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Hepatitis B virus transactivator protein X interacts with the TATA-binding protein

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ABSTRACT **Several viral transcriptional activators have been shown to interact with the basal transcription factor TATA-binding protein (TBP). These associations have been implicated in facilitating the assembly of the transcriptional preinitiation complex. We report here that the hepatitis B virus protein X (pX) specifically binds to TBP *in vitro*. While truncations of the highly conserved carboxyl terminus of TBP abolished this binding, amino-terminal deletions had no effect. Deletion analysis suggests that a domain consisting of 71 aa in the highly conserved carboxyl-terminal region of TBP is necessary for its interaction with pX. The minimal region in pX sufficient for its interaction with TBP includes aa 110–143. Furthermore, TBP from phylogenetically distinct species including *Arabidopsis thaliana, Saccharomyces cerevisiae, Drosophila melanogaster*, and *Solanum tuberosum* (potato) bound to pX. The pX–TBP interaction was inhibited in the presence of nonhydrolyzable analogs of ATP, suggesting a requirement for ATP. These results provide an explanation for the promiscuous behavior of pX in the transactivation of a large repertoire of cellular promoters. This study further implicates a fundamental role for pX in modulating transcriptional regulatory pathways by interacting with the basal transcription factor TBP.**

Infection by human hepatitis B virus (HBV) represents a major health problem. It is estimated that over 250 million people are chronically infected worldwide. Chronic infection has been associated with hepatocellular carcinoma (1). The HBV genome encodes four genes whose transcriptional activity is controlled by at least four promoters and two enhancer elements designated I and II. The 16.5-kDa protein X (pX), encoded by the X gene, can activate gene expression from diverse viral and cellular transcriptional control elements (2–7). pX does not interact directly with cis-acting DNA elements. The biochemical mechanism responsible for pX activity in the viral life cycle remains obscure. While little is known regarding the potential interactions between pX and cellular proteins, it is clear from numerous studies that pX may function by a number of distinct mechanisms (7). We have previously shown that pX mediates transcriptional transactivation by engaging in protein–protein interactions (8). pX was shown to enhance the DNA-binding specificity of the transcription factors ATF-2 and CREB. This interaction within the HBV enhancer element I leads to transactivation through the cAMP response element (8). Several cellular genes, including interleukin 8, intercellular adhesion molecule 1, c-*myc*, and major histocompatibility complex class I and class II genes, have been shown to be activated by pX via distinct transcription factors (9–13). Consistent with these findings, an NF-κB sequence located in the human immunodeficiency virus long terminal repeat was shown to be the target sequence of pX transactivation (5, 6). However, there is no direct evidence for a direct interaction between pX and these factors. pX has also been shown to increase an endogenous protein kinase C activity via signal transduction pathways (14, 15). A recent report demonstrated interactions of pX with the p53 tumor-suppressor gene, and it was proposed that this interaction may impede DNA repair of host genes (16). The mechanism(s) by which pX is able to activate gene expression through a variety of promoters/enhancers has been the subject of intense investigation. Of interest is the observation that the general transcriptional factor TFIID [or the TATA-binding protein (TBP)] is the target of transactivation by a large number of viral and cellular factors. These include adenovirus E1A (17), herpes simplex virus 1 VP16 (18), Epstein–Barr virus Zta (19), human cytomegalovirus IE2 (20), human T-cell lymphotropic virus 1 Tax (21), and human immunodeficiency virus Tat (22). In addition to these factors, the tumor-suppressor gene products p53 (23) and RB (24), the protooncoprotein v/c-Rel (25, 26), and the transcription factors c-Fos (27) and the ATF-2 (28) form stable associations with TBP. The binding of these factors to TBP suggests that these interactions play an important role in modulating the assembly of the transcriptional preinitiation complex and therefore transcriptional regulation (29). In this report, we demonstrate the physical interactions between pX and TBP. This study also delineates the regions of pX and TBP involved in the interaction. Finally, we provide evidence supporting the conclusion that pX–TBP interactions require ATP.

