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pX, the HBV-encoded coactivator, interacts with components of the transcription machinery and stimulates transcription in a TAF-independent manner

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The X protein of hepatitis B virus (HBV) coactivates activators bearing potent (mostly acidic) activation domains. Here, we investigated the molecular mechanisms of this coactivation. We show that pX interacts with general transcription factors TFIIB and TFIIH, as well as with the potent activation domain of VP16. TFIIB interacts with both pX and VP16 simultaneously. In addition, the RNA polymerase II enzyme itself binds to pX. By reducing the activity of cellular coactivators, through squelching, we intensify the dependence of the activator on pX-mediated coactivation. Squelching is essentially diminished in the presence of pX, both *in vivo* and *in vitro*. The target of pX in this activity is the template-bound activator, and not the squelcher. Furthermore, by following transcription in a TAF-deprived reaction, we demonstrate absolute dependence of the activator on the activity of pX. We propose that pX coactivates transcription by substituting cellular coactivators in activator–preinitiation complex interactions.

Keywords: activation domain/general transcription factors/squelching/viral regulators

Introduction

Hepatitis B virus (HBV) is a small DNA virus that, like a retrovirus, replicates by reverse transcription (reviewed in Ganem and Varmus, 1987). Despite its small size, the HBV genome contains at least three distinct promoters, all of which seem to be regulated by the viral enhancer (reviewed in Shaul, 1991) and its regulatory protein, pX. The open reading frame of pX is conserved among all mammalian hepadnaviridae, and it was shown to be essential for WHV infectivity (Chen *et al.*, 1993; Zoulim *et al.*, 1994). pX activates transcription of a vast number of genes through many different DNA elements (Faktor and Shaul, 1990; Cross *et al.*, 1993), implying a more general effect on the transcription machinery. One proposed mechanism assumes modulation of cellular signal transduction pathways (Kekule *et al.*, 1993; Benn and Schneider, 1994). However, it is difficult to distinguish direct and indirect effects of pX, in these studies. Other studies have demonstrated direct transcription regulation by pX. The X protein neither binds DNA directly nor activates basal promoter activity (Faktor and Shaul, 1990; Cross *et al.*, 1993; Haviv *et al.*, 1995), but rather its function depends fully on the cellular activators (Unger and Shaul, 1990; Haviv *et al.*, 1995). pX can not activate a DNA element, AP1 or UAS^G, without the corresponding activator. Furthermore, coactivation by pX depends absolutely on the activation domain (AD) of the activators (Haviv *et al.*, 1995).

Direct protein–protein interactions between activators and components of the transcription machinery are essential (Ingles *et al.*, 1991; Roberts *et al.*, 1993), but not sufficient (Tanese *et al.*, 1991; Boyer and Berk, 1993; Choy and Green, 1993; Walker *et al.*, 1993), for transcription activation. This implies that additional, auxiliary factors are required for transcription activation. Indeed such factors, termed coactivators or mediators, were demonstrated biochemically and enriched *in vitro* (Pugh and Tjian, 1990; Flanagan *et al.*, 1991; White *et al.*, 1991; Boyer and Berk, 1993; Brou *et al.*, 1993; Goodrich *et al.*, 1993; Kim *et al.*, 1994) or *in vivo* (Laux *et al.*, 1994). Some of those were demonstrated to interact directly, both with the general transcription factors (GTFs), and with the corresponding activation domain (AD) (Goodrich *et al.*, 1993; Chen *et al.*, 1994; Gill *et al.*, 1994; Kim *et al.*, 1994; Swaffield *et al.*, 1995). Few cellular activators (Maguire *et al.*, 1991; Feitelson *et al.*, 1993; Natoli *et al.*, 1994; Truant *et al.*, 1995), as well as RPB5 (subunit of eukaryotic RNA polymerases) (Cheong *et al.*, 1995) were reported to bind or to be affected by pX. We have recently described that pX coactivates activators bearing potent (mostly acidic) ADs (Haviv *et al.*, 1995). Here, we investigate the molecular mechanism of this coactivation. We show that GTFs TFIIB and TFIIH, the polymerase enzyme itself, as well as the VP16 AD, all bind pX. Furthermore, when the cellular coactivators (TAFs) are biochemically omitted from the reaction, transcription activation becomes dependent absolutely on pX. We propose that pX regulates transcription by mimicking the cellular coactivators' function, a novel mechanism that is distinct from that of other viral regulators.

Results

Preparation of a functional recombinant pX and Gal4-derived activators

We have previously shown that pX coactivates several activators *in vivo* (Haviv *et al.*, 1995). To explore the underlying molecular mechanism, we established a cell-free system that supports pX activity. For this purpose, we prepared recombinant proteins of pX (rphis-X), a set of Gal4-derived activators and a number of GTFs. To test the activity of our recombinant proteins we performed *in vivo* experiments, but instead of transfections, we applied the rpX and rpGal4-derived proteins directly on HepG2 cells. rpGal4p53 and rpGal4VP16 activators stimulated the expression of the transfected reporter plasmid (Figure 1, bars 3 and 5). In agreement with

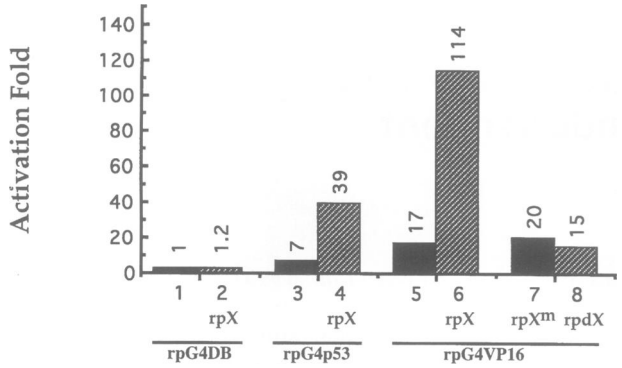

Fig. 1. Preparation of an active recombinant pX. HepG2 cells were transfected with 3 μg of the G5 luciferase reporter plasmid. The new medium applied to cells contained the following indicated recombinant proteins in a final concentration of 4 μg/ml; rphis-X, rpGal4Sp1Q, rpGal4VP16 and rpGal4p53. The obtained luciferase activity was calculated to show activation fold over the basal activity of the reporter plasmid with rpGal4DB(1-94), that was taken as 1. Each point is the average of three independent assays.

previous results (Haviv *et al.*, 1995), rpX had no effect when introduced with rpGal4DB, which lacks a functional AD (Figure 1, compare bar 1 with 2). Interestingly, both rpGal4p53 and rpGal4VP16, which bear a potent acidic AD, were coactivated by wild-type (wt) rpX (compare bar 4 with bar 3, and bar 6 with bar 5), but not by a mutant rpX (compare bar 7 with bar 6). To test the sensitivity of this assay to the folding of rpX a denatured rpX was also tested and found not to be active (compare bar 8 with bar 6). These results suggest that our preparations of rpX and activators are correctly folded, and are functional.

rpX interacts with hTFIIB, hTFIIH and POLII

A likely mechanism of coactivation by pX involves its binding to components of the transcription machinery. We therefore assayed direct binding of GTFs to rpX by employing immobilized protein interaction assay (IPIA; Goodrich *et al.*, 1993). Briefly, HeLa nuclear protein extracts were loaded on an immobilized rpX-affinity column (rphis-X covalently linked to Affi-Prep). Unbound fractions were collected, the column was washed extensively with relatively high salt buffer [5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 300 mM KCl] and the bound fractions were eluted. These fractions were analysed by immunoblotting, using antibodies raised against individual GTFs (Figure 2A). TFIIB (lanes 3 and 4), TFIIH (lanes 7 and 8), and the polymerase enzyme (lanes 9 and 10) bound to the rpX-column, whereas TATA-box Binding Protein (TBP) (lanes 5 and 6) and TFIIF (lanes 1 and 2) did not. The binding was EtBr-resistant (i.e. DNA-independent), and no binding was detected with control resin of Affi-Prep linked to a monoclonal antibody (data not shown).

