

# Hepatitis B Virus X Protein Induces RNA Polymerase III-Dependent Gene Transcription and Increases Cellular TATA-Binding Protein by Activating the Ras Signaling Pathway

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Received 2 July 1997/Returned for modification 13 August 1997/Accepted 21 August 1997

**Our previous studies have shown that the hepatitis B virus protein, X, activates all three classes of RNA polymerase III (pol III)-dependent promoters by increasing the cellular level of TATA-binding protein (TBP) (H.-D. Wang et al., *Mol. Cell. Biol.* 15:6720–6728, 1995), a limiting transcription component (A. Trivedi et al., *Mol. Cell. Biol.* 16:6909–6916, 1996). We have investigated whether these X-mediated events are dependent on the activation of the Ras/Raf-1 signaling pathway. Transient expression of a dominant-negative mutant *Ras* gene (*Ras-ala15*) in a *Drosophila* S-2 stable cell line expressing X (X-S2), or incubation of the cells with a Ras farnesylation inhibitor, specifically blocked both the X-dependent activation of a cotransfected tRNA gene and the increase in cellular TBP levels. Transient expression of a constitutively activated form of Ras (*Ras-val12*) in control S2 cells produced both an increase in tRNA gene transcription and an increase in cellular TBP levels. These events are not cell type specific since X-mediated gene induction was also shown to be dependent on Ras activation in a stable rat 1A cell line expressing X. Furthermore, increases in RNA pol III-dependent gene activity and TBP levels could be restored in X-S2 cells expressing *Ras-ala15* by coexpressing a constitutively activated form of Raf-1. These events are serum dependent, and when the cells are serum deprived, the X-mediated effects are augmented. Together, these results demonstrate that the X-mediated induction of RNA pol III-dependent genes and increase in TBP are both dependent on the activation of the Ras/Raf-1 signaling cascade. In addition, these studies define two new and important consequences mediated by the activation of the Ras signal transduction pathway: an increase in the central transcription factor, TBP, and the induction of RNA pol III-dependent gene activity.**

The hepatitis B virus (HBV) encodes a gene that produces a 154-amino-acid polypeptide, X. X has been shown to be essential for viral replication in animal hosts (7, 69), and it has also been implicated in the development of HBV-mediated carcinogenesis since X is able to induce liver tumors in certain transgenic mice strains (26, 29, 52). Although the exact role of X in the life cycle of the virus or in carcinogenesis is not known, it has been well established that X is a potent transactivator of a diverse number of viral and cellular promoters. X has been shown to activate RNA polymerase III (pol III)-dependent promoters (3, 58) and RNA pol II-dependent promoters which contain recognition sequences for AP-1, AP-2, ATF/CREB, c/EBP, NF- $\kappa$ B, serum response factor, and a variety of acidic activator proteins (for reviews, see 43 and 68).

There are at least two distinct mechanisms by which X appears to activate gene expression. For certain promoters, X may interact directly with the transcriptional machinery to augment RNA synthesis. Although X is not able to bind to DNA directly, it has been shown to bind to the CREB/ATF family of transcription factors and to enhance the affinity of the factor for its DNA recognition sequence (66) or alter its specificity (37). In addition, X has been shown to interact with the RPB5 subunit of the RNA polymerases (8, 33), the tumor suppressor p53 (15, 59), transcription factor IIB (TFIIB) (23, 33), TFIIF (23, 41), and the TATA-binding protein (TBP) in

vitro (40). Consistent with these studies, X has been shown to enhance transcription in vivo when targeted to specific promoters by its fusion to a heterologous DNA binding domain (45, 53). X has also been shown to mediate an increase in RNA pol II-dependent transcription that is activator dependent both in vivo (22) and in vitro (23). Together, these studies indicate that X directly modulates transcription of certain RNA pol II-dependent promoters by acting as a coactivator.

In contrast, other studies have shown that X can indirectly modulate gene expression. X has been shown to specifically interact with a cellular DNA repair protein (32) and with a subunit of the proteasome complex (16, 27), although direct modulation of the activities of these proteins by X has not yet been demonstrated. X has also been shown to activate protein kinase C (PKC) (28, 35, 58) and the Ras–Raf–MEK–mitogen-activated protein (MAP) kinase signal cascade (4, 38). The activation of cellular signaling has been shown to be required for X transactivation of AP-1- and NF- $\kappa$ B-dependent promoters (14). Induction of AP-1 DNA binding activity was specifically shown to be mediated by X activation of PKC (28, 35), Ras (4), Raf-1 (4, 12), and extracellular signal-related kinases and c-Jun N-terminal kinases (6). The ability of X to modulate gene activity by distinct mechanisms was elegantly shown by Doria et al. (14). When X was specifically targeted to the nucleus of cells, it maintained its ability to activate the HBV enhancer I, yet it was unable to activate signaling and the AP-1 or NF- $\kappa$ B transcription factor. Thus, X clearly possesses multiple functions which may be altered depending on its intracellular location. However, analyses of the activation of the Ras signaling cascade by X and its effect on cellular gene activity

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have so far been limited to the study of AP-1- and NF- $\kappa$ B-dependent promoters.

Previously, we have shown that X transactivates all classes of cellular RNA pol III-dependent promoters (58). RNA pol III is responsible for the synthesis of a variety of untranslated RNAs, including tRNA, 5S RNA, U6 RNA, and virus-associated RNAs such as VA1 and VA2 from adenovirus (67). The largest classes of transcripts, tRNA and 5S RNA, are essential components of the protein synthesis machinery. Although little is known as to how modulation of the levels of these RNAs may contribute to cell proliferation and tumor development, it is clear that the production of ribosomes and tRNAs is linked to the growth state of cells. Thus, understanding the mechanism by which the synthesis of these RNAs is regulated by X may provide insight into the potential role of X in cell transformation. Previously, we demonstrated that X transactivation of RNA pol III-dependent gene activity was a result of an increase in TFIIB activity. Furthermore, X was found to increase cellular levels of TBP, a transcription factor needed for the transcription of all cellular genes. This protein is a subunit of the TFIIB complex (24) which is a limiting component for RNA pol III-dependent transcription (49). Although other changes in the TFIIB complex may occur in response to X, an increase in cellular TBP, by itself, is sufficient for up-regulating gene activity. In this study, we have examined the mechanism for the X-mediated enhancement of RNA pol III-dependent transcription and the cellular increase in TBP levels. Our results demonstrate that both of these events are dependent on the ability of X to activate the Ras/Raf-1 signal transduction pathway.

