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Transcriptional Regulation of the TATA-Binding Protein by Ras Cellular Signaling

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Our previous studies have demonstrated that the level of the central transcription factor TATA-binding protein (TBP) is increased in cells expressing the hepatitis B virus (HBV) X protein through the activation of the Ras signaling pathway, which serves to enhance both RNA polymerase I and III promoter activities. To understand the mechanism by which TBP is regulated, we have investigated whether enhanced expression is modulated at the transcriptional level. Nuclear run-on assays revealed that the HBV X protein increases the number of active transcription complexes on the TBP gene. In transient-transfection assays with both transformed and primary hepatocytes, the human TBP promoter was shown to be induced by expression of the HBV X protein in a Ras-dependent manner, requiring both Ral guanine nucleotide dissociation stimulator (RalGDS) and Raf signaling. Transient overexpression of TBP did not affect TBP promoter activity. To further delineate the downstream Ras-mediated events contributing to TBP promoter regulation in primary rat hepatocytes, the best-characterized Ras effectors, Raf, phosphoinositide 3-kinase (PI-3 kinase), and RalGDS, were examined. Activation of either Raf or RalGDS, but not that of PI-3 kinase, was sufficient to induce TBP promoter activity. Both Raf- and RalGDS-mediated induction required the activation of mitogen-activated protein kinase kinase (MEK). In addition, another distinct Ras-activated pathway, which does not require MEK activation, appears to induce TBP promoter activity. Analysis of the DNA sequence requirement within the TBP promoter responsible for these regulatory events defined three distinct regions that modulate the abilities of Raf, RalGDS, and the Ras-dependent, MEK-independent pathways to regulate human TBP promoter activity. Together, these results provide new evidence that TBP can be regulated at the transcriptional level and identify three distinct Ras-activated pathways that modulate this central eukaryotic transcription factor.

The TATA-binding protein (TBP) is a central factor used in the transcription of all eukaryotic genes (18). TBP is assembled into at least three distinct protein complexes, SL1, transcription factor IID (TFIID), and TFIIIB, by its association with different TBP-associated factors, which then specifies its role in the transcription of the RNA polymerase I, II, and III promoters, respectively. In promoters containing a TATA element, these TBP complexes are recruited through direct interaction of TBP with the DNA. In contrast, TBP is recruited to promoters that lack a TATA element via protein-protein interactions. The recruitment of TBP to TATA-containing promoters has been shown to be a rate-limiting step for transcription activation (for a review, see reference 27). Thus, alterations in the cellular levels of TBP could produce global changes in cellular gene activity.

Although the role of TBP in the formation of transcription initiation complexes has been well studied, little is known regarding potential cellular events that might regulate this key transcription component or its ability to form various TBP-associated factor-associated complexes. Our previous studies have demonstrated that expression of the hepatitis B virus (HBV) X protein in both mammalian and insect cells produces an increase in the cellular levels of TBP (41). Since the X protein has been shown to be a promiscuous transcriptional transactivator, its ability to increase TBP likely contributes to its effect on a large and diverse number of viral and cellular promoters. Consistent with this notion, increases in cellular TBP have been shown to have pronounced effects on cellular transcription. We have demonstrated that all three classes of RNA polymerase III-dependent promoters are stimulated in response to increased levels of TBP, either directly (34) or indirectly by the expression of the HBV X protein (41) or the activation of protein kinase C by phorbol ester (13, 14). Evidence supports the idea that this is a result of increased numbers of functional TBP-containing TFIIIB complexes at these promoters (34, 41). The HBV X-dependent increase in TBP has also been shown to enhance RNA polymerase I-dependent ribosomal DNA promoters in both insect and mammalian cells (40). Initial studies that have examined the effect of TBP overexpression on the transcription of RNA polymerase II-dependent promoters have revealed that, depending on the promoter architecture, there are different effects. Overexpression of TBP in *Drosophila* cells was shown to generally stimulate TATA-containing promoters to various extents, while TATA-lacking promoters were either repressed or not affected (8). In mammalian cells, overexpression of TBP potentiates the transcriptional activation of certain activators and it represses the activation of others, depending on the core promoter structure (16, 31). Thus, the activities of RNA polymerase I- and III-dependent promoters are enhanced by TBP overexpression while RNA polymerase II-dependent promoters are differentially regulated.

The HBV X protein is necessary for viral replication in animal hosts (6, 48), and it has been extensively shown to be a transcriptional activator (46). X is believed to stimulate gene activity via two distinct mechanisms dependent on its subcel-

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lular location (9). Many X-responsive RNA polymerase II-dependent promoters, such as those containing sites for AP-1 and NF-κB, are induced by X through the activation of the Ras–Raf–mitogen-activated protein (MAP) kinase kinase (MEK)–MAP kinase (MAPK) and Jun-terminal kinase pathways (3, 4, 25). This function of X requires it to be located in the cytoplasm (9). Our studies have revealed that the X-mediated increase in TBP and its ability to induce both the RNA polymerase I and III promoters are also dependent on the activation of Ras signaling (39, 40). However, evidence also supports the idea that X directly interacts with certain transcription components in the nucleus, functioning as a transcriptional coactivator to induce specific cellular promoters (17, 22, 23, 44). While it is not understood how the transactivation function of X may contribute to the life cycle of the virus, the importance of the X-mediated activation of cellular signaling in the HBV replication process was recently demonstrated (21).

Ras proteins function as critical GDP/GTP-regulated switches that control cellular signaling from upstream growth factor receptor tyrosine kinases downstream to a cascade of serine/threonine kinases. Ras GTPases regulate cellular function through the activation of multiple signaling pathways mediated by distinct effector molecules (20). To date, three effector molecules that directly interact with Ras have been well characterized. The Raf serine/threonine protein kinases are key effectors of Ras that carry signals into the nucleus through the subsequent activation of MEK and MAPK. In addition to Raf, other proteins have been characterized that convey Raf-independent signaling responses. The Ral guanine nucleotide dissociation stimulator (RalGDS) family of guanine nucleotide exchange factors directly interacts with Ras (43), stimulating the GDP/GTP exchange of Ral proteins in a Ras-dependent manner (35). Ras-mediated cytoskeletal changes are dependent on a pathway involving phosphoinositide 3-kinase (PI-3 kinase) (30). These Ras effector pathways cooperate to exert the full transforming function of Ras (for a review, see reference 20). Although much progress has been made in identifying the proteins that are involved in mediating Raf, RalGDS, and PI-3 kinase signaling, less is known regarding the specific transcription factors that are ultimately targeted in these responses or the genes whose expression they regulate.

