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# The X Protein of Hepatitis B Virus Coactivates Potent Activation Domains

IZHAK HAVIV, DALIT VAIZEL, AND YOSEF SHAUL*

*Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, Israel*

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**Transactivation by hepatitis B virus X protein (pX) is promiscuous, but it requires cellular activators. To study the mode of action of pX, we coexpressed pX with Gal4-derived activators in a cotransfection system. Twelve different activators bearing different types of activation domains were compared for their response to pX. Because pX indirectly increases the amount of the activators, tools were developed to compare samples with equivalent amount of activators. We demonstrate that pX preferentially coactivates potent activators, especially those with acidic activation domains. Weak activators with nonacidic activation domains are not potentiated by pX. Interestingly, Gal4E1a, which is not rich in acidic residues but interacts with similar molecular targets, also responds to pX. The response to pX correlated with the strength of the activation domain. Collectively, these data imply that pX is a coactivator, which offers a molecular basis for the pleiotropic effects of pX on transcription.**

Hepatitis B virus (HBV) is a small DNA virus that replicates like a retrovirus by reverse transcription (for a review, see reference 27). Despite its small size, the HBV genome contains at least three distinct promoters, all of which seem to be regulated by the viral enhancer (73). Four different species of viral mRNA were identified. The smallest one, of 0.9 kb, codes for a 154-amino-acid polypeptide called pX. The sequence of pX is conserved among members of the *Hepadnaviridae* that infect mammals and has been shown to be expressed during viral infection (38, 57, 75) and to be essential for the viral life cycle (14, 90). The exact role of pX, however, remained obscure. A possible clue to pX function came from the finding that this protein has a capacity to stimulate transcription of a variety of viral enhancer-promoter units, as well as cellular genes. These include enhancers of the following: HBV (7, 24, 76), human immunodeficiency virus (75, 81), Rous sarcoma virus (76), simian virus 40 (SV40) (47, 71, 76), c-*myc*, c-*fos* (4, 5), c-*jun* (82), major histocompatibility complex class I, intercellular cell adhesion molecule, interleukin-8 (34, 35, 47, 51, 88), and even RNA polymerase III promoters (3). Furthermore, functional analysis revealed that the intact viral enhancers can be substituted with a single DNA element recognized by an activator such as NF-κB (24, 39, 51, 74, 81), AP-1 (4, 5, 18, 24, 48, 59, 60, 71), C/EBP (24, 51), activating transcription factor/cyclic AMP-responsive element-binding protein (ATF/CREB) (50), serum-responsive factor (SRF) (4, 5), and AP-2 (47, 71). It was demonstrated that the E element binds any of a number of bzip-type activators and that this binding is a prerequisite for pX to stimulate transcription (23, 84). It was found that the X protein neither binds DNA directly (29, 67, 70) nor activates basal promoter activity (18) but, rather, that its function fully depends on the cellular activators (84). This general ability of pX to stimulate transcription introduced much confusion regarding the underlying mechanism. It was reported that pX-mediated transcription stimulation is transduced through protein kinase C (18, 39), but others suggest that it is independent of protein kinase C (5, 39, 59, 61). A source of confusion could be that both the AP-1 components, c-*fos* and c-*jun* genes, are better expressed in the presence of pX (4, 5, 82). It seems that in the case of the NF-κB factor, pX does not work through protein kinase C (49, 55). Also, it is difficult to reconcile the signal transduction hypothesis with the observation that pX acts in in vitro transcription, using nuclear extracts (21, 43). A second working model involves protein-protein interaction of pX with the activators. Attempts to demonstrate direct stable binding of pX to DNA-bound AP-1 have mostly failed (5, 18), although in particular cells, pX may change the properties of the activators (61). pX does alter the DNA-binding specificity of cyclic AMP-responsive element-binding protein and activating transcription factor 2 by protein-protein interactions (50). Recently, pX was also reported to bind the p53 transcription factor and change its transcriptional properties (85). However, this binding did not elicit stimulation of transcription. This second model cannot account for the documented promiscuity of pX, and it is difficult to envisage direct binding to all the activators affected by pX. In the work described in this report, we used the Gal4-derived reporter, which in mammalian cells responds only to artificial activators. Twelve different Gal4 derivatives bearing different activation domains were assessed for response to pX by both controlling and monitoring the amount of the transcription activators during the pX effect. We revealed that pX activates transcription through these activators in a manner dependent on the potency and classification of the activation domains. The best response to pX is manifested by the activation domains rich in acidic residues (acidic activation domains). We propose that pX is a general viral transactivator which exerts its effect through the coactivation process.