To map the TFIIB-binding region of pX, deletion mutants of GST-X were assayed by IPIA for interaction with TFIIB (Figure 2B). All the GST-derived resins contained an equal amount (0.2 mg) of protein per ml resin. WT GST-X (lane 3), GST-XΔ22–36 (lane 2), GST-XΔ67–86, and GST-XΔ88–119 (data not shown), all bound TFIIB, whereas GST-XΔ104–154 did not (lane 1).

rpX interacts with rpGal4VP16

As pX function depends on the AD (Unger and Shaul, 1990; Haviv *et al.*, 1995), we assayed direct binding

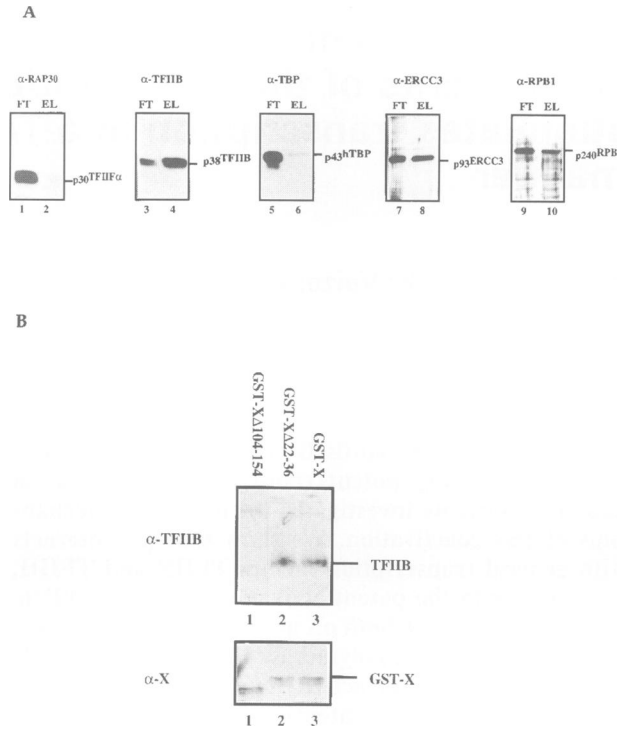

Fig. 2. rpX-interacting transcription factors in nuclear extracts. (**A**) IPIA was carried out with Affi-Prep X column as bait, and nuclear extract (120 μg in 0.2 ml) as interacting proteins. This concentration of nuclear extract was chosen as the minimum that supports *in vitro* transcription reactions. Unbound (FT) and eluted (EL) samples were subjected to SDS–PAGE, followed by immunoblotting using the indicated rabbit, or monoclonal IgGs. α-RAP30 recognizes the small subunit of TFIIF; α-TBP recognizes the central subunit of TFIID; α-ERCC3 recognizes a subunit of TFIIH; and α-RPB1 recognizes the large subunit of RNA polymerase enzyme. (**B**) GST-X, and derived deletion mutants, were used as bait for IPIA. The 12% SDS–PAGE mini gel fails to resolve GST-X wild-type, a 43.5 kDa protein, and GST-XΔ22–36, a 42 kDa protein. Recombinant TFIIB protein (instead of nuclear extract), was tested for binding. Bound fractions were analysed by SDS–PAGE immunoblotting with α-TFIIB IgG (top), and then with α-X (bottom).

of rpGal4VP16 to the GST-X column (Figure 3) or, reciprocally, binding of rpX to the GST-VP16 column (data not shown). Interestingly, wild-type rpGal4VP16 (lanes 2), but not mutant rpGal4VP16Δ456::F442P (lanes 8–14), was preferentially retained on the GST-X column. The finding that GST-X does not bind the inactive VP16 mutant excludes the possibility that rpX interacts with the Gal4 DNA-binding domain. A functionally inactive X deletion mutant (GST-XΔ104–154) did not bind rpGal4VP16 (lane 7). The loss of both TFIIB (Figure 2B) and VP16 binding, by deletion of the essential C-terminal region of pX (Unger and Shaul, 1990; Arii *et al.*, 1992; Runkel *et al.*, 1993), implies the functional significance of these interactions.

To test the effect of GTFs on rpX–VP16 interactions, IPIA of rpGal4VP16 on the GST-X column was carried out, in the presence of soluble TFIIB and TBP, and minor changes in the intensity of interactions were observed (lanes 3–6). The significance of these differences is not clear, as the immunoassay is semi-quantitative and allows only coarse comparison of the efficiency of binding.

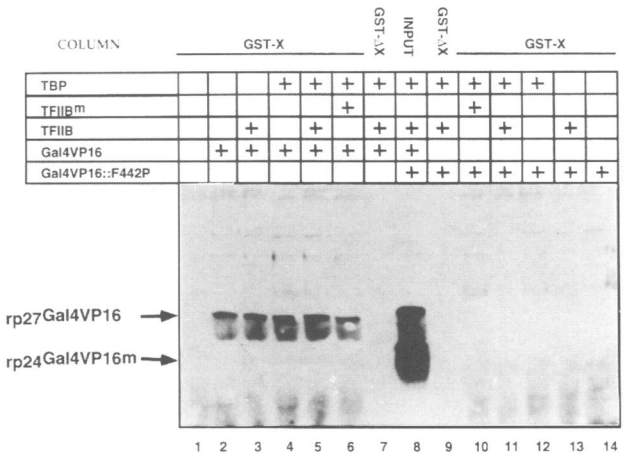

Fig. 3. rpX interacts with Gal4VP16. Resins that contain 2 μg of either GST-X or GSTΔX (Δ104–154) were analysed by IPIA, either with Gal4VP16 (lanes 1–7) or Gal4VP16Δ456–490(::F442P) (lanes 9–14). Both Gal4VP16 proteins were mixed and loaded in lane 8, as size and quantity controls. Bound fractions were analysed by SDS–PAGE immunoblotting with α-Gal4 IgG.

