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The Hepatitis B Virus X Protein Increases the Cellular Level of TATA-Binding Protein, Which Mediates Transactivation of RNA Polymerase III Genes

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The hepatitis B virus X gene product transactivates a variety of cellular and viral genes. The mechanism for X induction of RNA polymerase (pol) III genes was investigated. By using *Drosophila* S-2 cells stably transformed with the X gene, the transient expression of a tRNA gene is enhanced. Comparing the transcriptional activities of extracts derived from these cells, all three types of RNA pol III promoters are stimulated by X. Interestingly, both S-2 and rat 1A cells stably transformed with the X gene produce increased cellular levels of the TATA-binding protein (TBP). By using various kinase inhibitors, it was found that the X-mediated increases in both transcription and TBP are dependent upon protein kinase C activation. Since TBP is a subunit of TFIIIB, the activity of this component fractionated from extracts derived from control and X-transformed cells was analyzed. These studies reveal that TFIIIB activity is substantially more limiting in control cells and that TFIIIB isolated from X-transformed cells has increased activity in reconstitution assays compared with TFIIIB isolated from control cells. Conversely, comparison of TFIIIC from control and X-transformed cell extracts revealed that there is relatively little change in its ability either to reconstitute transcription or to bind to DNA and that there is no change in the catalytic activity of RNA pol III. Studies were performed to determine whether directly increasing cellular TBP alone could enhance RNA pol III gene transcription. Transient expression of a TBP cDNA in rat 1A cells was capable of stimulating transcription activity from the resultant extracts in vitro. Together, these results demonstrate that one mechanism by which X mediates transactivation of RNA pol III genes is by increasing limiting TBP via the activation of cellular signaling pathways. The discovery that X increases cellular TBP, the universal transcription factor, provides a novel mechanism for the function of a viral transactivator protein and may explain the ability of X to produce such large and diverse effects on cellular gene expression.

The hepatitis B virus (HBV) encodes four genes, one of which produces a 154-amino-acid polypeptide, X, which is known to transactivate a large variety of cellular and viral RNA polymerase (pol) II promoters as well as an RNA pol III promoter (51). There is evidence that X may play a significant role in the development of hepatocellular carcinoma, which occurs with high incidence following chronic infection by HBV (35). Hence, the mechanism of action of X has been the subject of intense study. The mechanism for X transactivation is not yet clear; however, several distinct models have been proposed. X does not bind to DNA directly, but several studies have provided evidence that X may interact with specific DNA-binding proteins to activate transcription. X has been shown to stimulate transcription when targeted to specific DNA-binding sites by fusion of X with heterologous DNA-binding domains (39, 45). Other studies have demonstrated direct protein-protein interactions between X and the CREB/ATF family of transcription factors in vitro (30, 49). In this case, X interacts specifically with the leucine zipper domain of CREB and increases the affinity of CREB for cyclic AMP response element (CRE) sites (49). Recently, X was shown to interact with human RPB5, a polypeptide shared by all RNA pols (5), and preliminary data indicate that X may also interact with the TATA-binding protein (TBP), a transcription factor used in the expression of all cellular genes (34). These studies suggest that at least one mechanism for transactivation by X may involve its direct interaction with the transcriptional machinery.

Alternative models have also been proposed whereby X functions indirectly to regulate transcription. X-mediated stimulation of AP-1-dependent promoters has been shown to occur through the activation of various cellular protein kinases, including protein kinase C (PKC), Raf-1, and Ras (3, 11, 25, 32, 33). X transactivation is abolished when various protein kinase inhibitors are used to block PKC activation (11, 25). X-induced increases in the DNA-binding activity of AP-1 (3) and transcriptional activation of c-Jun (33) are inhibited when dominant negative Ras mutants are used. However, the mechanism for the X-mediated activation of cellular signaling events remains unclear. X appears to modulate AP-1-dependent promoters in different cell lines, yet most cell lines do not exhibit enhanced AP-1 DNA-binding activity (32). Furthermore, both the HBV enhancer Enh 1 (31) and NF-κB-dependent promoters (29), which are inducible by phorbol ester activation of PKC, appear to be transactivated by X without requiring PKC activation. Thus, it is likely that stimulation of transcription of cellular genes by X occurs through different mechanisms, depending on the promoter and the cell system, involving both direct and indirect consequences on the transcription machinery.

RNA pol III is responsible for the transcription of a large

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variety of nontranslated cellular and viral RNAs. The three general classes of RNA pol III-transcribed genes in vertebrate cells include the tRNA/VA RNA, 5S RNA, and U6 RNA types of promoters. All of the RNA pol III genes analyzed to date require the transcription components TFIIIB and RNA pol III (50). The tRNA and 5S RNA genes additionally use TFIIIC. TFIIIC binds directly to the internal promoter of tRNA genes, leading to the assembly of TFIIIB and RNA pol III. The TFIIIA factor is additionally required for 5S RNA gene transcription. The U6 RNA gene promoter uses a unique factor complex that interacts with the proximal sequence element (19, 52) and does not require TFIIIC for transcription in vitro.

Both TFIIIB and TFIIIC are multisubunit proteins. The structures of these proteins from *Saccharomyces cerevisiae* have been best defined. In this system, TFIIIB is a complex containing three polypeptides, whereas TFIIIC contains six distinct subunits (50). The polypeptides that compose the TFIIIB and TFIIIC complexes in mammalian and insect systems remain unknown. Consequently, TFIIIB and TFIIIC are defined as biochemical fractions that elute from phosphocellulose chromatography and are required to reconstitute transcription from tRNA gene promoters (46, 50). The one polypeptide that has been shown to be an integral part of the TFIIIB complex is TBP (24, 27, 28, 47). TBP has been shown to be required for the transcription of all cellular genes (10, 20, 38, 43). In addition to TFIIIB, at least two other TBP-containing complexes have been characterized. TFIID, which is used in the transcription of RNA pol II genes, is composed of TBP and at least eight polypeptides or TBP-associated factors (TAFs) (15). Although TBP is sufficient to reconstitute basal transcription of RNA pol II TATA-containing promoters with the other general RNA pol II factors, the additional TAFs are required to mediate the action of upstream activators. SL1, a TBP-containing complex, is required for the transcription of RNA pol I genes (9). The TAFs that are assembled with TBP to form the SL1, TFIID, and TFIIIB complexes appear to be unique for each complex and thus determine the roles of TBP in the expression of RNA pol I, II, and III genes.