MATERIALS AND METHODS

Plasmid Constructions. Whenever necessary the termini of fragments and vectors were blunted by T4 DNA polymerase. pGST-X vector, encoding the full-length pX (aa 1–154), was constructed by inserting an *Nco* I–*Bgl* II fragment into the *Sma* I site of plasmid pGEX-2T (Pharmacia). pGST-XN, encoding the amino-terminal 66 aa of pX, was obtained by digesting pGST-X with *Rsr* II/*Eco*RI and self-ligating the plasmid in the presence of T4 DNA ligase. pGST-XC, containing aa 105–154 of pX, was produced by excising a *Hin*cII fragment from pSPX and ligating it into the unique *Sma* I site of pGEX-2T. pGST-XSM, encoding aa 110–143 of pX, was constructed by inserting a *Stu* I–*Mst* I fragment derived from pSPX into the *Eco*RI site of pGEX-3X (Pharmacia). pSPX was constructed by ligating an *Nco* I–*Bgl* II fragment into the *Sma* I site of pSP65 (Promega). pRCTBP was produced by inserting an *Eco*RI fragment derived from a human TBP cDNA plasmid pGPP21 (30), into the *Hin*dIII site of pRC/CMV (Invitrogen). To delete the entire amino terminus (aa 5–150) of TBP, pRCTBP was digested with *Alw* I/*Ple* I and self-ligated. Two synthetic oligonucleotides complementary to each other and encoding a 9-aa tag (YPYDVPDYA) from influenza virus hemagglutinin (HA) were introduced at the unique *Alw* I site (aa 5) of the TBP cDNA in pRCTBP to produce pHATBP. The TBP cDNA fragments from various species [an *Nco* I–*Nde* I fragment from pETAt2-14b (*Arabidopsis thaliana*) (31), an

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Abbreviations: HBV, hepatitis B virus; pX, HBV X protein; TBP, TATA-binding protein; GST, glutathione *S*-transferase; HA, hemagglutinin.

Nde I–*Bam*HI fragment from pGEM721A (*Saccharomyces cerevisiae*) (32), an *Eco*RI fragment from pFX29-7 (*Drosophila melanogaster*) (33), and an *Eco*RI fragment from pUBS (*Solanum tuberosum*) (potato) (34)] were excised from the respective plasmids and cloned into the *Hin*dIII site of pRC/CMV.

***In Vitro* Translation.** TBP and pX were translated *in vitro* from plasmids pRCTBP and pSPX, respectively, by using the TNT coupled *in vitro* transcription/translation kit (Promega). The expression of TBP and pX in these constructs is under the control of bacteriophage T7 and SP6 promoters, respectively. A series of sequential carboxyl-terminal deletion mutants of TBP were translated *in vitro* after digestion of pRCTBP vector with *Pst* I (aa 110), *Bsp*HI (aa 177), *Ssp* I (aa 210), *Stu* I (aa 281), or *Apo* I (aa 335).

Protein Interaction Assays. The glutathione *S*-transferase (GST) fusion proteins were purified as described (35). Four micrograms of GST or 2 μg of freshly extracted GST-X fusion protein immobilized on 20 μl of glutathione beads was incubated with 2 μl of ^{35}S-labeled TBP in 200 μl of buffer A [150 mM KCl/6 mM $MgCl_2$/25 mM Hepes, pH 7.9/10% (vol/vol) glycerol/0.1% Nonidet P-40/1 mM ATP/1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride] with gentle shaking at 4°C for 3–4 hr. Equal amounts of glutathione beads were incubated with TBP as a control. After incubation, the beads were washed six times with incubation buffer, and bound proteins were fractionated by SDS/PAGE. Protein bands were visualized by fluorography treatment with 1 M sodium salicylate. To study interactions between GST-XSM and the truncated TBP proteins (carboxyl-terminal deletions), 2 μl of full-length protein and 5 μl of mutant were incubated with 20 μl of glutathione beads containing either 16 μg of GST or 8 μg of fusion protein GST-XSM. The bound proteins were analyzed by SDS/13% PAGE followed by autoradiography.

Coimmunoprecipitation and Chemical Crosslinking. *In vitro* translated HA-tagged TBP and pX were mixed in a 100-μl reaction volume in buffer A (see above) and incubated for 2 hr. The mixture was immunoprecipitated with HA monoclonal antibody 12CA5 (BAbCO, CA) and anti-X serum by standard methods. For chemical crosslinking, ^{35}S-labeled *in vitro* translated TBP and pX were incubated in buffer A with gentle shaking at 4°C for 3–4 hr. Glutaraldehyde (grade I, 70% aqueous solution; Sigma) was added (0.05%) and incubation continued for 2 hr at 4°C with gentle rocking. The mixtures were immunoprecipitated with anti-X serum. The proteins were visualized after SDS/13% PAGE and autoradiography.