#### MATERIALS AND METHODS

**Cell culture.** *Drosophila* S-2 stable cell lines S2 and X-S2 (58) were propagated in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Inc.), 500 U of penicillin and 500  $\mu$ g of streptomycin (Gibco) per ml, and 250  $\mu$ M hygromycin B (Boehringer Mannheim). The rat 1A stable cell lines A1-Xr and A1-X, derived from rat 1A fibroblast cells (58), were maintained in low-glucose Dulbecco's modified Eagle medium (Gibco) with 10% FBS, 500 U of penicillin and 500  $\mu$ g of streptomycin per ml, and 400  $\mu$ g of G418 (Gibco) per ml.

**Plasmid DNAs.** To construct the *Drosophila* expression plasmids that express the H-Ras mutant proteins, pSP8 (containing a cDNA encoding the constitutively activated mutant human H-Ras-val12 gene) and pSP30 (containing the dominant-negative human H-Ras-ala15 gene) were used (55). The cDNAs within these plasmids were amplified by PCR using the *Bam*HI-containing primer P146 (5'-GGTTCGCGTGGATCCATGACGGAATATAAGCTGG-3') and the *Sac*I-containing primer P155 (5'-GGCCGGTCGACATGACGGAATATAAGCTG-3'), using plasmids pSP8 and pSP30, respectively, as templates. The amplified 598-bp fragments containing either the *Ras-val12* gene or *Ras-ala15* gene were digested with *Bam*HI and *Sac*I and subcloned into the *Bam*HI and *Sac*I sites of the *Drosophila* pAct5CpPA expression vector containing actin 5C promoter (21) to generate plasmids pAct-*Ras-val12* and pAct-*Ras-ala15*.

To express the *Ras* genes in mammalian cells, pAct-*Ras-val12* and pAct-*Ras-ala15* were used for further subcloning. pAct-*Ras-val12* and pAct-*Ras-ala15* were digested with *Sac*I, then treated with T4 DNA polymerase, and further digested with *Bam*HI. The resultant DNA fragments containing the *Ras* genes were subcloned into the *Bam*HI and *Eco*RV sites of pcDNA3 expression vector (Invitrogen) under the control of cytomegalovirus immediate-early gene promoter to generate pCMV-*Ras-val12* and pCMV-*Ras-ala15*.

The dominant active *Raf-1* gene (*Raf* $\Delta$ 2-334) is a mutant derivative of the mouse *c-Raf-1* gene that produces a truncated protein deleted of amino acid residues 2 through 334 (42). To construct a plasmid for expression of the mutant *Raf-1* gene in *Drosophila* cells, the mammalian expression plasmid EC12, containing the gene, was digested with *Apa*I, incubated with T4 DNA polymerase, and digested with *Eco*RI. The resultant DNA fragment containing the gene was subcloned into the *Eco*RI and *Hinc*II sites of pSK, and a 1-kb DNA fragment containing the gene was isolated after digestion of the DNA with *Bam*HI and *Kpn*I. This fragment was subcloned into the *Bam*HI and *Kpn*I sites of the *Drosophila* pAct5CpPA expression vector to generate plasmid *Raf* $\Delta$ 2-334.

Plasmid pArg-maxigene, which has been described previously (13), was used in the transient transfections and in vitro transcription assays as an RNA pol III-dependent reporter gene. The pBluescript SK+ vector DNA (Stratagene) was used to maintain a constant amount of DNA in the transient transfection

experiments. The *pcopia-lacZ* construct contains a  $\beta$ -galactosidase gene under the control of the *Drosophila copia* promoter (21).

**Transient transfections.** Transient transfections were performed by a calcium phosphate precipitation technique (50). *Drosophila* Schneider S2 and X-S2 cells were maintained at 25°C in Schneider medium (Gibco) containing 10% FBS (Gemini Bio-Products). For each transfection assay,  $0.5 \times 10^6$  to  $1.0 \times 10^6$  cells per ml, in a total of 5 ml, were cotransfected with 2  $\mu$ g of pArg-maxigene, 2  $\mu$ g of *pcopia-lacZ* (for measuring transfection efficiencies), and other DNAs as designated. The final DNA concentration was maintained at 20  $\mu$ g by using pBluescript SK+. After 24 h, the transfected cells were replaced with fresh medium and 4 h later induced with 250  $\mu$ M CuSO<sub>4</sub>; after an additional 24 h, the cells were harvested. Approximately half of the harvested cells were used for isolating RNA to analyze the transcription activity of the tRNA gene reporter (pArg-maxigene), and the remainder of the cells were used for the preparation of protein lysates for determining  $\beta$ -galactosidase activity to measure transfection efficiencies. The cell lysates were assayed for  $\beta$ -galactosidase activity by using an enzyme assay kit (Promega). For the data shown in Fig. 1 to 3, transfection efficiencies in the S2 and X-S2 cells did not vary by more than 10%.

Transient transfections of rat 1A cells were carried out by using calcium phosphate precipitation methods at a cell density of  $0.5 \times 10^5$  to  $1.0 \times 10^5$  cells/ml and by using 20  $\mu$ g of total DNA per 5 ml cell culture, including 2  $\mu$ g of pArg-maxigene and other DNAs as indicated. After 24 h, the cells were washed with Dulbecco's phosphate-buffered saline (Gibco), placed in fresh medium, and then collected after an additional 24 h.

**Transcription assays and RNase protection assays.** The in vitro transcription assays were carried out with cytoplasmic extracts derived from the S2 and X-S2 cells as described previously (58). For each reaction, 0.8  $\mu$ g of pArg-maxigene was used as the template, and 210  $\mu$ g of cytoplasmic extract was used in each reaction.