Our previous studies have identified an important consequence of HBV X-mediated Ras signaling: an increase in the cellular levels of TBP. Our present studies are the first to demonstrate that TBP can be regulated transcriptionally. Using both nuclear run-on assays and transient transfection of the human TBP promoter into a variety of different cell types, we show that X modulates TBP at the transcriptional level in a Ras-dependent manner. The overexpression of TBP does not appear to regulate TBP promoter activity in a transient-transfection context. We further found that the activation of either Raf or RalGDS signaling, but not that of PI-3 kinase signaling, contributes to the X- and Ras-mediated induction of human TBP (hTBP) promoter activity in primary rat hepatocytes. Induction of the hTBP promoter by both Raf and RalGDS requires the activation of MEK. In addition, our results support the notion that there is another Ras-activated, MEK-independent pathway that can stimulate hTBP promoter activity in hepatocytes. Analysis of DNA sequences within the hTBP promoter also revealed that there are three distinct regions that confer inducibility of the promoter by these three Ras-activated signaling events.

MATERIALS AND METHODS

Plasmids. All hTBP promoter-luciferase constructs i.e., p−4500/+66hTBP-luc, p−1120/+66hTBP-luc, p−736/+66hTBP-luc, p−176/+66hTBP-luc, p−84/+66hTBP-luc, p−84/−1hTBP-luc, and p−84/−1hTBP-luc-Mets, were the generous gift of Diane Hawley (12). Mammalian expression plasmids pCMV-X, pCMV-RasV12, and pCMV-RasA15 were described previously (39). The HBV X mutant containing a nuclear localization signal, pCMV-X-NLS, was provided by Robert Schneider (9). TBP expression plasmid pSRα-MSV-LTR-TBP (47) was provided by Arnold Berk. Thymidine kinase and human β-3 integrin promoter-luciferase constructs (TK-luc and β-3-luc) were described previously (7, 36) and were supplied by Chi Dang. Constitutively activated Ras and Ras effector mutants, pDCR-RasV12, pDCR-RasV12-S35, pDCR-RasV12-G37, and pDCR-RasV12-C40 (42), as well as constitutively activated Raf, pcDNA3-Raf-BXB (5, 37), were kindly provided by Michael A. White. Constitutively activated Rlf (pMT2-HA-Rlf-CAAX) and the Rlf mutant without a catalytic domain, pMT2-HA-Rlf-ΔCAT-CAAX (45), were supplied by Hendrick Gille and Johannes Bos. pCMV-Raf375M has been described previously (32) and was supplied by Jae-Won Soh. The Ral binding domain mutant (pRK5-RalBD), kindly provided by Jacques Camonis, is comprised of amino acids 397 to 518 of RLIP76 and was subcloned from pGEX-4T3-RalBD (2) into expression vector pRK5 using *Bam*HI and *Sal*I restriction sites. The pBluescript SK+ (pSK) vector DNA was obtained from Stratagene.

Primary rat hepatocyte preparation and cell culture. Hepatocyte cultures from male Sprague-Dawley rats (225 to 400 g; 6 to 12 weeks old) were obtained from the USC Liver Tissue Culture Core Facility. Surgery and isolation were done as described by Moldeus et al. (24). Cell number and viability (85 to 92%) were determined by trypan blue exclusion. Cells were initially isolated and cultured in William's E medium supplemented with 5% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, 0.1× ITS-X (insulin at 1 μg/ml, sodium transferrin at 0.55 μg/ml, 3.9 μM sodium selenite, ethanolamine at 0.2 μg/ml), penicillin at 200 U/ml, streptomycin at 200 μg/ml, amphotericin B (Fungizone) at 0.25 μg/ml, and gentamicin (Life Technologies) at 50 μg/ml. Cells were plated on Primaria plates (60 by 15 mm; Becton Dickinson) at 10^6/plate. Human hepatoblastoma HepG2 and human hepatoma Huh7 cells were propagated in high-glucose Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% FBS (Summit Biotechnologies, Fort Collins, Colo.), penicillin at 100 U/ml, and streptomycin at 100 μg/ml. All cells were maintained in a saturated, humidified environment of 5% CO_2–95% air at 37°C.

Transient transfections. Transient transfections of HepG2 and Huh7 cells were performed using a calcium phosphate method (40). For TBP promoter-luciferase reporter assays, cells were plated in 2 ml of medium per 35-mm well at a density of 150,000 to 200,000 cells per well on six-well plates. After 24 h, 0.5 μg of the TBP promoter-luciferase construct was transfected with other DNAs as indicated with a total final DNA amount of 6 μg maintained with pSK. Approximately 16 h later, cells were rinsed well with Dulbecco's phosphate-buffered saline (DPBS; Mediatech) and serum starved in high-glucose Dulbecco's modified Eagle medium with 0.5% FBS, penicillin at 100 U/ml, and streptomycin at 100 μg/ml for 48 h prior to harvesting.

Primary rat hepatocytes were transfected 4 h after isolation. Hepatocytes were rinsed well with DPBS and placed in William's E medium with 2 mM L-glutamine and 0.1× ITS-X. Cells were transfected with 13.5 μg of total DNA using Lipofectin (Life Technologies; 6.67 μg/μg of DNA) or Targefect F1 (Targeting Systems; 1 μg/μg of DNA) in accordance with the manufacturer's specifications. The TBP promoter-luciferase construct (5.5 μg) was transfected with other DNAs as indicated, and the total final DNA concentration was maintained with pSK. To determine the amount of each expression plasmid to use, various concentrations of each plasmid were initially tested in transfection assays to identify the minimal amount of DNA that would produce the most nearly optimal hTBP promoter activity response. While differences in the magnitude of the effect were observed, in all cases, the overall effect on TBP promoter activity was the same, irrespective of the amount of plasmid transfected. Cells were transfected overnight (≤14 h), rinsed with DPBS, and placed in William's E medium as described above for isolation without serum. Hepatocytes were refed after 24 h and harvested 48 h following transfection. When appropriate, a final concentration of 50 μM U0126 (Promega) or 0.5 μM wortmannin (Calbiochem) or 5 μl of dimethyl sulfoxide vehicle (control) was added to cells for 14 h prior to hepatocyte harvesting.

Preparation of cell extracts. For the preparation of total cell lysates from transfected cells for luciferase activity measurements, medium was aspirated from the cell culture and the cells were gently rinsed with DPBS. Cells were scraped from the plates and collected by centrifugation. Cell pellets were resuspended in Promega reporter lysis buffer. Cell suspensions were lysed by incubation on ice for 10 min, followed by freezing and thawing of the cell suspension. Cell lysates were centrifuged for 20 min at 10,000 × *g* at 4°C, and the supernatant was collected for protein and luciferase activity measurements immediately following lysate preparation. Protein concentrations of the resultant cell lysates were measured by the Bradford method using the Bio-Rad protein assay reagent. Lysates prepared from transfected cells were analyzed for luciferase activity using a luminometer and the Promega Luciferase Assay System as described by the manufacturer (Promega). Resultant luciferase activities were normalized to the amount of protein in each lysate. For all transient transfections with pro-

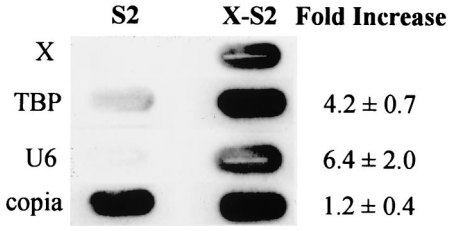

FIG. 1. Nuclear run-on transcription assay comparison of X-expressing and -nonexpressing *Drosophila* S-2 cells. Assays were performed as described in Materials and Methods. Plasmid DNAs used for hybridization contain the gene for X (HBV X) and the *Drosophila* genes for TBP, U6 (U6 RNA), and *copia*. Equal numbers of nuclei were isolated from X-S2 and S2 cells, and labeled nuclear transcripts generated from the reactions were hybridized to the designated plasmid DNAs immobilized on nitrocellulose filters.

moter-luciferase reporter constructs, the fold change in promoter activity was calculated by determining the level of luciferase specific activity in the presence of the empty expression vector DNA or functionally inactive mutant protein expression plasmid (pMT2-HA-Rlf-ΔCAT-CAAX only) and setting this value at 1 for each independent experiment. Values are means ± the standard error of the mean of at least three independent experiments.

