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Transcriptional Activation of Homologous and Heterologous Genes by the Hepatitis B Virus X Gene Product in Cells Permissive for Viral Replication

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The potential of the hepadnavirus X gene product to activate gene expression in *trans* was tested through a series of cotransfections of X expression vectors with a variety of potential targets for transactivation. The X gene products from human hepatitis B virus (HBV), woodchuck hepatitis virus, and ground squirrel hepatitis virus are all equally active in augmenting the expression of a wide array of target promoters in both permissive and nonpermissive cells. Using the HBV genome itself as the source of X protein, we demonstrate that transactivation of HBV and heterologous genes occurs when X protein is expressed in its native state during productive infection of permissive cells. Run-on transcription analysis indicates that this transactivation occurs at the level of primary transcription.

The hepadnaviruses are small DNA viruses that produce persistent infections of liver cells that in some cases strongly predispose host organisms to the development of liver cancer (for a recent review, see reference 6). Considerable progress has recently been made in understanding the structure of the viral genome and the overall program of viral gene expression. The genomes of the mammalian hepadnaviruses (14) contain four overlapping long open reading frames (Fig. 1A). Three of these coding regions have been assigned to known viral proteins: region preS/S encodes the viral surface proteins, region C (21) encodes the structural protein of the nucleocapsid (core), and the P open reading frame encodes the viral polymerase (24). The product of the fourth open reading frame, X, is a nuclear protein (2, 19) that displays no convincing homology with known protein sequences. Although the first study of an X gene nonsense mutant reported a wild-type phenotype for viral replication in cultured cells (28), the length and conservation of the open reading frame, the appearance of anti-X protein antibodies in the course of natural infection (9, 11, 13, 15), and the failure of X frameshift mutants to grow in animal hosts (C. Seeger, Abstr. Meet. Mol. Biol. Hepatitis B Viruses. 1988) all argue strongly that the X protein plays an important role in the viral life cycle.

Models for what the role of X protein in the viral life cycle might be are constrained by the absence of the X open reading frame from duck hepatitis B virus (DHBV), a related virus that shares all the major features of replication and gene expression with its mammalian homologs. Computer analysis of the sequence conservation, hydrophobicity, and potential secondary structures of the predicted X polypeptide paint the picture of a small, soluble, intracellular protein without a signal sequence or other obvious identifying sequence motifs (Fig. 1B). Consideration of the above constraints and comparison with other viral families suggested the hypothesis that the X protein might be a transactivator of gene expression. Experiments from several groups (17, 20, 27) have shown that X protein expression can increase the expression in *trans* of reporter genes in a variety of heterologous contexts. In these experiments, plasmids bearing chloramphenicol acetyltransferase (CAT) genes expressed from any of several nonhepadnavirus promoters or bearing the hepatitis B virus (HBV) enhancer and core promoter were cotransfected with plasmids bearing the HBV X gene into several nonpermissive cell lines. Transactivation of the CAT genes in an X protein-dependent fashion was observed. These studies clearly show a transactivating effect of the X gene product and are consistent with the hypothesis that transactivation of hepadnavirus promoters may be a natural function of the X protein.

We were interested in examining the effects of the X gene product in permissive cells undergoing productive viral infection. This is important because in heterologous systems in which no viral replication is ongoing, other viral proteins that could affect the structure or function of the X gene product are absent. Also, cellular proteins important for viral gene expression that are unique to permissive cells might also influence the magnitude or spectrum of X protein activity. For these reasons we have reexamined X protein-dependent transactivation in as natural a setting as possible, employing permissive cells (22, 26, 28) and using intact hepadnavirus genomes as both the source of and target for X activity. In addition, we examined whether the X products of the animal hepadnaviruses GSHV (ground squirrel hepatitis virus) and WHV (woodchuck hepatitis virus) also display transactivation activity. Our results demonstrate that transactivation by the hepadnavirus X gene product does indeed occur during authentic viral replication and that the X products of all mammalian hepadnaviruses possess transactivating activity.

MATERIALS AND METHODS

Cell culture. All cells were maintained at 37°C and 5% CO_2. Huh7 cells were grown in DM-160 medium (23) plus 10% fetal calf serum. Cells were maintained at a density sufficient to establish a nearly confluent monolayer and split 1:3 once every four days. All other cell lines were grown in standard Dulbecco modified Eagle medium plus 10% fetal calf serum and split at an appropriate density to achieve 50% confluence at the time of transfection.

