Hepatitis B Virus X Protein Activates Transcription by Bypassing CREB Phosphorylation, Not by Stabilizing bZIP−DNA Complexes†

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ABSTRACT: Although previous work has shown that the hepatitis B virus X protein (pX) stabilizes complexes between basic region leucine zipper (bZIP) proteins and target DNA, the relationship between enhanced binding and transcriptional activation has not been established. Here we show that interactions between CREB and pX, which coincidentally enhance DNA affinity, are necessary but not sufficient for increased transcriptional potency. Further, we show that transcriptional activation by pX requires a form of CREB in which Ser-133 is not phosphorylated. By stimulating the transcriptional potency of unphosphorylated CREB, pX can up-regulate the expression of cAMP-responsive genes implicated in hepatocyte proliferation, leading ultimately to the development of liver cancer after viral infection.

Over 350 million individuals worldwide are infected with the hepatitis B virus (HBV),[1] and epidemiological studies have identified a direct correlation between chronic HBV infection and development of hepatocellular carcinoma (HCC) (*1*−*3*). HBV is the smallest known DNA virus, containing four open reading frames that together encode seven proteins (*3*). One of these proteins, the X protein (pX), is conserved among most mammalian hepadnaviruses, but is not homologous to any other known protein sequence (*4*, *5*). pX is required for viral infection (*6*−*8*), transforms rodent cells (*9*, *10*), and induces HCC in transgenic mice (*11*, *12*). These data implicate pX as a critical factor in the development of HCC after HBV infection.

Early work demonstrated that pX contains a transcription activation domain (*13*, *14*). When brought to the DNA by a sequence-specific DNA binding protein, pX functions as a potent activator of various cellular and viral enhancers, including its own HBV enhancer (*8*, *15*, *16*). However, the precise events involving pX that result in transcriptional activation are unclear and controversial. In the cytoplasm, pX can influence the nuclear localization of NF-κB (*17*) and p53 (*18*) and increase the concentration of active GTP-bound Ras to activate the signal transduction cascade linking Ras, Raf, and MAP kinases (*19*). In addition, pX has been reported to function as a protein kinase (*20*), a protease inhibitor (*21*), and a ribo/deoxy ATPase (*22*). In the nucleus, pX can interact with general transcription factors, such as TFIIB (*23*), TFIIH (*24*), the TATA binding protein (TBP) (*25*), and RNA polymerase II (*26*). pX can also interact with transcriptional activators (discussed below) and can function as a transcription activation domain in vivo when fused to a Gal4 DNA binding domain (*13*, *14*).

Several lines of evidence suggest that the family of transcriptional activators known as basic region leucine zipper (bZIP) proteins are important targets of pX in the nucleus. First, transcriptional regulation of the HBV enhancer by pX requires an imperfect cyclic AMP response element (CRE) (*27*−*29*), the target of the CRE binding protein/activating transcription factor (CREB/ATF) subfamily of bZIP proteins. Second, methylation interference, nitrocellulose filter binding assays, and GST pull-down assays, as well as the results of two-hybrid screens, support a model where pX interacts directly with both CREB and ATF-2, two CREB/ATF family members (*30*, *31*). Studies with bZIP peptides identified the 60 amino acid DNA binding domain (termed the bZIP element) of CREB and ATF-2 as the primary site of interaction with pX (*30*, *32*, *33*). Moreover, quantitative electrophoretic mobility shift assays demonstrated that HBV pX increases the DNA affinity of bZIP element peptides derived from CREB and ATF-2 by enhancing both bZIP dimerization and DNA binding (*30*−*33*). The stabilizing effect of pX on bZIP·DNA complexes is DNA sequence-dependent, and as a result pX influences the DNA specificities of the proteins with which it interacts (*30*−*33*).

It has been proposed that HBV pX increases the transcriptional activity of a bZIP protein solely by enhancing the stability of the bZIP·DNA complex and hence the occupancy of the DNA target site at a given protein concentration (*30*, *31*, *33*, *34*). Here we use quantitative electrophoretic mobility shift assays (EMSA) and in vitro

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[1] Abbreviations: HBV, hepatitis B virus; pX, protein X of HBV; bZIP, basic region leucine zipper protein; CRE, cyclic AMP responsive element; CREB, CRE binding protein; P-CREB, phosphorylated CREB; HCC, hepatocellular carcinoma; TBP, TATA binding protein; ATF-2, activating transcription factor-2; P-ATF-2, phosphorylated ATF-2; EMSA, electrophoretic mobility shift assay; C/EBP, CAAT element binding protein; HTLV-1, human T-cell leukemia virus type 1; TRE, tax responsive element; CBP, CREB binding protein; KID, kinase inducible domain; Pol II, polymerase II; CREM, CRE modulating protein; TK, herpes virus thymidine kinase promoter; AdML, adenovirus major late promoter; PKA, cyclic AMP-dependent protein kinase.

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transcription assays to test this hypothesis. We find that the effect of pX on the transcriptional potency of CREB is maximal under conditions where the DNA is fully saturated with CREB in the absence of pX. We conclude that the effect of pX on transcription does not result from an enhancement in bZIP·DNA complex stability. Instead, our results suggest that pX augments and deregulates transcription by bypassing the need to phosphorylate CREB to achieve high transcriptional levels. By stimulating the transcriptional potency of unphosphorylated CREB, pX mimics the effect of elevated cAMP levels and could up-regulate expression of genes implicated in hepatocyte proliferation and the development of liver cancer after viral infection.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by use of standard solid-phase methods and an Expedite 8900 nucleic acid synthesizer. All DNA fragments described were double stranded, and the sequences were as follows: CRE_{33}: CTCTGCGTGGAGATGACGTCATCTCGTCTCTGC; $AP1_{32}$: CTCTGCGTGGAGATGACTCATCTCGTCTCTGC; TK promoter: AATTCAGCATGCTCTCGAGCCCATGGGTTCGCATATTAAGGTGACGCGTGTGGCCTCGAACACC; 3XCRE: CCTCTGCGTGGAGATGACGTCATCTCGTCTCTGCTCTGCGTGGAGATGACGTCATCTCGTCTCTCTCTGCGTGGAGATGACGTCATCTCGTCTCTGC; 3XAP1: CCTCTGCGTGGAGATGACTCATCTCGTCTCTGCTCTGCGTGGAGATGACTCATCTCGTCTCTCTCTGCGTGAGATGACTCATCTCGTCTCTGC; 146-base DNA standard: AGAGAATCTCGTAGAAAGAAGAAAGAATATGTGAAATCTT TAGAGAACAGAGTGGCAGTGCTTGAAAACCAAAACAAAACATTGATTGAGGAGCTAAAAGCACTTAAGGACCTTTACTGCCACAAGTCAGATTAATAGGATCCGCG; CRE26: GCAGTGGAGATGACGTCATCTCGTGC and its complement biotinylated at the 3′ end; AP125: GCAGTGGAGATGACTCATCTCGTGC and its complement biotinylated at the 3′end. The 3′-biotin was attached using Bio-TEG CPG obtained from Glen Research.