## MATERIALS AND METHODS

**Cell culture.** Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (GIBCO Laboratories) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), supplemented with 8% fetal bovine serum. Cells were seeded the day prior to transfection. At the time of transfection, the cells were 40 to 60% confluent. The F9 cells were grown according to published procedures (87).

**Plasmids.** The pX expressor plasmid pECE-flagX was constructed as follows. A synthetic oligonucleotide, with a sequence of 5′ TGCAGCCACCATGGC 3′, was self-annealed and inserted at the *Pst*I site of Bluescript plasmid. The *Nco*I-*Eco*RI fragment from the HBV genome was inserted into this Bluescript plas-

* Corresponding author. Phone: 972-8-342320. Fax: 972-8-344108.

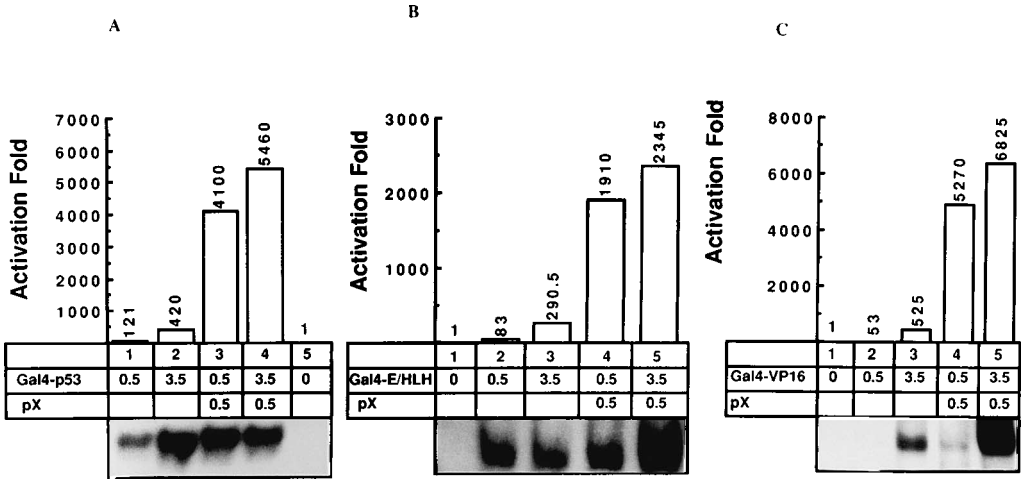


FIG. 1. pX coactivates through acidic activation domains. Cells were transfected with 1 μg of the G5Luc reporter plasmid with increasing amounts of the expressor plasmids pECEGal4p53 (A), pECEGal4E/HLH (B), and pECEGal4VP16 (C), each with or without $SV_2X$ (pX). Cytoplasmic extracts of the cells were assayed for luciferase activity. The fold activation obtained was calculated as luciferase activity of activator versus reporter-alone transfected cells. Each point is the average of six independent assays. The nuclear extracts were assayed by EMSA with the $UAS^G$ probe, with a typical EMSA shown.

mid, generating Bluescript-X. A synthetic oligonucleotide, with a sequence of 5′ CATGCCAGATCTGG 3′, was self-annealed and inserted at the *Nco*I site of Bluescript-X plasmid. The resulting plasmid was cut with *Bgl*II, to generate a 600-bp fragment containing the X open reading frame. This fragment was inserted into the pECE-flag plasmid. The expressor plasmids of all Gal4 derivatives, Gal4p53, Gal4Jun, Gal4fos, yflGal4, Gal4CTD, Gal4RFX, Gal4VP16, Gal4E1a, Gal4E2A, and Gal4Sp1Q, are based on the pECE expression vector, and their corresponding structures have been published elsewhere (2, 28, 45, 68, 83). The reporter was switched to G5luciferase (G5Luc) (22). G5Luc offers a few advantages over regular model systems of transcriptional activation. The enzyme assay is linear over a range of $10^6$. Also, background activity is very low and promoter dependent. The expressor plasmid of VP16 was constructed by reclonning the *Eco*RI-*Bgl*II fragment of Gal4VP16 plasmid in an SV2 vector downstream of a heterologous ATG and 71 irrelevant amino acids.