Previously, the HBV X protein was shown to transactivate the adenovirus VA RNA1 gene promoter (2). We have used the RNA pol III transcription system as a model for understanding the mechanism of X in the transactivation of cellular genes. By using both *Drosophila* S-2 and rat 1A cell lines, stable transformants that express the X gene were constructed. In both cases, expression of X results in a significant increase in the cellular levels of TBP. We have investigated whether this increase in TBP is responsible for upregulating RNA pol III gene transcription. In the S-2 cells, both the X-mediated increase in TBP and resultant RNA pol III gene activity in vitro require the activation of cellular protein kinases. Further analysis of the activities of the TFIIIB and TFIIIC components in extracts derived from X-transformed and nontransformed cells revealed that the expression of X increases TFIIIB activity, whereas no changes in TFIIIC or RNA pol III activities were observed. Increasing cellular TBP levels directly by transient expression of a TBP cDNA in rat 1A cells resulted in enhanced RNA pol III gene activity in vitro. Together, these results demonstrate that the X-mediated increase in TBP is one mechanism responsible for the activation of RNA pol III genes. The newly discovered function of X, its ability to regulate cellular levels of the universal transcription factor TBP, may also explain its pleiotropic effects on the activity of many RNA pol II genes.

MATERIALS AND METHODS

Plasmid DNAs. The plasmid used for expression of the X gene in the *Drosophila* S-2 cells, pMT-Hbx, contains the HBV X gene under the control of the metallothionein promoter. This plasmid was constructed by amplifying the X gene by PCR with the primers JN-3 (5′-AGTAGTCGACTTAGGCAGAGGTG AAAAAG-3′), which anneals to the initiation region, and JN-7 (5′-GACTTGA TCATGGCTGCTAGGCTGTACT-3′), which anneals to the termination region of the gene. The amplified 456-bp fragment containing the X gene was digested with *Bcl*I and *Sal*I restriction endonucleases and subcloned into the *Bam*HI and *Sal*I sites of the pMT expression vector (4). pCO-Hygro contains a hygromycin resistance gene driven by the copia promoter. pECE-X and pECE-Xr contain the X gene in the sense and antisense orientations, respectively, under the control of the simian virus 40 (SV40) promoter (40). pLTReTBP contains a human TBP gene under the control of the SV40 promoter (53). The pBluescriptSK$^+$ vector DNA (Promega) was used to maintain a constant amount of DNA in the transient-transfection assays.

The plasmids containing the RNA pol III genes used for the transcription assays are pArg-maxigene, a derivative of a *Drosophila* tRNA^{Arg} gene which contains an additional 12 bp inserted between the internal promoter regions (16); pDU6-2, a *Drosophila* U6 RNA gene (12); and pDm5SI, a *Drosophila* 5S RNA gene (42).

Stable and transient transfections. The X-S2 stable transformant cells were obtained by calcium phosphate transfection of *Drosophila* S-2 cells with a mixture of DNAs including 5 μg of pMT-Hbx, containing the HBV X gene under the control of the metallothionein promoter; 5 μg of pCO-Hygro, containing the hygromycin resistance gene under the control of the copia promoter; and 10 μg of pBluescriptSK$^+$ vector. After 24 h, the transfected cell medium was replaced with medium containing hygromycin B (250 μg/ml). The pooled cells, designated X-S2, were passaged every 3 days for 6 weeks. PCR was used to detect intact HBV X gene integration, with the transformant genomic DNA as the template.

For the transient transfections of the S-2 cells shown in Fig. 1B, control (S-2) and X-S2 cells were transfected by calcium phosphate precipitation methods with 20 μg of total DNA per 5 ml of cell culture with various amounts of pArg-maxigene and pBluescriptSK$^+$ plasmid DNAs. Cells were transfected at a density of 0.5×10^6 to 1.0×10^6 cells per ml. After 24 h, the medium of the transfected cells was replaced with fresh medium, and 500 μM $CuSO_4$ was added 6 h later. After an additional 24 h, the cells were harvested.

Rat 1A stable transformants were obtained by cotransfecting either pECE-X (containing the HBV X gene driven by the SV40 promoter) or pECE-Xr (containing the HBV X gene in the antisense orientation) and a neomycin resistance gene into rat 1A fibroblast cells, with G418 (400 μg/ml) selection.

Transient transfections of the rat 1A cells were carried out with calcium phosphate precipitation methods at a cell density of 0.5×10^5 to 1.0×10^5 cells per ml. The transfection assays were carried out with pLTReTBP (where designated) and pBluescriptSK$^+$ plasmids for 20 μg of total DNA per 10 ml of cell culture. Cells were harvested 48 h after transfection.

Transcription assays. The in vitro transcription assays were carried out with extracts or transcription factor fractions, as indicated. The final reaction mixture contained 0.4 μg of DNA template (unless otherwise indicated); 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9); 5 mM $MgCl_2$; 3 mM dithiothreitol (DTT); 100 mM KCl; 10% glycerol; 0.5 mM each ATP, CTP, and UTP; and 0.1 mM [α-^{32}P]GTP (6 Ci/mmol) in a final reaction volume of 60 μl. Reactions were incubated for 1 h at room temperature and stopped by the addition of 0.1% sodium dodecyl sulfate (SDS) and 400 μg of proteinase K per ml. After 15 min at 37°C, RNAs were purified by phenol extraction and ethanol precipitation and analyzed by electrophoresis on 8 M urea–8% polyacrylamide gels. Transcription products were visualized by autoradiography, and the resultant bands were quantitated by densitometry (Biosystem Image). The values given in the text for the relative levels of transcription stimulation were based on at least three independent experiments in each case.

For Fig. 4A and 5A, limiting TFIIIB and TFIIIC concentrations, respectively, were used. Conditions were experimentally determined so that the TFIIIB fraction was limiting in the reactions by keeping TFIIIC constant and varying the amount of the TFIIIB fraction. The concentrations of TFIIIB used for the assays shown in Fig. 4A were those concentrations of TFIIIB that were in the linear range of the assay and gave measurable levels of transcription. The same strategy was used for determining the concentrations of TFIIIC that were limiting in the assays shown in Fig. 5A.

For analysis of the expression of the transiently transfected pArg-maxigene in Fig. 1B, RNA was extracted after the cells were harvested by using TRIzol reagent (GIBCO). The isolated RNAs (0.1 μg of each sample) were hybridized with an excess of a ^{32}P-labeled antisense pArg-maxigene riboprobe and yeast tRNA as a carrier at 42°C overnight. RNase protection assays were carried out with an RPA II kit supplied by Ambion. The hybridized RNAs were digested with 200 μl of a 1:100 dilution of an RNase A-RNase T_1 mixture at 37°C for 30 min. The digestion was terminated by adding 300 μl of solution Dx and 250 μl of ethanol. The RNAs were precipitated and resuspended in 8 μl of RNA loading buffer and electrophoresed on 8 M urea–8% polyacrylamide gels. The transcripts were quantitated by optical densitometry of the resultant autoradiographs.