RESULTS

TBP Interacts with HBV pX. To investigate the interactions between TBP and pX, GST fusion proteins containing full-length pX and defined truncations of pX were prepared (Fig. 1*A*). In the initial analysis, human TBP was synthesized *in vitro* in the presence of [^{35}S]methionine and tested for its ability to interact with GST and GST-X fusion proteins immobilized on glutathione affinity beads (Fig. 1). The authenticity of the gene products was determined by SDS/PAGE followed by Western blot analysis (data not shown). While ^{35}S-labeled TBP interacted specifically with GST-X, no interactions with glutathione or GST beads in the absence of bound pX were observed (Fig. 1*B*). GST-X also did not show any interactions with basal transcription factor TFIIB or thyroid hormone receptor (Fig. 1*C*, lanes 2 and 3).

Highly Conserved Domain of pX Interacts with TBP. To delineate the regions of pX relevant for its interaction with TBP, we used several deletion mutants of pX. These included GST-XN (aa 1–66), GST-XC (aa 104–154), and GST-XSM (aa 110–143). While the mutants GST-XC and GST-XSM displayed qualitatively similar levels of binding with TBP as compared with full-length GST-X (Fig. 1*D*, lanes 2, 4, and 5),

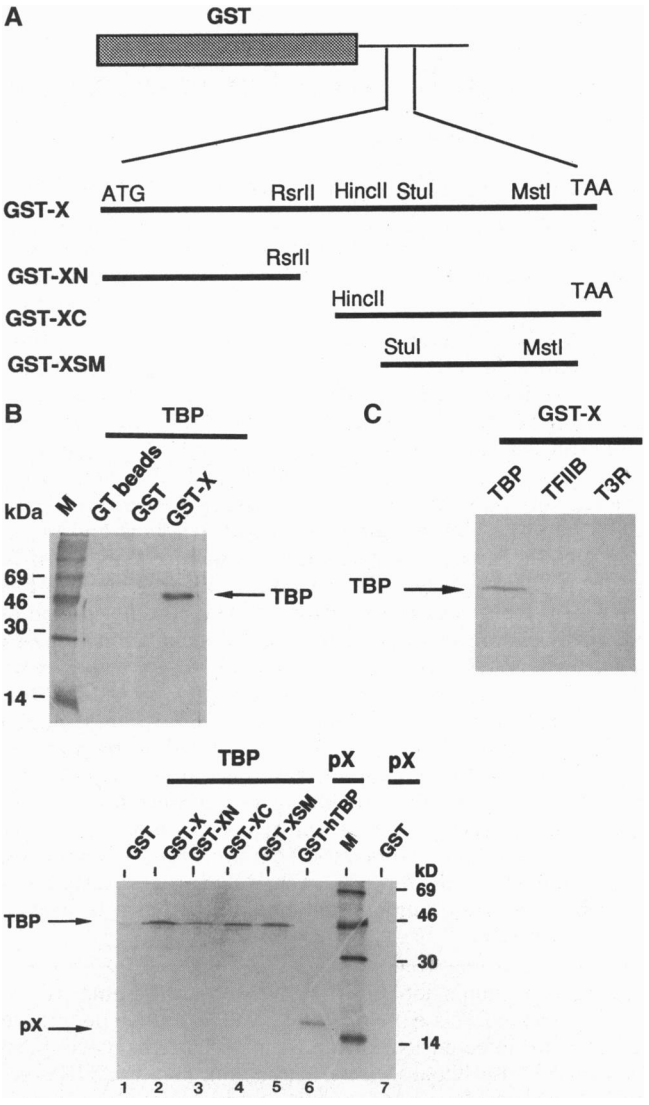

FIG. 1. (*A*) GST-X fusion protein constructs. Initiation (ATG) and termination (TAA) codons are indicated. (*B*) Binding of human [^{35}S]methionine-labeled TBP to GST and GST-X fusion proteins immobilized on glutathione (GT) beads (35). Lane M, size markers for SDS/PAGE. (*C*) Analysis of interactions of GST-X with [^{35}S]methionine-labeled TBP, TFIIB, and thyroid hormone receptor (T3R). (*D*) Binding of [^{35}S]methionine-labeled TBP to GST (lane 1), GST-X (lane 2), and its deletion mutants GST-XN (lane 3), GST-XC (lane 4), and GST-XSM (lane 5) immobilized on glutathione beads. Lanes 6 and 7, reciprocal binding of ^{35}S-labeled pX to either GST or GST-human TBP (hTBP) fusion-protein.

