

# The hepatitis B virus X protein targets the basic region–leucine zipper domain of CREB

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**ABSTRACT** The X gene product encoded by the hepatitis B virus, termed pX, is a promiscuous transactivator of a variety of viral and cellular genes under the control of diverse cis-acting elements. Although pX does not appear to directly bind DNA, pX-responsive elements include the NF- $\kappa$ B, AP-1, and CRE (cAMP response element) sites. Direct protein–protein interactions occur between viral pX and the CRE-binding transcription factors CREB and ATF. Here we examine the mechanism of the protein–protein interactions occurring between CREB and pX by using recombinant proteins and *in vitro* DNA-binding assays. We demonstrate that pX interacts with the basic region–leucine zipper domain of CREB but not with the DNA-binding domain of the yeast transactivator protein Gal4. The interaction between CREB and pX increases the affinity of CREB for the CRE site by an order of magnitude, although pX does not alter the rate of CREB dimerization. Methylation interference footprinting reveals differences between the CREB DNA and CREB–pX DNA complexes. These experiments demonstrate that pX alters the way CREB interacts with the CRE DNA and suggest that the basic, DNA-binding region of CREB is the target of pX. Transfection assays in PC12 cells with the CREB-dependent somatostatin promoter demonstrate a nearly 15-fold transcriptional induction after forskolin stimulation in the presence of pX. These results support the significance of the CREB–pX protein–protein interactions *in vivo*.

The 3.2-kb hepatitis B virus (HBV) genome contains four recognized open reading frames, three of which encode virion structural proteins. These include the surface and core antigens and viral polymerase (1). The fourth frame, conserved among all mammalian hepadnaviruses, encodes a 16.5-kDa protein, termed X antigen (1). The X gene is expressed during viral infection, producing a 1-kb mRNA (2). In transgenic mice, the HBV X protein (pX) induces liver cancer (3).

pX is a transactivator of transcriptional elements, such as those present within the HBV enhancer (4, 5), simian virus 40 enhancer (6), human immunodeficiency virus long terminal repeat (7–9), and the human interferon gene (10). X-responsive cis-acting elements include NF- $\kappa$ B (9), AP-1 (11), and cAMP response element (CRE) (12) sites. pX does not appear to bind DNA directly. Having multiple X-responsive cis-acting elements suggests that the mechanism of pX transactivation is pleiotropic (13). Activation by pX may involve targeting different regulatory steps in these signal transduction pathways or direct protein–protein interactions with diverse cellular transcription factors and the transcriptional machinery. Recent studies (14) with inhibitors of protein kinase C reported that pX acts indirectly by activating the protein kinase C transduction pathway, thereby increasing the activity of the cognate transcription factors. A more direct mechanism of pX transactivation is supported by earlier studies (11, 15) showing that pX possesses an activation domain. pX stimulates tran-

scription when targeted as a fusion with heterologous DNA-binding domains to specific DNA-binding sites (11, 15). This suggests that one mechanism of action of pX relies on protein–protein interactions with cellular transcription factors.

Maguire *et al.* (16) demonstrated direct protein–protein interactions between the viral pX and cellular transcription factors by showing *in vitro* that pX interacts directly with the CREB/ATF family of proteins. CREB is a leucine zipper transcription factor binding to the CRE motif as a homodimer (17, 18). The HBV enhancer contains a CRE-like sequence, TGACGCAA. In the absence of pX, CREB is incapable of binding *in vitro* to the CRE-like element of the HBV enhancer (16). In contrast, the CREB–pX protein complex is capable of recognizing and binding the variant CRE site of the HBV enhancer (16). The interactions between CREB–pX and the CRE-like site are not understood. Here we examine the mechanism of the interactions between the cellular transcription factor CREB and viral pX, employing recombinant CREB and pX in *in vitro* DNA–protein binding assays.