Preparation of extracts and transcription factor preparation. Cytoplasmic (S100) extracts were prepared from the *Drosophila* S-2 and X-S2 cells by the

method of Dingermann et al. (14). Nuclear extracts were prepared by the method of Dignam et al. (13). The TFIIIB and TFIIIC fractions were prepared as described previously (17). Briefly, cytoplasmic extracts were fractionated by phosphocellulose chromatography. TFIIIB activity was eluted at 0.35 M KCl, and TFIIIC activity was eluted at 0.7 M KCl. The fractions were dialyzed against 20 mM HEPES (pH 7.9)–0.1 mM EDTA–1 mM DTT–20% glycerol–100 mM KCl and stored at −70°C.

Gel shift assays. A 122-bp *Bsl*I-*Sau*96I DNA fragment derived from the pArg-maxigene plasmid containing the internal A and B box elements was labeled with ^{32}P at the 3′ end and used for a gel shift assay. The binding reactions contained 20 mM HEPES (pH 7.9), 83 mM KCl, 5 mM $MgCl_2$, 0.7 mM DTT, 0.02 mM EDTA, 8% glycerol, 2.5 μg of poly(dI-dC)-poly(dI-dC), 1 ng of the ^{32}P-labeled 122-bp fragment, and 4 μg of cytoplasmic extracts from either S-2 or X-S2 cells induced with 500 μM $CuSO_4$. The binding reactions were incubated at room temperature for 20 min and then subjected to electrophoresis on 4% polyacrylamide gels (acrylamide-bisacrylamide, 29:1) in a TBE (Tris-borate-EDTA) buffer system.

Western immunoblot analysis. Equal amounts of proteins from either *Drosophila* cell cytoplasmic extracts or rat cell nuclear extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblotting was carried out as described previously with anti-*Drosophila* TBP antibodies and anti-*Drosophila* eIF-2α antibodies (17). Anti-human TBP antibodies were generously provided by Arnold Berk (University of California, Los Angeles), and anti-bovine eIF-2α antibodies were provided by Stanley Tahara (University of Southern California). Horseradish peroxidase-linked anti-rabbit immunoglobulin antibodies and enhanced chemiluminescence reagents (Amersham) were used to detect bound antibodies.

RESULTS

Stably transformed S-2 cells expressing HBV X exhibit enhanced transcriptional activities for all three classes of RNA pol III promoters. We have constructed a *Drosophila* S-2 cell line that has been stably transformed with a cDNA encoding the X gene placed under the control of the metallothionein promoter. Reverse transcription (RT)-PCR analysis was carried out with RNA derived from pooled stably transformed cells containing both the X and hygromycin B expression plasmids (X-S2), stably transformed cells containing the hygromycin B resistance expression plasmid only (H-S2), and the parental (S-2) cells. As shown in Fig. 1A, the results revealed that X is expressed in the X-S2 cells even in the absence of a heavy metal ion inducer, but an approximately 20-fold increase in the levels of X mRNA is observed when the cells are incubated with copper. This can be compared with either the S-2 or H-S2 cells, which do not produce the X transcript. The effects of X expression on RNA pol III gene activity were further assessed (Fig. 1B). S-2 and X-S2 cells were transiently transfected with different amounts of a plasmid containing a $tRNA^{Arg}$ gene (pArg-maxigene). RNA was isolated from the transfected cells, and RNase protection assays were performed to determine the relative levels of expression from the pArg-maxigene with equal amounts of RNA. The X-S2 cells showed an increase of approximately fivefold in pArg-maxigene activity compared with that observed for the S-2 cells, regardless of the amount of pArg-maxigene used for transfection (Fig. 1B and Table 1). No difference was observed in the transcriptional activities of the control H-S2 and S-2 cells either in vivo, using transient-transfection assays, or in vitro, using extracts derived from these cells (data not shown). Therefore, all subsequent experiments were performed with the S-2 cells as a control.

In order to determine whether this stimulation could be reproduced in vitro, cytoplasmic extracts were prepared from S-2 and X-S2 cells and tested for RNA pol III transcription activities (Fig. 2). Using either tRNA, 5S RNA, or U6 RNA gene templates and equal amounts of cytoplasmic protein, significant enhancement of transcription activity from extracts derived from the X-S2 cells was observed (Table 1). Similar results were obtained with nuclear extracts derived from these cells (data not shown). A more pronounced increase in the transcriptional activities of the resultant extracts was obtained

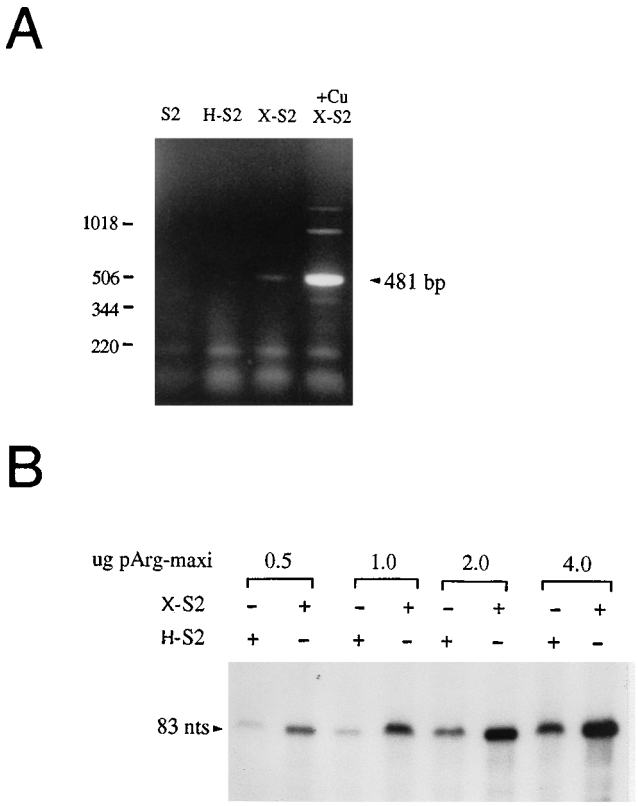

FIG. 1. Transactivation of a tRNA gene promoter in S-2 cells stably transformed with the X gene. (A) RT-PCR analysis of stably X-transformed S-2 cells. Stable transformants were constructed as described in Materials and Methods. S-2 (nontransformed cells), H-S2 (cells stably transformed with the hygromycin B resistance expression plasmid), and X-S2 (cells stably transformed with both the hygromycin B resistance and X expression plasmids) were analyzed for the presence of an X-specific transcript. Total RNA from the S-2, H-S2, and X-S2 cells was prepared by the acid guanidinium thiocyanate method (6). Total RNA (3 μg) from each cell type was used to generate the first-strand cDNA by using avian myeloblastosis virus reverse transcriptase and primer JN3 (5′-AGTAGTC GACTTAGGCAGAG GTGAAAAAG-3′), which anneals with the termination region of the HBV X gene. The reaction was incubated at 42°C for 1 h and then extracted with phenol-chloroform. This mixture, containing the first-strand cDNA, was ethanol precipitated and resuspended in 40 μl of distilled water. The resuspended DNA (5 μl) was used in the PCR with the primers JN3 and JN6 (5′-GGTACCATGGAGACCACCG TGAA-3′), which anneals with the HBV X initiation region. For PCR, 39 rounds of thermal cycling were carried out; each cycle consisted of 92°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and the last cycle consisted of 55°C for 2 min and 72°C for 7 min. Each PCR product was applied to a 1.5% agarose gel for electrophoresis. (B) Transient expression of a tRNA gene promoter in X-transformed cells. Transient transfections of increasing amounts of the pArg-maxigene-containing plasmid were performed as described in Materials and Methods. RNA was extracted 24 h after the addition of 500 μM $CuSO_4$ to the cells, and RNase protection assays (Ambion) were carried out with equal amounts (0.1 μg) of RNA and a ^{32}P-labeled antisense pArg-maxigene riboprobe. The resultant RNAs were separated by electrophoresis on 8% polyacrylamide gels containing 8 M urea, and the resultant ^{32}P-labeled RNAs were visualized by autoradiography.