For determining the amount of pArg-maxigene transcript generated in the transient transfection assays, RNA was extracted by using TRIzol (Gibco) as instructed by the vendor. RNase protection assays were carried out by using an RPA II kit (Ambion). The isolated RNAs (0.3  $\mu$ g of each sample) were hybridized with an excess of <sup>32</sup>P-labeled antisense transcript at 45°C overnight. The antisense transcript was generated from pArg-maxigene by using a Maxiscript kit (Ambion). The DNA was linearized and then transcribed with T7 RNA polymerase in the presence of [<sup>32</sup>P]CTP (specific activity, >600 Ci/mmol; ICN). The resultant riboprobe was treated with DNase I and ethanol precipitated. For each reaction,  $0.5 \times 10^6$  to  $1 \times 10^6$  cpm was used. The hybridized RNA was digested with 200  $\mu$ l of a 1:100 dilution of RNase A-T<sub>1</sub> mixture at 37°C for 30 min. The reaction was terminated by adding 300  $\mu$ l of stop buffer and 200  $\mu$ l of ethanol, and the RNA products were precipitated and resuspended in 8  $\mu$ l of RNA loading dye and electrophoresed on 8% polyacrylamide-8 M urea gels. The gel was exposed to X-ray film for 30 to 120 min at -80°C, and the resultant autoradiographs were quantitated by using a Bioimage scanner.

**Preparations of cell extracts.** For the preparation of total cell lysates, the transfected cells were collected and washed once with cold phosphate-buffered saline. The resultant cell pellets were lysed with triple-detergent lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 100  $\mu$ g of phenylmethylsulfonyl fluoride, 1  $\mu$ g of aprotinin per ml, 1% Nonidet P-40, 0.5% sodium deoxycholate) at 0°C for 20 min. Protein concentrations of the resultant cell lysates were measured by the Bradford method, using Bio-Rad protein assay reagent.  $\beta$ -Galactosidase assays of the resultant lysates were carried out as described above.

**Western blot analysis.** Equal amounts of protein from S2, X-S2, A1-Xr, and A1-X cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was carried out as described previously, using anti-*Drosophila* TBP antibodies and anti-*Drosophila* eIF-2 $\alpha$  antibodies for the *Drosophila* extracts and anti-human TBP antibodies and anti-bovine eIF-2 $\alpha$  antibodies for the rat 1A cell extracts (58). Horseradish peroxidase-linked anti-rabbit antibodies (Vector Laboratories) and enhanced chemiluminescence reagents (Amersham) were used to detect the bound antibodies. The resultant autoradiographs were quantitated by optical densitometry.

#### RESULTS

**Serum deprivation enhances the X-mediated increases in transcription and cellular TBP.** Our previous studies have demonstrated that X transactivation of RNA pol III-dependent genes requires the activation of cellular protein kinases (58). Since many cellular responses that result from the activation of signaling pathways are influenced by serum, the effects of serum concentration on the X-mediated increases in RNA pol III-dependent gene activity and TBP levels were first examined. To carry out these studies, we used two stable cell lines, S2 and X-S2, which were previously derived from a *Drosophila* S-2 line (58). Both cell lines were stably transfected with a hygromycin-resistant gene, and the X-S2 line addition-

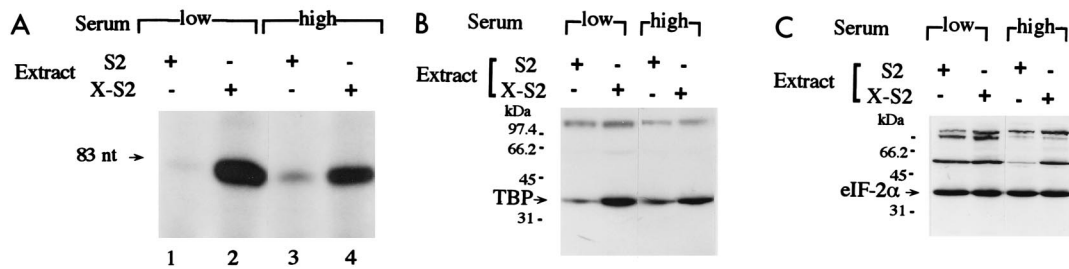


FIG. 1. Serum deprivation augments both the X-mediated induction of RNA pol III-dependent gene transcription and the increase in cellular TBP levels. S2 and X-S2 cells were propagated in media containing low (0.5% FBS) and high (10% FBS) serum concentrations for 3 days, and cytoplasmic extracts were prepared as described in Materials and Methods. (A) Serum deprivation decreases RNA pol III-dependent gene activity but further augments X-mediated transcription induction. In vitro transcription assays were carried out as described in Materials and Methods, using 210  $\mu$ g of cytoplasmic extracts derived from either S2 or X-S2 cells as designated and using 0.8  $\mu$ g of pArg-maxigene as the template. (B and C) Cellular level of TBP is serum dependent. Aliquots of 40  $\mu$ g each of cytoplasmic extracts derived from the S2 or X-S2 cells, as designated, were subjected to Western blot analysis as described in Materials and Methods. The TBP polypeptide was analyzed by using anti-*Drosophila* TBP antibodies (B), and the eIF-2 $\alpha$  polypeptide was analyzed by using anti-*Drosophila* eIF-2 $\alpha$  antibodies (C).

ally contains the X gene under the control of the metallothionein promoter. Both S2 and X-S2 cells were grown in either 10 or 0.5% serum for 4 days, cytoplasmic extracts were prepared from each set of cells, and the transcriptional activities of the extracts were determined by using a RNA pol III-dependent tRNA<sup>Arg</sup> reporter gene, pArg-maxigene, as the template (Fig. 1A). An approximate threefold decrease in the transcriptional capacity was observed when the S2 cells were serum deprived (compare lanes 1 and 3). In the presence of high serum concentrations, X produced an approximate 40-fold increase in RNA pol III-dependent transcription activity (compare lanes 3 and 4). Surprisingly, when the X-S2 cells were serum deprived, the X-mediated increase in transcription was approximately threefold higher than that for X-S2 cells grown in higher serum concentrations (compare lanes 2 and 4).