## MATERIALS AND METHODS

**Recombinant Proteins.** Bacterially expressed recombinant proteins used in this study were obtained via the T<sub>7</sub>/B121LysS system (19, 20) after induction with isopropyl  $\beta$ -D-thiogalactopyranoside. CREB<sup>327</sup> protein was expressed in bacteria and purified as described by Colbran *et al.* (21). Recombinant basic region–leucine zipper (bZip) CREB peptide, encompassing aa 259–327, was purified from bacteria according to the protocol described by Santiago-Rivera *et al.* (22). Recombinant CREB–Gal4 fusion protein was engineered to encode the DNA-binding domain of the yeast transactivator Gal4 (aa 1–147) in place of the bZip domain (aa 259–327) of CREB<sup>327</sup>. The bacterially expressed CREB–Gal4 protein was partially purified by chromatography on DEAE-Sephacel and eluted at 0.2 M KCl in buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol (DTT), and 0.5 mM EDTA.

Partially purified pX was obtained via the T<sub>7</sub>/B121LysS (DE3) system (19, 20). The recombinant pX was found in the insoluble pellet and was solubilized in buffer containing 6 M urea and 5 mM DTT (23, 24). After renaturation of pX, the sample was chromatographed on S-Sepharose. Elution was in 10 mM phosphate buffer (pH 7.2) containing 0.2 M KCl and 5 mM DTT. Throughout the DNA–protein binding assays, 10- $\mu$ l aliquots of renatured, S-Sepharose chromatographed pX were used. Ten-microliter aliquots contained  $\approx$ 300 ng of pX; however, the active fraction of pX is not known.

Baculovirus expression of pX was achieved by cloning X cDNA into the *Nco* I/*Bgl* II sites of the baculovirus expression vector pAcSG-His-NT-B from PharMingen. Recombinant baculovirus was obtained by using the BaculoGold transfection

Abbreviations: HBV, hepatitis B virus; bZip, basic region–leucine zipper domain.

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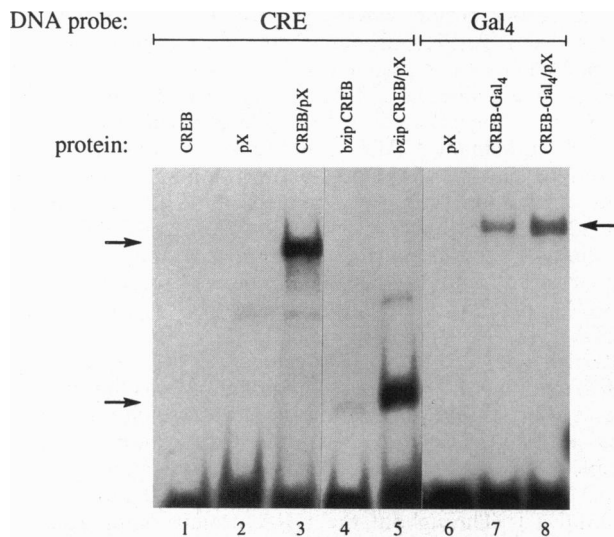
kit system from PharMingen. Sf21 cells were infected with 10 plaque-forming units per cell and cellular extracts were prepared 48 hr postinfection. pX encoded by the pAcSG-His-NT-B vector is a fusion protein containing a histidine tag, allowing rapid purification via nickel-chelate chromatography (25). The majority of the expressed pX is insoluble in the baculovirus system; the soluble fraction contains sufficient pX, which is purified by nickel-chelate chromatography. The identity of the purified protein was confirmed by Western blot analysis with pX-specific antiserum (data not shown).

**DNA-Protein Binding Assays.** Gel retardation and methylation interference assays were carried out as described by Andrisani *et al.* (26).  $K_d$  determination assays were performed as described by Williams *et al.* (27).

**Transient Transfection in PC12 Cells.** Expressor CMV4-X plasmid (1  $\mu$ g) was transfected with 10  $\mu$ g of indicator plasmid pBxSST (26) in PC12 cells by  $Ca_3(PO_4)_2$  coprecipitation using the BRL transfection kit as described (28).