Protein Expression and Purification. The complete sequence of rat CREB (amino acids 1−341), hereafter called CREB, was produced in BL21(DE3) cells using pET15b CREB (*35*). The complete human sequence of ATF-2 (amino acids 1−505), hereafter called ATF-2, was produced in BL21(DE3) cells using pET15b ATF-2 (*36*). Transformed cells were grown at 37 °C and the flasks rotated at 250 rpm until the solutions reached an optical density of 0.8 absorbance unit at 600 nm. IPTG was added to a final concentration of 1 mM, and incubation and shaking were continued for 3.5 h. The cells were harvested and resuspended in 1× CREB buffer [10 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol]. The cells were lysed using one freeze/thaw cycle and heated to 70 °C for 10 min. The soluble fraction of the lysate was collected and the bZIP protein precipitated upon addition of solid ammonium sulfate to 20% (CREB) or 30% (ATF-2) saturation (*37*). Precipitated bZIP protein was resuspended in 1× CREB buffer and purified further by ion exchange chromatography on a DEAE 650M toyopearl resin. The protein was eluted using a gradient of 30 mM KCl to 500 mM KCl in 1× CREB buffer. Fractions containing CREB or ATF-2 were pooled, concentrated by ultrafiltration using a centriprep 10 concentrator, dialyzed into 1× CREB buffer containing 15% glycerol, frozen on dry ice, and stored at −70 °C. HBV pX and the CREB bZIP element peptide (C69) were expressed and purified as described previously (*32*, *38*). Protein concentrations were determined by amino acid analysis.

Preparing Reporter Plasmids for Transcription Experiments. pTKCRE and pTKAP1 were prepared in two steps from the reporter plasmid p(C2AT)19 which contains a G-less region of 380 base pairs (*39*). Base pairs −35 to +1 of the herpes virus thymidine kinase (TK) gene were inserted between the *Eco*RI and *Sac*I restriction sites in p(C2AT)19 (*39*) to generate pTK. pTKCRE and pTKAP1 (Figure 2A) were prepared from pTK by inserting the 3XCRE or 3XAP1 fragments defined above between the *Nco*I and *Sph*I restriction sites located immediately upstream of the TK promoter (at position −35). pAdML190 contains base pairs −400 to +1 of the adenovirus major late (AdML) promoter upstream of a G-less region of 190 base pairs (Figure 2A) (*40*). Circular plasmids were purified on QAE Sephadex resins using a gradient elution of between 550 and 650 mM NaCl in TE buffer [50 mM Tris (pH 7.9), 10 mM EDTA]. Circular DNA was stored at 4 °C.

In Vitro Phosphorylation of CREB and ATF-2 with cAMP-Dependent Kinase. CREB and ATF-2 were phosphorylated in vitro by use of cAMP-dependent protein kinase (PKA). Reactions containing CREB and ATF-2 were performed identically. At least a 100-fold molar excess of each protein was incubated with PKA in 1× CREB buffer containing 2 mM ATP for 60 min at 30 °C. Phosphorylated proteins, hereafter referred to as P-CREB and P-ATF-2, were stored at −70 °C. The efficiency of CREB and ATF-2 phosphorylation was determined by comparing the extent to which the untreated and treated bZIP proteins reacted further with [γ-^{32}P]ATP and PKA. Analytical reactions contained 200 nM aliquots of the reacted or untreated bZIP protein, 200 nM PKA, and 10 μCi of [γ-^{32}P]ATP and were incubated for 1 h at 30 °C. The reactions were denatured by heating to 95 °C for 2 min and loaded onto a denaturing 10 × 8 cm gel with a separating layer containing 8.4% (29:1 acrylamide: bisacrylamide) in 50 mM Tris-HCl (pH 8.8) and 1% SDS and a stacking layer containing 4% (29:1 acrylamide: bisacrylamide) in 50 mM Tris-HCl (pH 6.8) and 0.1% SDS. Comparison of the untreated and treated proteins indicated that 91−98% of CREB and ATF-2 were phosphorylated.

Electrophoretic Mobility Shift Assays (EMSA). In general, EMSA were performed as described previously (*32*). Binding reactions performed with CRE_{33} or $AP1_{32}$ contained 5 μM pX, <50 pM duplex [5′-^{32}P]DNA, and between 1 μM and 1 nM bZIP protein (CREB, ATF-2, P-CREB, P-ATF-2) in PBS reaction buffer [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 10 mM EDTA, 0.034% NP40, 10% glycerol, 0.1 mg/mL BSA, 0.4 μg/mL poly dI-dC−poly dI-dC, 1 mM DTT (pH 7.4)]. The 9 or 10 bp CRE and AP1 sequences contained in CRE_{33} and $AP1_{32}$ are sufficient to support bZIP·pX interactions (*32*). Binding reactions were incubated for 45 min at room temperature (*32*) and applied to a 16 × 18 cm nondenaturing 5% (79:1 acrylamide: bisacrylamide) gel prepared in Tris−glycine (TG) buffer [20 mM Tris base, 153 mM glycine (pH 8.5)]. Gels were maintained at 25 °C during electrophoresis by immersion in a circulating, temperature-controlled water bath. The amount of complexed and free DNA was quantified on a Molecular Dynamics Storm PhosphorImager. ΔG_{obs} values were

calculated from the equation: $\Delta G_{obs} = -RT \ln (1/K_{app})$ where $R = 0.001987$ kcal·mol^{-1}·K^{-1}and $T = 298$ K. $\Delta\Delta G_{pX}$ values were calculated from the relationship: $\Delta\Delta G_{pX} = \Delta G_{obs}$(with pX) − ΔG_{obs}(without pX). Binding reactions performed with 3XCRE or 3XAP1 contained 10 nM duplex [5′-^{32}P]DNA and between 2 μM and 15 nM bZIP protein in transcription buffer [8.1 mM Hepes (pH 7.9), 5.4 mM Tris-HCl, pH 7.8, 39.3 mM KCl, 0.4 mM EDTA, 6.7 mM $MgCl_2$, 0.01% NP-40, 12.5% glycerol, 2.8 mM DTT] supplemented with 100 μg/mL BSA and 0.4 μg/mL poly dI-dC−poly dI-dC. Binding reactions were incubated for 30 min at room temperature and analyzed as described. Control experiments demonstrated that this length of time was sufficient to allow all binding reactions to reach equilibrium.

Depletion of HeLa Nuclear Extracts of CRE and AP1 Binding Proteins. HeLa nuclear extracts (Promega) were depleted of CRE or AP1 binding proteins upon treatment with magnetic beads (Dynal) bearing duplex CRE_{26} and $AP1_{25}$ oligonucleotides. Depletions were performed by incubating 320 μL (40 reactions) of extract with 4 mg beads loaded with 700 pmol of DNA for 45 min at 4 °C. The beads were removed, and the depletion cycle was repeated 2 additional times. The depletion process decreased the CRE and AP1 binding activities of the extracts by 1 order of magnitude as assessed by EMSA and diminished transcription from the pTKCRE reporter plasmid by 0.4-fold while not significantly affecting transcription from the control AdML190 reporter plasmid.

In Vitro Transcription Reactions. In vitro transcription reactions were performed in depleted HeLa cell nuclear extracts in a total reaction volume of 28 μL. Briefly, bZIP protein or peptide (CREB, ATF-2, P-CREB, P-ATF-2, or C_{69}) and pX (if added) were preincubated for 30 min at 25 °C with 500 ng of pTKCRE or pTKAP1 and 250 ng of AdML190 in transcription buffer supplemented with 20 units of RNase T1 (Calbiochem), 0.6 mM ATP, 0.6 mM UTP, 0.1 mM 3′-OMe-GTP, 4 μM CTP, and 10 μCi of [α-^{32}P]-CTP (*40*). Transcription was initiated upon addition of 4−7 μL of depleted HeLa nuclear extracts and allowed to progress for 1 h at 30 °C. Transcription was halted by addition of 7 volumes of stop buffer [0.3 M Tris-HCl (pH 7.4 at 25 °C), 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, and 3 μg/mL yeast tRNA]. An aliquot of a 5′-^{32}P-labeled 146 bp DNA standard was added at this time to monitor material loss during workup. Transcription reactions were extracted with phenol/chloroform, and the nucleic acid was precipitated with ethanol and applied to a denaturing 6% (19:1 acrylamide: bisacrylamide) gel in 1× TBE [45 mM Tris base, 45 mM boric acid, 1 mM EDTA (pH 8.0)]. Radioactivity was quantified using a Molecular Dynamics Storm PhosphorImager. The intensity of the 380-base RNA product produced from the pTKCRE or pTKAP1 reporter was background-corrected using an adjacent region on the gel and normalized versus the 190/210-base control RNA band from the AdML190 reporter.