We have previously shown that extracts derived from X-S2 cells expressing X contain increased TFIIB activity, and this is correlated with an increase in the cellular level of one of its subunits, TBP (58). Furthermore, by directly increasing cellular TBP, transcription from both TATA-containing and TATA-lacking RNA pol III-dependent promoters is enhanced (50). Thus, the X-mediated increase in TBP, a limiting RNA pol III transcription component, is a key event mediating gene activation. Therefore, the effect of serum concentration on the level of TBP was next assessed (Fig. 1B and C). Western blot analysis was performed with the cytoplasmic lysates that had been tested for transcription activity in the assay represented in Fig. 1A. Regardless of the serum concentration, X expression produced an increase in cellular TBP (Fig. 1B). By comparison, the level of the eIF-2 $\alpha$  polypeptide (Fig. 1C) does not change in response to X, consistent with our previous studies (58). The cross-reacting higher-molecular-weight bands observed in both blots are a result of interactions of the secondary antibodies with proteins in the lysates. Consistent with the transcriptional activities of the extracts, the level of TBP in the S2 cell line was decreased approximately twofold upon serum deprivation. The amount of TBP in the X-S2 cell extracts was approximately fourfold higher than that in the S2 extracts when the cells were grown in 10% serum. When the cells were grown in limiting serum concentrations, the X-S2 cell extracts contained sixfold more TBP than did the S2 cell extracts. The differences in TBP levels can be compared to the levels of eIF-2 $\alpha$ , which did not change in response to different serum concentrations in the media. Thus, these results indicate that both RNA pol III-dependent gene activity and cellular TBP levels are influenced by serum concentrations. Furthermore, we find that depriving the X-S2 cells of external growth factors

serves to augment the X-mediated increase in both transcription and TBP levels.

**X transactivation of RNA pol III-dependent genes requires the activation of Ras.** Previously, Benn and Schneider (4) showed that the expression of X stimulated the formation of Ras-GTP (active) complexes. We therefore investigated the possibility that the enhancement of RNA pol III-dependent gene transcription requires the activation of the Ras signaling pathway. We first examined whether inhibiting the function of activated Ras would affect RNA pol III-dependent gene activity. A gene encoding a dominant-negative mutant form of human Ras, *Ras-ala15*, was used to interfere with the activation of Ras that might occur in the S2 and X-S2 cells. Ras proteins are highly conserved between different eucaryotic systems, and human *Ras-ala15* has previously been shown to dominantly interfere with Ras function in yeast (39). The plasmid containing *Ras-ala15* was transiently cotransfected into the S2 and X-S2 cells with pArg-maxigene. pArg-maxigene was used as a reporter to analyze RNA pol III-dependent gene activity. A  $\beta$ -galactosidase gene driven by the *copia* promoter was additionally cotransfected to determine the relative transfection efficiencies. This promoter is not affected by X expression or by the level of activated Ras in these cells (see Fig. 5). To determine the level of tRNA gene transcription activity, RNase protection assays were carried out with RNAs extracted from each transfected set of cells. As shown in Fig. 2A, the X-S2 cells exhibited a significant increase in transcription activity compared to the S2 cells (compare lanes 1 and 3). Expression of *Ras-ala15* had no effect on tRNA gene transcription activity in the S2 cells (compare lanes 1 and 2). However, when *Ras-ala15* was expressed in the X-S2 cells, the X-mediated increase in tRNA gene transcription was abolished (compare lanes 3 and 4). The maximum inhibitory effect was observed when 2  $\mu$ g of the plasmid containing the *Ras-ala15* gene was transfected (data not shown).

An additional approach was used to block the ability of the cells to produce a functional activated Ras. S2 and X-S2 cells transfected with pArg-maxigene were incubated with the Ras farnesylation inhibitor L-739,749. This tetrapeptide has been previously shown to selectively block the farnesyltransferase enzyme, preventing the association of Ras with the membrane and the activation of downstream signaling events (11, 30). As shown in Fig. 2B, L-739,749 had no effect on the expression of the tRNA gene in the S2 cells (compare lanes 1 and 2). However, incubation with the Ras inhibitor substantially decreased the X-mediated increase in transcription in the X-S2 cells (compare lanes 3 and 4). Together, these results show that



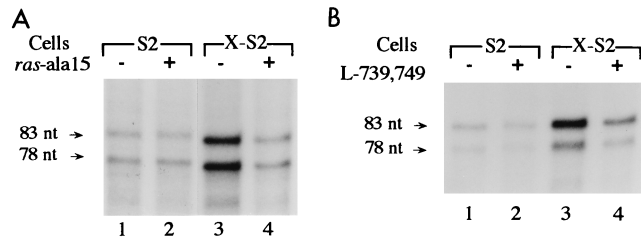


FIG. 2. The X-mediated transactivation of an RNA pol III-dependent promoter can be blocked by inhibiting the activation of Ras. (A) Expression of a dominant-negative mutant form of Ras blocks the X-mediated transactivation of a tRNA gene promoter. Transient transfections of S2 and X-S2 cells were performed with 2  $\mu$ g of pArg-maxigene, with or without 2  $\mu$ g of a plasmid containing a gene encoding a dominant-negative mutant form of Ras, Ras-ala15 (pAct-Ras-ala15), and 2  $\mu$ g of *pcopia-lacZ* as described in Materials and Methods. RNase protection assays were carried out with equal amounts (0.3  $\mu$ g) of extracted RNA and an excess of  $^{32}$ P-labeled antisense pArg-maxigene riboprobe. The resultant RNA was separated by gel electrophoresis and visualized by autoradiography. (B) The X-mediated transactivation of a tRNA gene promoter is blocked by the Ras farnesylation inhibitor L-739,749. S2 and X-S2 cells were incubated with 10  $\mu$ M L-739,749 3 h prior to transfection of the cells. The cells were transfected with 2  $\mu$ g of pArg-maxigene as described in Materials and Methods. After 24 h, fresh medium was added to the cells with or without 10  $\mu$ M L-739,749. The cells were incubated for 4 h, and then 250  $\mu$ M CuSO<sub>4</sub> was added to the medium to induce expression of X. After an additional 24 h, the cells were harvested and RNase protection assays were performed as described in Materials and Methods; the labeled pArg-maxigene transcript was visualized by autoradiography. nt, nucleotides.

activated Ras is required for X transactivation of RNA pol III-dependent genes.