## RESULTS

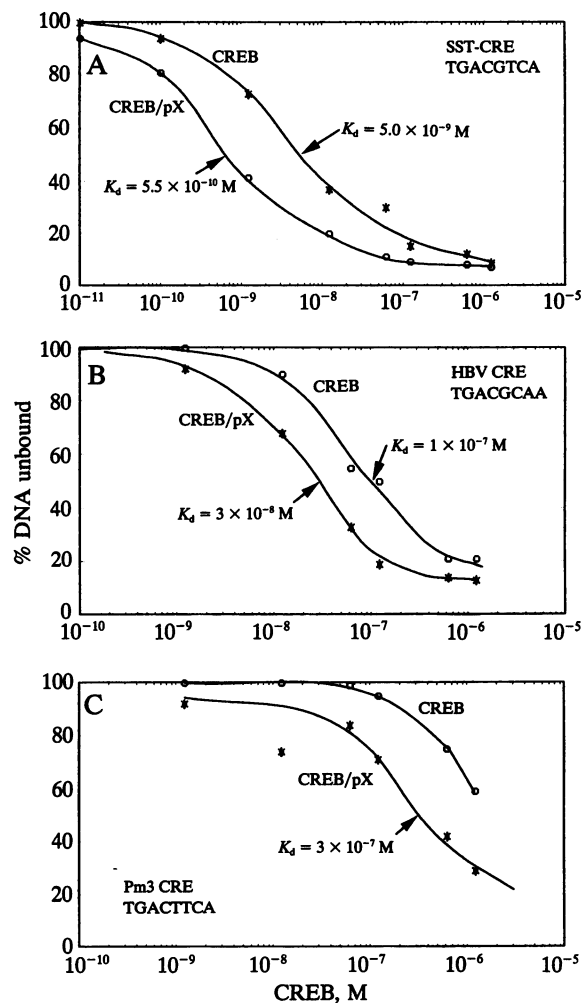
**The bZip Domain of CREB Interacts with pX.** To understand the mechanism of the CREB-pX interactions, we used recombinant CREB<sup>327</sup> and pX in *in vitro* DNA-protein binding assays (Fig. 1) (26). A defined amount of CREB, 10 ng (lane 1), displays enhanced binding to the CRE site in the presence of a defined amount of pX (lane 3). To determine the domain of CREB required for interaction with pX, recombinant CREB protein variants were used in DNA-protein binding assays. Purified recombinant bZip peptide of CREB<sup>327</sup> (22), used in DNA-protein binding assays with pX, effects  $\approx 100$ -fold enhancement in CRE binding (lanes 4 and 5). In contrast, a fusion protein of CREB<sup>327</sup> in which the bZip portion was replaced with the DNA-binding domain of the yeast transactivator Gal4 displays an insignificant enhancement in upstream activator sequence UAS<sub>GAL</sub> binding as a



**FIG. 1.** Gel retardation assays of pX with CREB protein variants. Ten nanograms of CREB<sup>327</sup> protein (lane 1) was incubated for 20 min with 15,000 cpm of CRE DNA in the presence of 1  $\mu$ g of poly[d(IC)] and 1  $\mu$ g of bovine serum albumin in buffer containing 10 mM HEPES (pH 7.4), 0.1 M KCl, and 10% (vol/vol) glycerol. Ten microliters of partially purified pX was added where indicated. Lanes 4–6, assays used recombinant bZip peptide CREB<sup>259–327</sup> (22). Lanes 7 and 8, recombinant CREB-Gal4 fusion protein contains the N-terminal portion of CREB<sup>327</sup> (aa 1–256) fused to the DNA-binding domain of the yeast transactivator Gal4 (aa 1–147). Ten nanograms of each CREB protein variant was used as a function of pX addition. The <sup>32</sup>P-labeled probe is either the somatostatin CRE or the UAS<sub>GAL</sub> DNA-binding site. Arrows on the left point to the CREB-pX complex.

function of pX addition (lanes 7 and 8). These results demonstrate that the bZip domain of CREB is the target of pX interactions. A synthetic peptide encoding the leucine zipper motif of CREB, spanning aa 293–327, when added to the pX, also failed to demonstrate X-mediated CRE binding (data not shown). This suggests that the dimerization domain of CREB is not sufficient for pX binding to the CRE, consistent with the demonstrated lack of direct DNA binding by pX (lane 2).