**Transfection luciferase, β-galactosidase, and EMSA analyses.** For luciferase assays, 6-cm plates were transfected with 1 μg of reporter G5Luc plasmid, 0.2 μg of SV2–β-Gal control plasmid, and various amounts of the expressor plasmids, together with a pECE carrier plasmid to keep the total amount of transfected DNA constant (5 μg). Calcium phosphate precipitation, cotransfection, and β-galactosidase assay were done by published procedures (69). Nuclear and cytoplasmic extracts were prepared with hypotonic buffer (modified from reference 72). The hypotonic buffer contained 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5), 0.75 mM spermidine, 0.15 mM spermine, 1 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid (CDTA), 0.5 mM EDTA, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM KCl, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, and 1 μg of leupeptin per ml. After a 10-min incubation on ice with 400 μl of hypotonic buffer, 40 μl of 75% sucrose–5% Triton X-100 was added, and nuclei were centrifuged briefly. Nuclei were extracted in 75 μl of high-salt buffer (20 mM HEPES-KOH [pH 7.9], 20% glycerol, 0.4 M KCl, 0.1 mM EDTA, 0.1 mM EGTA, 20 μM zinc acetate, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin A per ml, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml). The cytoplasmic fraction was assayed for luciferase and β-galactosidase, and nuclear fractions were assayed by $UAS^G$-electrophoretic mobility shift assay (EMSA). The luciferase assay involved a substrate buffer (Promega) and was read in Turner TD-20e luminometer. EMSA was also done by published procedures (13). Nuclear extracts (10 μg) were incubated with $10^4$ cpm of $UAS^G$ probe (5′ TTAAGGGAGGACAGTACTCCGCC 3′) labeled by a fill-in reaction. Alternatively, the nuclear extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted by standard procedures with α-Gal4 rabbit immunoglobulin G (protein G purified) produced in our laboratory.

## RESULTS

**pX coactivates cellular acidic-activation domains.** We investigated the effect of pX on the activation domains of cellular activators, in an isolated system. We cotransfected pX with Gal4-derived activators, which contain different functional activation domains. The reporter in these experiments is G5Luc, in which luciferase gene transcription is driven by a minimal E1b promoter and five copies of a 17-mer yeast enhancer-like DNA element from the GAL1/10 promoter ($UAS^G$). This reporter is inactive in mammalian cells even when cotransfected with $pSV_2X$ (data not shown). The DNA-binding domain of Gal4 (N-terminal amino acids 1 to 147; Gal4DB), when fused to a functional activation domain, can efficiently stimulate its expression. The expressor plasmid of Gal4DB did not activate the reporter and did not respond to cotransfected $pSV_2X$ (see Fig. 3B, bars 7 and 8). Functional activation domains are classified, according to their primary sequence, into at least three different classes (56). The p53 protein is a transcription activator with a strong acidic activation domain in its amino-terminal portion; therefore, we tested the effect of pX on activation by Gal4p53, which contains the first 65 amino acids of p53 (83).

To correct for the bias in Gal4-mediated activation that arises from differences in protein production, we concomitantly determined the steady-state amount of the chimeric Gal4 proteins along with the luciferase activity. Quantitative analysis of chimeric Gal4 proteins was carried out by EMSA with a $UAS^G$-specific probe. The EMSA with $UAS^G$ increases with the amount of DNA used for transfection (Fig. 1A, lanes 1 and 2). The addition of pX resulted in an increase in the level of the protein-DNA complex (compare lanes 1 and 3). This was expected, because pX highly activates the SV40 early promoter-enhancer expression vector, driving the expression of the Gal4 proteins (16, 71). EMSA shows that a similar amount of Gal4p53 was produced by 3.5 μg (lane 2) and 0.5 μg (lane 3) of the expressor in the absence and presence of pX, respectively. However, a remarkable ~10-fold-higher G5Luc activation is observed in the latter case. This finding strongly suggests that pX specifically coactivates the activation domain of p53. An additional acidic activation domain was taken from the E-box-binding protein E2A of the helix-loop-helix family (Gal4E/HLH) (2). This activator was somewhat weaker than p53 and exhibited a weaker response to pX (Fig. 1B, compare bars 5 and 3). We further found that the full-length yeast Gal4 protein, which bears two extremely potent acidic activation domains, behaves similarly (see Fig. 7). Control experiments were performed to show that the protein-DNA complex observed is sequence specific and is efficiently competed for by

