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 upregulated 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 Varma, 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 proinflammatory 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 transcription factor NF-AT in cells of the immune system (Okamoto et al., 1994; Tsai et al., 1996; Rao et al., 1997; Su and Schneider, 1996; Klein and Schneider, 1997) and consequently play a relevant role in the intrahepatic inflammatory processes. In this context, we investigated the transcriptional regulation of the TNF-α gene by HBx.
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; Iain 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 four AP-1-binding sites, and the transfected cells were activated the AP-1-dependent transcription induced by PMA (not shown) and by 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).
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.
Activation of NF-AT by HBx

**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) cells 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.

**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 32P-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...
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 32P-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 were competed by the homologous NF-AT oligonucleotide or an oligonucleotide containing an SP-1-binding site used. The three specific complexes formed in the presence of HBx are indicated. (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.

EBx 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
of HA-NF-AT are shown. (Luo et al., 1997). As previously characterized in the T-cell line Jurkat, where the mechanism of NF-AT activation/translocation is tightly controlled by NF-AT proteins (Tsai et al., 1997), whereas in lymphocytes the expression of TNF-α gene promoter can be interfered with by a dominant-negative mutant of NF-AT, but not by the cytoplasmic inhibitor of NF-kB proteins IκBα (Lara-Pezzi 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-kB/Rel and NF-κB/Rel 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-kB/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-kB 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-
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.

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
Activation of NF-AT by HBx

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.

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 pSH102Ca418, 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 pSH102Ca418. 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.
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 sequelae.

Materials and methods

Plasmid constructs

The expression vectors pSV-X and pSV-hygro, harboring the HBx ORF and the bacterial chloramphenicol acetyltransferase gene, respectively, under the control of the SV40 early promoter/enhancer have been described elsewhere (Chirillo et al., 1996). The expression vectors pMPTV-X and pMPTV-CAT, containing the HBx ORF and the bacterial hygromycin phosphotransferase gene, respectively, 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). The vectors pMMTV-X and pMMTV-CAT, containing the HBx ORF and the bacterial hygromycin phosphotransferase gene, respectively, under the control of the SV40 early promoter/enhancer have been described previously (Minden et al., 1989). The expression vectors pMMTV-X and pMMTV-CAT, containing the HBx ORF and the bacterial hygromycin phosphotransferase gene, respectively, 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 pSB5 and pSB102CM418 (a kind gift of Dr G. Crabtree, Stanford, CA) are derivatives of pSB5 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. pSB102CM418 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 pNFA-TLuc 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 has been described previously (Schreiber et al., 1989). The reporter plasmid pMMTV-X contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988). The plasmid pMMTV-Luc contains the minimal IL-2 promoter and was provided by Dr D. Faull (Durand et al., 1988).

Cell culture and reagents

CHL cells (ATCC CCL13) were grown at 37°C with a 5% CO2 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 pMPTV-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 transfected, 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 pNFA-T-Luc or pAP1-Luc, along with 5 μg of pSV-X or the negative control pSV-hygro, using the DOPSER reagent (Boehringer Mannheim), according to the manufacturer’s instructions. Where indicated, 2 μg of the dominant-negative NF-AT expression vector pSH102CM418 or varying amounts of the full-length NF-ATc expression vector pNF-ATcwt were added. The empty vector pB5 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 harvest. 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 pNFA-T-Luc, 0.2 μg of pNFA-Tcwt or pB5 and 5 μg of the carrier plasmid pGEM-7 (Promega, Madison, WI). Where indicated, 2 μg of pSH102CM418 were included. For Gal4-dependent transactivation experiments, 1 μg of the pGal4-Luc reporter construct, 5 μg of pRSV-Gal4-DBD or pGal4-NFAT(1–415) vector, and increasing amounts of pSV-X were transferred into CHL cells using the DOPSER reagent. CMO and CMX cells were transfected with 1 μg of reporter plasmid pGal4-Luc and increasing amounts of the pGal4-NFAT(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 transfected cells 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 MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 2.5 μg of poly(dI–dC), 0.75–1 ng of 32P-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 reactions were resolved by electrophoresis on 4% polyacrylamide gels. The nucleotide sequences of the oligonucleotides (and their complementaries) used are: 5′-gatGATAAAATTTT-CCAAATGAAA-3′ (NF-AT-binding site of the murine IL-4 promoter), 5′-gatGATAAAATTTAGTGATGAAA-3′ (mutated NF-AT-binding site of the murine IL-4 promoter), 5′-gatGATCGATCAGGGCGGGGGGCAG-3′ (SP-1 consensus binding site) and 5′-gatGATCGCTTGATGAGTGATGATCCGGGAA-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 NH2-CSPSPGSPAPYDDVLVGGKL (residues 53–70 of human NF-AT1) as previously described (Lynch et al., 1997); anti-NFATc monoclonal antibody (Alexis Corporation, San Diego, CA); anti-Jun and anti-Fos family antiserum (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 pSH102CM418, which expresses an HA-tagged NF-ATc mutant, and 8.5 μg of an irrelevant plasmid using the DOPSER reagent. At 24 h after transfection, cells were plated on coverslips and were grown overnight in the presence of...
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 HA-NF-AT was visualized 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 two independent clones of CMO and CMX cells.

CMO cells were co-transfected with 0.5 μg of pSH102Ca418 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 localization of HA-NF-AT was analyzed by immunofluorescence with the anti-HA antibody 12CA5.

**Western blots**

CMO and CMX cells were transfected with 1.5 μg of pSH102Ca418 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 (200 ng/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 H2O, membrane-bound antibody was visualized with the enhanced chemiluminescence (ECL) detection reagent (Amersham, Little Chalfont, UK).

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**References**


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