**pX Increases the Affinity of CREB for CRE Sites.** Since the bZip domain of CREB is the target for pX interactions, we examined the effect of pX binding on the affinity of CREB for CRE sites. We determined the  $K_d$  of CREB<sup>327</sup> (27) as a function of pX binding (Fig. 2). The radioactive DNA probe was either the palindromic CRE motif (TGACGTCA) of the somatostatin promoter (26) (Fig. 2A) or variant CRE motifs (Fig. 2B and C). The variant CRE motifs include the HBV enhancer (16) CRE (TGACGCAA) and a nonfunctional (27) CRE site containing a G  $\rightarrow$  T substitution of the central G residue (TGACTTCA). Table 1 summarizes the  $K_d$  values of CREB<sup>327</sup>, as a function of pX binding, using CRE and CRE-like DNA probes. These experiments demonstrate that the CREB-pX interactions induce an enhancement of  $\approx 1$  order of magnitude in the affinity of CREB<sup>327</sup> for both the palindromic and variant CRE motifs.



**FIG. 2.**  $K_d$  determination of CREB<sup>327</sup> in the presence of recombinant pX. Binding isotherms were carried out and analyzed as described by Williams *et al.* (27). The <sup>32</sup>P-labeled probe is CRE DNA used in the DNA-protein binding assays: (A) Somatostatin (SST)-CRE. (B) HBV enhancer CRE. (C) Synthetic CRE containing a G  $\rightarrow$  T substitution of the central G residue (pm3).

Table 1.  $K_d$  determination of CREB<sup>327</sup> as a function of HBV pX binding

	CRE	CREB, $K_d$	CREB-pX, $K_d$
SST CRE:	TGACGTC	$5.0 \times 10^{-9}$ M	$5.5 \times 10^{-10}$ M
HBV CRE:	TGACGCAA	$1.0 \times 10^{-7}$ M	$3.0 \times 10^{-8}$ M
pm-3 CRE:	TGACTTCA	$1.0 \times 10^{-6}$ M	$3.0 \times 10^{-7}$ M

To understand the increased affinity of CREB for the CRE site in the presence of pX, we examined whether the interaction alters the dimerization of CREB. CREB<sup>327</sup>, labeled with [ $\gamma$ -<sup>32</sup>P]ATP at the protein kinase A phosphorylation site, was used in glutaraldehyde crosslinking assays as a function of pX addition. The level of enhancement by pX, using equal amounts of phosphorylated (Fig. 3A) and unphosphorylated (Fig. 3B) CREB<sup>327</sup>, shown in lanes 2–6, indicates that both forms of CREB respond similarly to pX. Thus, the crosslinking experiments shown in Fig. 3C utilized <sup>32</sup>P-labeled CREB<sup>327</sup>. The assays shown in Fig. 3 were run in parallel, with 8 ng of CREB<sup>327</sup>, corresponding to a concentration of  $\approx 9 \times 10^{-9}$  M, which is within the  $K_d$  range of CREB<sup>327</sup> for the CRE site. The percentage CREB dimer formed as a function of increasing amounts of pX (Fig. 3C, lanes 3–6) was quantitated by scintillation counting of the excised gel bands corresponding to the monomeric and dimeric forms of CREB. Addition of increasing amounts of pX (Fig. 3A) shows an enhancement of CREB binding from 1.5-fold (lane 2) to 14-fold (lane 6). In contrast, lanes 3 and 6 in Fig. 3C demonstrate no effect on CREB dimerization. We conclude that the dimerization of CREB is not altered by pX. The increased CRE binding affinity of CREB in the presence of pX is mediated by a mechanism(s) other than enhanced CREB dimerization, suggesting that the leucine zipper motif is not the target of pX action.

**Methylation Interference of the CRE Bound to the CREB-pX Complex.** To further understand how the CREB-pX complex alters the  $K_d$  of CREB for CRE sites (Fig. 2) and allows binding to variant CRE sites (16), we examined by methylation interference how it contacts the CRE site. Methylation interference footprinting reveals the critical contact residues that occur between the DNA site and the bound transcription factor. The methylation interference patterns of the CRE DNA bound to CREB alone and to the CREB-pX