when the X-S2 cells were incubated with 500 μM $CuSO_4$ to induce high levels of X expression than with lower concentrations of $CuSO_4$ (Table 1). Therefore, all subsequent experiments were carried out by incubating control and X-S2 cells with 500 μM $CuSO_4$. As all three promoters share only RNA pol III and TFIIIB components and the U6 RNA gene does not use TFIIIC in vitro, this result provides the first evidence that TFIIIB or RNA pol III could be a target of X.

HBV X transactivation of RNA pol III genes depends on the activation of protein kinases. The increased transcriptional

TABLE 1. Enhancement of class III gene promoter activity in S-2 cells stably transformed with the HBV X protein

Promoter or gene	Fold enhancement (mean ± SD)[a]	
	250 μM $CuSO_4$	500 μM $CuSO_4$
tRNA[b]	ND[c]	5.1 ± 1.2 (3)
tRNA[d]	3.4 ± 1.2 (4)	14 ± 7.2 (6)
5S RNA[d]	11 ± 1.8 (4)	17 ± 9.1 (3)
U6 RNA[d]	6.2 ± 2.2 (3)	11 ± 3.7 (3)

[a] Transient-transfection and in vitro transcription assays were performed with the indicated DNAs as templates as described in Materials and Methods. The number of independent experiments performed for each template with either 250 or 500 μM $CuSO_4$ to induce X gene expression is indicated in parentheses.
[b] Data are based on transient transfection of the pArg-maxigene as described in the legend to Fig. 1B.
[c] ND, not determined.
[d] The data shown were derived from comparison of template activities in S-2 and XS-2 cell extracts as described in the legend to Fig. 2.

capacity of the extracts derived from the X-transformed cells could be ascribed to either a direct or an indirect effect of X on the RNA pol III transcription machinery. In order to assess whether X was acting indirectly on the transcription process, we analyzed whether the X-mediated enhancement of RNA pol III transcription activity was dependent upon the activation of cellular signaling pathways. X was previously shown to activate the PKC pathway (25), and our previous studies demonstrated that RNA pol III gene expression can be induced by the potent PKC activator tetradecanoyl phorbol acetate (TPA) (16). Therefore, the ability of X to act through PKC to increase transcription of these genes was examined. The protein kinase inhibitors HA1004, H7, and GF109203X were used at concentrations based on their relative K_is in vitro. Previous results have shown that, at a concentration of 0.1 mM, HA1004 completely inhibits PKA and PKG and partially blocks certain PKC isoforms (1). At the same concentration, H7 completely inhibits PKC, PKG, and PKA (21). The GF109203X inhibitor is a highly selective PKC inhibitor that acts as a competitive inhibitor of the ATP-binding site on PKC (44). Cytoplasmic extracts were prepared from X-S2 and S-2 cells incubated with the kinase inhibitors, and we subsequently determined the resultant transcriptional activities by using the pArg-maxigene as a template (Fig. 3A). Importantly, none of the inhibitors were found to decrease the transcriptional activities of the resultant S-2 extracts. This indicates that, at the concentrations of inhibitors used, no general toxic effects on the cells were observed. In fact, GF109203X produced a slight stimulatory effect of 1.5- to 3.6-fold. HA1004 produced a more reproducible increase in the transcription activity of the S-2 cell extracts of 2.6- to 5.2-fold. Examining the effect of these inhibitors on the X-S2 cell extracts, the PKA inhibitor HA1004 did not alter the X-mediated enhanced transcriptional activity of these extracts. However, the increased transcriptional activity of the X-S2 extracts was partially blocked by the PKC inhibitor H7 and fully inhibited by the more specific PKC inhibitor GF109203X. Similar results were obtained when the U6 RNA gene was used as the template (data not shown). These results indicate that X indirectly mediates an increase in the transcriptional capacities of the X-S2 cell extracts. Furthermore, these results suggest that this increase is dependent upon X-mediated activation of the PKC pathway.

HBV X produces an increase in the cellular levels of TBP. Our previous studies examined the mechanism for induction of RNA pol III transcription in *Drosophila* S-2 cells by the phorbol ester TPA, a potent activator of PKC. Biochemical analysis revealed that this regulatory event was mediated by an increase in TFIIIB activity, with no change in the activities of either RNA pol III or TFIIIC (16, 17). Further analysis of TFIIIB showed that TPA treatment of the cells produced a corresponding increase in the amount of the TBP subunit of TFIIIB (17). Consequently, we examined whether the X-mediated activation of the PKC pathway would similarly increase the cellular levels of TBP. As shown in Fig. 3B, Western blot analysis revealed that the amount of the TBP polypeptide was increased approximately fivefold in the X-S2 cell extracts. Incubation of the X-S2 cells with the PKC inhibitors H7 and GF109203X significantly blocked the X-mediated increase in TBP. As an internal control, the amount of the translational protein eIF-2α was determined. As shown (Fig. 3B), this polypeptide remained constant in each of the extracts. Thus, the amount of TBP in these extracts parallels the amount of transcription activity (compare Fig. 3A and B). It was also found that serum starvation of the S-2 and X-S2 cells significantly enhanced the X-mediated increases in both transcription and TBP levels (data not shown). These results provide the first evidence that X mediates an increase in the cellular level of TBP and suggest that the PKC pathway may regulate the cellular levels of TBP, a transcription component used by all cellular promoters.