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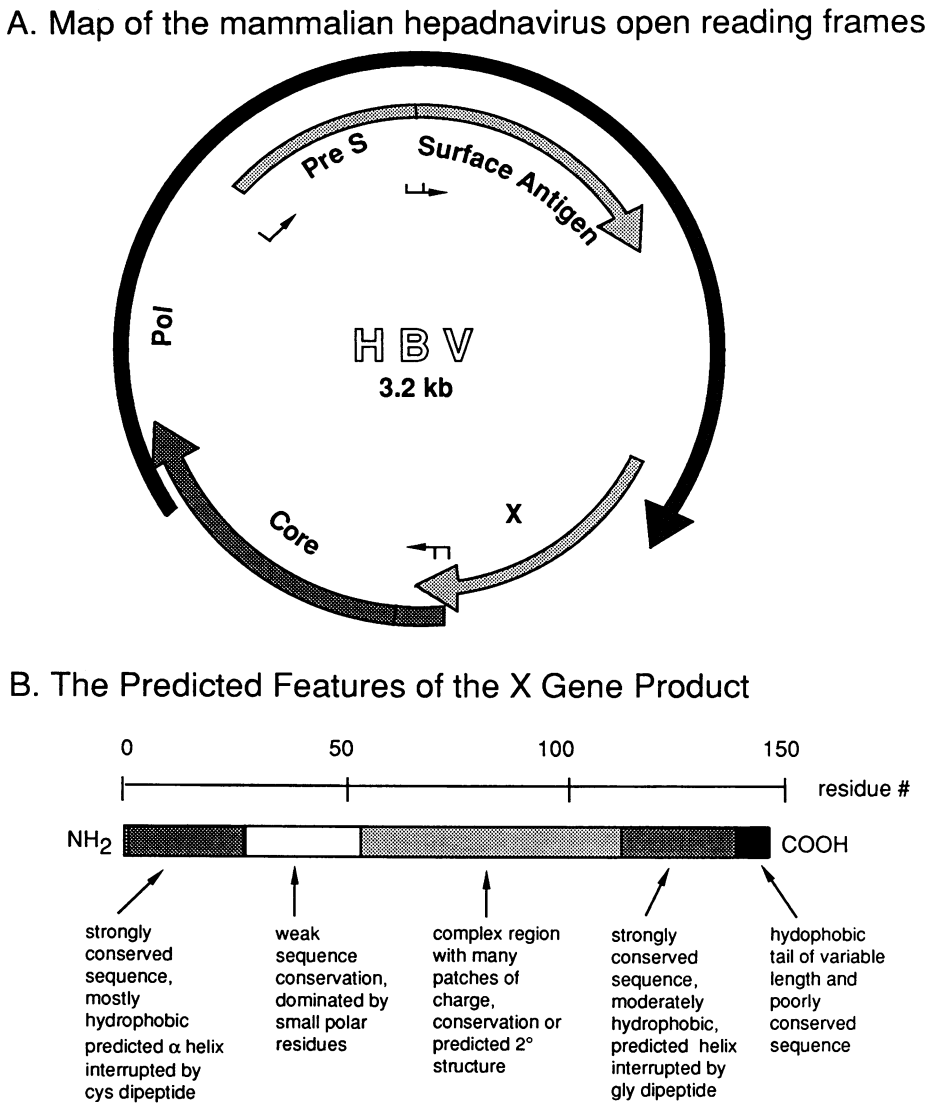

FIG. 1. (A) Map of the long open reading frames in the mammalian hepadnaviruses. Also shown are the major sites of transcription initiation (arrows). (B) Features of the X gene product predicted by sequence inspection. Secondary structures were predicted by the algorithm of Chou and Fasman (3).