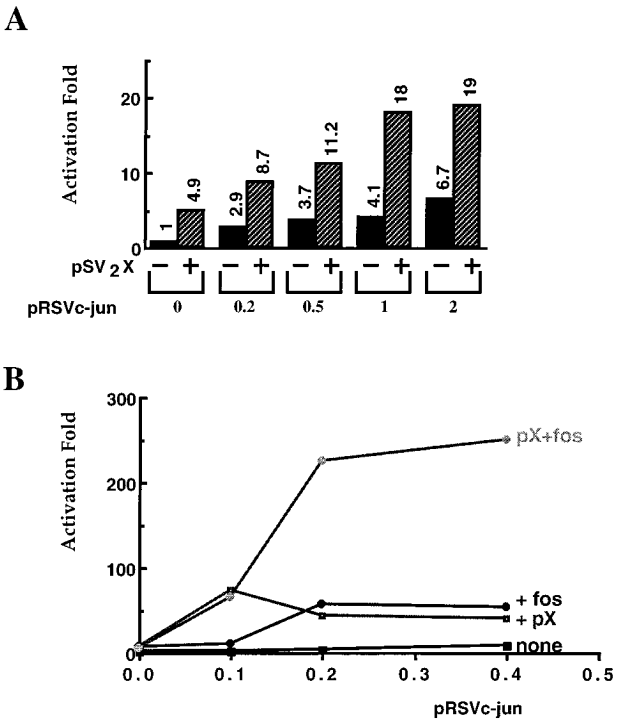


FIG. 2. pX requires the Fos and Jun proteins to activate transcription through the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE). Two different cell lines, HeLa (A) and F9 (B), were transfected with 1 μg of the Col-Luc reporter plasmid either alone or together with the expressor plasmids $SV_2X$ (pX), RSVc-jun, and RSVc-fos, as indicated. The amounts of plasmid RSVc-Jun used are shown in micrograms per dish. In panel B, 0.5 μg of plasmids RSVc-fos and $SV_2X$ was used per plate.

homologous cold DNA but not heterologous DNA. Also, our assay detects Gal4-specific proteins, as judged by the supershift induced by Gal4-specific rabbit antisera but not by control α-Abl sera (see Fig. 3C and D).

The data so far clearly demonstrate that distinct acidic activation domains are all potentiated by pX. Most of the mechanistic information on transcription activation was gained from the extensive study on the universal acidic activation domain of the herpes simplex virus VP16 gene (68). We therefore tested and found that pX potentiated Gal4VP16 up to ~13-fold (Fig. 1C, compare bars 2 and 3 with bars 4 and 5). Again, we observed a two- to fourfold increase in the amounts of Gal4VP16 in the presence of pX (compare lanes 3 and 5). However, the cotransfection of Gal4VP16 with pX improves activation, even when less Gal4VP16 protein is produced (compare bars 3 and 4). Therefore, pX is a potent coactivator of some acidic activation domains of cellular and viral origins.

**pX coactivates AP-1.** Next, we studied the effects of pX on AP-1, an authentic cellular activator. For this purpose we chose a reporter plasmid that contains the AP-1-responsive collagenase promoter. Cotransfection of pX with this reporter into HeLa cells resulted in up to fivefold activation of transcription (Fig. 2A). Cotransfection of an expressor plasmid of c-*jun* (a component of the AP-1 complex) in HeLa cells stimulated the reporter up to sevenfold in a dose-dependent manner. When $pSV_2X$ was cotransfected, the activation by c-*jun* was about three times more efficient. Because pX alone displayed a similar effect, it is possible that in the absence of ectopic c-*jun*, pX cooperated with the endogenous proteins. To eliminate this possibility, we used F9 cells, which lack endogenous AP-1 protein complex (87). We cotransfected increasing amounts of pRSVc-jun together with either pRSVc-fos or $pSV_2X$ or both. In these cells, pRSVc-jun alone activated the reporter very poorly (Fig. 2B) and pX did not itself affect the reporter (see point 0). Cotransfection of pRSVc-fos with pRSVc-jun resulted in a substantial (~50-fold) activation. pX increased the activation approximately a further fivefold.

**pX coactivates through the activation domain of c-*fos* but not c-*jun*.** To gain more information on the mechanism of action of pX on c-*fos* and c-*jun*, we used Gal4-derived activators. Either the amino-terminal portion of jun (Gal4jun) (6) or the portion immediately C-terminal to the bzip domain from c-*fos* (Gal4fos) (1) served as an activation domain; both lacked the bzip portions. The Gal4fos protein is a stronger activator than the Gal4jun protein. Interestingly, pX potentiated the activation by this small portion of *fos* in a Gal4-chimera context (~12-fold) (Fig. 3A), whereas the Gal4jun protein did not respond to pX (Fig. 3B).

