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Hepatitis B Virus X Protein Activates Transcription Factor NF-κB without a Requirement for Protein Kinase C

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The hepatitis B virus X protein stimulates transcription from a variety of promoter elements, including those activated by transcription factor NF-κB. A diverse group of extra- and intracellular agents, including growth factors and the human immunodeficiency virus *tat* protein, have been shown to require a functional protein kinase C (PKC) system to achieve activation of NF-κB. In this study we have investigated the molecular mechanism by which X protein activates NF-κB. We demonstrate that in hepatocytes, X protein induces a maximal activation of NF-κB corresponding to the sequestered pool of factor, which is also activated by phorbol esters. To determine whether X protein requires activation of PKC to stimulate transcription by NF-κB, we attempted to prevent transactivation by X protein in the presence of the PKC inhibitors calphostin C and H7. We show that PKC inhibitors do not block X protein activation of NF-κB, whereas they largely impair activation by phorbol esters. In addition, activation of PKC is correlated with its translocation from the cytoplasm to the plasma membrane. The subcellular distribution of PKC was investigated by introducing X protein from a replication-defective adenovirus vector, followed by immunochemical detection of PKC in cell fractions. These data also indicate that X protein stimulates transcription by NF-κB without the activation and translocation of PKC.

Chronic infection by hepatitis B virus (HBV) has long been recognized as a correlative agent in the development of hepatocellular carcinoma, although there is little understanding of the molecular mechanisms involved (4, 61). The failure to find a consistent molecular event by which HBV promotes neoplastic disease has suggested the involvement of indirect or secondary events in its mechanism of carcinogenesis, such as virus-mediated cell injury resulting from overexpression of the virion envelope proteins (9), or disruption of normal cellular gene regulation related to integration of viral DNA (reviewed in reference 18). It is not known to what extent the overexpression of virus-encoded structural proteins contributes to carcinogenesis in humans, but they are clearly transforming when expressed in transgenic mice (9). Although integration of viral genomes into cellular DNA is an event commonly associated with hepatocellular carcinoma, integration occurs randomly and there is little evidence to support the *cis* activation of proto-oncogenes by viral DNA (32, 53, 62; reviewed in reference 18). Thus, the integration of viral genomes into cellular DNA may be an important contributing factor but is unlikely to play a direct role in transformation. An alternative mechanism for altering cell growth potential may involve the activity of the HBV X gene, which has been shown to encode a transcriptional-regulatory (transactivating) protein that stimulates a variety of heterologous promoters (58, 65, 71). The X protein may potentially play a role in carcinogenesis during chronic viral infection by directly regulating cellular gene activity.

The X gene is highly conserved in HBV serotypes (38) and can encode at least one, 17-kDa polypeptide. The X protein is known to be synthesized during infection because antibodies to it are produced in many infected individuals (13, 41). Most studies have concluded that the X protein itself does not function as an oncogene because its expression in transgenic mice was not found to be associated with serious liver disease (1, 9, 16, 34) and an intact X gene has only occasionally been found integrated in many of the human hepatocellular carcinoma samples analyzed (see, e.g., references 42, 60, and 69). However, recent evidence now indicates that the X protein may possess oncogenic activity when expressed in transgenic mice at high levels for a sufficient duration (30) or in cell lines previously immortalized with simian virus 40 (SV40 T) antigen (25).

It is not currently known whether the effect of X protein on cell growth involves its transcriptional-regulatory activities or derives from unknown functions, such as a direct interaction with proteins that regulate cell proliferation. However, the X protein is a rather promiscuous transactivator of class II transcriptional elements including HBV enhancer 1, the SV40 enhancer, the human immunodeficiency virus (HIV) long terminal repeat, and major histocompatability complex I genes (52, 58, 64, 65, 71), as well as class III promoters (2). Because the X protein does not bind DNA directly (57, 70), it is widely believed to act indirectly by modifying or associating with cellular transcription factors and thereby altering their activities. Evidence to this effect has recently been presented in that X protein has been shown to participate in the binding of ATF/CREB factor to the HBV enhancer (39) and to modestly activate promoters when tethered to a specific protein DNA-binding domain (50, 67). Thus, X protein may alter cell growth properties by acting directly on the promoter elements of key regulatory genes.

In addition to the potential ability to directly participate in promoter activation, there is good reason to believe that X protein could act on components of cellular signal transduction pathways. In particular, X protein has been shown to stimulate promoters activated by transcription factors NF-κB (56, 66) and AP1 (50). Both factors are regulated through pathways which are induced in vivo by tumor promoters, growth factors, and cytokines, and NF-κB in

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particular is stimulated by activators of protein kinase C (PKC) (49), protein kinase A (54), and the double-stranded RNA-activated kinase DAI (68). NF-κB is regulated by sequestration in the cytoplasm in an inactive complex with its inhibitor (IκB), but is released and transported to the nucleus after inducer action. Initial studies conducted in vitro suggested that the direct phosphorylation of IκB by PKC might mediate the release and therefore activation of NF-κB (19, 55). However, several studies now indicate that activation of NF-κB may not involve the direct phosphorylation of IκB by PKC. For instance, activation of PKC and NF-κB by tumor necrosis factor alpha is not coupled in HL-60 cells (24, 40). In addition, evidence has recently been presented that reactive oxygen intermediates, which are generated by the action of a variety of biological agents including phorbol esters (reviewed in reference 7), may serve in some manner to mediate the release of IκB (47). Therefore, there are likely to be several pathways for activation of NF-κB, some of which are independent of PKC activity.