Extracts derived from X-transformed cells contain higher levels of TFIIIB activity than nontransformed cell extracts. The above results provided a strong correlation between the increased amount of TBP present in the X-S2 cell extracts and their resultant enhanced transcriptional activities, suggesting that X could be mediating an increase in the activity of the TBP-containing complex TFIIIB. Therefore, the potential differences in the activity of TFIIIB in the S-2 and X-S2 extracts were examined. We first determined whether TFIIIB was differentially limiting in these two extracts (Fig. 4A). Transcription reactions were performed with extracts derived from S-2 and X-S2 cells, with increasing amounts of an S-2 cell-derived TFIIIB fraction added to the reactions. The addition of TFIIIB to both extracts stimulated transcription from the tRNA gene template; however, the control extracts were more substantially stimulated when equivalent amounts of TFIIIB were added. In an examination of three different sets of extracts, TFIIIB was found to stimulate the S-2 extracts 5.8 ± 0.4-fold more than the X-S2 cell extracts. This indicates that TFIIIB activity is limiting in both the S-2 and X-S2 cell extracts but that TFIIIB is substantially more limiting in the S-2 cell extracts. To more directly assess potential differences in the level of TFIIIB activity in these extracts, TFIIIB was fractionated from both S-2 and X-S2 cell extracts. Transcription reconstitution assays were carried out with either an S-2 cell-derived or an X-S2 cell-derived TFIIIB fraction and saturating amounts of a TFIIIC fraction derived from S-2 cell extracts together

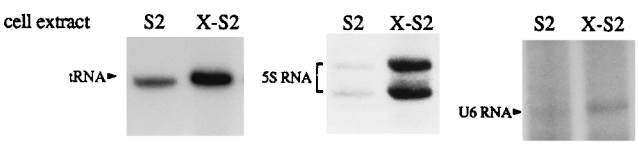

FIG. 2. Extracts derived from S-2 cells stably transformed with the X gene have enhanced transcriptional activities for all three classes of RNA pol III promoters. S-2 and X-S2 cells were treated with 250 μM $CuSO_4$ for 12 h, and cytoplasmic extracts were prepared as described previously (14). In vitro transcription assays were carried out as described in Materials and Methods with 162 μg of protein from either S-2 or X-S2 extracts and 0.8 μg of the pArg-maxigene (lanes 1 and 2), 0.8 μg of the 5S RNA gene (lanes 3 and 4), or 1.2 μg of the U6 RNA gene (lanes 5 and 6).

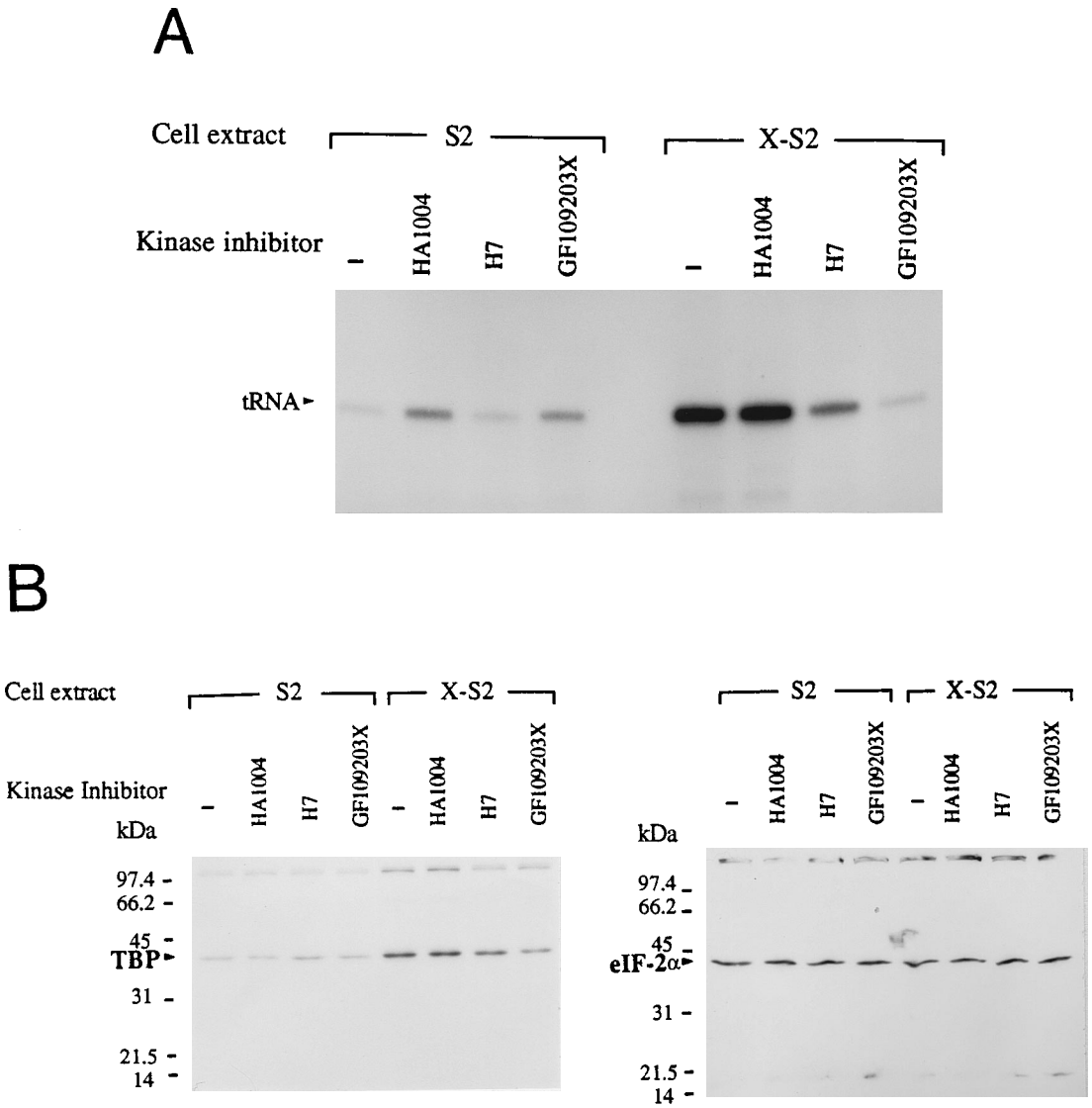

FIG. 3. X mediates an increase in RNA pol III transcription activity and the TBP that can be blocked by PKC inhibitors. Where designated, S-2 and X-S2 cells were treated with the PKA inhibitor HA1004 (0.1 mM) or the PKC inhibitor H7 (0.1 mM) or GF109203X (2 μM) for 6 h and then incubated with 500 μM $CuSO_4$ for another 24 h, and cytoplasmic extracts were prepared. (A) Analysis of transcription activities. Transcription assays were carried out as described in Materials and Methods with 129 μg of protein per extract and 0.6 μg of pArg-maxigene as the template. (B) Determination of the levels of TBP by Western blot analysis. The TBP polypeptide was analyzed with anti-*Drosophila* TBP antibodies and eIF-2α was analyzed with anti-*Drosophila* eIF-2α antibodies (15 μg of protein per lane). Horseradish peroxidase-linked anti-rabbit immunoglobulin antibodies and enhanced chemiluminescence reagents (Amersham) were used to detect bound antibodies.

with the pArg-maxigene template (Fig. 4B). For three different preparations of TFIIIB, a 4.0 ± 1.6-fold increase in transcription was observed in assays reconstituted with the X-S2 cell-derived TFIIIB fraction. These studies provide further evidence that X mediates an increase in TFIIIB activity which is responsible for the enhanced transcriptional capacity of the X-S2 cell extracts that was observed.