**X-mediated increase in cellular TBP requires Ras activation.** We next determined whether the X-mediated increase in TBP also requires Ras activation. Western blot analysis was carried out to determine the amount of TBP present in cell lysates derived from the transient transfection assays represented in Fig. 2. Expression of the dominant-negative *Ras-ala15* gene in S2 cells (Fig. 3A) or incubation of the S2 cells with the farnesylation inhibitor L-739,749 (Fig. 3B) had no effect on the level of cellular TBP (compare lanes 1 and 2). In the X-S2 cells, both expression of the *Ras-ala15* gene (Fig. 3A)

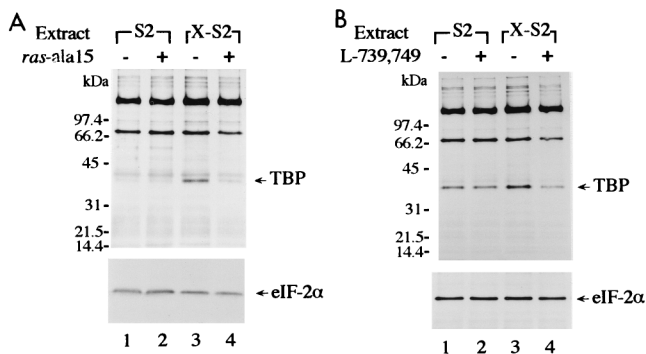


FIG. 3. The X-dependent increase in cellular TBP levels is blocked by inhibiting the activation of Ras. (A) Expression of a dominant-negative form of Ras inhibits the X-mediated increase in TBP. S2 and X-S2 cells were transiently transfected with the *Ras-ala15* expression plasmid, and cell lysates were prepared as described in Materials and Methods. Equal amounts of protein from the lysates (5  $\mu$ g) were used for Western blot analysis as described in Materials and Methods. The top panel depicts a blot probed with anti-*Drosophila* TBP antibodies; the bottom panel represents a blot probed with anti-*Drosophila* eIF-2 $\alpha$  antibodies. (B) The X-mediated increase in TBP is blocked by the Ras farnesylation inhibitor L-739,749. S2 and X-S2 cells were incubated with 10  $\mu$ M L-739,749 as described for Fig. 1B, the cells were harvested, and cell lysates were prepared as described in Materials and Methods. Western blot analysis was carried out as described for panel A.

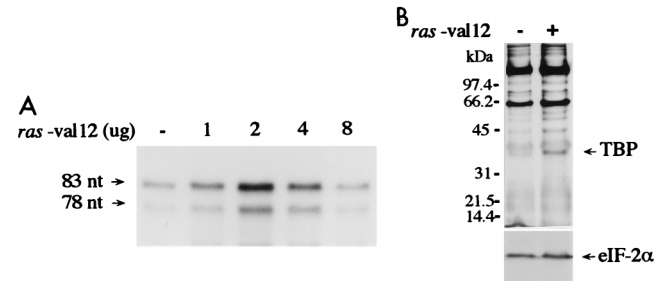


FIG. 4. Expression of activated Ras in S2 cells mimics the effect of X to stimulate RNA pol III-dependent gene activity and increase cellular levels of TBP. (A) Expression of a constitutively activated mutant form of Ras enhances the transcriptional activity of a RNA pol III-dependent promoter in a dose-dependent manner. S2 cells were cotransfected with increasing amount of an expression plasmid containing the *Ras-val12* gene as indicated, together with 2  $\mu$ g of pArg-maxigene as described in Materials and Methods. RNA was extracted from the transfected cells, RNase protection assays were performed, and the labeled RNA products were visualized by autoradiography as described in Materials and Methods. nt, nucleotides. (B) Expression of activated Ras increases the cellular level of TBP. Cell lysates were prepared from cells that were transfected with vector alone (-) or cells that were transfected with 2  $\mu$ g of the *Ras-val12* gene expression plasmid (+) as described in Materials and Methods. Equal amounts of protein from the cell lysates (7.5  $\mu$ g) were analyzed by Western blot to detect the amount of TBP (top) and eIF-2 $\alpha$  (bottom) as described in Materials and Methods.

and incubation of the cells with L-739,749 blocked the X-mediated increase in TBP (compare lanes 3 and 4). By comparison, the level of the eIF-2 $\alpha$  polypeptide was not affected by down-regulating the production of activated Ras. Thus, these results demonstrate that the X-dependent increase in TBP also requires Ras activation.

**Activated Ras mimics the effect of X to increase RNA pol III-dependent transcription and cellular TBP levels.** The results presented above revealed that the X-mediated increases in both RNA pol III-dependent gene transcription and TBP require Ras activation. If these X-mediated events are truly dependent on Ras activation, then expression of activated Ras should mimic the effect of X. To test this, a gene encoding a constitutively activated form of human Ras, *Ras-val12*, was transiently expressed in S2 cells, and the consequence on transcription and TBP levels was determined. When increasing amounts of the *Ras-val12* gene were transfected into S2 cells, a dose-dependent increase in the expression of the pArg-maxigene was obtained (Fig. 4A). The maximum enhancement of transcription was obtained when 2  $\mu$ g of plasmid containing the *Ras-val12* gene was transfected, consistent with the amount of *Ras-ala15* gene-containing plasmid required to produce the maximum inhibitory response (data not shown). By comparison, the activity of the *copia* promoter was unaffected by the expression of the *Ras-val12* gene in either the S2 or X-S2 cells (Fig. 5). Lysates prepared from the *Ras-val12*-expressing cells were analyzed by Western blotting to determine the amount of TBP present (Fig. 4B). As shown, expression of activated Ras produced a significant increase in the level of TBP in the S2 cells. In contrast, the eIF-2 $\alpha$  polypeptide level did not change with the expression of activated Ras.

The results of the experiments represented in Figs. 2 to 4 are examples of the data typically obtained. At least four independent experiments were performed for each set of transfections to analyze expression of the RNA pol III-dependent gene promoter, expression of the *copia* promoter, and TBP levels. The results were quantified and are summarized in Fig. 5. Together, these results demonstrate that both the X-mediated transactivation of RNA pol III-dependent genes and its ability