In this study we have investigated the activation of NF-κB-responsive promoters by the X protein. We show that X protein activates inducible NF-κB through a pathway which is stimulated by tumor promoters. However, several lines of evidence are presented to demonstrate that X protein acts independently of PKC. These include the inability to prevent X-protein function with inhibitors of PKC and the failure of active X protein to cause the translocation of PKC from the cytoplasm to the plasma membrane, which is correlated with PKC activation (33; reviewed in reference 29). Thus, our results indicate that transcriptional regulation by HBV X protein is mediated in part through its effects on a cellular signal transduction pathway, but that it acts independently of PKC in activating transcription factor NF-κB. Given that NF-κB regulates a number of genes involved in the control of cell proliferation, these results suggest that if HBV X protein alters cell growth, it may do so by influencing the regulation of an intracellular second-messenger system.

MATERIALS AND METHODS

Cell culture. Cell lines used in this study were obtained from the American Type Culture Collection. HepG2 cells were propagated in modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg of gentamicin sulfate per ml. Chang liver cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and gentamicin sulfate. Quiescent cells were accumulated by cultivation in serum-free medium for 48 h.

DNA transfections. Transfection of plasmid DNAs was performed by the calcium phosphate precipitation technique (22), with 10-cm plates of cells at 60 to 70% confluency. Total DNA concentrations of 20 μg per plate were maintained with salmon sperm DNA. The DNA from two plates for each sample was harvested and pooled 36 to 48 h after transfection. Transfection efficiencies were monitored as previously described (2), by immunofluorescence staining of a marker protein produced from a cotransfected, nonstimulated plasmid included in duplicate plates of cells. In the transfections we used 5 μg of reporter plasmids and 10 μg of X gene plasmids unless otherwise noted.

CAT assays. Preparation of cell lysates and analysis of chloramphenicol acetyltransferase (CAT) enzyme activity were performed as described previously (21). Equal amounts of protein were used and adjusted to ensure that enzyme activity remained within the linear (<50% conversion) range. Quantitation was carried out by liquid scintillation counting of spots on thin-layer chromatography plates, the percent conversion to acetylated chloramphenicol was calculated, and data are presented below each figure as the fold activation with respect to basal CAT activity levels. The quantitated results of CAT assays represent the average of at least three independent determinations, using pools of DNA from two plates per sample.

Analysis of PKC activity. (i) Translocation of PKC. Cells requiring the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were treated as follows. To activate PKC, cells were treated with 100 nM TPA for 30 min. To deplete PKC, cells were treated with 100 nM TPA for 24 h. Cytosolic and membrane cell fractions were prepared as described by Kraft and Anderson (33), incorporating the modifications of Struloici et al. (59). Approximately 3×10^6 cells per sample were harvested and sonicated in 2 ml of buffer A [20 mM Tris-HCl (pH 7.5), 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid (EGTA), 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride PMSF, 10 μg of leupeptin per ml] and centrifuged at 100,000 × *g* for 1 h at 4°C. The 2 ml of supernatant corresponding to the soluble cytoplasmic fraction was made 1% in sodium dodecyl sulfate (SDS) and 2% in β-mercaptoethanol. The high-speed pellet corresponding to the insoluble membrane fraction was resuspended in 1 ml of 1% SDS and 2% β-mercaptoethanol. Both fractions were denatured, and SDS-polyacrylamide gel electrophoresis was performed on aliquots as described previously (12), with a 25-μl cytoplasmic fraction and a 12.5-μl membrane fraction. Proteins were electrophoretically transferred to nitrocellulose and immunoblot analysis was performed as described previously (63), using an affinity-purified antiserum directed against PKC diluted 1:250 (59) followed by ^{125}I-protein A. Antiserum to PKC (a gift of A. Czernick, Rockefeller University) was prepared against a peptide that is common to isoforms α, β, and γ and that cross-reacts with all three species.

(ii) Use of PKC inhibitors. Inhibition of PKC activity was performed with the isoquinolinesulfonamide derivative H7 or calphostin C (Seikaga America, St. Petersburg, Fla.). Stock solutions of H7 (10 mM) and calphostin C (2.14 mM) were prepared in 100% dimethyl sulfoxide. Inhibitors were added to cells starting at 6 h posttransfection at the concentrations indicated and incubated with cells until preparation of extracts (36 h after transfection). Control plates received an equal concentration of dimethyl sulfoxide without inhibitor. PKC activity was stimulated by incubating cells in 20 nM TPA for 20 h, beginning 24 h after transfection. PKC activity was ablated by long-term treatment of cells with a high concentration of TPA, using 100 nM for 18 h prior to and throughout the period of transfection (45).

Plasmids. Plasmids pOP-HBV-1 (renamed pHBV-CAT2) and pOP-HBV-Acc/Nco (renamed pHBV-CAT1) were obtained from P. Hearing (SUNY, Stony Brook [43]). In pHBV-CAT2 the HBV*ayw* enhancer 1 fragment (*Stu*I [nucleotide (nt) 1117] to *Xmn*I [nt 1247]) was inserted upstream of the SV40 early promoter and CAT gene. Plasmid pHBV-CAT1 contains a larger enhancer 1 fragment (*Acc*I [nt 824] to *Nco*I [nt 1372]) in the same background. Plasmid pκB-CAT was derived from construct p59RLG-4κB (J. Habener, Harvard University [6]) and contains four tandem repeats of the NF-κB site linked to the rat minimal angiotensinogen promoter and CAT gene. Plasmid p59RLG-4κB was shown to be activated solely by the inducible factor NF-κB (6). To create pκB-CAT, the luciferase gene was removed from p59RLG-4κB and replaced by the CAT gene from pHBV-

CAT2, which was obtained by partial digestion with *Eco*RI and *Hin*dIII. Plasmid p*wt*X contains the HBV*ayw* *Bal*I (nt 303)-to-*Xba*I (nt 1992) X gene fragment cloned into pBR322. p*wt*X expresses the biologically active X protein under the control of its native transcription elements.