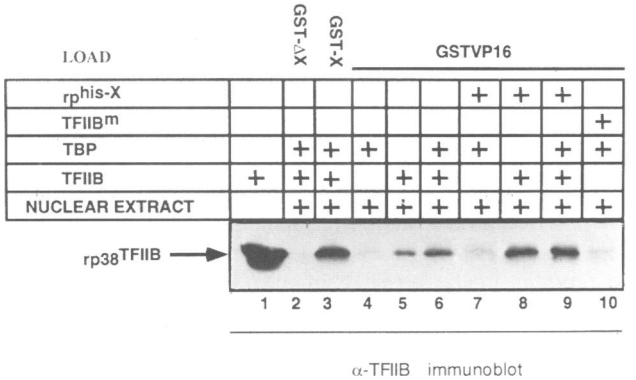

Fig. 4. rpX stabilizes VP16–TFIIB interaction. Three indicated columns were analysed by IPIA in the presence of nuclear extract proteins and with one or more of the following proteins; rphis-X, TBP, TFIIB or TFIIB::K189,200E (TFIIB^m), indicated by + (refer to Figure 2B for details of analysis). The GSTΔ104–154 served as a negative control (indicated as GST-ΔX).

rpX improves interaction of VP16 AD with its targets

Next we tested the effect of rpX on the documented binding of TFIIB and TBP to GST-VP16 (Ingles *et al.*, 1991; Lin *et al.*, 1991). GST-VP16 beads were incubated with recombinant TFIIB, TBP and rpX (rphis-X) for 60 min. After an extensive high salt wash, bound fractions were subjected to immunoblotting with α-TFIIB IgG (Figure 4). Approximately 2–5% of the TFIIB load was retained on the GST-VP16 column (compare lane 5 with 1). This binding was specific, as a TFIIB mutant reported not to bind VP16 (Roberts *et al.*, 1993) behaved as expected (compare lane 5 with lane 10). Significantly, under the stringent binding conditions employed by us, GST-X is more potent in TFIIB binding than GST-VP16 (compare lane 3 with lane 5). However, the unexpected observation was that, in the presence of rpX, increased TFIIB retention by GST-VP16 occurred (compare lane 5 with lane 8). These data argue for stabilization of VP16–TFIIB interaction by pX. TBP is known to bind both VP16 and TFIIB (Maldonado *et al.*, 1990; Ingles *et al.*, 1991). Interestingly, the stabilization effect of rpX was more pronounced than that obtained with TBP (compare lane 5 with lanes 6 and 8). However, rpX has no effect on TBP–VP16 interaction (as measured by immunoassay of the same blot with α-TBP; data not shown). As nuclear extract proteins were present in the binding reaction, the stabilization effect of rpX might be attributed to a cellular cofactor; however, this is unlikely as GST-X can bind TFIIB (Figure 2B) and VP16 (Figure 3) in the absence of additional proteins.

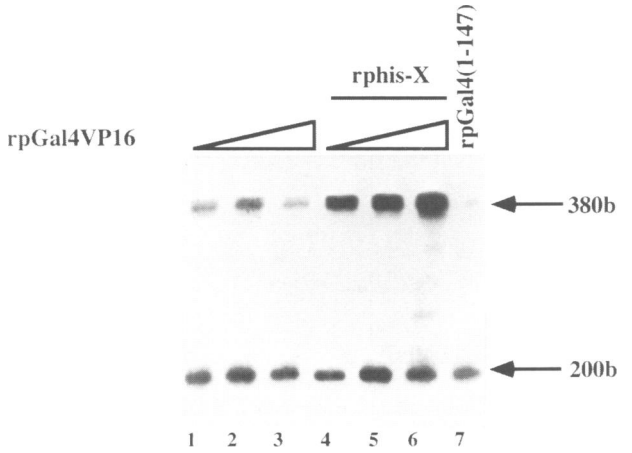

Fig. 5. rpX prevents self squelching *in vitro*. DNA templates bearing the G-less cassette of either 380 or 200 bases, under the regulation of Ad5-MLP and UASG-TATA elements, respectively, were used for *in vitro* reconstituted transcription reactions. 1 μg of rpGal4(1–147) was used as control (lane 7). Increasing amounts (1, 10 and 1000 ng) of Gal4VP16 proteins were added to *in vitro* transcription reactions, either without (lanes 1–3) or with rpX (rphis-X, lanes 4–6). Transcription products were purified and analysed on denaturing gel electrophoresis.

rpX prevents self-squelching in vitro

Having demonstrated that rpX stabilizes activator–TFIIB interaction, we next conducted *in vitro* transcription experiments to evaluate the transcriptional ramification of this effect. The molar ratio of the applied activator and template was determined by electrophoretic mobility shift assay (EMSA, data not shown). Transcription is stimulated by 1 ng of rpGal4VP16, but not rpGal4DB, which lacks a potent AD (Figure 5, compare lane 7 with lane 1). The optimum transcription activation was obtained by 10 ng of the rpGal4VP16 (lanes 1–3). Increasing rpGal4VP16 to an excess of 1 μg reduced transcription (lane 3). This reduction may reflect sequestration of transcription factors and cellular coactivators by the excess activators, i.e. a squelching condition. Transcription was improved in the presence of 30 ng of rpX (lanes 4–6). Remarkably, X-effect was most dramatic under squelched conditions (compare lane 4 with 6). The relief of VP16-mediated squelching was not affected by varying the order of addition or incubation periods of the reaction components (data not shown). Notably, the ability of the X protein to activate transcription in a cell-free system further confirms that rpX is functional and correctly refolded.

rpX supports transcription activation in the absence of TAFs and other coactivators

The enhancement of pX-effect under squelching conditions, in which cellular coactivators are likely to become

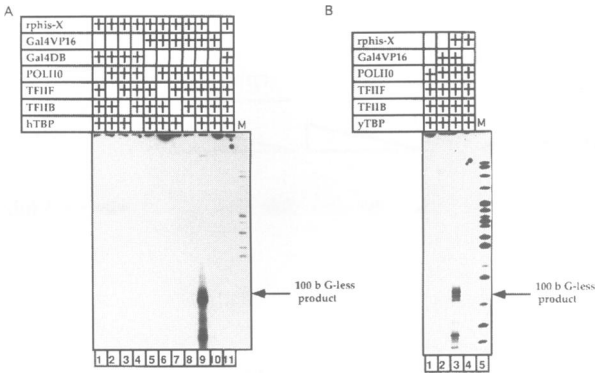

Fig. 6. rpX substitutes cellular coactivators in a fully-defined *in vitro* transcription. A DNA template bearing the G-less cassette of either 85 bases, under the regulation of UASG-TATA elements, was used for *in vitro* reconstituted transcription reactions (200 ng per reaction). The following indicated proteins were mixed in the indicated amounts; rphis-X (10 ng), rpGal4VP16 (6 ng), RNA POLII0 (50 ng), hTFIIB (5 ng), hTFIIF (RAP30/RAP74 heterotetramer, 10 ng) and (**A**) hTBP or (**B**) yTBP (10 ng). The mixtures were incubated for 1 h at 30°C; transcription was then initiated by the addition of nucleotides, and allowed to proceed for an additional 1 h. Transcription products were purified and analysed on denaturing gel electrophoresis. Molecular weight markers (M) are pBR322 cut by *Msp*I, and labelled by fill-in procedure. Sizes are: 622, 527, 404, 307, 240, 217, 200, 190, 180, 160, 147, 123, 110, 90, 76 and 67. The expected product size is indicated by an arrow.