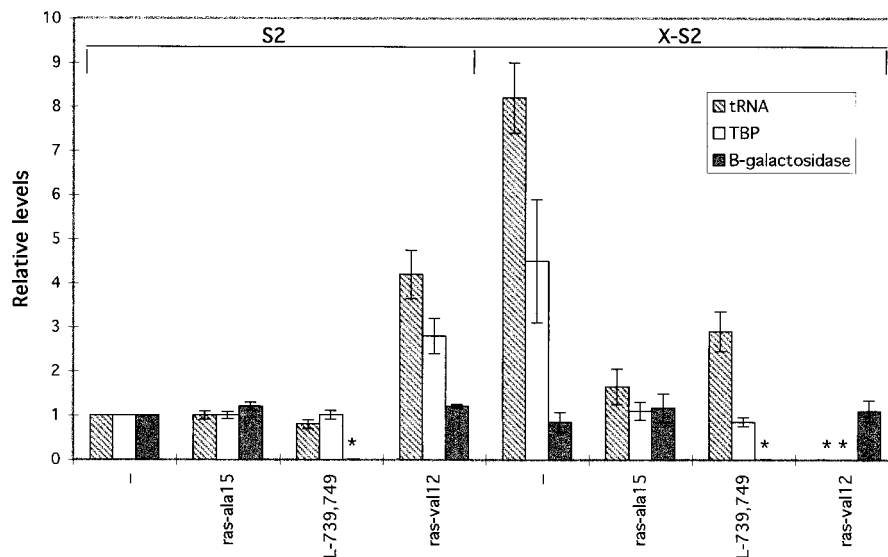


FIG. 5. The X-mediated increase in RNA pol III-dependent gene expression and cellular TBP is dependent on the activation of Ras. This graphical representation summarizes the data shown as examples in Fig. 1 to 3. S2 and X-S2 cells were transiently transfected as described in Materials and Methods with the tRNA gene reporter (pArg-maxigene), *pcopia-lacZ*, and, where designated, plasmids containing either the constitutively activated *Ras-val12* gene or the dominant negative *Ras-ala15* gene. One set of transfections was carried out in the presence of the Ras farnesylation inhibitor L-739,749 as designated. Transcription from the tRNA gene was determined by an RNase protection assay as described in Materials and Methods (striped bars). TBP levels from cell lysates were determined by Western blot analysis as described in Materials and Methods (open bars).  $\beta$ -Galactosidase activity was measured from the cell lysates (stippled bars). At least four independent sets of transfections were performed for each analysis. The activities or protein levels obtained for the S2 cells transfected with pArg-maxigene and *pcopia-lacZ* alone (-) were arbitrarily set to 1 and used to calculate the values shown on the y axis, depicting the fold change for tRNA gene transcription, TBP levels, and  $\beta$ -galactosidase activity. Measurements that were not determined or not shown are designated with asterisks.

to increase cellular TBP levels are dependent on the activation of Ras.

**Ras-mediated stimulation of RNA pol III-dependent genes by X is not cell type specific.** To determine whether the dependence on Ras activation observed for the X-mediated events was cell type specific or a general phenomenon, we additionally analyzed stable cell lines derived from rat 1A fibroblast cells. We have previously constructed an A1-Xr cell line that contains a stably integrated X gene in the antisense orientation relative to the simian virus 40 promoter and an A1-X cell line that contains a stably integrated X gene in the sense orientation (58). Consistent with our previous studies, transient expression of the tRNA<sup>Arg</sup> maxigene is greater in the A1-X cell line than in the A1-Xr cells (Fig. 6; compare lanes 1 and 4). Coexpression of the *Ras-ala15* gene did not affect expression of the tRNA gene promoter in the A1-Xr cells

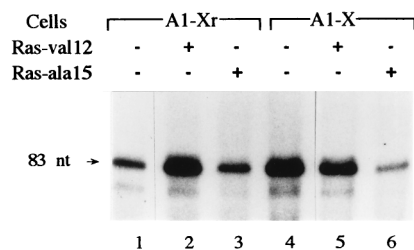


FIG. 6. The X-mediated increase in RNA pol III-dependent gene activity in rat 1A cells is dependent on Ras activation. A1-X (X-expressing) and A1-Xr (control) stable cell lines were transiently transfected with 2  $\mu$ g of pArg-maxigene and where designated, expression plasmids containing either the *Ras-ala15* gene or the *Ras-val12* gene. RNase protection assays were carried out with equal amounts (0.3  $\mu$ g) of extracted RNA and an excess of <sup>32</sup>P-labeled antisense pArg-maxigene riboprobe as described in Materials and Methods. The resultant RNA was separated by gel electrophoresis and visualized by autoradiography.

(compare lanes 1 and 3), yet it substantially decreased the X-mediated increase in RNA pol III-dependent gene activity in the A1-X cells (compare lanes 4 and 6). In this experiment, expression of the *Ras-ala15* gene reduced transcription to a level lower than that observed in the control (A1-Xr) cells. However, we generally found that expression of the *Ras-ala15* gene in the A1-X cells reduced transcription to approximately that observed in the A1-Xr cells. Expression of the dominant active form of *Ras*, *Ras-val12*, enhanced transcription of the RNA pol III-dependent gene in the A1-Xr cells (compare lanes 1 and 2). In the A1-X cells, expression of *Ras-val12* produced a slight but reproducible decrease in transcription (compare lanes 4 and 5). Since the response to activated Ras was found to be dose dependent in the A1-Xr cells (data not shown), similar to that observed for the S2 cells (Fig. 4A), this decrease likely represents the fact that the amount of activated Ras in the A1-X cells transfected with *Ras-ala15* exceeds the optimum level. Together, these results further substantiate the conclusion that the enhancement of RNA pol III-dependent gene activity by X is dependent on the activation of Ras. Furthermore, examining the levels of TBP in these transiently transfected cells revealed that the X-mediated increase in TBP is blocked by the expression of dominant-negative *Ras-ala15* in the A1-X cells and that TBP levels can be increased in the A1-Xr cells by the expression of *Ras-val12* (data not shown). Thus, by demonstrating that both X-dependent events occur in two distinct cell lines, we show that the requirement for activated Ras is not cell type specific.

**Expression of activated Raf-1 reverses the inhibitory effect of dominant negative Ras to restore the increase in RNA pol III-dependent gene activity and TBP levels in the X-expressing cells.** To further elucidate the downstream events transduced from the X-mediated activation of Ras, we examined the involvement of Raf-1, a downstream kinase that is activated in