Construction of recombinant adenoviruses. To construct an X protein expressing recombinant adenovirus vector, the X coding region was inserted into plasmid pNL3C (see Fig. 4) as described below. Plasmid pNL3C contains the adenovirus E1a enhancer-inverted terminal repeat sequence required for viral replication, fused to the adenovirus major late promoter, tripartite leader 5′ noncoding region, E1A poly(A)$^+$ signal, and E1B gene. The HBV*ayw* X gene was digested with *Xmn*I (nt 1351), and *Sal*I linkers were added, and then the gene was digested with *Sal*I and *Bgl*II (nt 1990). The X coding-region DNA fragment was inserted into the *Sal*I site at the 3′ end of the tripartite leader and the *Bgl*II site upstream of the E1B poly(A)$^+$ signal in pNL3C to create pNL3C-X. Subgenomic adenovirus 309 viral DNA was prepared by digestion with *Cla*I-*Xba*I, and the large (1.3 to 36-kb) fragment was purified by sedimentation in a 10 to 40% sucrose gradient in 1 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA for 14 h at 36,000 rpm at 4°C in a SW41 rotor. Approximately 5 μg of Ad309 DNA and 2 μg of pNL3C-X (linearized with *Nru*I) were introduced into 293 cells by the calcium phosphate precipitation technique. Viral recombinants were created by in vivo recombination between the defective 3′ end of the E1b gene in pNL3C-X and the E1b gene in viral genomic DNA. Cells were maintained under agar overlay, and individual plaques were selected for expansion and analysis. Recombinant adenoviruses were characterized by restriction enzyme analyses of genomic DNA, by Northern (RNA) analysis for X mRNA, and by the biological activity of X protein. Cells were infected with adenoviruses at 50 PFU per cell.

RESULTS

X protein activates inducible NF-κB. Previous studies have shown that the HBV X protein stimulates transcription of the HIV long terminal repeat (37, 57, 65, 71), which was mapped by deletion mutagenesis to the NF-κB-binding sequences contained within region U3 (15, 27, 56, 66). Therefore, we further explored the molecular mechanism by which X protein stimulates transcription mediated by factor NF-κB. In particular, we asked whether X protein acts through PKC to activate NF-κB, as described for phorbol esters and the HIV *tat* protein (26), or in a PKC-independent manner, as described for tumor necrosis factor alpha (40). To assess the requirements for activation of NF-κB by X protein, we used a functional assay (specific transcriptional induction) to assess the levels of transcriptionally active factor in vivo. Transcriptional activation studies used the plasmid pκB-CAT, which was shown previously to be activated solely by the binding of the inducible (sequestered) form of NF-κB (6, 46). Plasmid pκB-CAT contains four tandem copies of the NF-κB-binding sequence inserted immediately upstream of the rat minimal angiotensinogen promoter and CAT gene (Fig. 1). Control plasmid pκBM2-CAT contains a mutagenized repeat of this sequence that fails to bind inducible NF-κB, preventing transcriptional activation by phorbol esters (46).

In Fig. 1 we demonstrate that the target of X protein is the inducible form of NF-κB. First, X protein activated transcription of pκB-CAT to roughly the same extent (sevenfold) as did stimulation by TPA (fivefold [lanes 2 and 3]). In addition, TPA did not further stimulate CAT activity in cells cotransfected with p*wt*X and pκB-CAT (data not shown), demonstrating the efficient activation of sequestered NF-κB by X protein. Second, X protein failed to activate transcription of control plasmid pκBM2-CAT, which contains a mutated repeat of the NF-κB-binding site (lane 5), demonstrating that the target for activation in these studies is sequestered (inducible) NF-κB. Third, prolonged treatment of cells with a high concentration of TPA depletes the level of PKC (45) and results in resequestration of NF-κB in an