Plasmid constructs. pOOCAT, pSV2CAT, and pMSVCAT were gifts from the laboratory of Keith Yamamoto (S. D. Jones, Ph.D. dissertation, University of California, San Francisco, 1986). pOOCAT contains the CAT gene extending from the *Hin*dIII site 71 nucleotides upstream of the CAT gene AUG to the *Bam*HI site 0.8 kilobases (kb) downstream of the terminator. pSV2CAT contains the simian virus 40 (SV40) early promoter and enhancer from *Acc*I to *Hin*dIII, including both 72- and 21-base-pair repeats, fused to the *Hin*dIII site 5′ of the CAT gene. pMSVCAT contains the *Xba*I-to-*Xho*II fragment from the Moloney murine sarcoma virus long terminal repeat (LTR) placed 5′ to the CAT gene. pRSVCAT and pE3CAT were provided by the laboratory of J. M. Bishop, Department of Microbiology, University of California, San Francisco. pRSVCAT contains Rous sarcoma virus (RSV) LTR sequences from *Pvu*II to *Hin*dIII upstream of the CAT gene. pE3CAT contains the 635-base-pair *Sau*3A fragment bearing the adenovirus E3 control region 5′ to the CAT gene. pHGPCAT and pHEPCAT were constructed by standard cloning techniques (Fig. 2). pHGPCAT contains the HBV sequences from the *Bam*HI site in the X gene to the unique *Fsp*I site 11 nucleotides 3′ to the cap site of precore mRNA inserted 5′ to the CAT gene AUG. pGEPCAT shares the same 3′ extent of HBV sequence with pHGPCAT but extends 5′ to the *Xho*I site in the surface antigen gene. pHBV wt is an *Eco*RI monomer of the HBV genome inserted into pBR322 at its unique *Eco*RI site. The derivatives, pHBVp- and pHBVx-p-, were constructed from pHBV wt (see Fig. 4) by either filling in the four-nucleotide overhang of the *Nco*I site with T4 polymerase or digesting it away with mung bean nuclease, respectively. The HBV X expression vector containing the HBV sequences from the *Nco*I site at the X gene AUG to the *Bgl*II site in the core gene inserted at the *Bst*EII site immediately 3′ to the RSV LTR. pGSHVx contains the GSHV sequences from *Sac*I to *Eco*RI downstream of the SV40 early promoter (*Hin*dIII to *Bam*HI in SV40). pWHVx is the WHV analog of pGSHVx, containing the *Pvu*I-to-*Bgl*II sequences from

WHV. The frameshifts in the X genes used were made by digesting away the 3′ overhang from the *Aat*II site 31 nucleotides downstream of the X gene AUG with mung bean nuclease for the HBV X gene, filling in the *Bst*EII site 141 nucleotides 3′ to the initiator with Klenow fragment for the GSHV X gene, and filling in with Klenow fragment the overhang of the *Eco*RI site 13 nucleotides from the start in the WHV X gene.

Transfection. Cos 7 cells were transfected by replacing the growth medium with serum-free medium plus 200 μg of DEAE dextran per ml and the input DNA (1 μg) for 6 h, followed by two washes with phsophate-buffered saline and subsequent replacement of regular medium. All other cells were transfected with $CaPO_4$ precipitates as follows. The cells were fed with fresh medium 12 h before transfection. The precipitate (1 ml) was created by slowly adding 0.5 ml of 250 mM $CaCl_2$ plus 20 μg of plasmid DNA per 100-mm-diameter plate to 0.5 ml of 2× BBS (50 mM BES [pH 6.95], 280 mM NaCl, 1.5 mM Na_2HPO_4) (8) with mild bubbling. Cells were returned to the incubator, and after 12 h, precipitates were removed, cells were washed twice with phosphate-buffered saline, and returned to growth medium for another 12 h before harvesting.

CAT assay. Medium was removed from the plates, and cells were washed once with phosphate-buffered saline at 4°C and then resuspended in 2 ml of the same buffer by gentle scraping with a rubber policeman, followed by pipetting through a Pasteur pipette. Cells were pelleted, suspended in 200 μl of 250 mM Tris (pH 7.8), and disrupted by sonication. Fifty milligrams of total protein (determined by using a Bio-Rad protein assay kit) from the clarified lysate was added to a standard CAT reaction mix (7). Reactions were allowed to proceed for 1 h at 37°C with [^{14}C]chloramphenicol (0.4 mCi, Amersham Corp.). Reaction mix was extracted with ethyl acetate, dried, and analyzed by silica gel thin layer chromatography in 95:5 chloroform-methanol. Spots were visualized by autoradiography and quantitated by cutting out the appropriate regions of the chromatographic plate and counting ^{14}C in a scintillation counter. Results are expressed as fold transactivation, i.e., the ratio of the percent conversion of chloramphenicol to its acetylated forms in the presence of X^+ DNA to that observed in the presence of X^- DNA. Quantitative values shown in Table 1 were obtained by carrying out the experiments in sextuplicate and averaging the results.