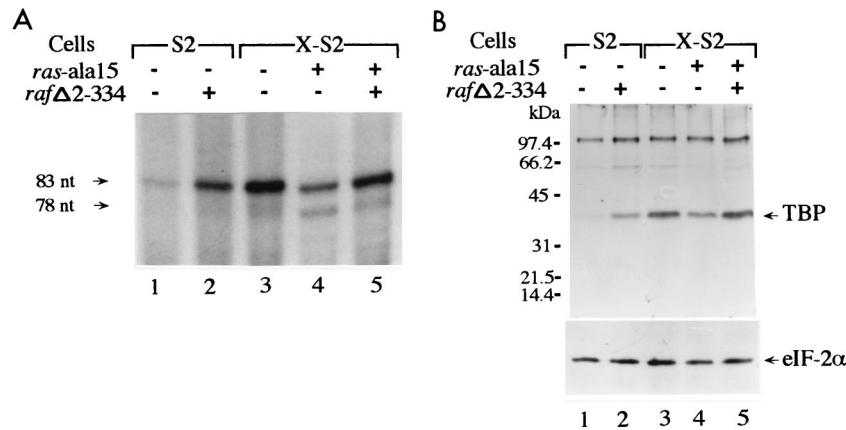


FIG. 7. Activated Raf-1 reverses the effect of dominant-negative Ras on the X-mediated increases in RNA pol III-dependent transcription and TBP levels. (A) Expression of activated Raf-1 augments transcription in S2 cells and in X-S2 cells that are cotransfected with a dominant-negative Ras mutant. S2 and X-S2 cells were cotransfected with 2  $\mu$ g of pArg-maxigene, *pcopia-lacZ*, and, where designated, 2  $\mu$ g of expression plasmids containing either the *Ras-ala15* or the *RafΔ2-334* gene or both. RNase protection assays were performed with RNA extracted from the transfected cells, and the labeled RNA products were visualized by autoradiography as described in Materials and Methods. (B) Expression of activated Raf-1 increases the cellular levels of TBP in S2 cells and in X-S2 cells that are cotransfected with a dominant negative Ras mutant. Cells were transfected as described for panel A, protein lysates were prepared from the transfected cells, and Western blot analysis was performed with anti-*Drosophila* TBP antibodies (top) or anti-*Drosophila* eIF-2 $\alpha$  antibodies (bottom).

this cascade. Transient expression of a gene encoding a constitutively activated form of mouse Raf-1, *RafΔ2-334*, in the S2 cells produced both an approximately fivefold increase in RNA pol III-dependent gene activity (Fig. 7A; compare lanes 1 and 2) and a threefold increase in TBP levels (Fig. 7B; compare lanes 1 and 2). We next determined whether the expression of activated Raf could induce an increase in gene activity and TBP levels in X-S2 cells that had activated Ras activity blocked. X-S2 cells expressing the dominant-negative Ras mutant gene, *Ras-ala15*, were inhibited in the ability to stimulate transcription (Fig. 7A; compare lanes 3 and 4) and increase TBP (Fig. 7B; compare lanes 3 and 4). However, when both *Ras-ala15* and *RafΔ2-334* were cotransfected in these cells, expression of activated Raf-1 was able to enhance transcription and increase TBP to approximately what was observed with the expression of X alone (Fig. 7A and B; compare lanes 3 and 5). These results provide additional evidence that the X-mediated events are dependent on the activation of the Ras/Raf-1 signaling cascade. We have further analyzed a downstream target of Raf-1, MAP kinase, which is activated by phosphorylation by MAP kinase kinase in this signaling cascade (2). An approximate fivefold increase in the phosphorylated state of the MAP kinase protein is observed in the X-expressing cells compared to the control cells (data not shown).

## DISCUSSION

The role of the HBV X protein in viral replication and its contribution to the development of HBV-associated hepatocellular carcinoma are still poorly understood. It has been well established, however, that the X protein has pleiotropic effects on cellular and viral gene expression. To better understand the function of X, it is necessary to both elucidate the cellular targets of X and determine the mechanisms that contribute to its profound effects on cellular gene activity. Previous studies have shown that X can induce gene activity either by directly interacting with the transcription machinery or by indirectly activating cellular signaling pathways. How each of these mechanisms contributes to the diverse effects that X has on cellular gene activity is not yet clear. X has been shown to produce an increase in the activated form of Ras, leading to

the activation of Raf-1 and MAP kinases (4), yet studies of the consequences of these events on cellular gene activity have so far been limited to analyses of the X-mediated induction of AP-1- and NF- $\kappa$ B-dependent promoters (4, 9, 12, 14, 38). In this study, we have identified two new important consequences of the X-mediated activation of Ras, by demonstrating that activation of this signaling cascade is responsible for the ability of X to increase cellular levels of TBP and to activate RNA pol III-dependent gene activity.

X induction of both AP-1- and NF- $\kappa$ B-dependent promoters requires that X be localized to the cytoplasm, supporting the view that these events are mediated by the activation of signal transduction pathways (14). Furthermore, X transactivation of AP-1-dependent promoters is blocked by inhibitors that prevent the activation of PKC (12, 28), Ras (4, 38), and Raf-1 (4, 12). However, X activation of NF- $\kappa$ B-dependent promoters does not require the activation of PKC (36). Although one study showed that X induction of NF- $\kappa$ B-dependent promoters was found to be dependent on the activation of Ras and Raf-1 (47), another study, using a different cell line, found no requirement for activated Raf-1 (9). Thus, the signaling pathways mediating X induction of AP-1 and NF- $\kappa$ B activities appear to be different. Our results demonstrate that RNA pol III-dependent promoters are activated by X similarly to the AP-1-dependent promoters in that they require the activation of PKC (58) and, as shown in the present study, the Ras/Raf-1 signaling cascade. The fact that X activates this signaling pathway in diverse cell types further supports the view that this is a general event which is not cell type specific.

Examining the effects of serum on RNA pol III-dependent gene activity and TBP levels, we find that both are down-regulated when the S2 cells are serum deprived. This finding is consistent with previous studies that have shown that serum stimulation can induce transcription of RNA pol III-dependent genes (25, 60) and increase TBP levels (56). Interestingly, the X-mediated increases in both gene activity and TBP are further augmented when the X-expressing cells are serum deprived. The induction of many events that occur via the activation of Ras is more pronounced when cells are serum deprived, which provides further evidence that the gene induction and increase in cellular TBP observed are modulated



by this pathway. These results further suggest that X may play a role similar to that of a growth factor and can substitute for certain growth factor(s) when they are limiting in the media. These results, however, differ from those of previous studies on X activation of AP-1-dependent promoters, where serum deprivation served to strongly decrease the X-mediated induction of these promoters (5, 12).