Although these results demonstrate that X mediates a change in the TFIIIB component, previous studies suggested that X may target TFIIIC and alter its DNA-binding activity (2). Therefore, the relative activities of TFIIIC derived from the S-2 and X-S2 cell extracts were determined (Fig. 5A). Transcription reconstitution assays with saturating amounts of S-2 cell-derived TFIIIB together with TFIIIC fractions purified from either S-2 or X-S2 cell extracts revealed that there was little difference (approximately 1- to 1.6-fold) in the ability of S-2 and X-S2 cell-derived TFIIIC to support tRNA gene transcription. The relative DNA-binding activities of TFIIIC from the S-2 and X-S2 extracts were also determined in a gel mobility shift assay (Fig. 5B). No apparent change in the ability of X-S2 cell-derived TFIIIC to bind to a tRNA gene template was found. Since the TFIIIC fraction also contains RNA pol III activity (17), these results also suggest that RNA pol III itself is not changed. To directly address this, the ability of RNA pol III from the S-2 and X-S2 extracts to catalyze nonspecific transcription was compared for four independent sets of extracts. Both the total RNA pol activity and the contribution of RNA pol III activity were measured as previously described (16). Comparison of the X-S2 and S-2 extracts revealed that there was a 1.3 ± 0.28-fold difference in total polymerase activity and a 1.5 ± 0.68-fold change in RNA pol III activity. When RNA pol III activity was measured in the TFIIIB fractions derived from these extracts, similar results were obtained. Thus, we find that there is no apparent change in the catalytic activity of RNA pol III in either the X-S2 extracts or TFIIIB-derived fractions. Together, these results indicate that the X-

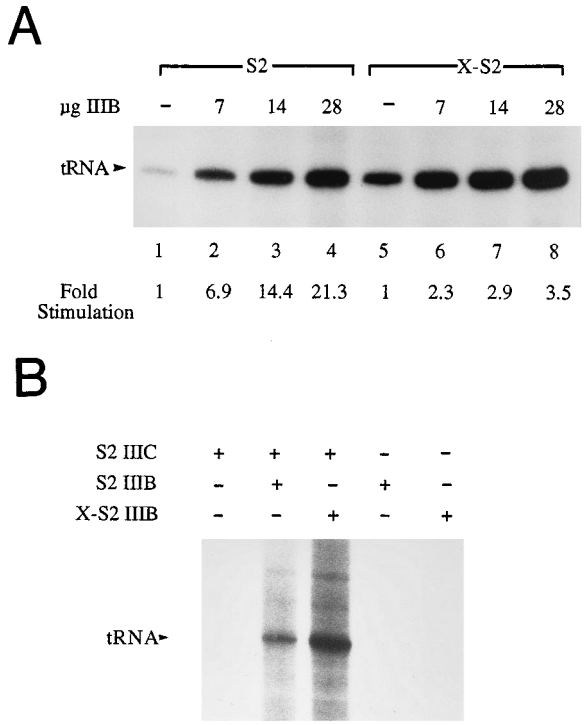

FIG. 4. Extracts derived from stably X-transformed S-2 cells contain increased TFIIIB activity. (A) Analysis of relative TFIIIB activities in S-2 and X-S2 extracts. Increasing amounts of a TFIIIB fraction were added to equal amounts of cytoplasmic extracts (60 μg of protein) derived from S-2 and X-S2 cells, as designated, and transcription assays were carried out with 0.4 μg of pArg-maxigene as the template. (B) Analysis of TFIIIB activity derived from S-2 and X-S2 extracts. The reconstituted transcription assays were carried out with 0.4 μg of pArg-maxigene as the template, and reactions contained one or more of the following fractions, as indicated, prepared as described previously (17): 24 μg of TFIIIC derived from S-2 cell extracts or 17.5 μg of TFIIIB derived from either S-2 or X-S2 cell extracts. Assay conditions were used so that TFIIIB concentrations were limiting in the reactions. These conditions were determined as described in Materials and Methods.

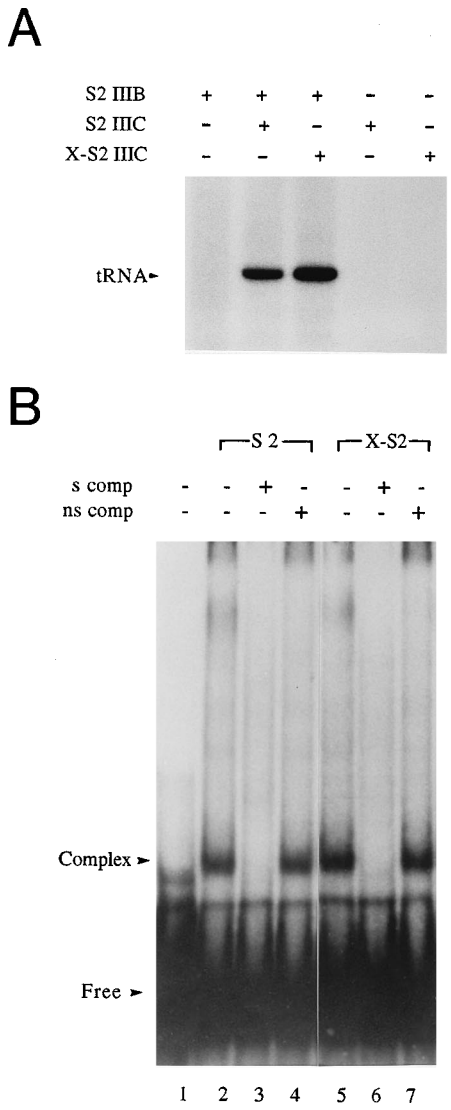

FIG. 5. Extracts from stably X-transformed S-2 cells do not exhibit changes in TFIIIC activity. (A) Analysis of TFIIIC activity derived from S-2 and X-S2 extracts. The reconstituted transcription assays were carried out with 0.4 μg of pArg-maxigene as the template, and reactions contained one or more of the following fractions, as indicated, prepared as described previously (17): 34 μg of TFIIIB derived from S-2 cell extracts or 11 μg of TFIIIC derived from either S-2 or X-S2 cell extracts. Assay conditions were used so that TFIIIC concentrations were limiting in the reactions. These conditions were determined as described in Materials and Methods. (B) Analysis of the relative DNA-binding activities of TFIIIC in S-2 and X-S2 extracts. A 122-bp *Bsl*I-*Sau* 96I DNA fragment derived from the pArg-maxigene plasmid containing the internal A and B box elements was 3′ end ^{32}P labeled and used for a gel shift assay with extracts derived from S-2 or X-S2 cells as described in Materials and Methods. Where designated, a 100-fold molar excess of the 122-bp *Bsl*I-*Sau* 96I fragment was included in the binding reaction as a specific competitor (s comp), or a 100-fold molar excess of a nonrelevant 26-bp oligonucleotide fragment was included as a nonspecific competitor (ns comp). The free DNA and TFIIIC-DNA complex separated by polyacrylamide gel electrophoresis are designated.

mediated enhancement of the transcriptional capacities of the X-S2 extracts is due to an increase in TFIIIB activity and occurs without alteration of the activities of TFIIIC or RNA pol III.