Nuclear run-on transcription assay. Nuclei were isolated as described by Garber et al. (15). Briefly, cells were washed and resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 150 mM KCl, 1 mM dithiothreitol) containing 0.3 M sucrose and 0.1% Nonidet P-40 and homogenized with a Dounce homogenizer. Nuclei were pelleted at 1,000 × *g*, resuspended in lysis buffer containing 0.25 M sucrose and 10 μg of RNase A, and incubated on ice for 30 min. Nuclei were washed twice in lysis buffer, collected by centrifugation, and resuspended in 200 μl of reaction buffer (50 mM Tris-HCl [pH 8.0], 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 200 U of RNase inhibitor per ml). For each reaction, 1.6×10^8 nuclei were used. The in vitro elongation reaction was initiated with the addition of ribonucleotides to a final concentration of 0.33 mM each ATP, GTP, and CTP and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol). The reaction was carried out for 10 min at 25°C. Isolation of ^{32}P-labeled RNA, preparation of nitrocellulose filters, and hybridization reactions were performed as previously described (15). One-tenth of the labeled nuclear transcripts was hybridized to 10 μg each of the indicated DNAs immobilized on nitrocellulose to ensure a DNA excess.

RESULTS

The HBV X protein enhances transcription of the endogenous TBP gene in S-2 cells. To begin to examine how the HBV X protein and oncogenic Ras regulate the cellular levels of TBP, nuclear run-on assays were carried out to determine whether TBP is regulated transcriptionally in X-expressing cells. We previously constructed a *Drosophila* Schneider S2 stable cell line, X-S2, that expresses X under the control of the metallothionien promoter (41). As shown in Fig. 1, there was a significant increase in the number of engaged transcription complexes on the TBP gene in the X-expressing cells compared to the control cells. As suggested from our previous studies using transiently transfected genes (39), transcription from the endogenous *copia* gene was unaffected. Transcription of the endogenous U6 RNA gene, an RNA polymerase III promoter, was substantially enhanced by the expression of X, consistent with our previous in vitro studies (41). Thus, these results indicate that at least one mechanism by which X increases cellular TBP is at the level of transcription. Since previous studies have only examined the effect of X on the transcription of transiently transfected genes, these results importantly demonstrate that X expression can affect the activity of endogenous genes as well.

The HBV X protein induces expression of the hTBP promoter in primary and transformed hepatocytes in a Ras-dependent manner. To examine the mechanism by which X regulates TBP at the transcriptional level, we obtained a plasmid containing 4.5 kb of the genomic sequence upstream of the human TBP gene linked to a luciferase reporter (12). To test whether the human TBP promoter could be regulated by X and activated Ras, we transiently cotransfected HepG2 and Huh7 human liver cells with the hTBP promoter-reporter plasmid and expression plasmids containing either the X-encoding gene or a constitutively activated form of Ras, RasV12 (Fig. 2A and B). The expression of either X or activated Ras significantly stimulated the hTBP promoter in both cell lines. The X-mediated induction was inhibited by coexpression of a dominant negative form of Ras, RasA15.

Studies have shown that X is largely cytoplasmic and targeting it to the nucleus abolishes its ability to stimulate cellular signaling (9). Expression of a mutant form of X containing a nuclear localization signal, X-NLS, in HepG2 cells failed to stimulate the TBP promoter (Fig. 2B), regardless of the amount of DNA transfected (data not shown). This confirmed that X exerts its effect in the cytoplasm. To examine whether the effect of X and Ras on TBP promoter activity is specific to the transformed phenotype of the cell lines we used, we assessed whether hTBP promoter activity could be similarly regulated in primary rat hepatocytes. The hTBP promoter was substantially induced by X, but not by X-NLS, in a Ras-dependent manner (Fig. 2C). Together, these results demonstrate that X induces the human TBP promoter in both transformed and nontransformed mammalian hepatocytes by its ability to activate Ras signaling. Since primary rat hepatocytes represent a more biologically relevant system and we were able to develop a transient-transfection protocol yielding highly reproducible results, the remaining studies were conducted using these cells.

Transient overexpression of TBP does not regulate hTBP promoter activity in primary rat hepatocytes. The results described above demonstrate that the X- and Ras-mediated increase in TBP is produced, at least in part, by enhanced transcription. We therefore examined whether overexpression of TBP could serve to regulate the activity of the TBP promoter. The hTBP promoter was cotransfected with increasing amounts of an hTBP expression plasmid into primary hepatocytes (Fig. 3). hTBP promoter activity was not affected at any concentration of the transfected TBP expression plasmid used. This can be compared to the thymidine kinase promoter, which was significantly stimulated by overexpression of TBP, and the human β-3 integrin promoter which, like the hTBP promoter, was not affected. These results indicate that the hTBP promoter is not autoregulated by overexpression of TBP in rat hepatocytes, at least when transiently expressed.

Inhibition of either RalGDS or Raf signaling blocks HBV X transactivation of the hTBP promoter. In order to begin to identify the downstream Ras-activated signaling events mediated by X that regulate TBP promoter activity, the contributions of three well-characterized Ras effectors, PI-3 kinase, Raf, and RalGDS, were examined. While X has been shown to activate the Raf-MEK-MAPK pathway, its ability to activate either PI-3 kinase or RalGDS has not been determined. Hepatocytes were cotransfected with the hTBP promoter construct and the X expression plasmid and either cotransfected with dominant interfering mutants or incubated with specific inhibitors to prevent signaling from each downstream pathway. As shown in Fig. 4, incubation of hepatocytes with the PI-3 kinase inhibitor wortmannin (26) did not affect X induction of the hTBP promoter. Expression of RafM375, a dominant negative form of Raf (32), was able to significantly inhibit X-mediated induction of the hTBP promoter. In addition, the role of MEK, an immediate downstream target of Raf, was examined. Incubation of hepatocytes with the MEK inhibitor U0126 was shown to block hTBP promoter induction by X. To determine the role of RalGDS signaling on X-mediated hTBP promoter