complex are compared in Fig. 4. Residues of the CRE palindrome are numbered 1–4—i.e., T<sup>1</sup>G<sup>2</sup>A<sup>3</sup>C<sup>4</sup>G<sup>4</sup>T<sup>3</sup>C<sup>2</sup>A<sup>1</sup>. The critical contact residues of the CRE site required to interact with CREB are residues G<sup>2</sup> and G<sup>4</sup> (Fig. 4A) (26, 31). In contrast, Fig. 4B shows that the CREB-pX complex yields an altered methylation interference pattern. Only the central G<sup>4</sup> residue is unmethylated upon CREB-pX binding to the CRE site, pointing to the importance of the G<sup>4</sup> residue in the formation of the critical contacts. The observation that CREB-pX allows methylated G<sup>2</sup> residues within the CRE is interpreted to mean that either CREB-pX does not contact the G<sup>2</sup> residue or that the contact with the G<sup>2</sup> residue is no longer critical for DNA-protein complex formation. These results directly demonstrate that the interaction of pX with CREB alters the way CREB contacts the CRE site, suggesting that pX targets the basic region of CREB. This is in agreement with the crosslinking experiment (Fig. 3), which suggests that the dimerization domain of CREB is not the target of pX interactions.

**Methylation Interference of Variant CRE Motifs Bound to the CREB-pX Complex.** CREB binds to the CRE symmetrically (26, 31) with a  $K_d$  of  $10^{-9}$  M (27). The  $K_d$  of CREB to variant CRE sites, which contain bases other than G at position G<sup>2</sup> of the CRE half-site, is  $\approx 10^{-8}$  M (27), suggesting that maintenance of the symmetric contacts with CREB is important in DNA-protein complex formation. The results of the methylation interference analysis, employing the CREB-pX complex (Fig. 4), show that pX alters the way CREB contacts the CRE site; the G<sup>4</sup> residue is the primary critical contact with CREB in the presence of pX. This suggests that base variation at the G<sup>2</sup> position is less crucial in DNA-protein complex formation with the CREB-pX complex.

We examined the methylation interference patterns of two variant CRE motifs bound to CREB or to CREB-pX (Fig. 5). The variant CRE motifs contained either a G  $\rightarrow$  C (Fig. 5A) or a G  $\rightarrow$  A (Fig. 5B) substitution at the G<sup>2</sup> residue of the CRE. The remaining sequence is identical to the sequence of the palindromic CRE used in the analysis of Fig. 4. The methylation interference pattern of the variant CRE motifs bound to the CREB protein (Fig. 5) shows that the critical contact residues of the CRE half-site—namely, G<sup>2</sup> and G<sup>4</sup>—are important in complex formation, although they display only partial methylation interference. This is attributed to the lack