Remarkably, 0.5 μg of transfected Gal4fos plasmid, together with pX, elicits 10-fold-higher activation than does 3.5 μg of activator alone (Fig. 3A, bars 4 and 5), even though the latter produced larger amounts of the Gal4fos protein, as detected by immunoblotting (Fig. 3E, lanes 2 and 3), and $UAS^G$-binding activity (Fig. 3C, lanes 2 and 3). These data suggest that pX functions as a transcriptional coactivator of the *fos* activation domain. EMSA shows that Gal4jun protein, expressed from the same SV40-based vector, was also induced by pX (Fig. 3D). Thus, although pX was functional in these cells and activated the SV40 regulatory unit, it failed to activate the Gal4jun protein (Fig. 3B). The inability of pX to coactivate the Gal4DB and Gal4jun proteins suggests a dependence of pX on the activation domain of c-*fos*.

To further investigate the coactivation of the c-*fos* activation domain by pX, we dissected the activation domain into two fragments, one designated BN and containing the N-terminal portion (residues 206 to 317), and the other designated NS (residues 317 to 380). We found that both are very weak activators and that the obtained activation is lower than that of the c-*jun* activation domain (Fig. 4). Remarkably, these subfragments of the c-*fos* activation domain are no longer pX responsive, suggesting that coactivation by pX requires an intact and potent c-*fos* activation domain. EMSA confirmed that Gal4 fusion proteins were produced and that in the cotransfected cells pX was functional in coactivating the SV40 regulatory units (Fig. 4, compare lanes 1 to 3 with lanes 4 to 6). Thus, the emerging picture suggests that pX acts only on potent activation domains.

**Cellular weak activation domains do not respond to pX.** We further extended the tested activation domains to the glutamine-rich domain of Sp1 (Gal4Sp1Q) (28). Activation was weaker than that observed with the acidic activators, although Gal4Sp1Q accumulated to high levels in transfected cells (Fig. 5A, lanes 1 to 3). Interestingly, pX did not stimulate activation by Gal4Sp1Q (compare bars 3 and 5). However, pX was active in the transfected cells, as evident from a comparison of the amounts of Gal4Sp1Q (compare lanes 2 and 5). This strongly suggests that Sp1Q is not pX responsive.

An additional non-acidic activation domain of the E-box-binding E2A protein (Gal4AD2) (2) was tested. This activation domain is unresponsive to the effect of pX on the activation level (Fig. 5B, compare bars 3 and 5), although the protein level was increased by pX (compare lanes 5 and 3). We further found that an activator, which bears the C-terminal domain (CTD) of the largest subunit of RNA polymerase II, which is an activation domain, is also not affected by pX (see Fig. 7).