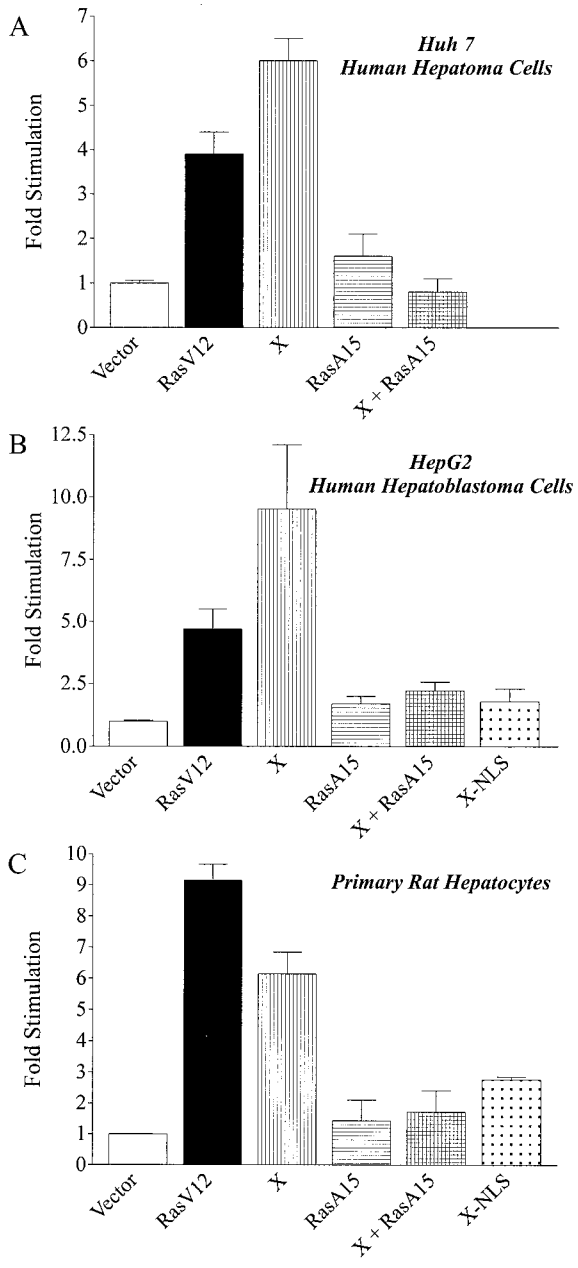

FIG. 2. HBV X protein induces hTBP promoter activity through its ability to activate Ras. The designated cells were transiently cotransfected as described in Materials and Methods with 0.5 μg (for Huh-7 and HepG2 cells) or 5.5 μg (for rat hepatocytes) of the TBP promoter-luciferase construct p−4500/+66hTBP-luc and one or more of the expression plasmids, as indicated. (A) pCMV-X, X (4 μg); constitutively activated Ras, RasV12 (0.5 μg); and dominant negative Ras, RasA15 (1 μg). (B) X (2.75 μg), RasV12 (0.75 μg), RasA15 (1.5 μg), and X containing a nuclear localizing signal, NLS-X (2.75 μg). (C) X (6 μg), RasV12 (1.7 μg), RasA15 (1.7 μg), and NLS-X (6 μg).

induction, the effect of a Ral binding domain mutant, RalBD, was analyzed. RalBD contains only the Ral binding domain of RLIP76, a downstream effector of Ral, and it interacts specifically with the GTP-bound forms of RalA and RalB (2). Thus, in RalGDS-activated cells, RalBD inhibits RalGDS downstream signaling by sequestering activated Ral proteins. Expression of RalBD significantly reduced X-mediated stimulation of the hTBP promoter. These initial results suggest that

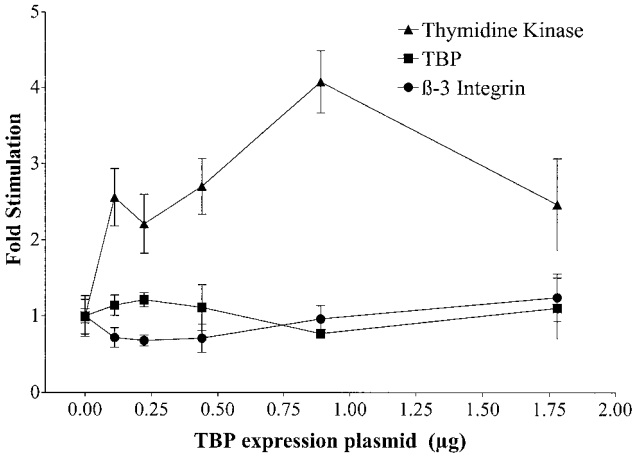

FIG. 3. Transient overexpression of hTBP does not regulate TBP promoter activity in rat hepatocytes. Primary rat hepatocytes were transiently transfected with increasing amounts of an hTBP expression plasmid together with 5.5 μg of a plasmid containing either the TBP promoter, the thymidine kinase promoter, or the β-3 integrin promoter, each of which was linked to a luciferase reporter.

both Raf signaling and RalGDS signaling play important roles in X-mediated transactivation of the hTBP promoter in primary rat hepatocytes.

The RalGDS and Raf pathways, but not the PI-3 kinase pathway, mediate induction of the hTBP promoter through the activation of MEK. To further elucidate which downstream Ras-mediated signaling events potentially regulate the hTBP promoter, three Ras effector loop mutants were used. Each contains the V12 mutation that renders it constitutively activated, and in addition, each harbors another distinct mutation which allows the resultant protein to interact selectively with one of the three defined downstream Ras targets but not the other two (20, 43, 45). To determine if the activation of PI-3

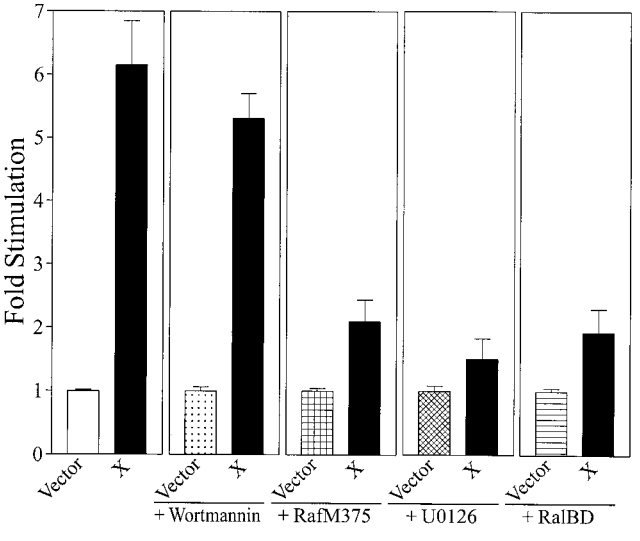

FIG. 4. Inhibition of Raf or RalGDS signaling blocks activation of the hTBP promoter by the HBV X protein. Primary rat hepatocytes were transiently transfected with 5.5 μg of p−4500/+66hTBP-luc and 6 μg of pCMV-X together in the presence or absence of the following inhibiting agents: 0.5 μM wortmannin, 2 μg of RafM375, 50 μM U0126 (a MEK inhibitor), or 2 μg of RalBD. Cells treated with U0126 or wortmannin where exposed to inhibitor 14 h prior to harvesting of the transfected cells.