RNA preparation. For each sample, the medium from a confluent 100-mm-diameter plate of transfected cells was aspirated, washed with phosphate-buffered saline, and lysed with 1 ml of lysis solution (0.5 M NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 200 mg of proteinase K per ml). The DNA of the lysate was sheared by passage three times through a 19-gauge needle, followed by passage three times through a 26-gauge needle; an additional 100 mg of proteinase K per ml was added and allowed to incubate for 1 h at 37°C. Ten milligrams of oligo(dT)-cellulose (Collaborative Research, Inc.) was added, and the mix was rocked at room temperature for 1 h. The cellulose pellet was washed three times with lysis buffer minus proteinase, followed by one wash with the same buffer plus 0.1 M NaCl. Poly(A)$^+$ RNA was eluted with two washes with 10 mM Tris (pH 7.5)–1 mM EDTA and precipitated with sodium acetate-ethanol.

Northern (RNA) blotting. RNA was run on 1% agarose–2.2 M formaldehyde gels and blotted by standard methods (10). Blots were hybridized to the indicated ^{32}P-labeled DNA probes (prepared by nick translation) and washed as described by Church and Gilbert in 1984 (4). Hybridizing species were visualized by autoradiography at −70°C with an intensifying screen.

In vitro transcriptions. In vitro transcription reactions were performed by using purified SP6 polymerase (Promega Biotec) as described in the Promega catalog. Templates for in vitro transcription were derived from pSP65 (Promega) and contained the HBV 1.9-kb *Eco*RI-*Bgl*II fragment cloned into the polylinker region in either the sense or antisense orientation. Control template contained an unrelated insert from the genome polyprotein of tobacco etch virus. After transcription, template DNA was degraded with DNase I digestion (Promega).

Run-on transcription assay. For each reaction, two 150-mm-diameter plates of Huh7 cells were used. At 24 h after transfection, the plates of cells were placed on ice, medium was removed, and cells were washed twice with ice-cold buffer A (10 mM Tris [pH 8.0], 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 M sucrose). Cells were permeabilized by incubation in 4 ml per plate of buffer B (buffer A plus 200 mg of digitonin [Sigma Chemical Co.]) for 2 min on ice. Buffer B was removed, and cells were washed once with buffer A scraped off the plate with a rubber policeman, dispersed by pipetting, and transferred to prechilled 15-ml conical tubes. Cell suspensions were centrifuged for 5 min at 2,000 rpm in a small refrigerated centrifuge at 4°C, and supernatant was removed. Cell pellets were suspended in 280 μl of reaction mix (160 ml of buffer C, 80 ml of buffer D, 1 ml each of 100 mM rATP, rCTP, and rGTP). (Buffer C consists of 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mg of bovine serum albumin per ml, and 25% glycerol [vol/vol]. Buffer D consists of 100 mM HEPES, 100 mM $MgCl_2$, 6.6 mM dithiothreitol, 2 mg of bovine serum albumin per ml, and 500 mM ammonium chloride.) To each reaction, [α-^{32}P]UTP (250 mCi; 300 Ci/mmol; Amersham) was added and reactions were incubated for 20 min at 30°C. To each reaction was then added 1.5 ml of GHCL solution (7.5 M guanidine hydrochloride, 25 mM sodium acetate [pH 7.0]). Mixtures were sheared five times through a 26-gauge needle, and 44 μl of acetic acid was added. Suspensions were ethanol precipitated twice, followed by one wash of the pellet in ethanol. Pellets were suspended in 200 μl of sterile TE (10 mM Tris [pH 8.0], 1 mM EDTA) and a 2-μl aliquot was used to calculate the total incorporated label by scintillation counting. For each hybridization, 2×10^7 cpm (approximately 50% of total reaction mix) was added to a 15-ml conical tube with 2 ml of hybridization mix (50% formamide, 3× SSPE [10], 1× Denhardt solution, 0.4% SDS), 1.5 μg of heat-denatured yeast tRNA, and a strip of nylon membrane (GeneScreen) onto which 1 μg of the appropriate in vitro-transcribed RNA had been spotted. Hybridizations were allowed to proceed for 2 days at 42°C with rotary shaking. Nylon filters were washed as follows: three times for 5 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS at room temperature, twice for 15 min each in 0.3× SSC plus 0.1% SDS at 65°C, twice for 15 min each in 2× SSC at room temperature, once for 30 min in 2× SSC plus 5 mg of RNase A per ml at room temperature, and once for 5 min in 2× SSC plus 0.1% SDS at room temperature. Hybridized spots were visualized by autoradiography at −70°C for 1 week with an intensifying screen.