The constitutive activation of the Ras/Raf-1 mitogenic signaling pathway has been widely shown to promote oncogenic transformation and human tumorigenesis (34). Elucidating the transcription factors that are the downstream targets of this pathway and the genes that they regulate is important in understanding the cellular changes that accompany this response. The activation of Ras has been extensively shown to modulate the activity of a variety of transcription factors, including *c-fos*, *c-jun*, *c-myc*, and certain ETS family members (2). Our present study identifies another transcription factor, TBP, that is targeted by this pathway. Since TBP is required for the transcription of all cellular genes (24), the increase of this protein, mediated by both X and Ras, likely has profound consequences on the activities of many cellular genes. Overexpression of TBP has been shown to differentially affect the transcription of RNA pol II-dependent promoters. In mammalian cells, overexpressed TBP potentiates some activators and inhibits others (44). In *Drosophila* cells, increased cellular TBP enhances the transcription of TATA-containing promoters, whereas the transcription of TATA-lacking promoters is either not affected or repressed (10). For RNA pol III-dependent promoters, both tRNA and U6 RNA genes are induced when TBP is overexpressed in *Drosophila* cells (50). How the X- and Ras-mediated increases in cellular TBP levels and subsequent changes in gene expression might contribute to cell transformation and tumorigenesis needs to be further explored. Interestingly, TBP mRNA has been found to be overexpressed in lung and breast carcinomas compared to normal human tissue (56).

Our results show that the level of RNA pol III-dependent transcription parallels the cellular level of TBP. We have previously demonstrated that the X-mediated increase in TBP is sufficient for enhancing RNA pol III-dependent transcription (50, 58). There are several possibilities for how the increase in TBP could modulate transcription. This enhanced transcription could result from an increased number of active TFIIB complexes. Alternatively, an increase in the number of functional TFIID complexes could stimulate RNA pol II-dependent gene activity and indirectly enhance RNA pol III-dependent transcription. A third possibility is that the enhanced transcription could be a result of antirepression by TBP, through its interaction with molecules such as Rb or Dr-1, known repressors of RNA pol III-dependent transcription (61, 65). Evidence so far supports the view that TBP acts directly on RNA pol III-dependent promoters by increasing functional TFIIB complexes. First, analysis of the activities of TFIIB, TFIIC, and RNA pol III in X-expressing cells revealed that only TFIIB activity is increased (58). We have also analyzed a mutant TBP protein that was found to be specifically defective in RNA pol III-dependent transcription, yet it fully retained its ability to activate certain RNA pol II-dependent promoters (50). This result demonstrates that the enhanced activity of RNA pol II-dependent promoters is not sufficient to activate RNA pol III-dependent promoters. Furthermore, this mutant TBP was shown to be defective in its ability to stably associate with one of the TFIIB subunits, demonstrating that it cannot form functional TFIIB complexes (54). Together, these results provide evidence to support the view that the X-mediated increase in the cellular levels of TBP produce an increase in

the number of functional TFIIB complexes which stimulates RNA pol III-dependent transcription. However, we cannot rule out the possibility that the increase in cellular TBP produces an antirepression effect which further contributes to the enhancement of RNA pol III-dependent transcription.

The mechanism by which X or Ras increases cellular TBP remains to be addressed. However, preliminary studies indicate that the steady-state level of TBP mRNA is increased in X-expressing cells (51). This finding suggests that the activation of the Ras/Raf-1 signaling pathway may stimulate transcription of the TBP promoter. The human TBP promoter has been recently shown to contain several putative ETS transcription factor binding sites, and at least one of these elements is essential for transcription (17). Interestingly, several ETS transcription factor family members have been shown to be activated by the Ras pathway (49), and the function of the ETS proteins appears to be conserved between vertebrates and *Drosophila* (1). Thus, consistent with our results, it is possible that TBP levels are modulated by a common mechanism in both mammalian and insect cells.

These results represent the first demonstration that RNA pol III-dependent gene activity is regulated by the Ras/Raf-1 signaling pathway. Since this signaling cascade plays a central role in cell cycle and growth control, these results support the notion that regulation of the synthesis of RNA pol III transcripts, which includes tRNAs and 5S RNAs, contributes to the growth potential of the cell. This is consistent with previous studies that have shown that RNA pol III-dependent gene expression is regulated in response to agents that affect cell growth or the cell cycle. Down-regulation of RNA pol III-dependent transcription has been observed in cells due to cycloheximide treatment or growth into stationary phase (48), during the differentiation of F9 embryonic carcinoma stem cells into endoderm (63), and in cells that express the tumor suppressor retinoblastoma protein (65). Conversely, the tumor-promoting agent tetradecanoyl phorbol acetate has been shown to activate RNA pol III-dependent promoters (18, 19). Simian virus transformation also has been shown to enhance RNA pol III-dependent transcription activity (64). Furthermore, RNA pol III-dependent gene expression has been shown to be cell cycle regulated and dependent on the mitotic state of the cell (20, 62).

Since X has been shown to induce focus formation in certain cells (46) and to promote the development of tumors in certain transgenic mice strains (26, 29, 52), it is thought that X may be an important factor in the development of hepatocellular carcinoma in individuals chronically infected with HBV. Consistent with these observations, X stimulates DNA synthesis (4, 31) and cell cycle progression (5, 31). Since increased tRNA and rRNA synthesis is required to sustain enhanced rates of cell proliferation, it is possible that the X-mediated induction of tRNA and rRNA synthesis is a necessary event leading to cell transformation and tumorigenesis. By responding to changes in growth rate, the transcription of tRNAs and rRNAs could regulate the level of ribosome production and the translational potential of the cell. Consistent with this idea, the X protein also transactivates RNA pol I-dependent promoters which are responsible for the synthesis of the 28S, 18S, and 5.8S rRNAs. Furthermore, X induction of RNA pol I-dependent gene activity is mediated by the activation of Ras (57). Whether the Ras-mediated induction of RNA pol I- and III-dependent genes by X and the subsequent increase in tRNAs and rRNAs contributes to cell transformation and proliferation remains to be determined.

## ACKNOWLEDGMENTS

We thank Adrian Vilalta for helpful discussions and Chiou-Hwa Yuh for help with the construction of expression plasmids. We are grateful to Michael Stallcup, Axel Schonthal, and Daniel Broek for critical review of the manuscript. We thank Ray Mosteller for providing pSP8, pSP30, P146, and P155 DNAs; Axel Schonthal for providing EC12; and James Manley for providing *pcopia-lacZ*. L-739,749 was kindly provided by Alan Oliff and Jay Gibbs (Merck & Co.).

This work was supported by grants from the United Liver Association, by American Cancer Society research grant NP-907, and by National Institutes of Health grant CA74138 to D.L.J. H.-D.W. was supported by predoctoral fellowships from the Pharmaceutical Research and Manufacturers of America Foundation and the Charles & Charlotte Krown Foundation.

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