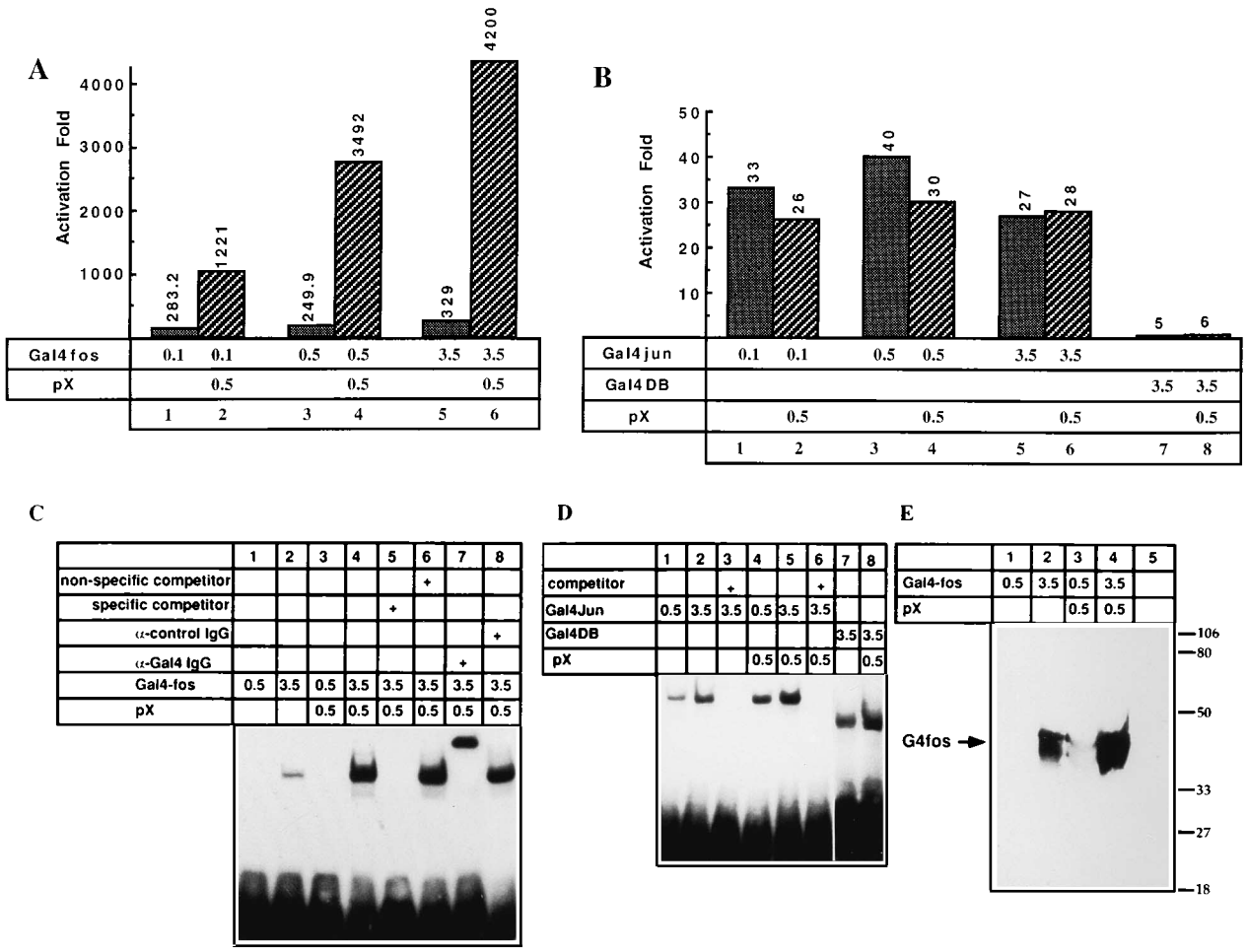


FIG. 3. The *fos* activation domain can mediate pX response when fused to the heterologous DNA-binding domain. G5Luc reporter plasmid (1 μg) was cotransfected with increasing amounts of expressor plasmid encoding Gal4-derived activator, either pECEGal4fos (A) or pECEGal4Jun (B), each with or without $SV_2X$ (pX). Cytoplasmic extracts of the cells were assayed for luciferase activity. Refer to the legend to Fig. 1 for analysis. Each bar is the average of four independent assays. Nuclear extracts from the same plates were used for EMSA with the $UAS^G$ probe (C and D) or for immunoblotting with α-Gal4 immunoglobulin G (E).

**pX works on a target of potent activation domains.** A characteristic feature of potent activation domains is their ability to directly bind the general initiation factors TFIID and TFIIB (36, 66, 77). The pX-mediated coactivation can arise from direct interaction either with the acidic sequence of the activation domain or with their targets. To distinguish between these possibilities, we used the adenovirus E1a activation domain (Gal4E1a). This activation domain is not acidic in sequence. On the basis of competition assays, it was suggested that it interacts with similar targets (9, 42, 52). Here, too, pX induced the activity of Gal4E1a ~10-fold (Fig. 6, compare bars 3 and 5) without causing a significant change in the Gal4E1a protein level. We also determined, using RNase protection analysis, that pX coactivates Gal4E1a on the transcriptional level (data not shown). This suggests that pX coactivates the activators not by directly interacting with the acidic activation domains but by modifying the interaction with their "targets."

## DISCUSSION

This study addresses mechanistic aspects of transcription regulation by pX of HBV. Our findings strongly suggest that the effect of pX on transcription involves a coactivation process. pX cannot activate a DNA element, AP-1 or $UAS^G$, in the absence of the corresponding activator. Furthermore, pX is unable to stimulate transcription with a DNA-binding protein, Gal4DB, that lacks an activation domain. Thus, coactivation by pX is absolutely dependent on the activation domain of the factor, and the mere presence of DNA binding is not enough. However, as exemplified by the c-*fos* deletion analysis, pX requires potent activation domains to coactivate transcription. These observations with the AP-1 and $UAS^G$ elements agree with our previous observation with the C/EBP activator (84). The potentiation of transcription by pX can result from two distinct effects of pX: an increase in the amount of activator protein, and potentiation of the activation. To separate these two effects, it is crucial to compare the amounts of activator in the transfected cells. By comparing cells with similar amounts of the activator protein, a net potentiation by pX is observed.