RESULTS

pX Enhances the Affinities of CREB and ATF-2 for the CRE Site, but Not the AP1 Site. As a first step toward assessing the relationship between the effect of pX on bZIP·DNA complex stability and transcriptional activation, we performed quantitative electrophoretic mobility shift assays to determine the effect of pX on the equilibrium stabilization of complexes between DNA and full-length CREB or ATF-2. Experiments were performed with a 33 bp oligonucleotide containing the CRE site (ATGACGTCAT) recognized preferentially by CREB/ATF proteins (*41*, *42*) as well as a 32 bp oligonucleotide containing the AP1 site (ATGACTCAT) recognized by proteins related to Fos/Jun and GCN4 (*41*, *42*). The 9 or 10 bp CRE and AP1 sequences are known to be sufficient for formation of a DNA·bZIP dimer·pX quaternary complex (*30*−*32*).

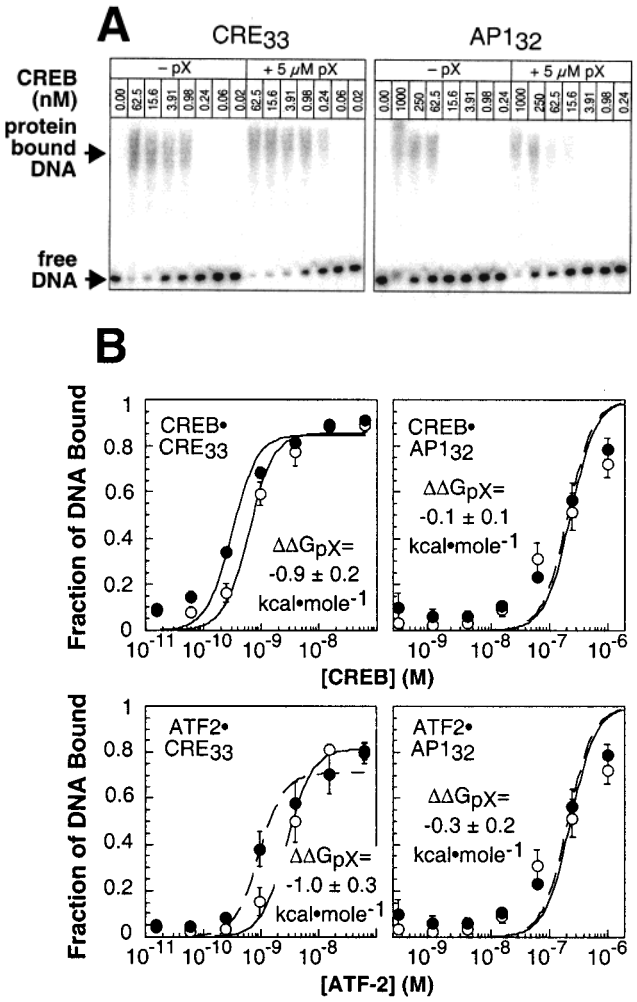

FIGURE 1: pX enhances the stability of bZIP·CRE complexes, but not bZIP·AP1 complexes. (A) EMSA experiments performed with ^{32}P-labeled CRE_{33} or $AP1_{32}$ duplex oligonucleotides in the presence of the indicated amount of CREB and pX. The locations of free DNA and protein-bound DNA are indicated with arrows. (B) Shown are binding curves illustrating the fraction of CRE_{33} or $AP1_{32}$ bound (Θ) as a function of $[CREB]_T$ or $[ATF\text{-}2]_T$ in the presence (filled circles) and absence (open circles) of 5 μM pX. The curves shown represent the best fit of the data to the equation: $(\Theta) = 1/(1 + K_{app}/[protein]_T^2)$ where K_{app} is the adjustable parameter (*76*). Each data point represents the average of at least 3 independent determinations. The error bars denote the standard error (*77*). $\Delta\Delta G_{pX}$ values were calculated from the relationship: $\Delta\Delta G_{pX} = \Delta G_{obs}$(with pX) − ΔG_{obs}(without pX).

As anticipated from experiments utilizing bZIP element peptides from CREB, ATF-2, and GCN4 (*32*), pX enhanced the binding free energies of bZIP protein·DNA complexes in a DNA sequence-dependent manner (Figure 1). These changes in the binding free energies are referred to as $\Delta\Delta G_{pX}$ values. pX stabilized the CREB·CRE_{33} complex by $\Delta\Delta G_{pX}$

$= -0.9 \pm 0.2$ kcal·mol^{-1} but had little effect on the stability of the CREB·AP1$_{32}$ complex ($\Delta\Delta G_{pX} = -0.1 \pm 0.1$ kcal·mol^{-1}). Similarly, pX stabilized the ATF-2·CRE$_{33}$ complex by $\Delta\Delta G_{pX} = -1.0 \pm 0.3$ kcal·mol^{-1} but had little effect on the stability of the ATF-2·P1$_{32}$ complex ($\Delta\Delta G_{pX} = -0.3 \pm 0.2$ kcal·mol^{-1}). Because both CREB and ATF-2 prefer the CRE site to the AP1 site in the absence of pX, this DNA sequence-dependent change in equilibrium stability in the presence of pX exaggerated the natural selectivity of CREB and ATF-2; pX increased the preferences of these proteins for the CRE site over the AP1 site by 0.7−0.8 kcal·mol^{-1}. Similar, although not identical, results were observed in experiments with bZIP element peptides derived from these proteins (*32*, *33*). We reasoned that if the effect of pX on transcription were limited to its effect on the stability of a bZIP·DNA complex at equilibrium, then this enhancement in CRE/AP1 specificity should manifest itself as a predictable change in transcription from CRE- and AP1-dependent reporter plasmids.

pX Augments the Transcriptional Potency of CREB, but Not ATF-2. To determine if pX influences transcriptional activation by CREB and ATF-2 in a manner that correlates with its effect on bZIP·DNA complex stability, we performed in vitro transcription reactions using HeLa nuclear extracts that were depleted of CRE and AP1 binding proteins (Figure 2). We used reporter plasmids containing three tandem CRE$_{33}$ (pTKCRE) or AP1$_{32}$ (pTKAP1) sites located immediately upstream of the thymidine kinase promoter (Figure 2A). These plasmids directed synthesis of a 380 nucleotide G-less RNA transcript (*39*). Transcription from these plasmids was inhibited by α-amanitin, confirming that these RNA transcripts were synthesized by RNA polymerase II (data not shown). The transcriptional efficiency of each reaction was determined by addition of a control plasmid (AdML190) which produced two G-less RNA transcripts containing 210 and 190 bases (Figure 2A). In the absence of exogenous bZIP protein, pX had little effect on transcription from the CRE- or AP1-containing reporter plasmids or from the activator-independent control plasmid (Figure 2B, compare lanes 1 to 2, 7 to 8, 13 to 14, and 19 to 20). In the absence of pX, CREB had a small, concentration-dependent effect on CRE- or AP1-dependent transcription (Figure 2B, compare lanes 1, 3, 5 and 7, 9, 11). At 0.5 μM CREB, transcription from the CRE or AP1 reporters was marginally stimulated 2-fold, while at 1.0 and 2.0 μM CREB, transcription from these reporters was inhibited 2−3-fold. The inhibition of promoter-specific transcription was due to high protein concentrations in the transcription reactions (*43*). ATF-2 had no effect on transcription from either the pTKCRE or the pTKAP1 constructs at any concentration tested.