limiting, suggests that rpX may substitute fully for the cellular coactivators. We next examined this possibility in a fully reconstituted transcription assay, employing recombinant GTFs and homogeneous RNA polymerase II. It is documented that under these conditions activator-dependent transcription was not achieved unless accessory factors, i.e. TAFs (Pugh and Tjian, 1990; Chen *et al.*, 1994), and other coactivators (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991) are added. As expected, transcription is not detectable (Figure 6A, lane 11 and 6B, lane 1), even when the activator is added before GTFs (data not shown). Remarkably, when rpX is added transcription is achieved (Figure 6A, lane 9 and 6B, lane 3). The activity of pX depends on the AD of VP16, as rpGal4DB lacking this domain did not support transcription (Figure 6A, lanes 1–4). Furthermore, all the different added components are crucial for the function of pX, and omitting each abolishes transcription (lanes 5–8). The pX activity is supported by human TBP (Figure 6A), as well as yeast TBP (Figure 6B), reconfirming that TFIIB and not TBP is the pX target. Alternatively, it is possible that the conserved C-terminal portion of TBP (Peterson *et al.*, 1990) is responsible for the observed pX-effect. Collectively, our data suggest that rpX functions directly as a coactivator.

pX prevents self-squelching in vivo

To compare pX function *in vitro* and *in vivo*, we reproduced the squelching-relief experiments in tissue culture cells. To do so, we co-transfected increasing amounts of the yeast Gal4 activator–expression vector, with constant amounts of the G5 luciferase (Gal4-responsive) reporter, in the presence or absence of pX-expressor (Figure 7). This reporter is fully dependent on co-transfection with a Gal4-derived activator, and is not affected by pX in the absence of a Gal4-derived activator (Haviv *et al.*, 1995).

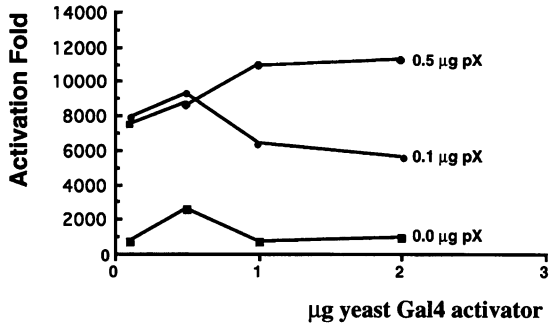

Fig. 7. pX prevents the inhibitory phase of an activator. 1 μg of the G5 luciferase reporter plasmid was transfected into SK-Hep1 cells by the CaP_i method, with the indicated amount of the yeast Gal4 activator expression plasmid, without or with increasing amounts of an X-expressor plasmid (pECEfX, indicated as pX). At 48 h post-transfection, cytoplasmic extracts of the cells were assayed for luciferase activity. The obtained values of activation fold were calculated as luciferase activity of activator versus reporter-alone transfected cells. Each point is the average of six independent assays.

In the absence of pX, transcription activation peaked at 0.5 μg Gal4 activator plasmid per plate (2500-fold activation). With larger amounts of activator, activation declined to 800-fold, probably due to squelching. In the presence of 0.1 μg pX-expressor plasmid, an overall higher activation was observed (further 5- to 15-fold), while with 0.5 μg transcription was no longer susceptible to squelching. Squelching was most apparent with potent activators, such as Gal4E1a, Gal4fos, Gal4VP16 or Gal4p53 (data not shown). Squelching induced by all these activators was relieved by pX. pX neither activated the reporter in the absence of a Gal4-derived activator, nor reduced the production of the Gal4 activators (Haviv *et al.*, 1995). When larger amounts of pX were introduced, the effect of pX gradually diminished, while not affecting the activity of Gal4, in agreement with our published observation (Faktor and Shaul, 1990).

The ability of pX to relieve squelching was further challenged by co-expression with activators that do not bind the reporter DNA (13S E1a, p53 and VP16). Inclusion of 0.2 μg of the 13S E1a plasmid in the transfection mixture resulted in 12-fold reduction of the Gal4E1a activity (Figure 8A, compare bar 3 with 1). We obtained similar results when Gal4VP16 served as the activator, and potent AD of VP16 served as a squelcher (Figure 8B, bar 2). Interestingly, in the presence of pX, not only is squelching diminished and activation fully recovered, but a net 3-fold increase is observed as compared with the non-squelched situation (Figure 8A, bars 4 and 5 and 8B, bar 3). Under these conditions, an overall 20-fold effect of pX is obtained. Squelching relief by pX can also be obtained when the potent AD of VP16 is co-transfected with the Gal4E1a activator (Figure 9A). Finally, we show that pX can not relieve squelching if the activator is not pX-responsive (Gal4Sp1Q; Figure 8C, bar 3) (Haviv *et al.*, 1995).

Two regions of pX are required for squelching relief

Deletion mutants of pX enabled the identification of three functional regions for transactivation (Unger and Shaul, 1990; Arii *et al.*, 1992; Runkel *et al.*, 1993). Transfection experiments revealed that at least two such regions,

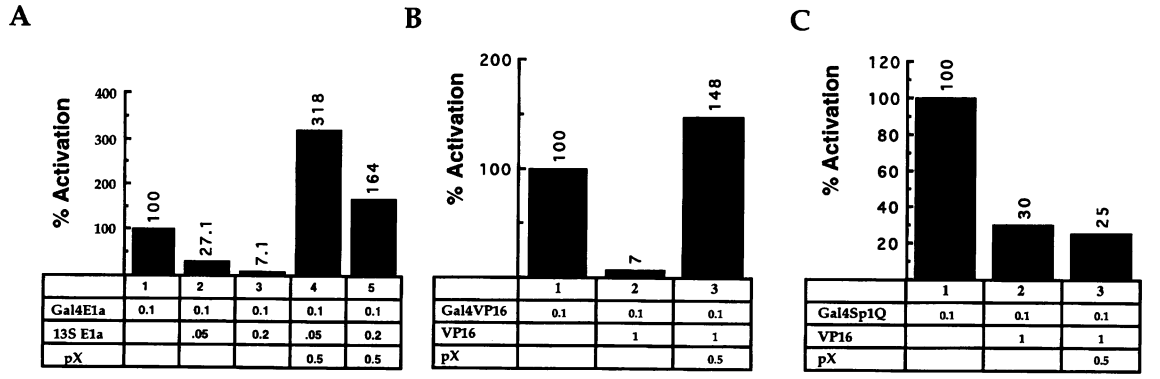

Fig. 8. pX prevents squelching *in vivo*. Transfections with G5 luciferase, Gal4-derived activators, and X expression plasmids, were carried out essentially as in Figure 7. The amount of plasmids employed (μg) are indicated under the corresponding bars. Relative activity was calculated by dividing activation fold, obtained in the presence of squelcher, with that obtained in its absence. The AdV 13S E1a was co-transfected as squelcher of Gal4E1a activity (**A**). HSV VP16 AD was co-transfected as squelcher of Gal4VP16 activity (**B**) or of Gal4Sp1Q activity (**C**).

residues 67–86 and residues 110–143, are also essential for squelching relief by pX (Figure 9A). Thus, the C-terminal portion of pX, which is necessary for interaction with its transcription targets (Figure 2B), is also necessary for squelching relief (Figure 9A). The wild-type expression vector and the two mutant vectors produced similar amounts of steady-state pX protein, as judged by an immunoblot with a commercial α-Flag antibody (Figure 9B, compare lane 6 with lanes 1 and 3).