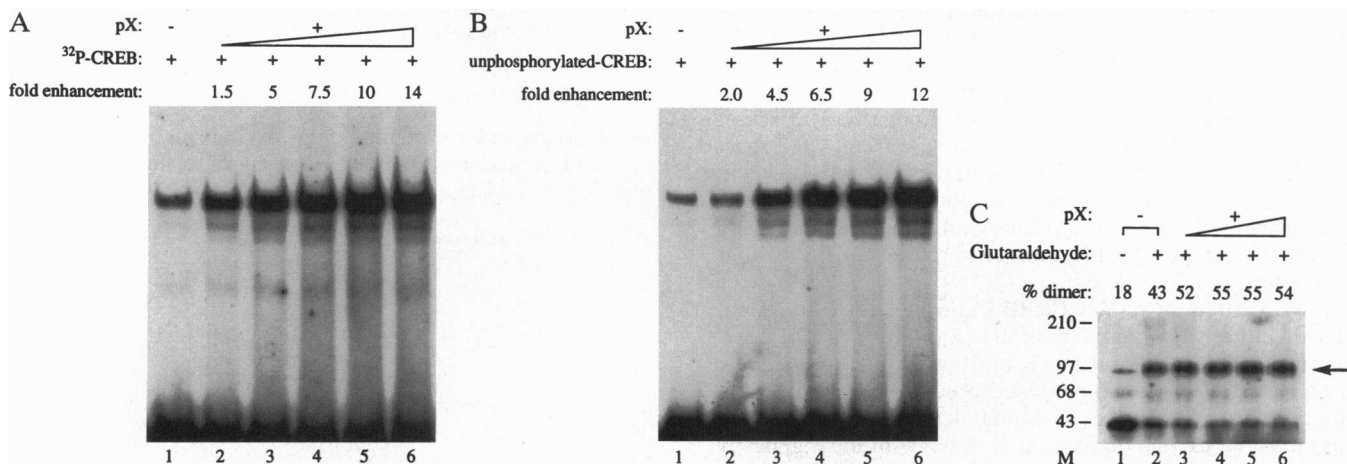


FIG. 3. Dimerization of CREB<sup>327</sup> in the presence of the HBV pX. Gel retardation assays with 8 ng of CREB<sup>327</sup> protein, phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP by protein kinase A (A) and 8 ng of unphosphorylated CREB<sup>327</sup> (B). CRE was the probe in both A and B. DNA-protein binding assays were carried out as described (26) with increasing amounts of baculovirus-expressed pX (approximate concentration, 1–20 ng/ $\mu$ l). (A and B) Lanes: 1, no pX; 2–6, 2, 5, 7, 10, and 15  $\mu$ l of pX, respectively. Enhancement in CREB-CRE binding by pX is shown above each lane. (C) <sup>32</sup>P-labeled CREB<sup>327</sup> crosslinked with 0.02% glutaraldehyde (29) for 4 min at room temperature as a function of pX addition. Before crosslinking, CREB-pX proteins were incubated for 15 min under DNA-protein binding assay conditions (26). Crosslinking reactions were analyzed by SDS/7% PAGE and autoradiography. All lanes contain 8 ng of <sup>32</sup>P-labeled CREB<sup>327</sup>; pX (+), increasing amounts of baculovirus-expressed pX. Lanes: 1 and 2, without pX in the absence (-) and presence (+) of glutaraldehyde, respectively; 3–6, 2, 5, 10, and 15  $\mu$ l of pX, respectively. Percentage dimer formed is shown above each lane.

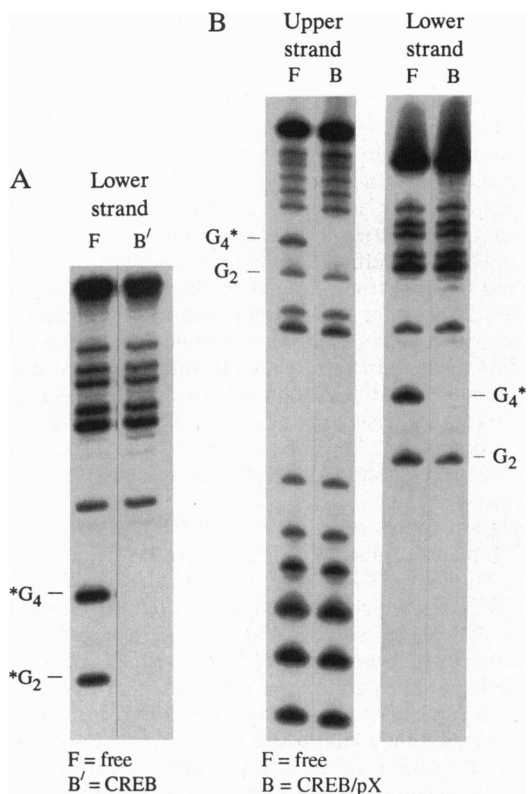


FIG. 4. Methylation interference assay of CRE bound to the CREB-pX complex. (A) Methylation interference of the somatostatin CRE DNA (lower strand) bound (lane B') to CREB<sup>327</sup> protein. Lane F, Maxam and Gilbert (30) G reaction of unbound CRE DNA. The critical G residues  $G_4^*$  and  $G_2$  in the CREB-CRE complex are shown by asterisks. (B) Methylation interference of CREB-pX complex bound (lanes B) to somatostatin CRE DNA. Upper and lower strands are shown. The critical contact G residue  $G_4^*$  is indicated by an asterisk. The sequence of the CRE DNA probe is shown in Andrisani *et al.* (26).

of complete symmetry within these variant CRE sites, which is reflected in the increased  $K_d$  of CREB for these sites (27). Interestingly, the CREB-pX complex, when bound to the variant CRE motifs (Fig. 5), imparts upon the critical contact residue  $G_4$  complete methylation interference in comparison to that bound to the CREB protein alone. In contrast, the  $G_2$  residue of the CRE half-site displays the same level of partial methylation whether bound to CREB protein or CREB-pX. The results in Fig. 5 suggest that pX strengthens the interaction of CREB with the central  $G_4$  residue of the variant CRE sites. In agreement with the conclusions derived from Fig. 4, the above results also show that pX alters the way CREB interacts with variant CRE sites. The primary target interaction of the CREB-pX complex is the central  $G_4$  residue of the variant CRE sites.