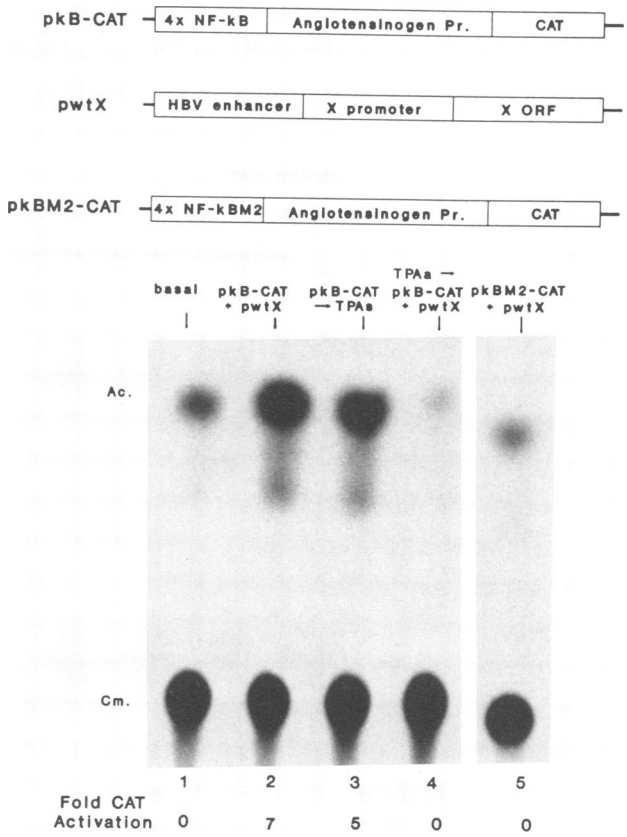

FIG. 1. X protein transactivates authentic NF-κB. Plasmid pκB-CAT contains four contiguous NF-κB-binding sites inserted immediately upstream of the rat minimal angiotensinogen promoter (6) and CAT gene. This transcription element was previously shown to be specifically activated by inducible NF-κB (6, 46). Plasmid pκBM2-CAT contains a mutated NF-κB repeat that is not activated by NF-κB (46). Plasmid p*wt*X expresses the HBV X gene under the control of its native transcriptional elements (2). HepG2 cells were transfected in duplicate with plasmids, and extracts were prepared 36 to 48 h later for analysis of CAT enzyme activity (21). Transfection efficiencies were monitored by immunofluororescent staining of a marker protein transcribed from a cotransfected, nonstimulated plasmid (data not shown) included in duplicate samples as described previously (2). Stimulation of PKC activity by TPA (TPAs) was performed 24 h after transfection by incubating cells for 20 h in 20 nM TPA. Ablation of PKC activity (TPAa) was performed by including 100 nM TPA 18 h prior to and throughout the period of transfection. Further specific details can be found in Materials and Methods. Quantitation of CAT assays was performed by liquid scintillation counting of spots in thin-layer chromatography plates. Data were averaged from at least three independent experiments and calculated as the percentage converted to the acetylated-chloramphenicol form. Results are presented (numbers below lanes) as the fold activation above the basal level (pκB-CAT alone).

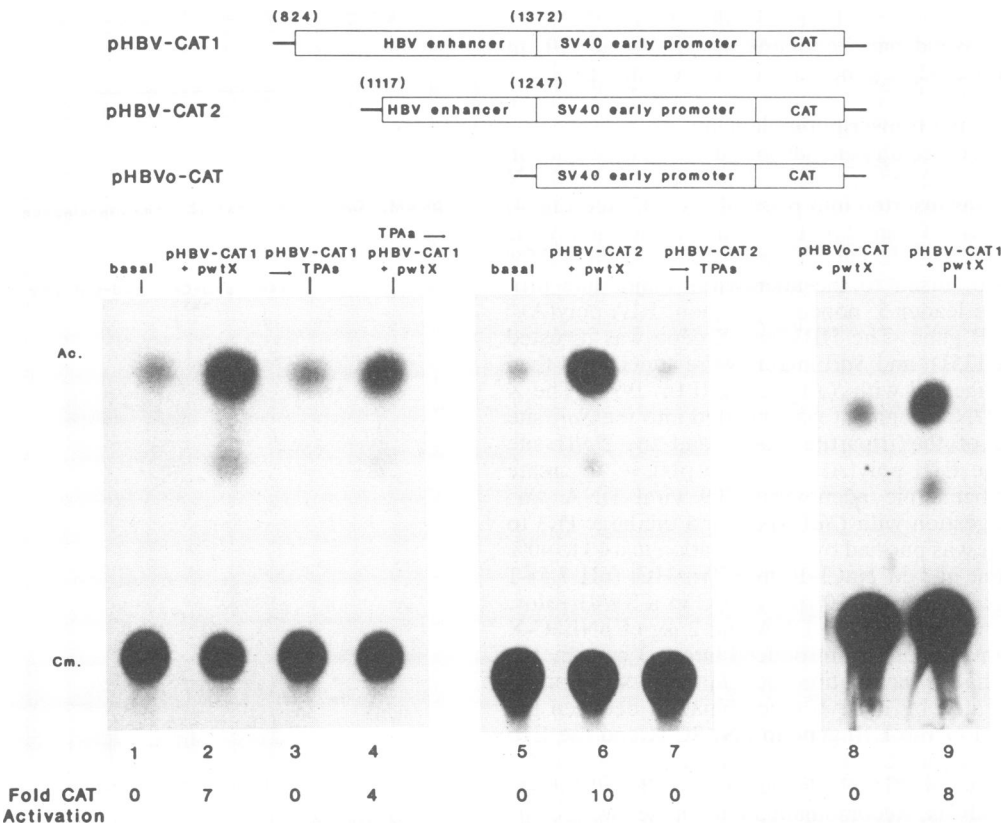

FIG. 2. X protein activates the HBV enhancer in cells depleted of PKC. Plasmids pHBV-CAT1 and pHBV-CAT2 (43) contain the HBV*ayw* enhancer fragment with different amounts of flanking sequence (boundaries marked in parentheses) inserted adjacent to the SV40 early promoter and CAT gene. Plasmid pHBVo-CAT lacks the HBV enhancer. Transfection of HepG2 cells, treatment with TPA, and analysis of CAT enzyme activity are described in the legend to Fig. 1. Basal refers to cells transfected with only pHBV-CAT1.

inactive cytoplasmic complex that is resistant to further stimulation in vivo (3, 49). X protein, like phorbol esters, was unable to reverse the block to further stimulation of NF-κB created by prolonged treatment with TPA (lane 4). Therefore, both X protein and phorbol esters stimulate transcription by activating the latent pool of NF-κB, and the activity of both is blocked by prolonged exposure of cells to TPA. Because PKC activity is ablated by long-term treatment with phorbol esters, we next addressed whether transactivation of the diverse transcriptional elements stimulated by X protein is mediated through an ability to activate PKC.