Discussion

In cultured cells, the X protein of HBV coactivates potent acidic activators (Haviv *et al.*, 1995); here, we describe the molecular mechanism responsible. We found that rpX simultaneously binds components of the transcription machinery both on the enhancer and on the promoter, i.e. the acidic AD of VP16, and GTFs TFIIB and TFIIH, as well as RNA polymerase II enzyme. In addition, rpX improves interaction of the acidic AD of herpes virus VP16 with TFIIB. These *in vitro* interactions are sensitive to the same mutations that abolish either VP16's or pX's transcription activities *in vivo*, implying their functional significance. The VP16 protein is certainly not a natural target for pX and serves as a model acidic activator. However, interaction of rpX with the CREB/ATF and p53 activators was previously reported (Maguire *et al.*, 1991; Feitelson *et al.*, 1993; Truant *et al.*, 1995). Such protein–protein interactions with DNA-binding proteins may target pX to distinct pX-responsive elements. We also observed interaction of rpX with the polymerase II enzyme, though the nature of this interaction is not defined in this study; however, it may be mediated through RPB5 binding (Cheong *et al.*, 1995). rpX was recently reported to bind TBP in an ATP-dependent manner (Qadri *et al.*, 1995). This binding could not be detected in our assays, possibly due to the absence of ATP.

Viral transcription regulators can be broadly classified according to their mode of macromolecular interaction. A first class consists of proteins that recognize a specific cognate DNA sequence. Proteins of the second class potentiate cellular DNA-binding proteins by hooking to them an additional potent AD. VP16 binds the Oct-1 cellular activator, and hooks onto it an AD, which binds GTFs TBP, TFIIB and TFIIH, as well as TAFII40 (Figure

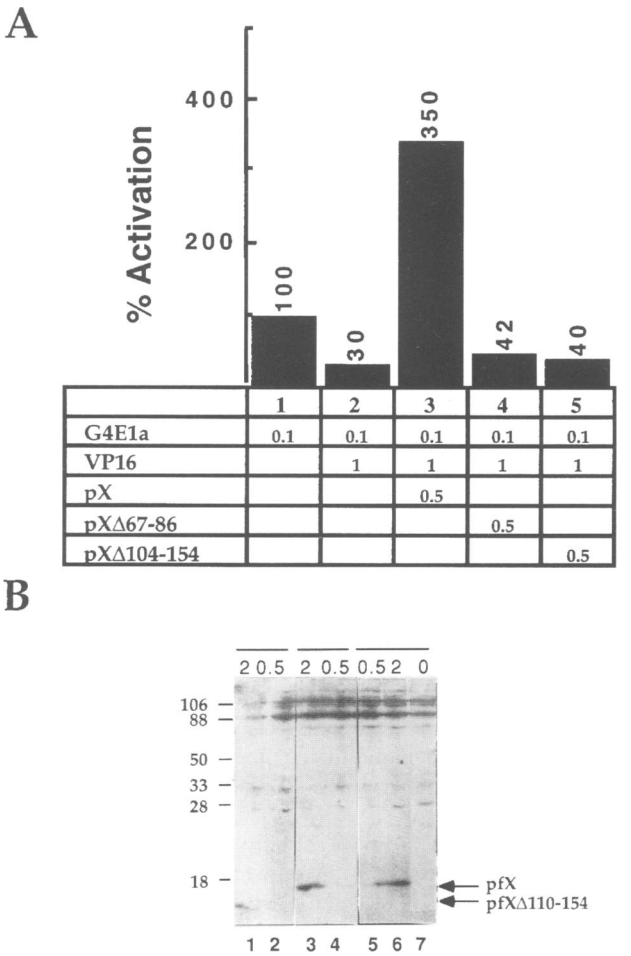

Fig. 9. Two regions of pX are required for squelching relief. (**A**) HSV VP16 AD was used as squelcher of Gal4E1a activity. Two deletion mutants of pX are compared for squelching relief. (**B**) Cells were transfected with pfXΔ110–154 (lanes 1 and 2), pfXΔ67–86 (lanes 3 and 4) or pfX wild-type (lanes 5–7). Cell extracts were examined by immunoblot, using α-Flag antibodies. Standard pre-stained molecular weights are indicated in kDa. The SDS–PAGE employed cannot resolve pfXΔ67–86 (17 kDa) from the wild-type protein (18 kDa).

10B) (Gerster and Roeder, 1988; Ingles *et al.*, 1991; Lin *et al.*, 1991; Goodrich *et al.*, 1993; Xiao *et al.*, 1994). E1a binds several activators as well as TBP (Figure 10C) (Liu and Green, 1990, 1994; Horikoshi *et al.*, 1991; Boyer

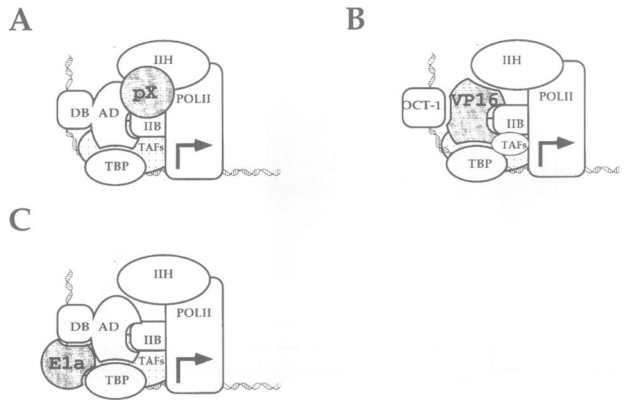

Fig. 10. Molecular model for mechanism of transcription regulation by viral proteins. The activator is represented by DB and AD for DNA binding, and ADs respectively. POLII is RNA polymerase enzyme, TAFs represent TBP-associated factors. The site of interaction with the cellular transcription machinery is indicated for: (**A**) HBV pX; (**B**) HSV VP16 or EBV EBNA-2; (**C**) AdV E1a or HTLV tax1.

and Berk, 1993). Like E1a, Tax1 binds a cellular factor at TxRE 21 bp and TBP but not TFIIB (Matthews *et al.*, 1992; Caron *et al.*, 1993; Adya and Giam, 1995). We show here that pX differs substantially from both these classes, and establishes a new class that resembles the cellular coactivators. This conclusion is based on two unique features of pX: squelching relief, and coactivation in reconstituted transcription assays.

Co-expression of ADs results in mutual interference (Martin *et al.*, 1990), whereas co-expression of either of Gal4VP16 or Gal4E1a with pX results in potentiation of their activity (Haviv *et al.*, 1995). Furthermore, the mutual interference between E1a and VP16 ADs is alleviated by co-expression of pX (Figures 5, 8 and 9). In the *in vitro* transcription experiments (Figure 5), rpX was remarkably potent in squelching relief; on 50 ng of template (10^{-8} M of Gal4-binding sites), ~30 ng of rpX (10^{-8} M) prevents the competitive inhibitory effect of 1 μg of rpGal4VP16 (10^{-5} M) protein. Assuming that all protein molecules in the system are equally active (EMSA verified that the template is fully occupied), this implies that the amount of rpX required for complete squelching relief is determined by the template-bound activator molecules (10^{-8} M) and not by the amount of free activator (10^{-5} M), implying that pX discriminately recognizes its targets on the template.

The unique ability of pX to bind its targets, preferentially on the DNA template, is in agreement with the simultaneous binding to GTFs and the activator. As pX-mediated stimulation of transcription can benefit from sequestration of cellular coactivators and TAFs, it is possible that pX can in fact substitute these missing components. This rationale led us to establish an *in vitro* system that lacks TAFs and other coactivators. It is documented that when transcription is driven by recombinant GTFs and homogeneous RNA polymerase II, activator-dependent transcription depends on accessory factors, i.e. TAFs (Pugh and Tjian, 1990; Chen *et al.*, 1994) and other coactivators (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991). Remarkably, in this completely defined reconstituted transcription system, pX is essential for rpGal4VP16 to act upon the basal transcription apparatus (Figure 6). We therefore conclude that pX is a coactivator *per se*.

