibody (12CA5) supernatant (diluted 1:5 in TBS-T) for 2 h at room temperature. Membranes were washed three times in TBS-T and incubated with a peroxidase-labeled rabbit anti-mouse IgG (DAKO, Glostrup, Denmark), 1:3000 in TBS-T, for 2 h at room temperature. After three washes in TBS-T and one wash in H_2O, membrane-bound antibody was visualized with the enhanced chemiluminescence (ECL) detection reagent (Amersham, Little Chalfont, UK).

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