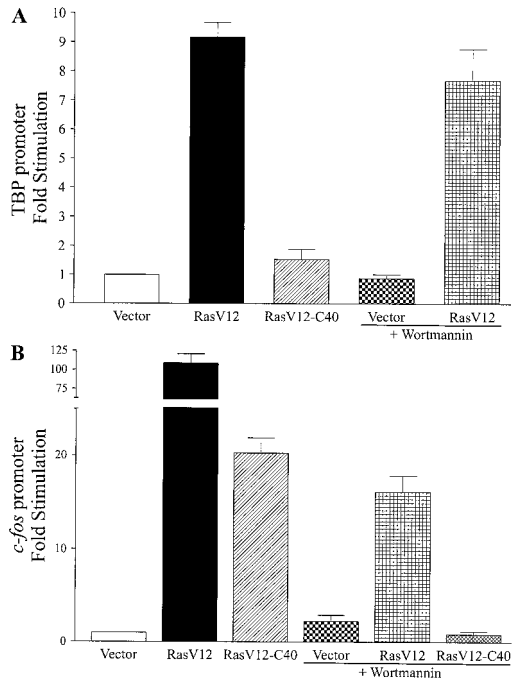

FIG. 5. hTBP promoter activity is not regulated by the PI-3 kinase signaling pathway. Primary rat hepatocytes were transiently transfected with the following DNAs: A, 5.5 μg of p−4500/+66hTBP-luc together with 1.7 μg of RasV12 or RasV12-C40; B, 5.5 μg of c-*fos* promoter-luc together with 1.7 μg of RasV12 or RasV12-C40. Wortmannin (0.5 μM) was added where indicated to cells 14 h prior to harvesting of the transfected cells.

kinase could independently induce hTBP promoter activity, the Ras effector mutant RasV12-C40, which activates PI-3 kinase (30), was cotransfected with the hTBP promoter in hepatocytes (Fig. 5A). Compared to that of RasV12, expression of RasV12-C40 did not have any effect on promoter activity. In addition, incubation of hepatocytes with the PI-3 kinase inhibitor wortmannin did not inhibit hTBP promoter activity either in the absence or in the presence of RasV12 expression. To ensure that PI-3 kinase signaling was being activated or repressed in these cells by the reagents and conditions used, we tested a well-characterized reporter construct that contains the c-*fos* promoter, which is known to be regulated by this signaling pathway (19). As shown in Fig. 5B, RasV12-C40 stimulated c-*fos* promoter activity and this response was effectively blocked by wortmannin. These results indicate that PI-3 kinase signaling was being regulated in the hepatocytes and that hTBP promoter activity was unaffected by either activation or inhibition of PI-3 kinase signaling.

We next ascertained whether the activation of Raf and RalGDS signaling could contribute to the Ras-mediated hTBP promoter induction observed. To determine whether activation of the RalGDS signaling pathway could affect hTBP promoter activity, expression of the Ras effector mutant RasV12-G37, which interacts with and activates both RalGDS (43) and the RalGDS-related factor Rlf (45), or expression of a constitutively activated form of Rlf, Rlf-CAAX (45), was examined. As shown, the expression of either of these proteins produced a substantial increase in hTBP promoter activity (Fig. 6A). Analysis of the ability of the Raf signaling pathway to induce hTBP promoter activity revealed that the expression of either RasV12-S35, which activates Raf signaling (30), or Raf-BXB, a constitutively activated form of Raf (5, 37), significantly

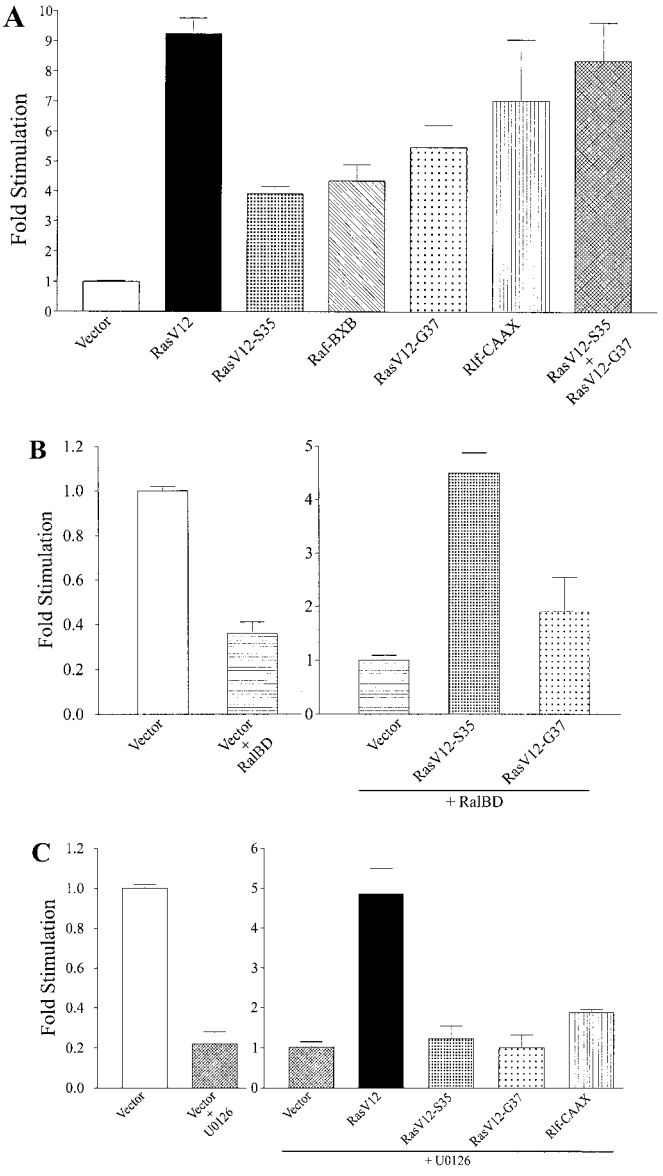

FIG. 6. Both Raf signaling and RalGDS signaling independently stimulate hTBP promoter activity in a MEK activation-dependent manner. Primary rat hepatocytes were transiently cotransfected with p−4500/+66hTBP-luc (5.5 μg) together with the following DNA(s): A, RasV12 (1.7 μg), RasV12-S35 (1.7 μg), RafBXB (0.5 μg), RasV12-G37 (1.7 μg), or RasV12-S35 plus RasV12-G37 (0.85 μg of each); B, RasV12 (1.7 μg), RasV12-S35 (1.7 μg), or RasV12-G37 (1.7 μg) in the presence and absence of RalBD (2 μg). In the left graph in panel B, promoter activity is compared in the presence and absence of cotransfected mutant RalBD. Fold change was calculated based on the promoter activity measured after cotransfection with the empty pRK5 vector alone. For the right graph, the fold stimulation of promoter activity was calculated by normalizing the results to the activity of the promoter construct after cotransfection with RalBD. (C) RasV12 (0.85 μg), RasV12-S35 (1.7 μg), or RasV12-G37 (1.7 μg) in the presence or absence of 50 μM U0126, a MEK inhibitor. For the left graph, promoter activity was compared in the presence and absence of 50 μM U0126.