**In Vivo Effect of pX on CREB-Dependent Transcription.** The quantitative assessment of the  $K_d$  values of CREB<sup>327</sup> shown in Fig. 2 suggests that one of the effects of the viral pX *in vivo* is to effect CREB-mediated transcriptional induction, especially from CRE-like, cis-acting elements. To investigate this effect, we carried out transfection assays of the X gene, by using the somatostatin promoter (26), a well-documented CREB-dependent assay system (32). Expression of pX *in vivo* was via the CMV4 mammalian expression vector.

The somatostatin-chloramphenicol acetyltransferase reporter plasmid was cotransfected in PC12 cells (28) with CMV4-X plasmid DNA, in the presence of 10  $\mu$ M forskolin, for 48 hr. Fig. 6 shows that expression of the pX resulted in an  $\approx$ 15-fold induction of the CRE-mediated transcription in the presence of forskolin. Absence of forskolin induction (lanes 1

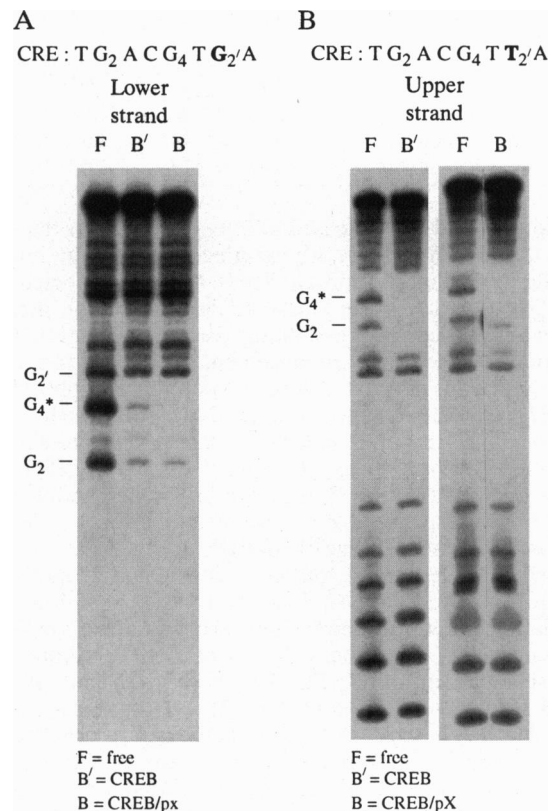


FIG. 5. Methylation interference of variant CRE bound to CREB-pX complex. Lane F, Maxam and Gilbert (30) G reaction of unbound CRE DNA. Lanes B' and B, methylation interference of variant CRE bound to CREB<sup>327</sup> protein and CREB-pX complex, respectively. (A) Variant CRE contains a G  $\rightarrow$  C substitution at a  $G_2'$  site, shown in boldface type. (B) Variant CRE contains a G  $\rightarrow$  A substitution at a  $G_2$  site, shown in boldface type. Critical contact residues are indicated. The central  $G_4$  residue is also indicated by an asterisk.

and 2) resulted in no induction of CRE-mediated transcription, supporting the hypothesis that the cAMP mediating transactivator CREB is targeted by pX *in vivo*. This experiment confirms the transactivator role of pX using the CRE-dependent somatostatin promoter.