HBV X stably transformed rat 1A cells exhibit enhanced RNA pol III transcriptional activities and increased TBP levels. In order to assess whether the X-mediated increase in TBP was a general consequence of X function or a cell type-specific process, two stable rat 1A cell lines were constructed. The parental cell line was transfected either with an expression plasmid containing the SV40 promoter and a cDNA encoding the antisense X transcript (A1-Xr) or with an expression plasmid containing a cDNA encoding the sense X transcript (A1-X). Stable transformants were selected, and cells were pooled for further analysis. RT-PCR analysis verified that only the A1-X cell line was expressing the functional X transcript (data not shown). Nuclear extracts were prepared from the A1-X and A1-Xr cells to assess their relative transcriptional activities and TBP levels. As shown in Fig. 6A, the A1-X cell extracts exhibited 8.5-fold higher transcriptional activity with the pArg-maxigene template than the A1-Xr cell extracts. Furthermore, the A1-X cells had an approximately fourfold increase in the amounts of TBP; this can be compared with the level of the eIF-2α polypeptide, which remained constant in both cell types (Fig. 6B). Thus, the increased levels of TBP correlate with the enhanced transcriptional activity in the A1-X cells. Since X produces an increase in TBP in both a mammalian and an insect cell line, these results suggest that this X-mediated event may be universal.

Transient transfection of a TBP expression plasmid into rat 1A cells stimulates RNA pol III gene transcription. In addition to increasing the cellular levels of TBP, X activation of signal

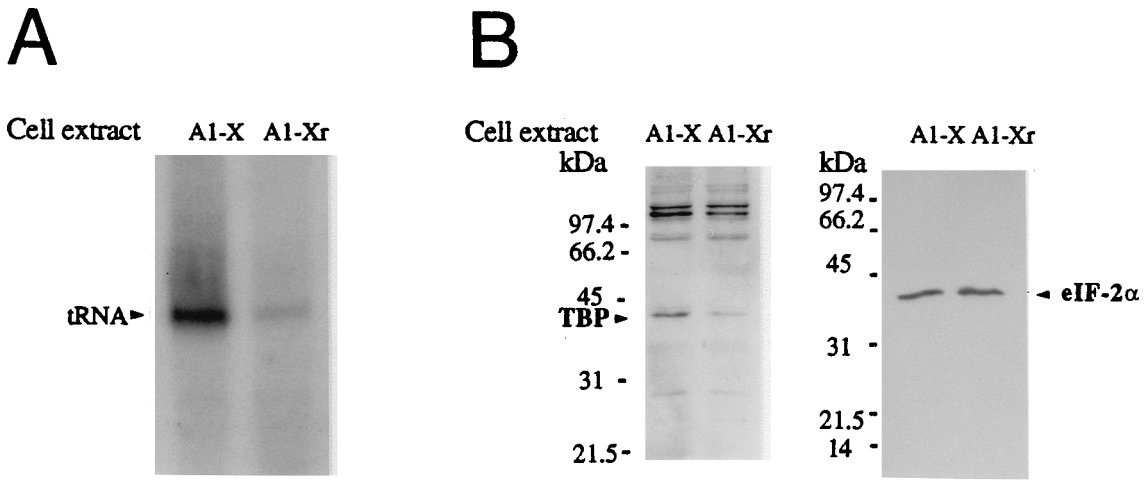

FIG. 6. Extracts derived from rat 1A cells stably transformed with the X gene exhibit enhanced transcriptional activities and increased TBP levels. Rat 1A stable transformants containing the expression plasmid carrying the HBV X gene in the sense orientation (A1-X) or the antisense orientation (A1-Xr) were constructed as described in Materials and Methods. (A) Analysis of transcription activities of A1-X and A1-Xr cell extracts. Nuclear extracts of the rat 1A cell line expressing X (A1-X) or antisense X (A1-Xr) were prepared as previously described (13). For each reaction, 84 μg of protein from each extract and 0.4 μg of pArg-maxigene as the template were used for the transcription assays as described in Materials and Methods. (B) Determination of the levels of TBP in A1-X and A1-Xr cell extracts by Western blot analysis. Equal amounts of protein (15 μg) of nuclear extract from either A1-X or A1-Xr were analyzed by Western blot. TBP was detected by antibodies against human TBP, and monoclonal anti-bovine eIF-2α antibodies were used as an internal control.

transduction could conceivably produce other effects which could serve to increase RNA pol III transcription. We determined whether directly increasing the cellular levels of TBP could alone enhance RNA pol III gene expression. An expression plasmid containing a human TBP cDNA was transiently transfected into the rat 1A cell line, and nuclear extracts were prepared from cells transfected with the vector DNA alone or the TBP expression plasmid. Analysis of these extracts revealed that the transcriptional activity was increased approximately fivefold (Fig. 7A) and the amount of TBP was increased threefold (Fig. 7B) when the cells were transfected with the TBP expression plasmid. These results demonstrate that increases in cellular TBP can stimulate RNA pol III gene transcription.

DISCUSSION

In the course of viral infection, certain viral transactivator proteins either activate or repress cellular gene expression. RNA pol III gene expression has been shown to be enhanced by the adenovirus E1A protein (18, 22) and the SV40 large T antigen (48) and repressed by the poliovirus proteinase 3C (7). In all cases, the mechanism for regulating gene expression by these viral proteins was shown to involve changes in the activity