enhanced hTBP promoter activity. Thus, the activation of either Raf or RalGDS signaling events can serve to regulate hTBP promoter activity. We further determined the effect on promoter activity when the two signaling pathways were simultaneously activated. Cotransfection of RasV12-G37 and RasV12-S35 expression plasmids produced an additive effect on hTBP promoter induction compared to the expression of

either one alone. Coexpression of either of these Ras effectors with RasV12-C40 (which activates PI-3 kinase) did not significantly change the level of promoter induction (data not shown). Thus, activation of either the Raf or the RalGDS pathway is capable of regulating hTBP promoter activity in primary rat hepatocytes and these pathways work together to enhance TBP transcription.

The role of RalGDS signaling in hTBP promoter activity was further analyzed by expressing the Ral binding domain mutant RalBD to inhibit downstream signaling through Ral. As shown in Fig. 6B, expression of RalBD significantly reduced hTBP promoter activity, even in the absence of activated Ras signaling proteins. This result is consistent with the notion that, even without expression of the activated signaling proteins, there is already some activation of the RalGDS pathway within the hepatocytes that can be blocked by expressing RalBD. In the presence of RalBD, RasV12-G37-mediated induction of the promoter was inhibited yet RasV12-S35-mediated induction was not, confirming its selectivity for blocking of RalGDS signaling. Together, these results demonstrate that the RalGDS-Ral pathway regulates hTBP promoter activity.

To further explore the signaling pathways involved in Ras-mediated induction of the hTBP promoter, we determined whether the activation of MEK is required. The U0126 inhibitor was chosen, as it inhibits MEK directly by blocking the catalytic activity of the active enzyme (11), compared to the PD098059 inhibitor, which inhibits MEK activation indirectly by binding to the inactive enzyme, preventing its activation by Raf (10). As shown in Fig. 6C, incubation of the U0126 inhibitor with hepatocytes transfected with the hTBP promoter alone significantly decreased promoter activity. In addition, both Raf- and RalGDS-mediated induction of the hTBP promoter was abolished in the presence of the MEK inhibitor. However, inhibition of MEK activation did not completely reduce the approximately ninefold hTBP promoter induction by RasV12 (compare to Fig. 6A). These results indicate that MEK activation is required for both Raf- and RalGDS-induced stimulation of hTBP promoter activity and support the idea that there is at least one additional Ras-activated but Raf- and RalGDS-independent pathway that regulates TBP.

Three distinct regions within the hTBP promoter confer Ras-mediated inducibility. In order to fully delineate the signaling events, downstream of Ras, that ultimately target specific transcription components that regulate the human TBP promoter, we have begun to identify the regions within the promoter that are important in these Ras-mediated events. A series of hTBP promoter deletion constructs have been analyzed (Fig. 7A). The relative activities of the resulting promoters were first compared in the absence of any transfected signaling proteins to the construct containing 4,500 bp of the genomic sequence upstream and 66 bp of that downstream of the translation start site (−4500 to +66). The unstimulated (basal) hTBP promoter activity was essentially maintained within the sequence including positions −84 to +66 relative to the translation start site (Fig. 7A). Removal of the 66-bp region downstream of the start site, which includes a putative Ets transcription factor binding site, decreased promoter activity approximately twofold. Additional mutation of a putative Ets site within the −84 to −1 region reduced transcription approximately 20-fold less than that of the −4500 to +66 construct. These results are consistent with previous studies conducted with human HeLa S3 and Namalwa cells, where the minimal hTBP promoter was also shown to be contained within these sequences (12).

To determine the sequences necessary for regulation of the promoter by Ras signaling, the relative abilities of these pro-