Experiments to assess the increase in the transcriptional potency of a bZIP protein in the presence of pX contained 5 μM pX and between 0.25 and 2 μM CREB or ATF-2. The greatest fold increase (6 ± 1.2-fold) in CRE-dependent transcription with pX was observed at 1 μM CREB (Figure 2B, compare lanes 5 and 6). A more modest fold increase (2.6 ± 0.5-fold) was observed at 0.5 μM CREB, and little increase (1.4 ± 0.4-fold) was observed at a CREB concentration of 2 μM. In addition, pX augmented transcription in the presence of 1.5 μM CREB by 4.0-fold (data not shown). By contrast, transcription from the AP1 reporter was only modestly affected by pX (Figure 2B, compare lanes 9 to 10 and 11 to 12). Only at 2 μM CREB was a small pX-dependent increase in transcription (2.8 ± 1.3-fold) observed. Importantly, the augmentation of CRE-dependent transcription by pX with CREB was independent of the inhibition of transcription observed at high CREB concentrations. Despite the fact that high CREB concentrations inhibited transcription from both the CRE and AP1 promoters, pX only significantly augmented transcription by CREB with the CRE reporter. pX had no effect on CRE- or AP1-dependent transcription at any concentration of ATF-2 tested (Figure 2B).

A comparison of the in vitro DNA binding and transcription results suggests a correlation between the two in certain cases. In the case of CREB, the effects of pX on DNA binding and transcription appear to correlate. As predicted, pX stimulated CRE-dependent transcription but had little or no effect on AP1-dependent transcription. This result is consistent with the relative effects of pX on the equilibrium stabilities of CREB·CRE ($\Delta\Delta G_{pX} = -0.9$ kcal·mol^{-1}) and CREB·AP1 ($\Delta\Delta G_{pX} = -0.1$ kcal·mol^{-1}) complexes. However, in the case of ATF-2, the effects of pX on DNA binding and transcription do not correlate; although pX increased the equilibrium stability of the ATF-2·CRE complex, it had no effect on CRE-dependent transcription. ATF-2 is known to be transcriptionally silent in the absence of an interaction with a coactivator such as the adenovirus E1a protein (*44*). E1A disrupts the protein−protein interactions that silence the transcriptional potential of ATF-2. Our studies show that pX cannot also supply this function. Because the transcriptional potency of ATF-2 was insensitive to pX, subsequent experiments were performed only with CREB.

Effect of pX on Transcription Is Independent of Promoter Occupancy by CREB. Our studies with CREB described above support the hypothesis that the effect of pX on transcription results solely from the increase in stability it provides to bZIP·DNA complexes. The physical ramification of this effect at a given promoter is an increase in DNA occupancy within a defined range of bZIP protein concentrations, illustrated hypothetically in Figure 3A. Therefore, if the effect of pX on CRE-dependent transcription described above results solely from an increase in DNA occupancy by CREB, then the increase in transcription should be largest at CREB concentrations where the DNA is minimally saturated. Importantly, the increase in transcription due to pX should be minimal under conditions where the DNA reporter is fully saturated with CREB, as illustrated hypothetically in Figure 3A.

Electrophoretic mobility shift assays were performed to evaluate the fractional saturation of the CRE and AP1 reporter constructs with CREB under precisely those conditions used in the transcription experiments and in the presence and absence of HeLa nuclear extracts (Figure 3B). Figure 3D displays the fraction of DNA molecules that were fully saturated with three CREB dimers as a function of protein concentration. The stoichiometries of the complexes identified in Figure 3B were assigned on the basis of side-by-side experiments with DNA molecules containing one or two CRE sites (Figure 3C). As expected, the fraction of fully occupied DNA molecules increased with increasing protein concentration. Notably, the CRE reporter was fully bound at a CREB concentration of 0.5 μM in the presence and absence of HeLa nuclear extracts (Figure 3B). By contrast, the AP1 reporter was bound minimally at the highest