Two regions in X-ORF were found to be essential for transcription stimulation activity (including residues 46–72 and 105–142), on the basis of sequence conservation, deletion and insertion, and residue substitution analyses (Unger and Shaul, 1990; Arii *et al.*, 1992; Runkel *et al.*, 1993). These participate in distinct protein–protein interactions (Takada and Koike, 1994). The more N-terminal domain was recently reported to mediate interactions with RNA POL II fifth subunit (Cheong *et al.*, 1995), and partial deletion in this region hampered the ability of pX to relieve squelching (Figure 9A, bar 4). Here, we show that the C-terminal region is also required for binding to TFIIB and VP16, and for squelching relief. More accurate mapping of the binding sites to TFIIB and the ADs is being carried out by amino acid substitutions.

Hepadnaviridae rely on transcription initiation for both their replication and differential gene expression. On the other hand, the relatively small HBV genome transcribes multiple mRNA molecules, initiating from more than four different promoters. This crowded promoter usage is problematic in terms of transcription complex assembly on the promoters, due to promoter occlusion and elongating polymerases that run through downstream promoters, and increase the turnover rate of preinitiation complex. A potent coactivator is perhaps a preferred strategy to meet these particular requirements of hepadnaviridae.

The X protein transforms immortalized cells (Shirakata *et al.*, 1989; Seifer *et al.*, 1991; Seifer and Gerlich, 1992), and induces liver tumours in transgenic mice (Kim *et al.*, 1991). Therefore, pX was implicated in the progression of hepatocellular carcinoma. Transcription stimulation by pX occurs in infected liver cells (Balsano *et al.*, 1994; Schluter *et al.*, 1994), and may mediate its effect on the cell malignancy. Whether or not general coactivation plays a role in accelerating tumour progression is an open question that deserves further investigation.

Materials and methods

Cell cultures and protein feeding

Cell maintenance and transfections were carried out as described previously (Haviv *et al.*, 1995). For the protein feeding experiment (Figure 1), cells were washed from CaP_i. After 6 h, cells were placed in 2.5 ml fresh DMEM/10% FCS and treated with 2 μl of 5 mg/ml stocks of the designated recombinant proteins. After a 12 h incubation, cells were harvested and processed as described by Haviv *et al.* (1995).

Protein production

HeLa cell nuclear extract was prepared from 3×10^{10} cells (10^6 cells/ml) according to published procedures (Dignam *et al.*, 1983). Protease inhibitors were included in all buffers (Haviv *et al.*, 1995). The extract was then fractionated on heparin–agarose (Sigma) and phosphocellulose (Whatmann) as described by Brou *et al.* (1993). Both the 0.5 M KCl and 0.85 M KCl fractions of the phosphocellulose were further fractionated on Sephacryl S-300 [Pharmacia; K_{av} = 0 for P-11(0.85 M); K_{av} = 0.2 for P-11(0.5 M)] and concentrated on DEAE–Sephacell (Pharmacia; 0.1–0.3 M KCl fraction), resulting in the DEA fraction (from the P-11 0.5 M fraction) and the DEB fraction (from the P-11 0.85 M fraction).

Recombinant TFIIB was produced in bacteria as described by Wang *et al.* (1992), and further purified on ϕ-Sepharose, HAP, S-200, mono-S (Ha *et al.*, 1991). Recombinant hTBP was produced as described by Lee *et al.* (1991); recombinant hTFIIF was produced as described (Wang *et al.*, 1995) for the RAP74–RAP30 heterotetramer, including the gel filtration step. The rpGal4DB and rpGal4VP16 proteins were produced using the protocol of Chasman *et al.* (1989), with the modification that rpGal4VP16 was first purified on 20 ml Macro-Prep high S (Bio-Rad; 200–1000 mM NaCl gradient), followed by 20 ml *tert*-butyl Macro-Prep

[Bio-Rad; 0.5 M $(NH_4)_2SO_4$ load, 0.2 M $(NH_4)_2SO_4$ wash and buffer A(0) for elution], before the standard DE-52, and heparin–agarose columns (Chasman *et al.*, 1989). Typically, 20 mg of ~90% pure rpGal4VP16 protein was attained in a 6 mg/ml concentration.

Recombinant rphis-X was produced from pRSETc::X plasmid, in BL21::plysS bacteria, in a 12 l fermenter. Expression was induced at OD_{600} 0.5, by inclusion of 0.5 mM IPTG, for 90 min. Cells were collected, washed and sonicated in 100 ml of lysis buffer (50 mM HEPES–KOH pH 7.9, 5% glycerol, 2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 200 mM LiCl). Debris and inclusion bodies were pelleted in SS-34 (16 000 *g* × 10 min). The pellet was Dounce homogenized in 20 ml of lysis buffer with 1% Triton X-100, 0.05% DOC. Inclusion bodies were repelleted and Dounce homogenized three times in 20 ml of buffer S (10 mM HEPES–KOH pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT) plus 3 M urea. The pellet was solubilized by shaking for 1 h at 37°C (following homogenization) in 20 ml buffer S plus 6 M guanidinium HCl and 10 mM DTT. The mixture was sonicated twice (30 s maximum output) and debris was pelleted. The supernatant was dialysed overnight against 5 l of buffer SB (20 mM HEPES–KOH pH 7.4, 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 5 mM imidazole, 5 mM β-mercaptoethanol) plus 3 M urea. Dialysate was cleared by spinning as above, and loaded on 5 ml NINTA–agarose, pre-equilibrated with equivalent buffer (0.2 ml/min). The column was then subjected to three consecutive linear gradients of urea in buffer SB; 2–0.5, 0.5–0.1 and 0.1–0. The rphis-X protein was step-eluted with buffer SB, with 200 mM imidazole and dialysed against buffer D (20 mM HEPES–KOH pH 7.9, 20% glycerol, 100 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF). For the partially renatured rphis-X the protein was eluted from the column in 3 M urea and dialysed three times against 0.1 M Na acetate pH 5.2, for 8 h each; further dialysis was against buffer D. An H139P substitution which was inactive in co-transfection experiments was taken as an rphis-X mutant control (data not shown).

In vitro transcription

Each reaction vial contained 1.5 µl of 10× preinitiation (0.2 M HEPES–KOH pH 8.2, 80 mM $MgCl_2$, 1 M KO-glutamate, 3 µM ZnOAc), 1.5 µl 40% PEG, 100 ng pGEM3, 50 ng $pMLATC_2(200)$ and 50 ng $pG5ETATC_2(377)$, in a total volume of 8 µl. Proteins (8 µl per reaction) were added: 4 µg from the DEA fractions (2 mg/ml) and 4 µg from the DEB fractions (1 mg/ml) were mixed with 25 ng of recombinant TFIIB, to give the transcription activity used for the experiments shown in Figure 5. Reactions were incubated at 30°C for 20 min (1 h for the recombinant reaction); 4 µl nucleotide mix was then added per vial: 2 µl rNTP mix (2.5 mM ATP, 2.5 mM CTP, 40 µM UTP), 0.5 µl RNasIn (Promega), 0.5 µl 10× preinitiation buffer, 0.5 µl [α-^{32}P]UTP (400 Ci/mmol). The reaction was allowed to proceed for 50 min at 30°C. Reactions were stopped and processed as in Shapiro *et al.* (1988).