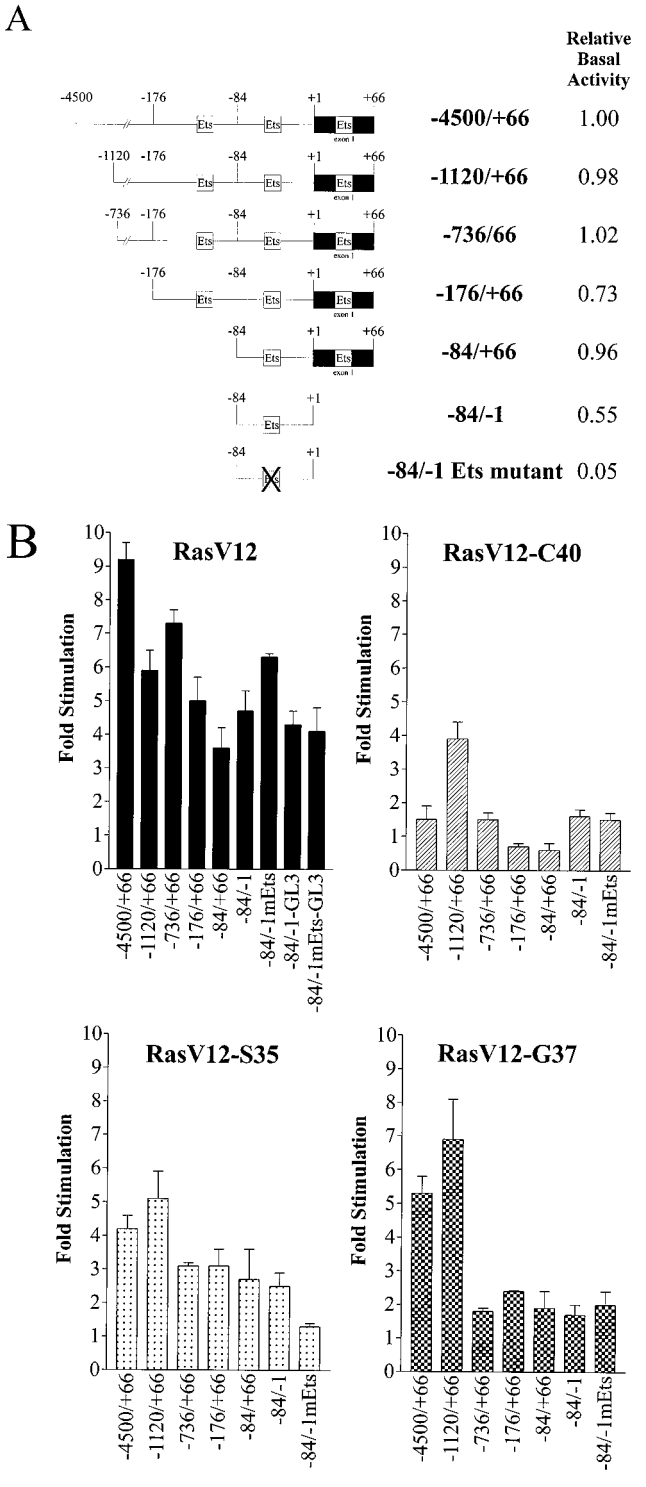

FIG. 7. Analysis of sequences required for basal activity and Ras inducibility of the hTBP promoter. Primary rat hepatocytes were transiently cotransfected with 5.5 μg of one of the following hTBP promoter constructs: p−4500/+66hTBP-luc, p−1120/+66hTBP-luc, p−172/+66hTBP-luc, p−84/+66hTBP-luc, p−84/+1hTBP-luc, p−84/+1 mETS hTBP-luc, p−84/+1hTBP-luc-pGL3, and p−84/+1mETShTBP-luc-pGL3. (A) The various hTBP promoter constructs shown were cotransfected with 1.7 μg of the vector alone. Relative basal, unstimulated promoter activities were calculated based on the full-length TBP promoter activity (p−4500/+66hTBP-luc), which averaged 18.2 ± 3.4 U of luciferase activity/mg of protein. (B) The hTBP promoter constructs were cotransfected with either 1.7 μg of the vector alone, RasV12, RasV12-S35, RasV12-C40, or RasV12-G37 as indicated.

moter constructs to be stimulated with RasV12, RasV12-S35, RasV12-C40, and RasV12-G37 were compared (Fig. 7B). With the exception of −1120/+66, none of the promoter constructs tested were stimulated by RasV12-C40, supporting the notion that PI-3 kinase signaling does not regulate hTBP promoter activity in these cells. Although it is not clear why the −1120/+66 promoter is induced with RasV12-C40, it is possible that when the deletion mutant was constructed, a PI-3 kinase-responsive site was fortuitously created. The ability of RalGDS activation to stimulate promoter activity required sequences between −1120 and −736, as deletion of these sequences significantly reduced RasV12-G37 inducibility. Raf inducibility of the promoter was largely maintained until a putative Ets binding site at positions −50 to −41 was mutated. These initial results reveal that this Ets site may be at least one site important for Raf-mediated stimulation of the promoter.

RasV12, which activates all downstream pathways, was capable of significantly inducing all of the promoter constructs. Although mutation of the Ets binding site within the −84 to −1 fragment completely abolished RasV12-S35 inducibility of the TBP promoter, RasV12 was still capable of stimulating promoter activity. These results, together with the results described above, provide further evidence that there is a separate Raf- and RalGDS-independent pathway, activated by Ras, that regulates hTBP promoter activity. Since DNA sequence analysis of the −84 to −1 region of the promoter did not reveal any obvious DNA binding sequences that might confer Ras inducibility of this fragment (12), we considered that sequences within the plasmid might be responsible for conferring the Raf-, RalGDS-, and MEK-independent response observed. To address this issue, the −84/−1 and the −84/−1 mEts fragments were each subcloned into a pGL3 vector. This vector introduces a polyadenylation sequence upstream from the genomic fragment that reduces background luciferase expression caused by nonspecific transcription initiation (Promega). The resulting constructs were then transiently expressed in hepatocytes and tested for RasV12 inducibility. As shown in Fig. 7B (left side), introduction of these fragments into a different vector did not change their response to RasV12. These results indicate that it is the genomic promoter fragment itself, rather than the plasmid, which confers RasV12 inducibility.

DISCUSSION

Our studies demonstrate that the central transcription factor TBP can be regulated transcriptionally. As our previous work showed that both the HBV X protein and oncogenic Ras produce increases in cellular TBP (39, 41), our new results reveal that this is mediated at the transcriptional level. Nuclear run-on and transient-transfection assays revealed that X expression induces both the transcription of the endogenous TBP gene in insect cells and transient expression of the hTBP promoter in primary rat hepatocytes and human liver cell lines. The increased transcription of the TBP gene by X is dependent on the activation of Ras signaling. Since the Ras signal transduction pathway is strongly conserved among the yeast, *Drosophila*, and mammalian systems (1), it is not surprising that transcriptional regulation of TBP by X-mediated activation of this signaling pathway occurs in both insect and mammalian systems. Although we cannot rule out the possibility that there are other regulatory events that contribute to the increased expression of TBP observed in X- and oncogenic Ras-expressing cells (39, 41), our results support the notion that transcriptional regulation is an important mechanism that controls the level of cellular TBP.

To determine how the increase in TBP promoter activity is regulated, we first considered the possibility that increased expression of TBP could regulate TBP promoter activity. Previous studies by Zhou et al. (47) provided evidence that TBP regulates itself. Clonally selected HeLa S3 cell lines were obtained that expressed a stably transfected hTBP cDNA, yet the endogenous TBP protein levels were down-regulated. However, in the present study, when the same hTBP cDNA expression plasmid was transiently overexpressed in hepatocytes, no significant change in TBP promoter activity was observed. Therefore, our results do not support the idea that overexpression of TBP, at least transiently, in primary hepatocytes can regulate TBP promoter activity. The autoregulation previously shown may be a result of long-term overexpression of TBP, or alternatively, the autoregulation may occur at the protein level rather than at the transcriptional level. Given the fact that neither the hTBP promoter (12) nor the human β-3 integrin promoter (36) contains a discernible TATA element, these results are consistent with the previous study of Colgan and Manley (8) that revealed that TATA-lacking RNA polymerase II-dependent promoters are generally unaffected by the transient overexpression of TBP in *Drosophila* cells, whereas TATA-containing promoters are stimulated. Likewise, we found that the thymidine kinase promoter, a TATA-containing promoter, is induced when TBP is overexpressed. Thus, the ability of TBP to differentially affect RNA polymerase II-dependent gene activity when it is overexpressed may be a general feature of vertebrate cells.

Our results clearly show that the HBV X protein activates the TBP promoter through Ras signaling. Initial analysis revealed that this X-mediated response involved both Raf- and RalGDS-dependent signaling events. These results suggest that, in addition to the activation of Raf-dependent signaling (3), X may also regulate RalGDS signaling. Inhibition of PI-3 kinase did not appear to affect promoter inducibility by X. This result suggests either that the X-mediated activation of Ras does not confer PI-3 kinase activation or that the PI-3 kinase pathway does not regulate hTBP promoter activity in primary rat hepatocytes. To more conclusively analyze these Ras-activated pathways contributing to hTBP promoter regulation, the activation of each downstream Ras effector was analyzed for the ability to induce hTBP promoter activity. These experiments revealed that the activation or inhibition of PI-3 kinase does not alter TBP promoter activity, indicating that, at least in primary rat hepatocytes, this pathway does not regulate TBP. Modulation of the RalGDS pathway was found to substantially regulate TBP promoter activity independently of the activity of other Ras effectors. Using two approaches, expression of either the Ras effector domain mutant RasV12-G37 or a constitutively activated form of a RalGDS-related protein, Rlf, we found that activation of the RalGDS pathway was able to induce TBP promoter activity. Furthermore, expression of a mutant that blocks RalGDS signaling through Ral proteins not only significantly inhibited promoter activation by RasV12-G37 but also decreased promoter activity even in the absence of transfected signaling proteins. Similar results were also obtained using a dominant negative form of RalB (data not shown). This suggests that the level of endogenous activated RalGDS signaling proteins is already significant. Together, these results demonstrate that the RalGDS pathway is involved in the regulation of TBP promoter activity by both X and oncogenic Ras.