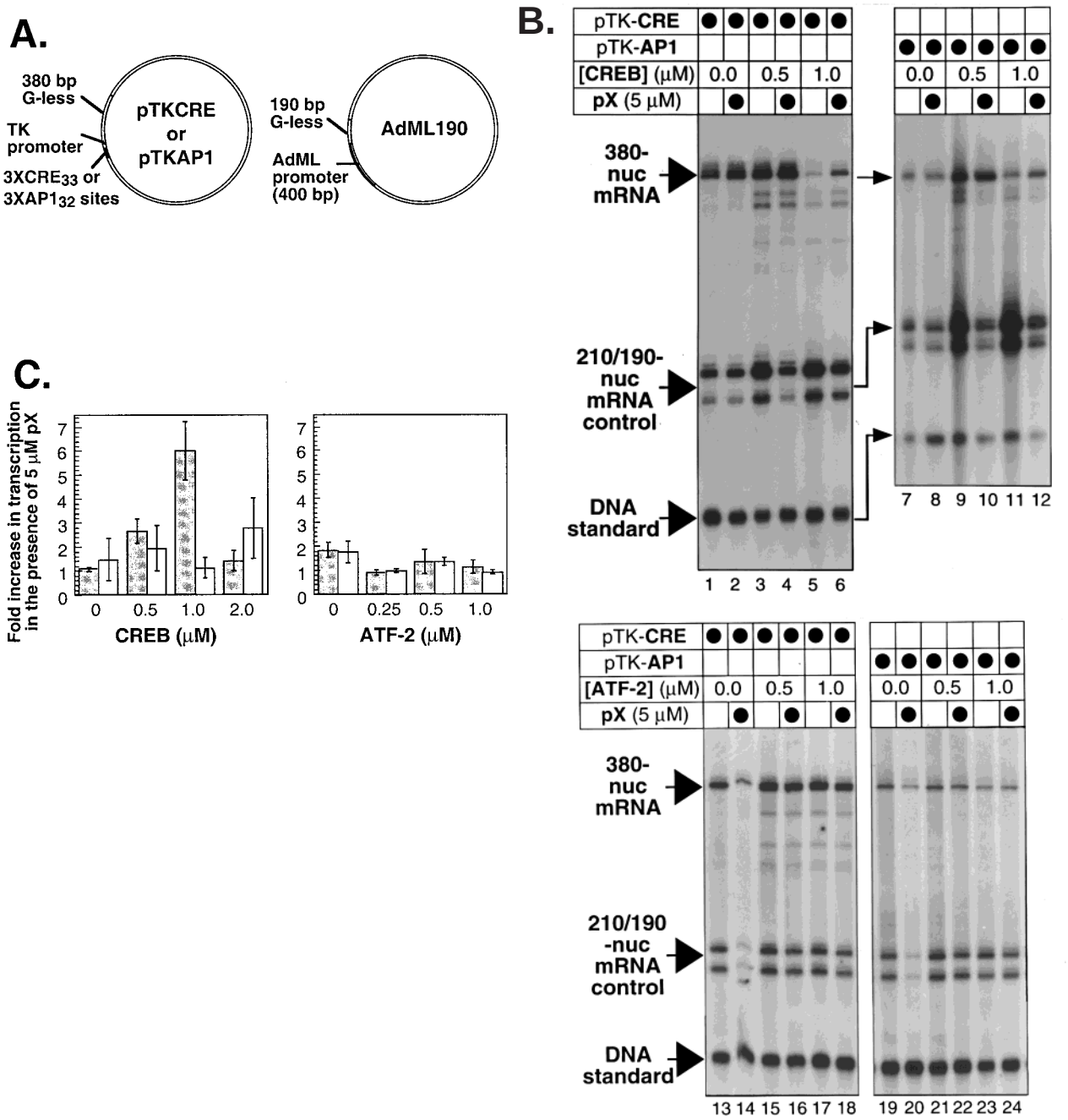

FIGURE 2: pX increases the transcriptional potency of CREB, but not ATF-2, in a DNA sequence-dependent manner. (A) Schematic representation of pTKCRE, pTKAP1, and AdML190 reporter plasmids used in transcription reactions. pTKCRE and pTKAP1 each contain 3 tandem CRE_{33} or $AP1_{32}$ sites, respectively, and the thymidine kinase promoter upstream of a G-less cassette of 380 bp. The control plasmid AdML190 contained 400 bp of the adenovirus 2 major late promoter upstream of a G-less cassette of 190 bp. (B) In vitro transcription reactions contained 0, 0.5, or 1 μM CREB and ATF-2, either the pTKCRE or pTKAP1 reporter plasmid, and 5 μM pX (when added). The reactions also contained AdML190 and a 146-nucleotide DNA fragment as an internal control. The RNA and DNA fragments are indicated with arrows. (C) Shown are histograms representing the fold increases in CRE-dependent (shaded bars) or AP1-dependent (open bars) transcription as a function of bZIP protein concentration. Fold increase in transcription represents the ratio of mRNA yield from identical reactions performed in the presence and absence of pX. The data are an average of at least 3 trials, and the standard error is indicated with error bars (*77*).

concentration of CREB studied (24 ± 1%). Thus, the CRE reporter was fully bound by three CREB dimers at a concentration below that where pX maximally enhanced transcription (0.5 or 1 μM). Moreover, occupancy of the CRE reporter was equivalent at 1.0 and 2.0 μM CREB, whereas the fold increase in transcription due to pX was very different at these concentrations (Figure 2C). Taken together, these results argue that interactions between CREB and pX, which coincidentally enhance DNA affinity, are necessary but not sufficient for an increase in transcriptional potency.

Regions Outside the bZIP Element Peptide of CREB Are Required for pX-Dependent Increase in Transcription. To determine if regions of CREB outside the bZIP element are required for transcriptional activation by pX, in vitro transcription reactions were performed with a peptide comprising the minimal bZIP element of CREB, amino acids 273−341 (C_{69}), in place of the CREB protein (*38*, *45*). EMSA as well as direct protein−protein interaction assays have previously demonstrated that C_{69} is sufficient to effectively recruit pX to the bZIP·DNA complex (*30*, *32*,