GST-XN displayed a reduced level of binding (about 30%) (lane 3). These results localize a domain consisting of aa 110–143 involved in interactions with TBP. Since the truncated fusion protein GST-XSM showed binding that was equivalent to that of full-length GST-X, GST-XSM was used for subsequent analyses. These findings are consistent with previous studies from several laboratories, which have shown that aa 104–154 of pX are critical for transcriptional transactivation activity (6). A comparison of the X gene sequences from various mammalian hepadnaviruses reveals a highly conserved, moderately hydrophobic domain within this domain. This domain constitutes the putative α-helical structures that are interrupted by glycine residues (4). A reciprocal analysis was also carried out using *in vitro* synthesized ^{35}S-labeled pX and the GST-human TBP fusion proteins. While pX interacted with GST-human TBP, no binding was observed with GST (Fig. 1*D*, lanes 6 and 7).

Identification of the TBP Domain Which Interacts with pX. To identify the TBP domain involved in its interaction with pX, carboxyl-terminal deletions of TBP were generated by using convenient restriction endonuclease sites (Fig. 2*A*). ^{35}S-labeled mutant and full-length (positive control) TBP proteins in the same reaction were synthesized *in vitro* and incubated with either GST or GST-XSM immobilized on glutathione affinity beads. A 4-fold higher amount of GST and GST-XSM fusion proteins was used in this experiment to allow for noncompetitive simultaneous binding of full-length and truncated TBP. Polypeptides generated by cleavage of templates at the *Stu* I and *Apo* I sites (aa 281 and 335, respectively) formed stable complexes with GST-XSM (Fig. 2*B*, lanes 8 and 10). In contrast, polypeptides generated by cleavage of the TBP gene at *Pst* I, *Ssp* I, and *Bsp*HI sites (aa 110, 177, and 210, respectively) showed no binding with GST-XSM (Fig. 2*B*, lanes 2, 4, and 6; note that the full-length TBP bound effectively to GST-XSM). The interaction of pX with truncated TBP polypeptides containing aa 1–335 and 1–281 but not with 1–210 or shorter derivatives, indicates that the 71 aa domain including aa 211–281 of TBP is critical for pX binding. We subsequently used an amino-terminal deletion mutant of TBP (Δ5–150) which lacked the entire region encompassing the nonconserved aa 5–150. When compared with the full-length TBP, mutant Δ5–150 interacted with pX in a qualitatively comparable manner (Fig. 2*C*, lanes 2 and 4).

A further test of specificity of pX–TBP interaction was provided by using TBPs from several phylogenetically divergent species: *A. thaliana* (isoform 2), *S. cerevisiae, D. melanogaster*, and *Solanum tuberosum* (31–34). These TBPs share a highly conserved carboxyl-terminal region displaying 81–88% homology with human TBP (29–34). The proteins were translated *in vitro* and analyzed for their ability to interact with pX. TBP from these species showed strong binding to GST-XSM (which contains the carboxyl-terminal domain including aa 110–143 of pX) (Fig. 2*D*, lanes 2, 4, 6, and 8). Taken together, these studies demonstrate that the highly conserved carboxyl-terminal domain of TBP is involved in the interaction with pX.

Identification of pX–TBP Complexes by Coimmunoprecipitation and Chemical Crosslinking. In a complementary approach, we also detected protein–protein interactions between pX and TBP by using a coimmunoprecipitation assay. The TBP used in this experiment contained an influenza virus (HA) tag fused at its amino terminus. *In vitro* translated HA-tagged TBP was incubated with GST-XSM, and the protein complexes were immunoprecipitated with either the HA monoclonal antibody 12CA5 (Fig. 3*A*, lanes 2, 3, and 5) or polyclonal anti-X serum (lanes 1, 4, and 6). Neither anti-X nor 12CA5 antibodies showed crossreactivity to TBP or pX, respectively (Fig. 3*A*, lanes 3 and 4). Each antiserum interacted with the corresponding protein (Fig. 3*A*, lanes 1 and 2). When pX and TBP polypeptides were mixed together in a binding reaction mixture, either antiserum was able to immunoprecipitate both proteins (Fig. 3*A*, lanes 5 and 6). A glutaraldehyde-mediated crosslinking experiment was performed to further confirm the pX–TBP interaction. A 56-kDa crosslinked protein product consisting of TBP and pX heterodimers was observed by immunoprecipitation with anti-X serum (Fig. 3*B*, lane 3). Neither pX nor TBP homodimers were observed (Fig. 3*B*, lanes 1 and 4).