Since the expression of either RasV12-S35 or constitutively activated Raf was found to be sufficient for induction of TBP promoter activity, these results support that the activation of Raf signaling is another important contributor to TBP regulation. This agrees with our previous studies with *Drosophila* S-2

cells that showed that TBP levels were significantly increased when a constitutively activated form of Raf was expressed (39). Both RalGDS and Raf signaling events require MEK activation in order to stimulate TBP promoter activity. This is consistent with previous studies in which expression of either RasV12-S35 or RasV12-G37 in NIH 3T3 cells was shown to activate MEK2 (42). While these results appear to suggest that the Raf- and RalGDS-mediated induction of the TBP promoter occurs via overlapping or converging pathways, additional experimental evidence indicates that they in fact regulate TBP promoter activity via distinct signaling pathways. First, there are at least seven distinct MEK protein family members that have been identified to date that target unique sets of downstream signaling proteins (33). The specificity of the U0126 MEK inhibitor for all of these different MEK members is unclear, but at the concentrations used, it has so far been shown to inhibit both MEK1 and MEK2 (11). Thus, it is likely that U0126 does not distinguish between the MEK proteins that are differentially involved in mediating Raf and RalGDS signaling. In addition, deletion analysis of the TBP promoter revealed that different regions within the promoter are required to mediate induction by RalGDS and Raf signaling events. While a gradual loss of Raf inducibility was observed with progressive deletion of sequences between −1120 and +66, the most significant loss of RasV12-S35 induction was obtained when a putative Ets site was mutated between positions −84 and −1. In contrast, sequences between positions −1120 and −736 are required for RalGDS-dependent promoter activation. RalGDS-responsive elements have not yet been identified; however, certain serum response elements have been shown to confer RalGDS inducibility (29). Examination of the TBP promoter sequence did not reveal any serum response element-like elements within this region. Further studies are in progress to further define the DNA element(s) and transcription factor(s) responsible for regulating TBP promoter activity through RalGDS signaling.

In addition to the Raf and RalGDS signaling events that regulate hTBP promoter activity, our data support the idea that there is at least one other Ras-mediated pathway that also regulates TBP promoter activity when oncogenic Ras is expressed. We found that treatment of cells with the MEK inhibitor U0126 did not completely reduce the ability of RasV12 to stimulate hTBP promoter activity (Fig. 6C). In contrast, U0126 was able to completely block X-mediated induction of the hTBP promoter (Fig. 4). In addition, when we examined the promoter deletion constructs for inducibility by RasV12 and the Ras effector mutants, we observed that sequences within −84 to −1 were sufficient to confer RasV12- or Raf-mediated stimulation of the promoter but not RalGDS inducibility. Mutation of a putative Ets binding site within this region abolishes Raf inducibility but not RasV12 activation of the hTBP promoter. We tested the possibility that the vector itself introduces sequences that might confer RasV12 inducibility. However, subcloning of the genomic fragment into a modified vector did not eliminate RasV12 stimulation of the promoter. In contrast to these results, neither of these constructs was able to confer X inducibility of the promoter (data not shown). Together, these results indicate that there is an additional signaling pathway(s), distinct from those mediated by Raf and RalGDS, that is activated by RasV12 in hepatocytes, which can regulate hTBP promoter activity. This pathway, however, does not appear to be activated by the HBV X protein. Thus, our studies strongly support the notion that there are at least three independent Ras-activated pathways that can contribute to hTBP promoter regulation in primary rat hepatocytes. Both X and oncogenic Ras can stimulate promoter activity through the Raf and RalGDS pathways, and an additional RasV12-activated pathway may also contribute to hTBP promoter regulation. A model that summarizes these results is shown in Fig. 8.

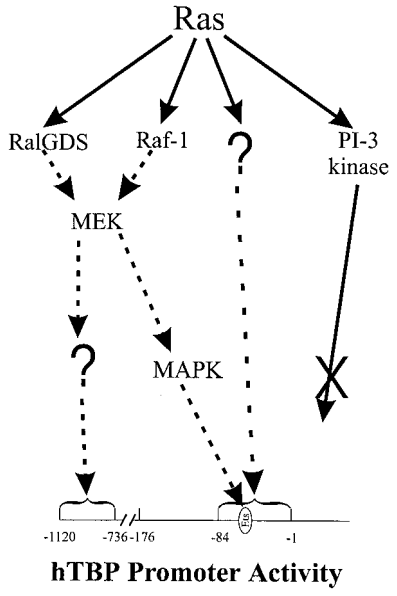

FIG. 8. Schematic model for Ras pathway regulation of hTBP promoter activity in primary rat hepatocytes. The model is based on the data in Fig. 4 to 7. Raf-, and RalGDS-MEK-dependent pathways and at least one MEK-independent pathway activate hTBP promoter activity. Three distinct sites within the TBP promoter further define these distinct Ras signaling events.

An essential step in the expression of eukaryotic genes is the assembly of transcription complexes at the promoter. There is evidence that the recruitment of TBP, which is required for the transcription of all cellular promoters, is rate limiting for transcription in vivo. TBP is limiting for both the RNA polymerase I (40) and III (32) promoters, while RNA polymerase II promoters are differentially regulated by the overexpression of TBP (8, 16, 31; Fig. 3). Thus, mechanisms which regulate the function and production of TBP can profoundly alter cellular gene expression. Our previous studies have provided several lines of evidence that the levels of TBP are up-regulated in the cellular transformation process. Expression of the HBV X protein, a transcriptional transactivator that is thought to be an important contributor to the ability of HBV to transform hepatocytes (46), has been shown to significantly increase cellular TBP levels in both insect and mammalian cell lines (39). This increase in TBP is dependent on the ability of X to activate specific protein kinases (41) and Ras (39). In addition, the activation of protein kinase C by the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate also increases cellular TBP levels (13). The relevance of these findings to human tumorigenesis is supported by results that revealed that TBP mRNA levels are significantly increased in lung and breast carcinomas (38). Furthermore, initial studies have revealed that colon tumor cell lines have substantially increased levels of TBP compared to nontumor colon epithelial cells (S. S. Johnson and D. L. Johnson, unpublished data). Together, these studies suggest the intriguing possibility that increases in the cellular levels of TBP are important in the transformation process. Our studies will continue to define the molecular events transmitted from the Ras-activated pathways to the hTBP promoter that regulate its activity and to discern

whether the resultant increase in cellular TBP levels contributes to Ras-mediated transformation.

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