## DISCUSSION

In this study, we have examined the mechanism of the protein-protein interactions occurring between the cellular transcription factor CREB and the HBV pX. We used recombinant

% Conversion: 1.9 2.0 0.8 13.3

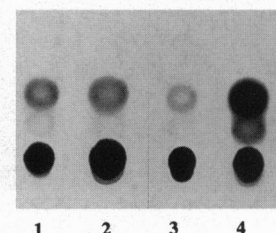


FIG. 6. Transcriptional induction by the HBV pX of the CREB-dependent somatostatin promoter. Transient transfection assays in PC12 cells using 10  $\mu$ g of somatostatin-chloramphenicol acetyltransferase (pBxSST) (26, 28) indicator plasmid and cotransfected with 0  $\mu$ g (lane 1) and 1  $\mu$ g (lane 2) of CMV4-X expressor in the absence of forskolin. Lanes 3 and 4, 0  $\mu$ g and 1  $\mu$ g of CMV4-X expressor, respectively, in the presence of 10  $\mu$ M forskolin. Percentage chloramphenicol conversion is shown above each lane.

proteins and *in vitro* DNA-binding assays to monitor these interactions. We demonstrate via the use of recombinant CREB protein variants that pX targets the bZip domain of CREB.

The increase in the affinity of CREB for CRE-like sites (Table 1) via interaction with pX is toward physiologically relevant (nM) concentrations of CREB. In conjunction with the *in vivo* transfection results (Fig. 6), this suggests that pX expression *in vivo* will result in transcriptional induction of both CRE- and CRE-like-containing genes. The increased affinity of CREB due to interactions with pX will effect activation of a repertoire of cellular genes, even those containing CRE-like elements that are normally unresponsive to the physiological levels of CREB. It is not yet known whether other CRE-binding transcription factors, such as Jun/fos (33) and CREMs (34), which function in response to different cellular signals (35–37), will respond to the enhancing effect of the viral pX toward binding to CRE sites. One can thus appreciate the magnitude of the transcriptional induction/depression brought about within the cell via the action of the HBV pX by altering the affinity of CRE-binding transcription factors.

The mechanism by which pX alters the affinity of CREB for CRE sites is not yet understood. Wagner and Green (29) have demonstrated that the HTLV-I Tax1 protein stimulates the DNA binding of bZip proteins by enhancing the rate of dimerization. Similar analysis of the CREB–pX system (Fig. 3) demonstrated that pX does not enhance the dimerization of CREB. The lack of enhanced dimerization of CREB by pX suggests that the leucine zipper motif is not the target of pX. The altered methylation interference pattern of the CRE motif bound to the CREB–pX complex demonstrates that pX modifies how CREB contacts the CRE motif. It strongly suggests that the basic DNA-binding domain of CREB is the target of pX action. Santiago-Rivera *et al.* (22) demonstrated that the basic domain of CREB, as with the other leucine zipper proteins studied to date, assumes an  $\alpha$ -helical configuration upon binding to the cognate CRE motif. Based on our interpretation of the results here—namely, that pX targets the basic domain of CREB—our current working hypothesis is that pX promotes and maintains the correct folding of the basic domain of CREB, thus contributing to its enhanced binding affinity. We propose that pX may act as a molecular chaperone by preventing the formation of incorrect folding intermediates or by stabilizing the active CREB conformation.

Whether pX in the CREB–pX complex binds DNA is not known. The interaction of CREB with pX may unmask a putative DNA-binding domain of pX. Contacts of DNA by both components of CREB–pX can also explain the altered methylation interference footprints obtained by the CREB–pX complex in comparison to CREB alone. Based on the methylation interference analyses (Figs. 4 and 5), we conclude that the CREB–pX complex targets primarily the G<sup>4</sup> residue of the CRE half-site. The primary G<sup>4</sup> contact by the CREB–pX complex and additional DNA contact(s) by pX can explain the increased affinity of CREB–pX for the palindromic and variant CRE sites.

Our results lend further support to the proposed pleiotropic mechanism of pX action (13). It has been reported that HBV pX participates in the protein kinase C signaling pathway to effect AP-1-mediated transcription (14, 38, 39). pX also has the potential to transactivate when directed as a fusion protein via heterologous DNA-binding domains to specific DNA-binding sites, suggesting that it can interact with the cellular transcriptional machinery. The direct protein–protein interactions of pX, initially reported by Maguire *et al.* (16) and further analyzed here, support the notion that pX alters the affinity of cellular bZip (CREB/ATF) transactivators by targeting the basic region of their bZip domain. The pleiotro-

pic action of the HBV pX is surely linked to its oncogenic properties (3).

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