pX–TBP Interaction Requires ATP. Another line of evidence for the specificity of the pX–TBP interaction is provided by the requirement for ATP. pX–TBP interaction studies were carried out in the presence of the nonhydrolyzable ATP analogs adenosine 5′-[γ-thio]triphosphate and adenosine 5′-[β,γ-imido]triphosphate, the ATPase inhibitor carbonyl cyanide *m*-chlorophenylhydrazone, and the ATP-hydrolyzable enzyme apyrase. In all cases, pX–TBP interactions were abolished when compared with a reaction mixture in which

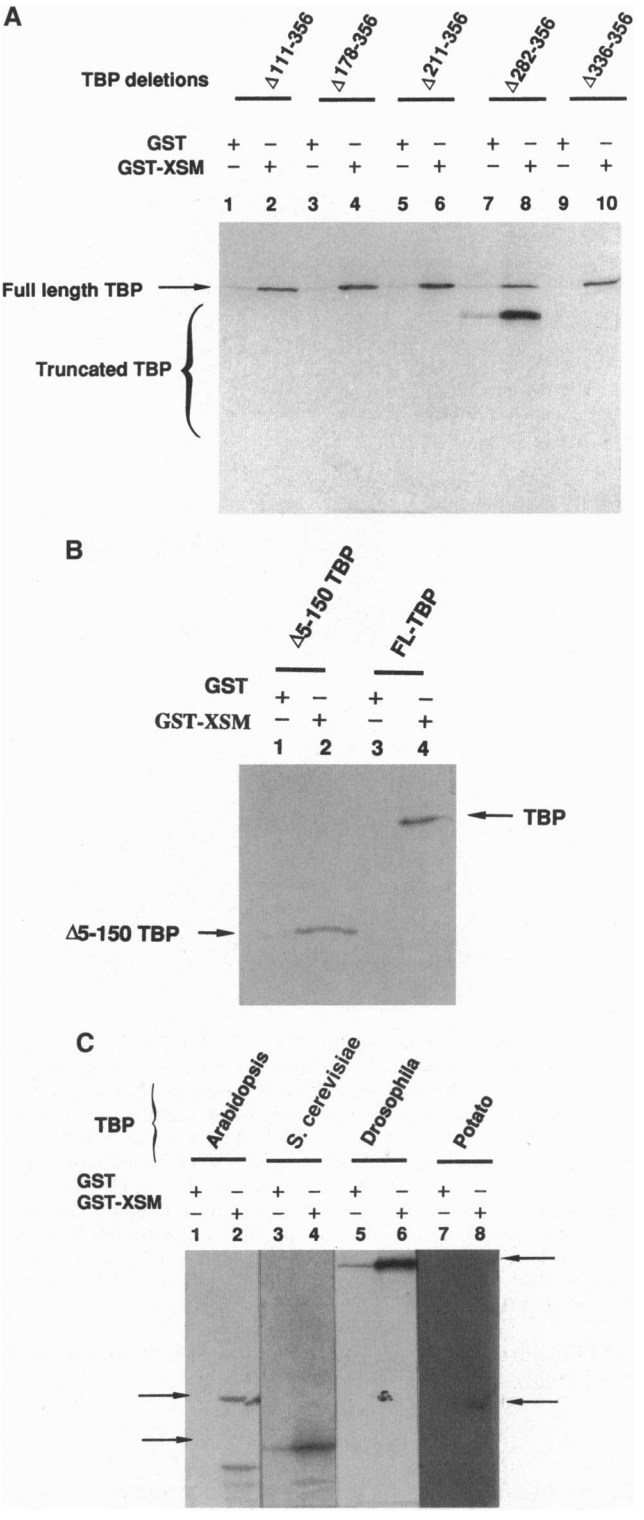

FIG. 2. (*A*) Binding of pX to various TBP deletion mutants. Full-length TBP and truncated TBP proteins Δ111–356 (lanes 1 and 2), Δ178–356 (lanes 3 and 4), Δ211–356 (lanes 5 and 6), Δ282–356 (lanes 7 and 8), and Δ336–356 (lanes 9 and 10) were bound on glutathione beads containing either GST or GST-XSM. (*B*) Binding of the full-length (FL) TBP and an amino-terminal deletion mutant (Δ5–150) of TBP to GST or GST-XSM. (*C*) Interactions of ^{35}S-labeled TBP derived from phylogenetically divergent species to glutathione beads containing either GST or GST-XSM: lanes 1 and 2, *A. thaliana* (isoform 2); lanes 3 and 4, *S. cerevisiae*; lanes 5 and 6, *D. melanogaster*; lanes 7 and 8, *Solanum tuberosum* (potato).