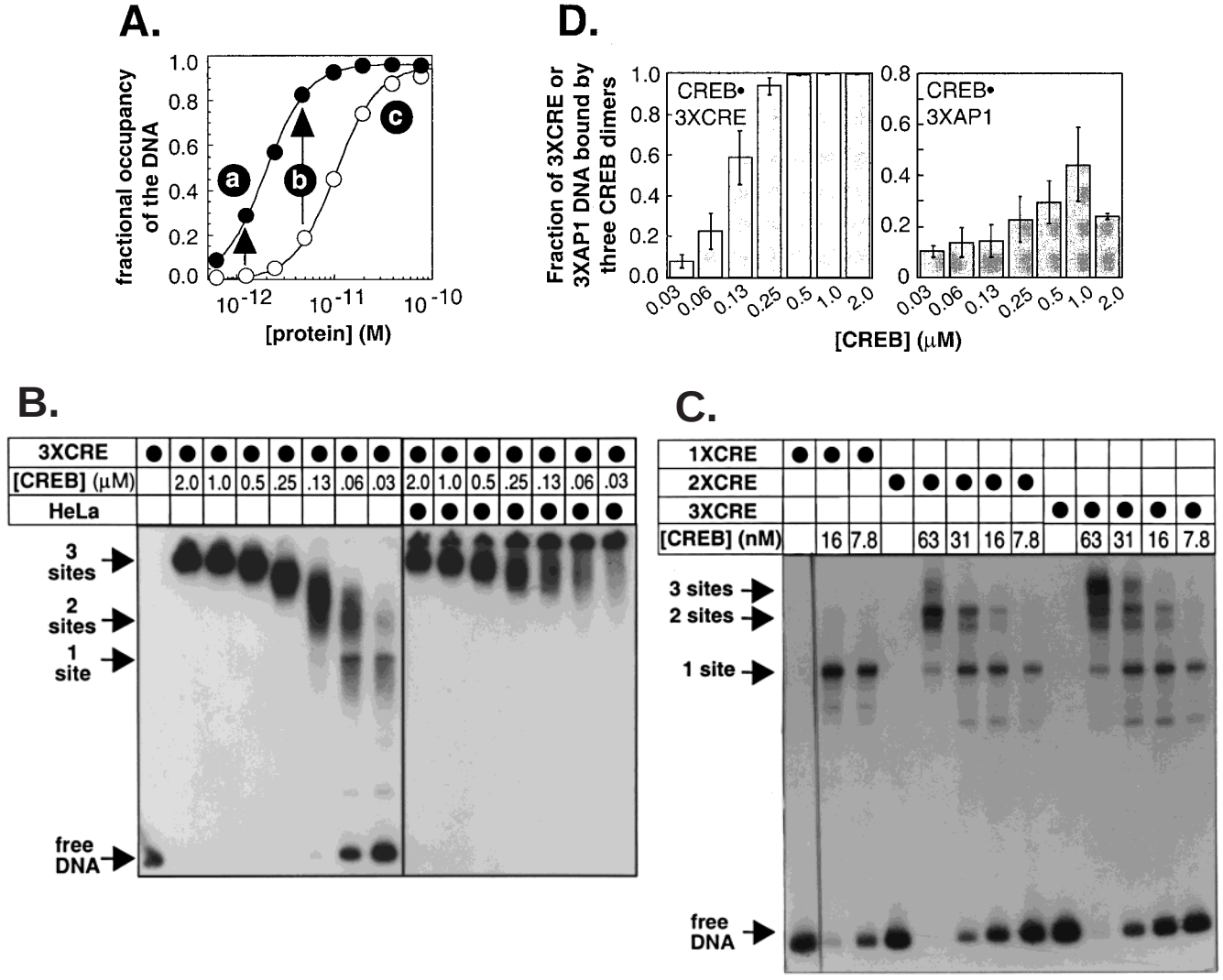

FIGURE 3: DNA site occupancy of CREB and ATF-2 under transcription conditions. (A) Hypothetical illustration of how the effect of pX on the occupancy of a given DNA site increases and then decreases as the protein concentration increases near the apparent K_d of the protein·DNA complex. Open symbols follow the hypothetical binding isotherm in the absence of pX; the solid symbols follow the hypothetical binding isotherm expected if pX were to increase the stability of the protein·DNA complex by approximately 2 kcal·mol^{-1}. In this hypothetical example, the effect of pX on DNA occupancy is small at [protein] = 10^{-12} M [see (a)], large at [protein] = 10^{-11} M [see (b)], and small again at [protein] = 10^{-10} M [see (c)]. (B) Shown are EMSA representing the binding of CREB to a 97 bp fragment of the pTKCRE reporter containing 3 tandem CRE_{33} (3XCRE) sites and various concentrations of CREB. The bands representing DNA fragments with 1, 2, or 3 sites bound by protein are indicated with arrows. Analogous experiments were performed with a 94 bp fragment of the pTKAP1 reporter (data not shown). (C) Shown are EMSA representing the binding of CREB to a 97 bp fragment of the pTKCRE reporter containing 1(1XCRE), 2(2XCRE), or 3(3XCRE) tandem CRE_{33} sites and various concentrations of CREB. (D) Histograms representing the fraction of 3XCRE or 3XAP1 DNA bound at the concentrations of CREB shown in the absence of HeLa nuclear extract. The data are an average of at least 3 experiments, and the standard error is indicated (*77*).

34). As expected, pX had no effect on transcription in the absence of C_{69} (Figure 4A, compare lanes 1 and 2). Whereas pX stimulated CRE-dependent transcription 6-fold in the presence of 1.0 μM CREB, pX had no effect on transcription in the presence of C_{69} at any concentration tested (Figure 4B). To provide additional evidence that C_{69} did not support pX-dependent transcription, we monitored the effect of increasing concentrations of C_{69} on transcription in the presence of 1 μM CREB and 5 μM pX. The effect of pX on CRE-dependent transcription by CREB was attenuated to a basal level upon addition of an equimolar concentration of C_{69} (Figure 4C). This result is consistent with the observation that a peptide comprising the bZIP element of C/EBP inhibited pX-dependent transcription from a reporter containing three C/EBP target sites (*13*). Taken together, these results demonstrate that regions of CREB that lie outside the minimal DNA binding domain are required for transcriptional stimulation by pX.

pX-Dependent Transcriptional Activation Requires the Unphosphorylated Form of CREB. Phosphorylation of CREB at Ser-133 by cAMP-dependent protein kinase significantly increases its transcriptional potency (*46, 47*). To determine if the effect of pX on transcriptional activation by CREB is phosphorylation-dependent, we evaluated the effect of pX on (1) the DNA affinity of phosphorylated CREB and (2) CRE-dependent transcription in the presence of phosphorylated CREB.

CREB was phosphorylated in vitro with cAMP-dependent protein kinase, and the affinity of P-CREB for CRE_{33} and $AP1_{32}$ DNA in the presence and absence of pX was

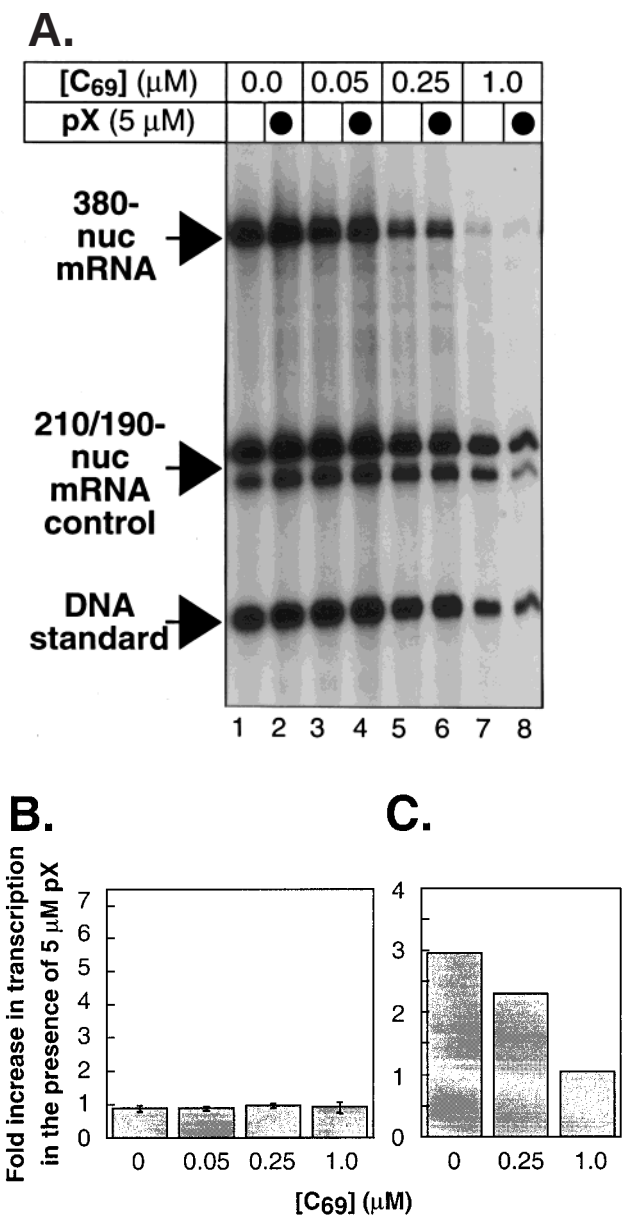

FIGURE 4: bZIP element of CREB does not support transcriptional activation by HBV pX. (A) In vitro transcription reactions contained the pTKCRE reporter plasmid, the bZIP element peptide of CREB (C_{69}) at the concentrations indicated, and 5 μM pX (where indicated). The bands corresponding to the expected 380 and 210/190 nucleotide mRNA products and the internal DNA standard are indicated with arrows. (B) A histogram representing the fold increase in CRE-dependent transcription due to pX in the presence or absence of C_{69} at the concentrations shown. For comparison, the fold enhancement was displayed using the same scale as in Figure 2C. The data are an average of at least 3 trials, and the standard error is indicated with error bars (*77*). (C) A histogram representing the fold enhancement in CRE-dependent transcription due to pX in the presence of 1 μM CREB and the indicated concentrations of C_{69}. Note that the preparation of pX used here was less active than the average preparation used in Figure 2B.

monitored by EMSA. Parallel experiments with P-ATF-2 were also performed as a control. Phosphorylation did not change the effect of pX on bZIP·DNA complex stability (Figure 5A). As was observed with unphosphorylated proteins, pX significantly enhanced the stabilities of P-CREB·CRE_{33} and P-ATF-2·CRE_{33} complexes ($\Delta\Delta G_{pX} = -1.3 \pm 0.4$ and -1.1 ± 0.4 kcal·mol^{-1}, respectively) but had a reduced effect on P-CREB·$AP1_{32}$ and P-ATF-2·$AP1_{32}$ complex stabilities ($\Delta\Delta G_{pX} = -0.0 \pm 0.1$ and -0.7 ± 0.2 kcal·mol^{-1}, respectively). The similarity of the $\Delta\Delta G_{pX}$ values measured before and after phosphorylation implies that recognition of the bZIP·DNA complex by pX and the effect of this recognition on complex stability are independent of phosphorylation.