For the recombinant reaction in Figure 6, the Gal-stimulated reporter was 200 ng $pG5ETATC_2(85)$, and proteins providing transcription activity mixed in the following amounts; rphis-X (10 ng), rpGal4VP16 (6 ng), RNA POLII0 (50 ng), hTFIIB (5 ng), hTFIIF (RAP30/RAP74 hetero-tetramer, 10 ng) and yTBP or hTBP (10 ng). In addition, the nucleotide mix was supplemented with 2 ng creatine kinase (Sigma).

Immobilized protein interaction assay (IPIA)

Nuclear extract proteins (Figure 2; 120 µg in 0.2 ml), were incubated with 10 µl of Affi-Prep-X column (0.5 mg/ml) for 8 h on ice. After collection of unbound proteins, and washing the resin extensively (5× 5-min incubation with 1 ml), bound proteins were step-eluted in 0.1 M glycine–HCl pH 2.5, 10% ethyleneglycol, 1% Tween-20. The wash buffer contained 30 mM HEPES–KOH pH 7.4, 0.2 mM EDTA, 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 20 µM $ZnCl_2$, 300 mM KCl, 10% glycerol, 0.05% Triton X-100. Loaded and eluted samples were subjected to SDS–PAGE and immunoblotting.

GST-derivatives were expressed in bacteria and purified on glutathione–Sepharose beads, resulting in GST-X affinity resins (0.2 mg/ml, with the exception of GST alone, which contained 1.5 mg/ml). 5 µl of the indicated resin (bearing 1 µg of the corresponding protein) was incubated as above with 5 µg recombinant proteins (instead of nuclear extract). Following washes, the resin was eluted in 50 mM reduced glutathione in TE, boiled in SDS sample buffer, resolved on 12% SDS–PAGE and analysed by immunoblotting.

Immunoblotting was carried out with the following IgGs: α-X, α-TFIIB, α-TFIIF, α-Gal4DB and α-TBP produced (by us from rabbit sera), with α-ERCC3 (Drapkin *et al.*, 1994) and with commercial α-RPB1 (8WG16; Thompson *et al.*, 1989) and α-Flag (M2, IBI) monoclonal antibodies.

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References

Adya,N. and Giam,C.-Z. (1995) Distinct regions in human T-cell lymphotrophic virus type I tax mediate interactions with activator protein CREB and basal factors. *J. Virol.*, **69**, 1834–1841.

Arii,M., Takada,S. and Koike,K. (1992) Identification of three essential regions of hepatitis B virus X protein for trans-activation function. *Oncogene*, **7**, 397–403.

Balsano,C., Billet,O., Bennoun,M., Cavard,C., Zider,A., Grimber,G., Natoli,G., Briand,P. and Levrero,M. (1994) Hepatitis B virus X gene product acts as a transactivator *in vivo*. *J. Hepatol.*, **21**, 103–109.

Benn,J. and Schneider,R.J. (1994) Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. *Proc. Natl Acad. Sci. USA*, **91**, 10350–10354.

Boyer,T.G. and Berk,A.J. (1993) Functional interaction of adenovirus E1A with holo-TFIID. *Genes Dev.*, **7**, 1810–1823.

Brou,C., Chaudhary,S., Davidson,I., Lutz,Y., Wu,J., Egly,J.M., Tora,L. and Chambon,P. (1993) Distinct TFIID complexes mediate the effect of different transcriptional activators. *EMBO J.*, **12**, 489–499.

Caron,C., Rousset,R., Beraud,C., Moncollin,V., Egly,J.M. and Jalinot,P. (1993) Functional and biochemical interaction of the HTLV-I Tax1 transactivator with TBP. *EMBO J.*, **12**, 4269–4278.

Chasman,D.I., Leatherwood,J., Carey,M., Ptashne,M. and Kornberg,R.D. (1989) Activation of yeast polymerase II transcription by herpesvirus VP16 and GAL4 derivatives *in vitro*. *Mol. Cell. Biol.*, **9**, 4746–4749.

Chen,H.S. *et al.* (1993) The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. *J. Virol.*, **67**, 1218–1226.

Chen,J.L., Attardi,L.D., Verrijzer,C.P., Yokomori,K. and Tjian,R. (1994) Assembly of recombinant TFIID reveals differential coactivator requirements for distinct transcriptional activators. *Cell*, **79**, 93–105.

Cheong,J., Yi,M., Lin,Y. and Murakami,S. (1995) Human RPB5, a subunit shared by eukaryotic nuclear RNA polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. *EMBO J.*, **14**, 143–150.

Choy,B. and Green,M.R. (1993) Eukaryotic activators function during multiple steps of preinitiation complex assembly. *Nature*, **366**, 531–536.

Cross,J.C., Wen,P. and Rutter,W.J. (1993) Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen-activated cellular serine/threonine kinases. *Proc. Natl Acad. Sci. USA*, **90**, 8078–8082.

Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.

Drapkin,R., Reardon,J.T., Ansari,A., Huang,J.C., Zawel,L., Ahn,K., Sancar,A. and Reinberg,D. (1994) Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature*, **368**, 769–772.

Faktor,O. and Shaul,Y. (1990) The identification of hepatitis B virus X gene responsive elements reveals functional similarity of X and HTLV-I tax. *Oncogene*, **5**, 867–872.

Feitelson,M.A., Zhu,M., Duan,L.X. and London,W.T. (1993) Hepatitis Bx antigen and p53 are associated *in vitro* and in liver tissues from patients with primary hepatocellular carcinoma. *Oncogene*, **8**, 1109–1117.

Flanagan,P.M., Kelleher,R.III., Sayre,M.H., Tschochner,H. and Kornberg,R.D. (1991) A mediator required for activation of RNA polymerase II transcription *in vitro*. *Nature*, **350**, 436–438.

Ganem,D. and Varmus,H.E. (1987) The molecular biology of the hepatitis B viruses. *Annu. Rev. Biochem.*, **56**, 651–693.

Gerster,T. and Roeder,R.G. (1988) A herpesvirus trans-activating protein interacts with transcription factor OTF-1 and other cellular proteins. *Proc. Natl Acad. Sci. USA*, **85**, 6347–6351.

Gill,G., Pascal,E., Tseng,Z.H. and Tjian,R. (1994) A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. *Proc. Natl Acad. Sci. USA*, **91**, 192–196.

Goodrich,J.A., Hoey,T., Thut,C.J., Admon,A. and Tjian,R. (1993) *Drosophila* TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell*, **75**, 519–530.

Ha,I., Lane,W.S. and Reinberg,D. (1991) Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature*, **352**, 689–695.

Haviv,I., Vaizel,D. and Shaul,Y. (1995) The X protein of hepatitis B virus coactivates potent activation domains. *Mol. Cell. Biol.*, **15**, 1079–1085.

Horikoshi,N., Maguire,K., Kralli,A., Maldonado,E., Reinberg,D. and Weinmann,R. (1991) Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. *Proc. Natl Acad. Sci. USA*, **88**, 5124–5128.

Ingles,C.J., Shales,M., Cress,W.D., Triezenberg,S.J. and Greenblatt,J. (1991) Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature*, **351**, 588–590.