ATP was present (Fig. 4). Since no interaction was detected in the presence of nonhydrolyzable analogs of ATP, we believe

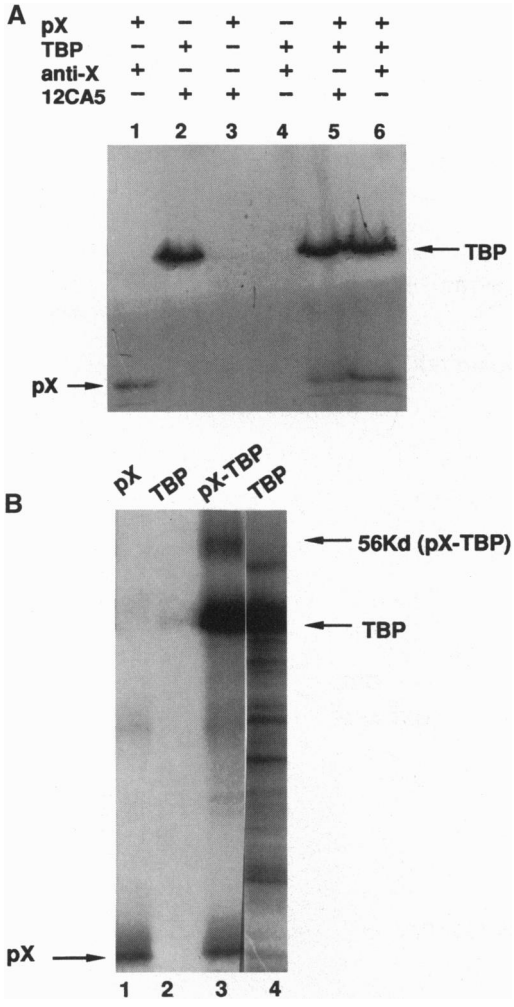

FIG. 3. (*A*) Coimmunoprecipitation of ^{35}S-labeled HA-tagged TBP and pX. HA monoclonal antibody 12CA5 or polyclonal anti-X serum was used to immunoprecipitate the mixture. Lanes 1 and 2, immunoprecipitation of positive control; lanes 3 and 4, immunoprecipitation of pX with monoclonal antibody 12CA5 and TBP with anti-X serum; lanes 5 and 6, complexes of pX–TBP immunoprecipitated with either 12CA5 or anti-X serum, respectively. (*B*) Chemical crosslinking of TBP with pX in the presence of glutaraldehyde. Crosslinked complexes were immunoprecipitated with anti-X serum except for lane 4. Lane 1, pX; lane 2, TBP; lane 3, pX and TBP; lane 4, input TBP without immunoprecipitation. Arrow indicates the 56-kDa pX–TBP complex.

that the hydrolysis of ATP is required for pX to interact with TBP *in vitro*.

DISCUSSION

One curious feature of pX is its ability to transactivate a large number of viral and cellular promoters/enhancers. The precise mechanism(s) by which pX accomplishes this function has been the subject of intense scrutiny. Here we present evidence that pX can associate with TBP. This interaction is inhibited in the presence of nonhydrolyzable ATP analogs, suggesting a requirement for ATP. The functional significance of this observation is not understood.