The potential role of PKC in X protein activity was first investigated by determining whether its depletion also blocks transactivation of promoters which do not require NF-κB for activation. The HBV enhancer does not utilize NF-κB and is strongly transactivated by the X protein (10, 15, 58, 69). Several transcription factors have been shown to play a role in activation of the HBV enhancer, including EF-C (43), an AP1-like and C/EBP-like factor (5, 14, 15), and possibly ATF/CREB (39). It has also been reported that in some cell lines the HBV enhancer is weakly induced (ca. threefold) by phorbol esters (5, 14, 15). In the following experiments we first demonstrated that the HBV enhancer is not stimulated by phorbol esters in HepG2 cells. It was then shown that X protein transactivation of the HBV enhancer does not involve activation of PKC.

HBV enhancer constructs consisted of pHBV-CAT2, which contains the enhancer fused to the SV40 early promoter and CAT gene, and pHBV-CAT1, which contains the enhancer with additional upstream sequences (Fig. 2) that may include a potential binding site for factor NF-κB (unpublished observations). X protein strongly transactivated both enhancer constructs about 7 to 10-fold (lanes 2 and 6). Transcriptional activation was mediated through the HBV enhancer, as demonstrated by the failure to stimulate transcription from pHBVo-CAT, which contains only the SV40 early promoter and CAT gene (lanes 8 and 9). Treatment of cells with a low concentration of TPA did not stimulate transcription from either HBV enhancer construct (lanes 3 and 7), suggesting that activation by X protein probably does not involve a pathway mediated by PKC. This was tested directly by depleting cells of PKC with a high concentration of TPA, followed by cotransfection of pHBV-CAT1 and p*wt*X. Depletion of PKC only slightly (ca. twofold) impaired X protein activation of the HBV enhancer (lane 4). The slight decrease in transcriptional activity is probably a result of secondary effects of the TPA during the long incubation period (29). These results are consistent with the notion that X protein does not act through PKC to stimulate the HBV enhancer. We therefore asked whether X-protein activation of NF-κB also occurs independently of PKC, unlike that of the HIV *tat* protein.

Inhibitors of PKC do not block X-protein activation of NF-κB. The isoquinolonesulfonamide derivative H7 (23) and the microbial compound calphostin C (31) are inhibitors of PKC that have been widely used to assess its involvement in

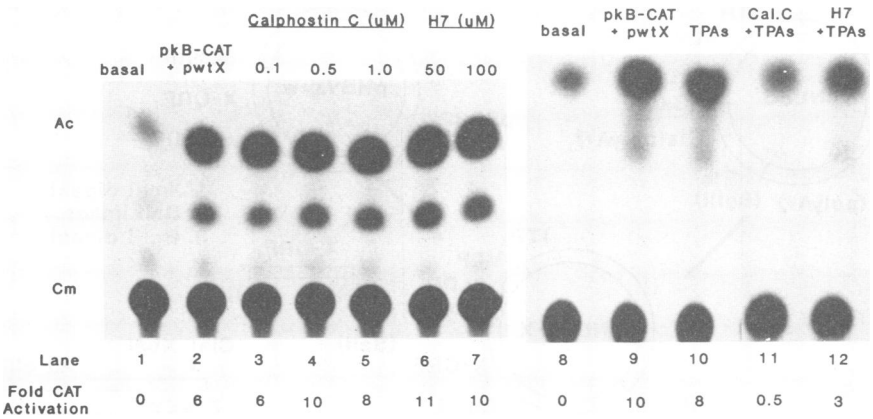

FIG. 3. PKC inhibitors do not block X protein activity. HepG2 cells were transfected with plasmids p*wt*X and pκB-CAT and, 6 h later, were treated with PKC inhibitors until harvest at 36 h posttransfection. Control cells were treated with inhibitors followed by stimulation with 20 nM TPA for 16 h. Analysis of CAT enzyme activity is described in the legend to Fig. 1. Basal refers to cells transfected with pκB-CAT alone. The final concentration of inhibitors present in medium is shown above each lane.