As expected, phosphorylation of CREB at Ser-133 increased transcription from the pTKCRE reporter 4−6-fold in the absence of pX (Figure 5B, compare lane 5 to lane 11)(*46, 47*). However, under conditions where pX increased the transcriptional potency of 0.5 μM unphosphorylated CREB by 2.6-fold and 1 μM unphosphorylated CREB by 6-fold (Figures 2B and 5B, compare lanes 5 and 6), pX had no effect on the transcriptional potency of P-CREB (Figure 5B, compare lanes 11 and 12). Interestingly, the combination of 5 μM pX and 1 μM CREB produced the same yield of 380-nucleotide RNA product as did 1 μM P-CREB alone (Figure 5B, compare lane 6 to lane 11 or 12). Thus, pX increased the affinity of P-CREB for the CRE site (Figure 5A), but this increase had no effect on transcriptional potency. Similar results were obtained in experiments with ATF-2. Despite the ability of pX to increase saturation of CRE and AP1 sites by P-ATF-2, pX had no effect on the transcriptional potency of P-ATF-2 at any concentration tested (Figure 5C). These results emphasize that the effects of pX on transcription are not due to an increase in promoter occupancy. Moreover, because the level of transcription with P-CREB was similar to that observed with a combination of pX and CREB, we conclude that pX bypasses the need for CREB phosphorylation to achieve full transcriptional potency.

DISCUSSION

DNA Binding versus Transcription Activation in the Presence of pX. Because pX interacts with bZIP proteins to enhance their affinities for DNA (*32*), it has been proposed that pX augments the transcriptional potencies of bZIP proteins by increasing their affinities for DNA target sites at equilibrium (*30, 31, 33*). Our initial goal was to test this hypothesis by determining if a correlation existed between the extent of in vitro stabilization of bZIP−DNA complexes by pX and the pX-dependent fold increase in transcription.

Two lines of evidence suggest that this correlation does not exist. If the effect of pX on the transcriptional potency of a bZIP protein were due solely to its ability to increase occupancy of the promoter by the bZIP protein, we would expect the effect of pX on transcription to be maximal at concentrations where the reporter is not fully saturated and minimal under conditions where the promoter is fully bound (Figure 3A). This trend was not observed. The fold increase in transcription due to pX was maximal under conditions where the CRE reporter was fully saturated by CREB and minimal under conditions where the reporter was bound partially. We would also expect that pX would have comparable effects on the transcriptional potencies of CREB and P-CREB, since it has comparable effects on the DNA affinities of these proteins. Again, this trend was not observed. While pX had little effect on CRE-dependent transcription by P-CREB, it significantly stabilized the P-CREB·CRE complex. Taken together, our results exclude the possibility that transcriptional stimulation by pX results

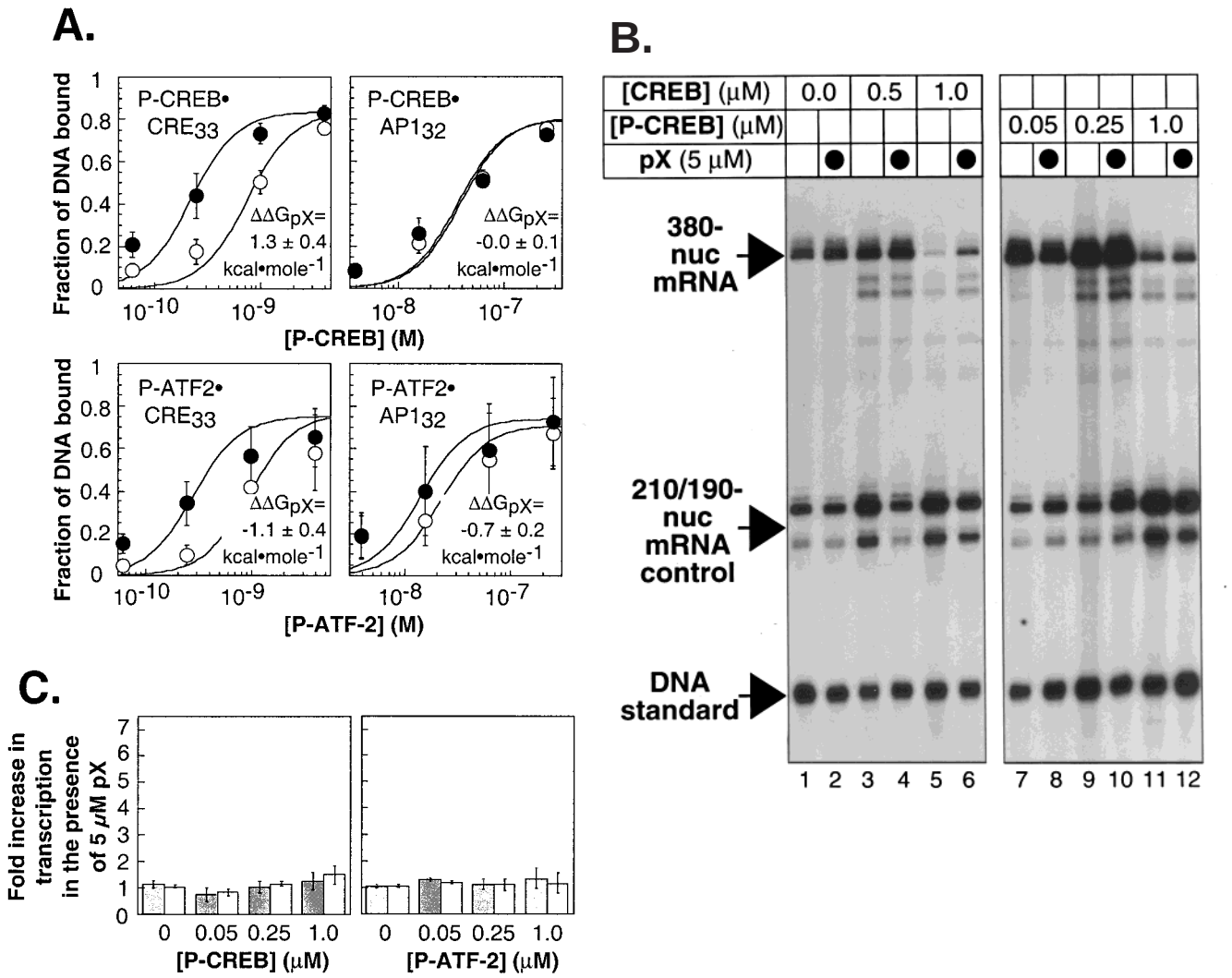

FIGURE 5: Phosphorylation of CREB abolishes the effect of pX on transcription but not its effect on bZIP•DNA stability. (A) Shown are binding curves illustrating the affinities of P-CREB or P-ATF-2 for CRE_{33} or $AP1_{32}$ DNA in the presence (filled circles) and absence (open circles) of 5 μM pX. The plots illustrate the fraction of DNA bound as a function of total protein concentrations as described in Figure 1 (*76*). The binding isotherms shown represent the average of at least 3 independent determinations, and error bars denote the standard error (*77*). (B) In vitro transcription reactions contained the pTKCRE reporter plasmid and the indicated concentrations of CREB or P-CREB in the presence and absence of 5 μM pX. The DNA standard and mRNA products are indicated with arrows. An aliquot of CREB that had been heated to 30 °C was as responsive to pX as unheated CREB, ensuring that the conditions used for in vitro phosphorylation did not alter transcriptional potency (data not shown). Additionally, transcription levels either in the presence or in the absence of pX were not altered when PKA was included in the reactions (data not shown). The gel illustrating data for CREB is identical to that shown in Figure 2B. (C) Shown are histograms representing the fold increases in CRE-dependent (shaded bars) or AP1-dependent (open bars) transcription as a function of bZIP protein concentration. Fold increase in transcription represents the ratio of mRNA yield from identical reactions performed in the presence and absence of pX. For comparison, the fold enhancement is displayed using the same scale as in Figure 2C. The data are an average of at least 3 trials, and the standard error is indicated with error bars (*77*).

solely from increased DNA occupancy. The interactions between CREB and pX, which coincidentally enhance DNA affinity, are not sufficient for a pX-dependent increase in transcription.