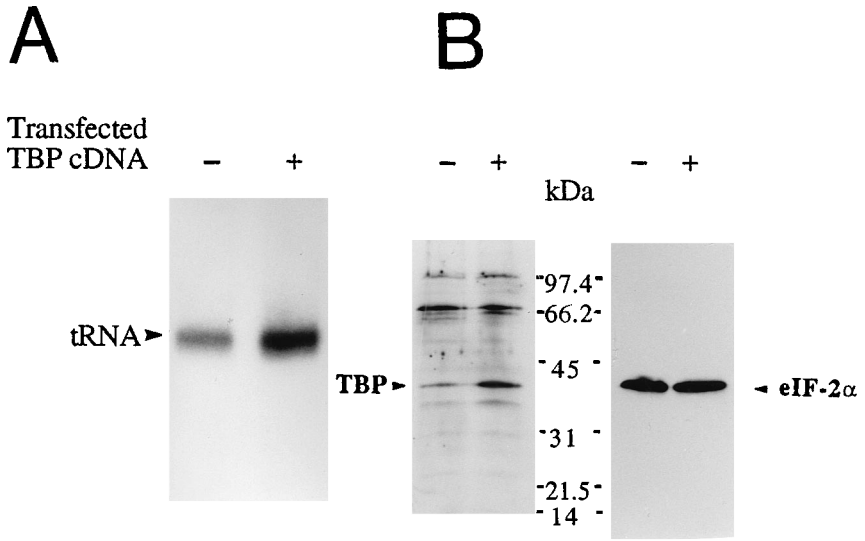

FIG. 7. Directly increasing TBP levels in rat 1A cells by transient expression of TBP enhances RNA pol III transcription. Rat 1A cells were transiently transfected with 1.6 μg of a plasmid for expression of human TBP, pLTReTBP (+), or with vector alone (−), and nuclear extracts were prepared as described previously (13). (A) Analysis of transcription activities of extracts. For each reaction, 57 μg of protein from each extract was used, and 0.4 μg of pArg-maxigene was used as the template for the in vitro transcription assays. (B) Determination of the levels of TBP by Western blot analysis. The amounts of TBP and eIF-2α present in the extracts were determined by Western blot analysis as described in the legend to Fig. 3B, with 20 μg of protein from each extract.

of TFIIIC. Our studies represent the first example of a virus-mediated event that induces RNA pol III gene activity through TFIIIB. Preliminary studies suggested that the HBV X protein enhances RNA pol III gene activity by targeting TFIIIC (2). In this study, results obtained from template commitment assays suggested that the DNA-binding activity of TFIIIC might be altered in Chang liver (CL) cells stably transformed with the X gene. Our studies, which used X-transformed *Drosophila* S-2 cells and directly measured both the relative levels of TFIIIC activity in vitro and TFIIIC DNA-binding activity, do not support this result (Fig. 5). Because it was not determined whether TFIIIC was the limiting component in the CL extracts, it is possible that the differential abilities of the X-transformed and nontransformed cell extracts to form stable transcription complexes could reflect differentially limiting TFIIIB activity.

In examining how X ultimately affects the RNA pol III transcriptional machinery, by activating protein kinase signaling pathways, we have discovered an indirect target of X, the transcription component TBP. Our results provide the first evidence that X mediates an increase in the cellular level of TBP. These results are consistent with our previous studies demonstrating that TPA activation of PKC produces an increase in the TBP subunit of TFIIIB and results in an increase in TFIIIB activity with no alterations in the activities of either TFIIIC or RNA pol III (16, 17). However, the relationship between X- and TPA-mediated induction of the PKC pathway remains controversial. It has been well documented that AP-1-dependent promoters are inducible by TPA, resulting in an increase in AP-1 DNA-binding activity (23). In contrast, X-mediated transactivation of AP-1-dependent promoters via activation of PKC does not correlate with increased AP-1 DNA-binding activity in most cell lines (32). Moreover, both the Enh 1 HBV enhancer (31) and NF-κB-dependent promoters (29), known to be inducible by TPA, are transactivated by X without requiring activation of PKC. Although the use of protein kinase inhibitors suggests that PKC may be activated in response to X, the rather nonspecific nature of these inhibitors, along with the large number of PKC isoforms (26), makes it difficult to clearly delineate the specific X-mediated effects. Thus, how X activates signal transduction and its targets in these pathways remains to be determined. Recent experiments suggest that X mediates activation of Ras (3, 32), which occurs upstream of PKC in the signal transduction pathway. Thus, it is likely that PKC is activated as a consequence of Ras activation and is not directly activated by X. How the X-mediated activation of signaling events results in increased cellular TBP is not yet known; however, stably X-transformed cells have higher levels of TBP mRNA (45a), suggesting that X could be indirectly enhancing the expression of the TBP gene. Initial experiments also demonstrated that X can directly interact with TBP in vitro (34). The biological significance of this finding awaits further investigation but could provide an additional mechanism for X modulation of TBP.

We have provided several lines of evidence that the X-mediated increase in TBP is responsible for its ability to transactivate RNA pol III genes: (i) all types of RNA pol III promoters that share TBP are stimulated by X; (ii) the relative transcriptional activities of resultant cell extracts parallel the levels of TBP; (iii) X-transformed cell extracts contain increased TFIIIB activities; and (iv) directly increasing cellular TBP levels by transient transfection of a TBP expression plasmid stimulates RNA pol III transcription. We also find that both tRNA and U6 RNA gene promoters are limiting for TBP in the *Drosophila* S-2 cell line to an even greater extent than what is observed in the rat 1A cell line (44a). The variation in the transcription enhancement by X or TBP in these two systems is consistent with previous studies showing that the level of X transactivation varies significantly depending on the cell line used (11, 41). In addition, we have investigated whether increases in cellular TBP could be stimulating RNA pol III transcription either directly at the promoter or indirectly by stimulating the transcription of RNA pol II-transcribed genes encoding specific RNA pol III transcription factor subunits. We have constructed a TBP mutant gene that, when transiently expressed in S-2 cells, retains its ability to stimulate specific RNA pol II promoters but is defective for stimulating either tRNA or U6 RNA gene promoters (44a). These results are consistent with the notion that increased cellular TBP produces an increase in TFIIIB complexes that directly stimulates RNA pol III transcription at the promoter. Together, these studies suggest that the X-mediated increase in the cellular levels of TBP plays an important role in the ability of X to transactivate this class of genes. Although there may be additional consequences on the TFIIIB complex mediated by X, we have found that increases in TBP alone can enhance RNA pol III gene activity.

TBP is an essential component for the transcription of all cellular genes (10, 20, 38, 43). TBP is associated with additional polypeptides that form the SL1, TFIID, and TFIIIB complexes, which specify its role in the transcription of RNA pol I, II, and III genes, respectively. It is therefore intriguing to consider that the X-mediated increase in cellular TBP levels could play a pivotal role in the X-mediated transactivation of many other cellular promoters transcribed by RNA pol II. It is possible that TBP is a limiting factor in the assembly and function of TFIID complexes. Although little is yet known in this regard, it has been shown that certain TATA-containing (but not TATA-less) RNA pol II promoters are limiting for TBP in *Drosophila* S-2 cells and can be stimulated to various extents by increasing cellular TBP levels (8). In mammalian cells, it has been shown that overexpression of TBP can potentiate the effect of certain activators, such as VP16, while inhibiting others, such as Sp1 and NF-1 (36). Thus, our discovery that X mediates an increase in TBP may explain how X is able to transactivate so many different types of RNA pol II promoters. It may be possible to identify new genes that are transactivated by the X protein by further determining other types of cellular promoters that are limiting for TBP.

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