mediating signal transduction pathways. For example, H7 was shown to prevent transactivation by HIV *tat* protein (26), demonstrating that an active PKC system is required for *tat* function. Calphostin C is reported to be a highly potent and more specific inhibitor of PKC activation than H7 (31). These inhibitors were therefore used to determine whether X protein activates NF-κB independently of PKC. Cells were transfected with the X protein expression vector p*wt*X and the NF-κB-dependent reporter plasmid pκB-CAT in the presence of different concentrations of PKC inhibitors (Fig. 3). Control experiments indicated that the viability of cells was not detectably altered during the period of treatment (data not shown). H7 has been shown to effectively inhibit PKC activation in HeLa cells at a concentration as low as 10 μM (see, e.g., reference 44). Calphostin C, which is more specific than H7, strongly blocks PKC activation at levels as low as 0.1 μM (31). Inhibitors were therefore titrated over a 10-fold range to determine whether they effected X protein activation of NF-κB. Neither inhibitor blocked X protein activation of NF-κB, even when they were present at concentrations approximately 5- to 10-fold higher than that required to suppress PKC activity in vivo. The activity of pκB-CAT was typically stimulated approximately sixfold by X protein (lane 2). Treatment with calphostin C at 0.1 μM did not detectably affect transactivation by X protein, which was slightly but reproducibly enhanced at 0.5 μM (10-fold; lane 4) and 1 μM concentrations (8-fold; lane 5). Accordingly, X protein transactivation of pκB-CAT was not impaired by treatment with H7, which also resulted in a slight but reproducible stimulation at high concentrations (lanes 6 and 7). In contrast, calphostin C and H7 impaired the ability of TPA to induce PKC activation of pκB-CAT by 75% (compare lanes 5 and 9), and by 60% (lanes 7 and 10), respectively, and largely blocked activation by *tat* protein (data not shown) as described previously (see, e.g., reference 26). The extent of reduction in activation by TPA in the presence of PKC inhibitors was similar to levels observed by others (see, e.g., references 17 and 19). A slight increase in the activation of NF-κB was also observed in cells exposed to tumor necrosis factor alpha (which is PKC independent) in the presence of PKC inhibitors (24), but the significance of this effect is unclear. Thus, X protein did not apparently require functional PKC to achieve activation of NF-κB.

X protein activates NF-κB without inducing translocation of PKC. Activation of PKC has been shown to correlate with its translocation from the cytoplasm to the plasma membrane (33). For example, transient activation of PKC by tumor promoters (TPA and phorbol myristate acetate) or oncogenes (*ras* and *src*) results in the quantitative subcellular redistribution of the kinase from the cytoplasm to the plasma membrane (reviewed in reference 29). Thus, the translocation of PKC provides a reliable marker for the detection of PKC activation. We therefore examined the subcellular distribution of PKC in cells expressing biologically active X protein. Because only a small percentage of cells are transfected by plasmids, it was not feasable to measure PKC translocation by introducing an X protein expression vector by this technique. In addition, some plasmids generate double-stranded RNAs which could potentially activate PKC directly or after induction of interferon synthesis. To circumvent these limitations, the X gene was instead inserted into a replication-defective adenovirus vector, which was then used to infect cells in culture. Construction of the recombinant adenovirus is shown in Fig. 4 and described in detail in Materials and Methods. Briefly, the X gene coding region was first inserted into a plasmid downstream of the adenovirus replication signal (the inverted terminal repeat) and under the control of the viral major late promoter. The X gene was then inserted into an intact viral genome by the method of homologous recombination between overlapping regions of the viral E1b gene present in the plasmid and viral genomic DNA. Insertion of the X gene into adenovirus DNA results in inactivation of the adenovirus E1a and E1b genes, producing a recombinant virus (Ad-X) which is defective for adenovirus gene expression and therefore does not replicate in hepatocytes. The Ad-X recombinant virus can be propagated in 293 cells, which complement for the lost gene functions.

X protein expressed from the Ad-X recombinant was found to be biologically active and to strongly activate NF-κB (Fig. 5). The activity of X protein expressed from the Ad-X vector was titrated by transfecting Chang liver cells with plasmid pκB-CAT and then infecting them with either the replication-defective Ad-X recombinant virus or control virus *dl*312, which is also deleted in regions E1a and E1b (28). Approximately 36 h after infection, cell extracts were prepared and tested for stimulation of pκB-CAT transcription. X protein expressed from the Ad-X recombinant virus