One of the documented features of pX is its ability to activate a variety of enhancer-promoter units. We observed a similar promiscuity on the activator level with the Gal4 experimental system. Five different activators, each bearing an acidic activation domain, are all responsive to pX (Fig. 7). Evidently, the stronger the activator, the stronger is the effect of pX. Similar results were obtained with many cell lines, including HeLa, F9, SK-Hep1, HepG2, COS7, and 293 (data not shown),

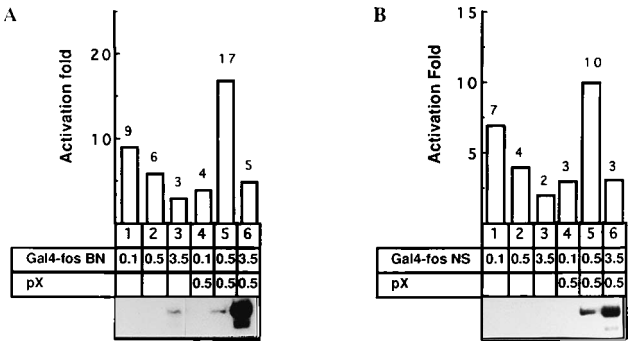


FIG. 4. Coactivation of the *fos* activation domain by pX requires a full-length activator. Cells were transfected with 1 μg of the G5Luc reporter plasmid with increasing amounts of expressor plasmids pECEGal4fos (*fos* residues 206 to 317) (A) and pECEGal4fos (*fos* residues 317 to 380) (B), each with or without $SV_2X$ (pX). Refer to the legend to Fig. 1 for analysis.

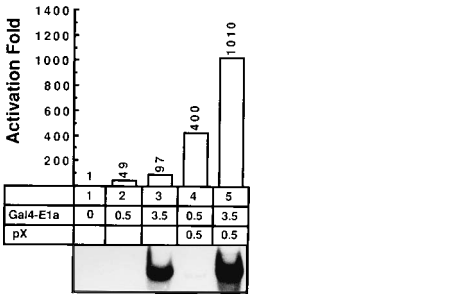


FIG. 6. pX coactivates through the nonacidic activation domain of E1a. Cells were transfected with 1 μg of the G5Luc reporter plasmid and increasing amounts of the expressor plasmid pECEGal4E1a, with or without $SV_2X$ (pX). Refer to the legend to Fig. 1 for analysis.

which suggests that the activity of pX is not cell type specific, as predicted from the role we assign to pX. The effect of pX is X open reading frame dependent: both an ATG knockout mutant and a deletion mutant with a mutation of the C-terminal portion (amino acids 110 to 145) were inactive (data not shown). The direct involvement of pX in the activation step is further supported by the correlation between pX response and activation domain classification. A set of weak nonacidic activation domains, including the c-*jun* activation domain (Fig. 3B), two subdomains of the c-*fos* activation domain (Fig. 4), the glutamine-rich domain of Sp1 and the tissue-specific activation domain of the E/HLH E2A protein (Fig. 5), the proline-rich CTD of the largest subunit of RNA polymerase II (Fig. 7), and the class II major histocompatibility complex X-box-binding protein RFX (data not shown), are all refractory to the effect of pX. A single nonacidic but potent activator, Gal4E1a, was also potentiated by pX. The ability of pX to coactivate Gal4E1a implies that the effect of pX does not depend on the acidic sequence of the acidic activation domains. Although E1a cannot be classified as an acidic domain, based on sequence, it is possible that E1a shares with the acidic activation domains some crucial residues positioned to function in a similar manner. Indeed, competition experiments (52), as well as direct protein-protein interactions (9, 33, 42), suggest a common target for these proteins.