The pX–TBP interaction implies that pX should reside in the nucleus. Although previous work in our laboratory showed that pX was predominately localized in the cytoplasm in transiently transfected COS cells (36), nuclear distribution has been reported by others (7). In light of the role of pX in signal transduction pathways, its interaction with important compo-

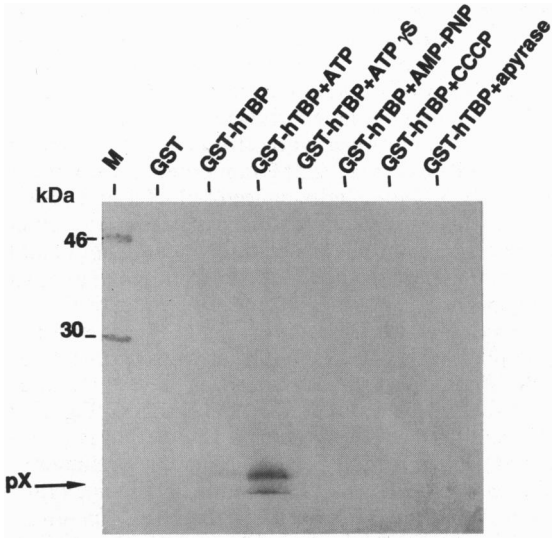

FIG. 4. Inhibition of pX binding to GST-human TBP (hTBP) in the presence of analogs and inhibitors of ATP. GST-hTBP protein and *in vitro* translated ^{35}S-labeled pX were incubated with hexokinase (1 unit/μg of protein) and glucose (1 mM) in 70 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM dithiothreitol for 20 min at room temperature to deplete endogenous ATP. ATP, adenosine 5′-[γ-thio]triphosphate (ATPγS), adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP), or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added at 1 mM, and apyrase was added at 10 units/μg of protein, as indicated above the lanes. Binding reactions were carried out as described.

nents of the transcriptional machinery and ATF-2/CREB, such patterns of cellular distribution would probably be dictated by its function.

TBP binds to the minor groove of the DNA and appears to promote DNA bending (29). The x-ray crystal structure of TBP shows that it forms a saddle-like structure, where the concave underside binds DNA and the convex upper surface interacts with regulatory proteins (29, 37, 38). In the yeast TBP, direct repeat sequences (aa 61–150 and 151–240) contribute to the functional activity of this protein (38). An analogous domain corresponds to aa 155–355 in the carboxyl-terminal region of human TBP. The subdomains designated S4/S4′, S5/S5′, S1′, and H2 that appear on the convex surface of TBP are deleted in the mutant Δ211–356 used in the present analysis. Not surprisingly, this mutant and the sequential shorter deletion mutants of TBP which lack the putative protein interaction sites fail to interact with pX (Fig. 2*A*, lanes 2, 4, and 6).

The pX domain consisting of aa 110–143 displays a striking resemblance with a domain (aa 125–145) of the adenovirus E1A protein that was identified as the minimal region required for interaction with TBP (6, 17). This analogous domain in pX has also been shown to be indispensable for transactivation function (7). Our recent studies in which the E1A gene was replaced with the HBV X gene in the background of adenovirus indicate that pX can substitute for E1A by restoring early gene expression to wild-type levels (J. S. Schaack, H.F.M., and A.S., unpublished results). In the present context, another feature shared by both pX and E1A is their ability to interact with ATF-2. In that capacity, these proteins may act as adapter molecules that link TBP with ATF-2 (7, 17). In a similar model, a heteromeric complex of the transcriptional activators MBF-1 and MBF-2 has been proposed to serve as an adapter between BmFTZ-F1 and TBP, which functions to stabilize the BmFTZ-F1–DNA complex (39).

Studies were carried out to demonstrate a functional role for pX in cotransfection experiments using TBP, pX, and the luciferase reporter gene under the control of the TATA box.

However, these combinations did not produce any effect on luciferase expression. This observation suggests that pX–TBP-mediated activity requires coactivators (i.e., ATF-2, NF-κB, or an unknown factor) (5–8). Therefore, multiple interactions with the promoter complex may be crucial for transcriptional transactivation by pX. A similar theme is also echoed for the human cytomegalovirus IE2 protein, whose ability to transactivate correlates not only with its interaction with TBP, but also with other upstream regulatory factors (e.g., Sp1 and Tef-1) (20, 40). Gel mobility-shift experiments indicate that pX does not appear to interfere with TBP–TATA box interactions (data not shown). Based on the models of TBP interactions with various factors, we suggest that pX may exert its transactivation function by operating in conjunction with the universal transcriptional factor TBP and other upstream regulatory factors that influence the transcriptional machinery. In this capacity, pX could influence transcription of a large repertoire of genes, which is consistent with the observed promiscuous behavior of pX (7). In a recent report, an ATPase activity has been shown to be associated with pX (41). It will be of particular interest to determine the functional relevance of pX's ATPase activity in the context of preinitiation complex assembly and pX–TBP interaction.

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