Mechanism of Transcriptional Activation by pX. We considered several different alternative mechanisms that might describe the effect of pX on transcription. First we considered the possibility that CREB simply recruits pX to the DNA and transcriptional activation is modulated by the activation domain within pX. This model implies that the only region of a bZIP protein required for a pX-dependent increase in transcription is the bZIP element, which interacts with pX (*30*, *32*, *34*). This model is consistent with the observation that pX (1) functions as a potent activation domain when fused to a Gal4 DNA binding domain (*13*, *14*), and (2) interacts in vitro with proteins such as TFIIB, TFIIH, TBP, and RNA polymerase II within the general transcription machinery (*23*−*26*, *48*). According to this model, pX functions in a manner similar to the human T-cell leukemia virus type I (HTLV I) Tax protein (*49*), another viral protein that interacts with bZIP proteins to enhance their affinities for DNA (*50*−*54*). Tax activates transcription from the viral CRE-like tax response element (TRE) in the presence of a minimal CREB bZIP element peptide, demonstrating that transcriptional activation does not require the activation domain of CREB (*55*). To determine if this model best described the mechanism of transcription activation by pX, transcription experiments with the CREB bZIP element peptide were performed. Our results indicate that the minimal bZIP element of CREB is not sufficient to support pX-

dependent transcription activation. Thus, we conclude that regions outside of the bZIP element are necessary for transcriptional activation by pX.

A second possibility is that CREB recruits pX to the promoter and the increase in transcription results from simultaneous action by the transcription activation domains on CREB and pX. Implicit in this model are interactions of CREB and pX with separate transcriptional targets that each individually contribute, perhaps synergistically (*56*), to the observed increase in transcription. This model is supported by experiments that monitored the effect of pX on the transcriptional potencies of fusion proteins containing a Gal4 DNA binding domain and the activation domain from various proteins, including p53, jun, fos, and herpes simplex virus VP16 (*57*). The extent of transcriptional stimulation by pX was dependent on the strength of the activation domain tested; pX potentiated the transcription of stronger activation domains, like those contained within p53, fos, and VP16, to a greater extent than it potentiated the transcription of weaker activation domains, like that contained within jun. To determine if this model best describes the mechanism of transcriptional activation by pX, transcription experiments were performed with phosphorylated CREB, the transcriptionally active form of CREB. If CREB and pX stimulate transcription through interactions with separate targets, then pX should augment the activity of phosphorylated CREB to the same or greater extent as it augmented the activity of unphosphorylated CREB. However, pX failed to augment the transcriptional activity of phosphorylated CREB (Figure 5C). Thus, the transcriptional activation domains of P-CREB and pX do not function additively. The observation that pX neither augments nor inhibits the transcriptional potency of P-CREB suggests one of three scenarios. It is possible that the interactions of P-CREB that result in enhanced transcription (such as its interaction with CBP) block the interactions of pX that result in enhanced transcription, or visa versa. A third possibility is that P-CREB and pX contact a common target.

Which member of the transcriptional machinery might CREB and pX interact with to augment transcription? As assessed by coimmunoprecipitation, CREB interacts with TFIIB and the human TBP-associated factor hTAF130, a component of the TFIID complex, through a glutamine-rich activation domain (Q2) (*43, 58*). Upon phosphorylation by the cAMP-dependent protein kinase, CREB also interacts with its coactivator, the CREB binding protein (CBP), through a kinase inducible domain (KID), and transcription levels are increased due to recruitment of RNA polymerase II (Pol II) by CBP (*59*−*63*). Because hTAF130 antisera inhibit the transcriptional activity of P-CREB, the association of P-CREB with CBP and hTAF130 must be required for activated transcription (*63*). These studies suggest that pX might stimulate transcription by stabilizing the association of CBP, Pol II, hTAF130, or TFIIB with the CREB·DNA complex (*64*). Alternatively, pX might structurally alter or globally orient the CREB activation domain, preorganizing it for association with members of the preinitiation complex. The fact that pX stimulates transcription by the viral activator VP16 and stabilizes the interaction of VP16 with its transcriptional target, TFIIB, suggests that pX augments transcription by recruitment of TFIIB to the promoter complex (*48*). Indeed, previous studies have shown that pX interacts with members of the preinitiation complex, including TFIIB (*23, 48*), TBP, a component of TFIID (*25*), and Pol II (*26*). Therefore, it is feasible that the stabilized association of TFIID, TFIIB, Pol II, or CBP with CREB through direct or indirect interaction with pX could increase the activated transcription mediated by CREB (*65*).

pX May Bypass the cAMP Pathway. Phosphorylation of CREB in vivo is catalyzed by the cAMP-dependent protein kinase, PKA, which is itself activated in response to elevated cellular levels of cAMP (*66*). The observations that transcriptional activation by pX requires the unphosphorylated form of CREB and that the level of transcription in the presence of pX approximates that seen with P-CREB in the absence of pX suggest that pX bypasses the need to phosphorylate CREB in order to produce a potent transcriptional signal. As P-CREB is the downstream product of the cAMP pathway, these data suggest that pX mimics activation of the cAMP pathway by up-regulating the function of unphosphorylated CREB.

The possibility that pX up-regulates CREB in the absence of signals that induce phosphorylation has implications regarding development of hepatocellular carcinoma (HCC). Regenerating livers, a model system for hepatocyte proliferation, contain significantly higher intracellular cAMP levels than do asymptomatic livers (*67, 68*). As a result, regenerating livers contain high levels of CREM (CRE modulating protein) and CREB (*69*) and up-regulated expression of genes implicated in hepatocyte proliferation, including crem (*69, 70*) and c-fos (*71*). Hepatocyte proliferation contributes strongly to HCC development as cellular proliferation correlates with the propensity of HBV transgenic mice to develop HCC (*72*). Therefore, by stimulating the transcriptional potency of unphosphorylated CREB, pX mimics the effect of elevated cAMP levels and up-regulates the expression of genes implicated in hepatocyte proliferation. In this way, pX resembles the recently discovered tissue-specific coactivator protein, ACT (activator of CREM in testis), which stimulates transcription by CREB and CREM in a phosphorylation-independent manner (*73*). Like pX, the presence of ACT bypasses the need for CREB and CREM phosphorylation by stimulating the level of transcription observed with unphosphorylated protein to the levels observed with phosphorylated proteins.

HBV pX and HTLV-I Tax May Use Different Mechanisms To Effect Up-regulation of CRE-Dependent Genes. The characteristics of pX are often compared to those of another viral transcription factor, the HTLV-1 tax protein (*33, 49*). Like pX, tax stimulates transcription from CRE-like promoters called tax response elements (TRE), within its viral enhancer and interacts with bZIP proteins, including CREB and ATF-2, to enhance their affinities for DNA (*50*−*54*). Our results point to a significant difference in the mechanisms by which cellular CRE promoters are activated by these two viral proteins. Transcriptional activation of cellular CRE promoters by tax requires CREB that is phosphorylated at Ser-133 (*74, 75*), whereas transcriptional activation of CRE sites by pX requires unphosphorylated CREB. Thus, although HBV pX and HTLV-1 tax both interact with common bZIP transcription factors, the current evidence suggests that they make use of different means to affect up-regulation of CRE-dependent genes.

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