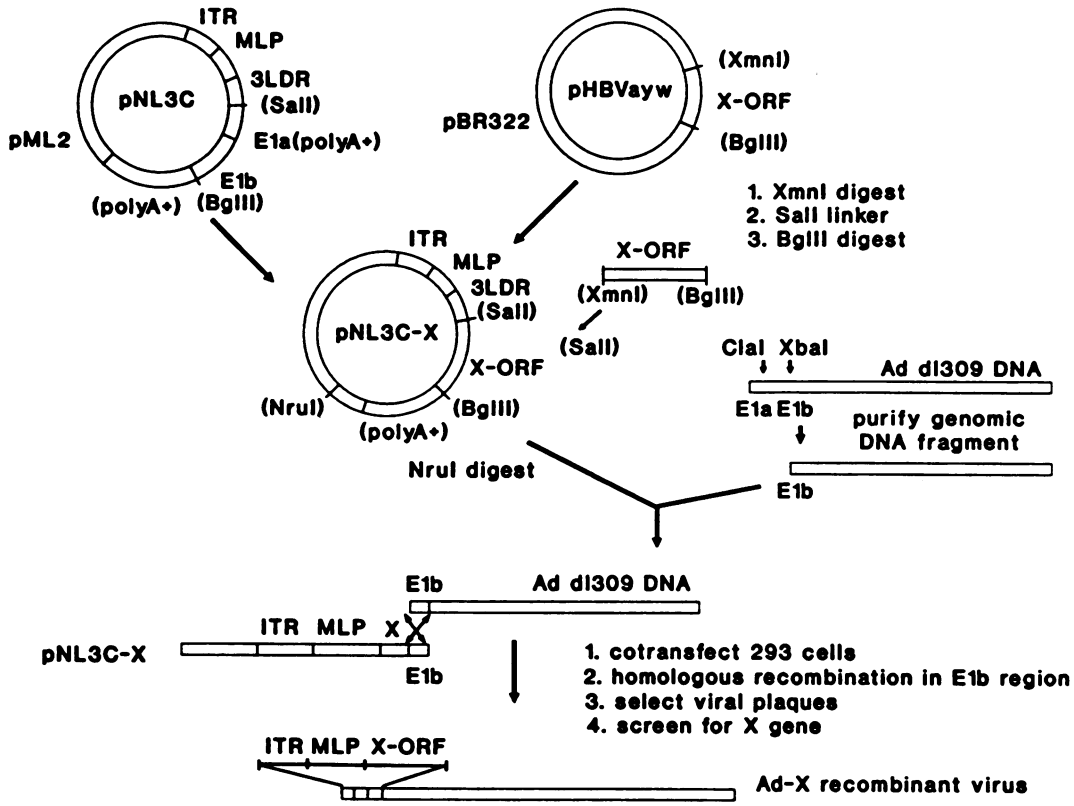

FIG. 4. Strategy for construction of the X protein-expressing recombinant adenovirus vector Ad-X. Plasmid pNL3C contains the Ad5 E1a enhancer/inverted terminal repeat (ITR) sequence required for viral replication, the Ad2 major late promoter (MLP) and tripartite leader 5′ noncoding region cDNA, the E1a poly(A)$^+$ signal, and E1b gene inserted in plasmid pML2. The HBV*ayw* X coding region was inserted between the tripartite leader and E1b poly(A)$^+$ signal. A detailed description of cloning procedures is provided in Materials and Methods. Ad5 wild-type variant *dl*309 (28) genomic DNA was digested with *Cla*I and *Xba*I, and the large fragment was purified. Plasmid pNL3C-X was linearized by digestion with *Nru*I and coprecipitated with the large fragment of *dl*309 on 293 cells, recombinants were created by homologous recombination, and viruses were selected by the plaque assay (28). Viral plaques were propagated, and the presence of the X gene was analyzed by restriction enzyme digestion of genomic viral DNAs and Northern (mRNA) blot analysis.

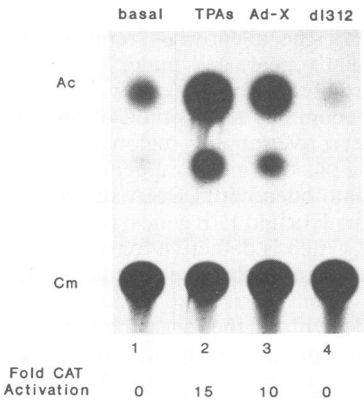

FIG. 5. X protein expressed from Ad-X recombinant virus transactivates NF-κB. HepG2 cells were transfected with plasmid pκB-CAT and then infected 6 h later at 50 PFU per cell with replication-defective adenovirus Ad-X or with *dl*312 virus which lacks regions E1a and E1b. At 36 h postinfection cells were harvested and extracts were prepared for CAT enzyme analysis. Stimulation by TPA was performed by using 20 nM TPA for 16 h, beginning 24 h after infection. Basal refers to cells transfected with pκB-CAT alone.

stimulated NF-κB-dependent transcription about 10-fold, but not quite as well as TPA did (15-fold; compare lanes 2 and 3). Infection with Ad *dl*312 did not result in stimulation of pκB-CAT transcription (lane 4), demonstrating that activation of NF-κB was due to expression of biologically active X protein rather than to low-level expression of adenovirus-encoded gene products.

The subcellular distribution of PKC in cells expressing X protein was then examined by infecting the cells with either Ad-X or *dl*312 virus. At 36 h after infection, cytoplasmic and membrane fractions were prepared from cells as described previously (33; also see Materials and Methods) and the proteins in each fraction were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with an antiserum directed against the three major isoforms (α, β, and γ) of PKC. Figure 6A demonstrates that in control cells not stimulated by TPA, PKC was quantitatively retained in the cytoplasmic fraction (lane 2) and was not detectable in the membrane fraction (lane 5), as expected. In contrast, stimulation of cells with TPA resulted in the loss of PKC from the cytoplasmic fraction (land 1) and its appearance in the membrane fraction (lane 4). Treatment of cells with a high concentration of TPA largely depleted both the cytoplasmic (lane 3) and membrane (lane 6) fractions of immunodetectable PKC. Thus, the compartmental-

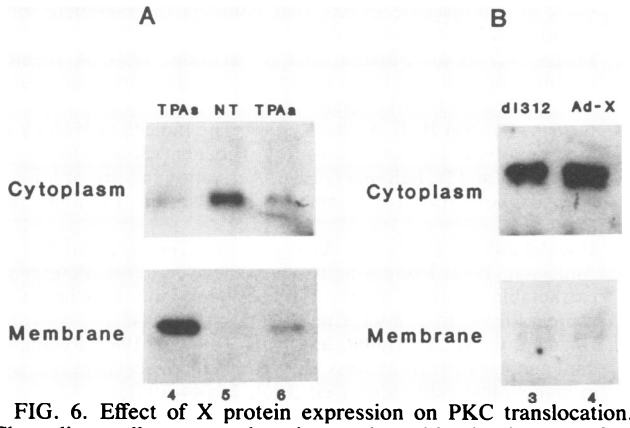

FIG. 6. Effect of X protein expression on PKC translocation. Chang liver cells were made quiescent by cultivation in serum-free medium for 48 h. (A) PKC activity was stimulated by incubating quiescent cells in 100 nM TPA for 30 min. For ablation of PKC activity, cells were incubated in 100 nM TPA for 24 h. (B) Quiescent cells were infected with Ad-X or *dl*312 virus for 36 h. Cytosolic and membrane cell fractions were prepared as described in Materials and Methods, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Western immunoblotting was performed by using an affinity-purified antibody directed against a common epitope of PKC isotypes α, β, and γ, followed by ^{125}I-protein A and autoradiography. Lanes: NT, not treated; TPAs, TPA stimulated; TPAa, TPA ablated.