Kekule,A.S., Lauer,U., Weiss,L., Luber,B. and Hofschneider,P.H. (1993) Hepatitis B virus transactivator HBx uses a tumour promoter signalling pathway. (Comments). *Nature*, **361**, 742–745.

Kim,C.M., Koike,K., Saito,I., Miyamura,T. and Jay,G. (1991) HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature*, **351**, 317–320.

Kim,Y.J., Bjorklund,S., Li,Y., Sayre,M.H. and Kornberg,R.D. (1994) A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell*, **77**, 599–608.

Laux,G., Adam,B., Strobl,L.J. and Moreau,G.F. (1994) The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein RBP-J kappa interact with an Epstein–Barr virus nuclear antigen 2 responsive cis-element. *EMBO J.*, **13**, 5624–5632.

Lee,W.S., Kao,C.C., Bryant,G.O., Liu,X. and Berk,A.J. (1991) Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. *Cell*, **67**, 365–376.

Lin,Y.S., Ha,I., Maldonado,E., Reinberg,D. and Green,M.R. (1991) Binding of general transcription factor TFIIB to an acidic activating region. *Nature*, **353**, 569–571.

Liu,F. and Green,M.R. (1990) A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1a protein. *Cell*, **61**, 1217–1224.

Liu,F. and Green,M.R. (1994) Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. *Nature*, **368**, 520–525.

Maguire,H.F., Hoeffler,J.P. and Siddiqui,A. (1991) HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein–protein interactions. *Science*, **252**, 842–844.

Maldonado,E., Ha,I., Cortes,P., Weis,L. and Reinberg,D. (1990) Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex. *Mol. Cell. Biol.*, **10**, 6335–6347.

Martin,K.J., Lillie,J.W. and Green,M.R. (1990) Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. *Nature*, **346**, 147–152.

Matthews,M.A., Markowitz,R.B. and Dynan,W.S. (1992) *In vitro* activation of transcription by the human T-cell leukemia virus type I Tax protein. *Mol. Cell. Biol.*, **12**, 1986–1996.

Meisterernst,M. and Roeder,R.G. (1991) Family of proteins that interact with TFIID and regulate promoter activity. *Cell*, **67**, 557–567.

Meisterernst,M., Roy,A.L., Lieu,H.M. and Roeder,R.G. (1991) Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. *Cell*, **66**, 981–993.

Natoli,G., Avantaggiati,M.L., Chirillo,P., Costanzo,A., Artini,M., Balsano,C. and Levrero,M. (1994) Induction of the DNA-binding activity of c-jun/c-fos heterodimers by the hepatitis B virus transactivator pX. *Mol. Cell. Biol.*, **14**, 989–998.

Peterson,M.G., Tanese,N., Pugh,B.F. and Tjian,R. (1990) Functional domains and upstream activation properties of cloned human TATA binding protein. *Science*, **248**, 1625–1630. (Published erratum appears in *Science*, **249**, 844.)

Pugh,B.F. and Tjian,R. (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell*, **61**, 1187–1197.

Qadri,I., Maguire,H.F. and Siddiqui,A. (1995) Hepatitis B virus transactivator protein X interacts with the TATA-binding protein. *Proc. Natl Acad. Sci. USA*, **92**, 1003–1007.

Roberts,S.G., Ha,I., Maldonado,E., Reinberg,D. and Green,M.R. (1993) Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature*, **363**, 741–744.

Runkel,L., Fischer,M. and Schaller,H. (1993) Two-codon insertion mutations of the HBx define two separate regions necessary for its trans-activation function. *Virology*, **197**, 529–536.

Schluter,V., Meyer,M., Hofschneider,P.H., Koshy,R. and Caselmann,W.H. (1994) Integrated hepatitis B virus X and 3′ truncated preS/S sequences derived from human hepatomas encode functionally active transactivators. *Oncogene*, **9**, 3335–3344.

Seifer,M. and Gerlich,W.H. (1992) Increased growth of permanent mouse fibroblasts in soft agar after transfection with hepatitis B virus DNA. *Arch. Virol.*, **126**, 119–128.

Seifer,M., Hohne,M., Schaefer,S. and Gerlich,W.H. (1991) *In vitro* tumorigenicity of hepatitis B virus DNA and HBx protein. *J. Hepatol.*, **13**, 5.

Shapiro,D.J., Sharp,P.A., Wahli,W.W. and Keller,M.J. (1988) A high-efficiency HeLa cell nuclear transcription extract. *DNA*, **7**, 47–55.

Shaul,Y. (1991) Regulation of HBV transcription. In Maclachlan,A. (ed.), *Molecular Biology of Hepatitis B Virus*. CRC Press, Inc., Boca Raton, FL, pp. 193–213.

Shirakata,Y., Kawada,M., Fujiki,Y., Sano,H., Oda,M., Yaginuma,K., Kobayashi,M. and Koike,K. (1989) The X gene of hepatitis B virus induced growth stimulation and tumorigenic transformation of mouse NIH3T3 cells. *Jpn. J. Cancer Res.*, **80**, 617–621.

Swaffield,J.C., Melcher,K. and Johnston,S.A. (1995) A highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein. *Nature*, **374**, 88–91.

Takada,S. and Koike,K. (1994) Three sites of the hepatitis B virus X protein cooperatively interact with cellular proteins. *Virology*, **205**, 503–510.

Tanese,N., Pugh,B.F. and Tjian,R. (1991) Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.*, **5**, 2212–2224.

Thompson,N.E., Steinberg,T.H., Aronson,D.B. and Burgess,R.R. (1989) Inhibition of *in vivo* and *in vitro* transcription by monoclonal antibodies prepared against wheat germ RNA polymerase II that react with the heptapeptide repeat of eukaryotic RNA polymerase II. *J. Biol. Chem.*, **264**, 11511–11520.

Truant,R., Antunovic,J., Greenblatt,J., Prives,C. and Cromlish,J.A. (1995) Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. *J. Virol.*, **69**, 1851–1859.

Unger,T. and Shaul,Y. (1990) The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. *EMBO J.*, **9**, 1889–1895.

Walker,S., Greaves,R. and O'Hare,P. (1993) Transcriptional activation by the acidic domain of Vmw65 requires the integrity of the domain and involves additional determinants distinct from those necessary for TFIIB binding. *Mol. Cell. Biol.*, **13**, 5233–5244.

Wang,B.Q., Lei,L. and Burton,Z.F. (1995) Importance of codon usage preference for production of human RAP74 and reconstitution of the RAP30/RAP74 complex. *Protein Exp. Purif.*, **5**, 476–485.

Wang,W., Gralla,J.D. and Carey,M. (1992) The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. *Genes Dev.*, **6**, 1716–1727.

White,J.H., Brou,C., Wu,J., Burton,N., Egly,J.M. and Chambon,P. (1991) Evidence for a factor required for transcriptional stimulation by the chimeric acidic activator GAL-VP16 in HeLa cell extracts. *Proc. Natl Acad. Sci. USA*, **88**, 7674–7678.

Xiao,H. *et al.* (1994) Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol. Cell. Biol.*, **14**, 7013–7024.

Zoulim,F., Saputelli,J. and Seeger,C. (1994) Woodchuck hepatitis virus X protein is required for viral infection *in vivo*. *J. Virol.*, **68**, 2026–2030.

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