Acidic activation domains are functional in a wide evolutionary range from yeasts to humans (63, 64), suggesting that the target of this universal domain is a general component of the transcription machinery. Direct physical interaction between VP16 and the general initiation factors TFIID (36, 77) and TFIIB (15, 66) was demonstrated. This interaction is not enough for transcription activation; hence, the need for auxiliary factors was proposed (10, 15, 25, 30, 32, 44, 52, 65, 80). These include factors that associate with the initiation factor TFIID (30, 89) or factors that govern topological and structural behavior of the DNA in chromatin (19, 21, 37, 40, 41, 53, 54). Interestingly, activation domains which do not respond to pX, such as Sp1Q, bind alternative targets (11, 28, 32, 80) and use alternative TFIID complexes (10). Furthermore, pX also stimulates polymerase III transcription (3), a process in which the TATA-binding protein and a homolog of TFIIB participate (17, 46, 86). An intriguing possibility, raised by the coactivation with pX and potent activation domains, would be that the site of action of pX is at these basic components. We are currently pursuing this possibility by characterizing direct interactions of pX with TBP, TFIIB, holo-TFIID, and additional factors. Preliminary results support this possibility.

The data presented here imply that pX is a novel kind of viral transcriptional activator with a characteristic coactivator phenotype. The term "coactivator" has been defined in various ways: as a component essential for transcriptional activation in vitro and as a protein which potentiates an activator rather than being an essential component for activation in vivo (8, 12, 26, 31, 58, 62). The effects of pX, demonstrated in this work, fit with the latter definition.

There are three documented biochemical activities of pX, which may mediate coactivation. First, hydrolysis of the β-γ

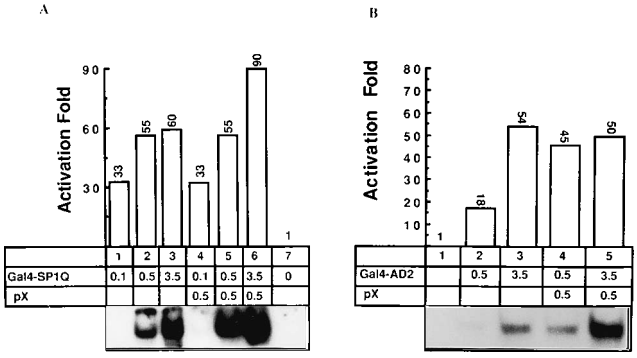


FIG. 5. pX does not coactivate nonacidic activation domains. Cells were transfected with 1 μg of the G5Luc reporter plasmid and increasing amounts of expressor plasmids pECEGal4Sp1Q (A) and pECEGal4AD2 (B), each with or without $SV_2X$ (pX). Refer to the legend to Fig. 1 for analysis.

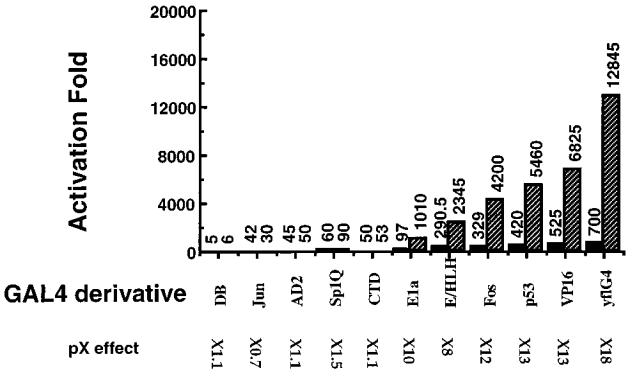


FIG. 7. Comparative summary of the Gal4-derived activators, assayed for response to pX. The fold pX-mediated coactivation is shown below. A summary of 5 to 10 different experiments for each activator is shown.

phosphoanhydride bond of ATP ($r/d^{ATPase}$). We have previously shown that this activity is efficiently carried out by highly purified recombinant glutathione *S*-transferase (GST)–X fusion protein (20). Second, an essential domain for pX transcriptional function is homologous to Kunitz-type serine protease inhibitors (79) and mediates stable interaction with the tryptase TL2 (78). Third, it was suggested that pX changes the DNA-binding specificities of certain activators (50, 61). This last effect cannot be accounted for by the observations described here, because the Gal4 DNA binding seems not to be induced by pX.

In this study, we used a categorical approach to define the requirements for pX-mediated activation. These requirements include a DNA-bound activator with a potent, mostly acidic activation domain. The molecular mechanism of coactivation by pX remains to be elucidated. We are currently developing an in vitro transcription system that responds to recombinant X protein. This system should help to distinguish among the three possibilities proposed above.

Finally, our findings provide a molecular explanation for the promiscuity of pX. pX activates many promoter/enhancer units, all of which contain elements recognized by acidic activators. In contrast, the SV40 early promoter is not responsive to pX (16, 24). This promoter is composed of Sp1 element, which bears an activation domain refractory to pX (Sp1Q). Assuredly, this model deserves further investigation.

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