ization of PKC in nonstimulated cells and the correlation between PKC activation and translocation to the plasma membrane occur as expected in these cells. Cells were therefore infected with the replication-defective control adenovirus *dl*312 or Ad-X recombinant, and the subcellular distribution of PKC was determined (Fig. 6B). In cells infected with *dl*312, PKC is quantitatively retained in the cytoplasmic fraction (lane 1) and only trace amounts are immunologically detectable in the membrane fraction (lane 3). Importantly, cells infected with Ad-X were also found to display no evidence for the translocation of PKC to the plasma membrane. PKC was fully retained in the cytoplasmic fraction (lane 2) with no evidence of kinase in the plasma membrane (lane 4). We can therefore conclude that X protein activation of NF-κB occurs without concomitant activation or translocation of PKC.

DISCUSSION

It is well established from the work of several laboratories that the HBV X protein activates transcription factor NF-κB, but the mechanism of activation has not been explored. There is ample evidence for involvement of PKC in activation of NF-κB by various autocrine factors (reviewed in reference 29). In addition, proteins that act intracellularly in postreceptor signaling pathways can activate PKC, such as the HIV *tat* protein which in turn activates NF-κB (26), and *src* and *ras* oncogenes (11). Because NF-κB represents an important second messenger involved in immune cell growth and activation of PKC can influence cell proliferation, the mechanism by which X protein activates NF-κB is of significant biological importance.

In this investigation we provided several lines of evidence demonstrating that X protein efficiently activates the pool of sequestered NF-κB without a requirement for PKC. We showed that PKC activation can be largely blocked by the inhibitors H7 or calphostin C without preventing the transactivation of NF-κB by X protein. Even high concentrations of calphostin C, a potent and specific inhibitor of PKC, did not impair activation of NF-κB by X protein. A potential limitation of these results is that PKC consists of several related enzymes that may differ in their activation by various agents (29). However, calphostin C was shown to inhibit all three major isoforms (α, β, and γ) of PKC to an equal extent (31). Furthermore, even at concentrations five times that required to largely inhibit PKC activity in HeLa cells (31) and hepatocytes (Fig. 3, lane 9), calphostin C did not impair X protein activation of NF-κB. We also showed that the subcellular distribution of PKC in cells expressing biologically active X protein is not consistent with its activation (Fig. 6). Activation of PKC by a variety of agents is generally linked to its quantitative translocation to the plasma membrane. As Fig. 6 demonstrates, X protein activated NF-κB without a concomitant translocation of PKC from the cytoplasmic to the membrane fraction. The immunochemical detection of PKC is quite sensitive, and analysis of its subcellular distribution has been shown to be a reliable measure of its activation (33). Although it is possible that X protein activates PKC in a transient manner that involves a weak and easily reversible association with the plasma membrane, as observed upon treatment with diacylglycerol (20), this mechanism of activation is inconsistent with the results obtained with PKC inhibitors.

The results presented here pose questions concerning the intracellular distribution of X protein. Unfortunately, the subcellular location of X protein is currently a matter of some confusion, reported to be cytoplasmic (8, 57), nuclear (25, 48), and both (37, 57). It should be noted that the studies which found exclusively nuclear localization of X protein were performed in cells expressing SV40 T antigen. It has been subsequently shown that T antigen, which is nuclear protein, associates with X protein in a fairly stable complex (51) and therefore may have sequestered much of the X protein in the nucleus. Thus, the distribution of X protein may be compatible with a dual role in transcriptional regulation. Perhaps X protein located in the nucleus functions at the promoter level, as suggested by its ability to facilitate the binding of ATF/CREB to DNA (39) and to stimulate promoters when tethered to a specific DNA-binding domain (50, 67). X protein located in the cytoplasm might then be involved in influencing the regulation of second-messenger systems, such as activation of NF-κB. Clearly, a more rigorous analysis of the distribution of X protein should help to establish the subcellular location in which it acts.

The experiments described here have demonstrated that X protein does not utilize PKC to activate NF-κB, but they do not address the mechanism by which activation occurs. Conceivably, X protein could destabilize the association between IκB and NF-κB by directly binding the inhibitor and altering its conformation. Destabilization could also be directly mediated by X protein modification of IκB. In this regard, IκB has been shown to be inhibited by phosphorylation (reviewed in reference 35) or in some manner by an oxidation reaction (47). Alternatively, X protein could indirectly activate NF-κB by stimulating components of the signaling pathways involved in its activation. Activation of NF-κB by tumor necrosis factor alpha (40), by protein synthesis inhibitors (49), and by double-stranded RNA (36, 68) appears to be mediated independently of PKC. It is not known whether mechanisms of activation by these agents converge on a common, unknown mediator or involve a variety of mediators. It is therefore important to determine

whether reactive oxygen intermediates, recently shown to potentially account for activation of NF-κB by both PKC-dependent and -independent mechanisms (47), might also account for activation by X protein.

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