TABLE 1. Transactivation by the X gene products of mammalian hepadnaviruses[a]

CAT vector	Fold CAT transactivation[b] by:		
	pHBVx	pWHVx	pGSHVx
pRSVCAT	6.8 ± 0.5	6.5 ± 0.6	6.6 ± 0.9
pSV2CAT	6.4 ± 0.6	6.0 ± 0.9	6.4 ± 0.4

[a] X expression vectors, pHBVx (RSV LTR-driven HBV X gene), pWHVx (SV40-driven WHV X gene), and pGSHVx (SV40-driven GSHV X gene) were compared with their frameshifted derivatives when cotransfected with either pRSVCAT or pSV2CAT into PLC/PRF/5 cells.

[b] Each value represents the ratio of the percent conversion of chloramphenicol to the acetylated form when the cotransfectant carries a wild-type X gene versus a frameshift mutant X gene. All values are the average from experiments carried out in sextuplicate.

RESULTS

Transactivation by mammalian hepadnavirus X products. As noted above, earlier work has established that the product of the HBV X gene can augment gene expression in *trans*. We have further explored the generality of this phenomenon by asking whether the X gene products of related animal hepadnaviruses also display a similar activity. For these experiments we constructed plasmids in which the X coding regions of GSHV or WHV were cloned downstream of the SV40 early promoter; in the control plasmid, pHBVx, the HBV X gene is expressed from the RSV LTR. Cells of the human hepatoma line PLC/PRF/5 were cotransfected with the indicated X gene plasmid and either pRSV CAT or pSV2CAT; in parallel, each CAT construct was transfected with an identical X expression plasmid bearing a frameshift lesion in the X gene. The CAT assay was performed 24 h posttransfection, and the results were expressed as the ratio of CAT activity produced in the presence of X protein to that produced in its absence. All three X expression plasmids augmented CAT expression from both targets to a comparable degree (approximately sixfold) (Table 1).

Next, the range of X-dependent transactivation was explored by assaying a number of different cell lines and reporter gene constructs. In these experiments, CAT plasmids bearing the promoter of interest were again cotransfected with X expression vectors carrying either a wild-type or frameshift mutant X gene. After 24 h, cell extracts were assayed for CAT activity, and the results were expressed as

TABLE 2. Broad host range of GSHV X product transactivation[a]

Plasmid	Fold CAT transactivation[b] by GHSV X protein in cell line:	
	Alexander	L
pSV2CAT	6.4 ± 0.4	4.4 ± 1.1
pRSVCAT	6.6 ± 0.9	4.0 ± 0.9
pMSVCAT	3.4 ± 0.3	ND[c]
pE3CAT	8.5 ± 0.6	5.6 ± 0.7

[a] pGSHVx, an SH40-driven GSHV X expression vector, is compared with its frameshift mutant derivative, pGSHVx-fs, as a cotransfectant with CAT vectors pSV2CAT (SV40 early promoter and enhancer), pRSVCAT (RSV LTR), pMSVCAT (murine sarcoma virus LTR), and pE3CAT (adenovirus E3 promoter) in PLC/PRF/5 cells (Alexander cells, nonpermissive but HBV DNA-containing human hepatoma line) and in L cells (mouse fibroblast-derived cell line).

[b] Each value represents the ratio between the percent conversion of chloramphenicol to the acetylated form when the cotransfectant carries a wild-type X gene versus a frameshift mutant gene. All values are the average from experiments carried out in sextuplicate.

[c] ND, Not done.

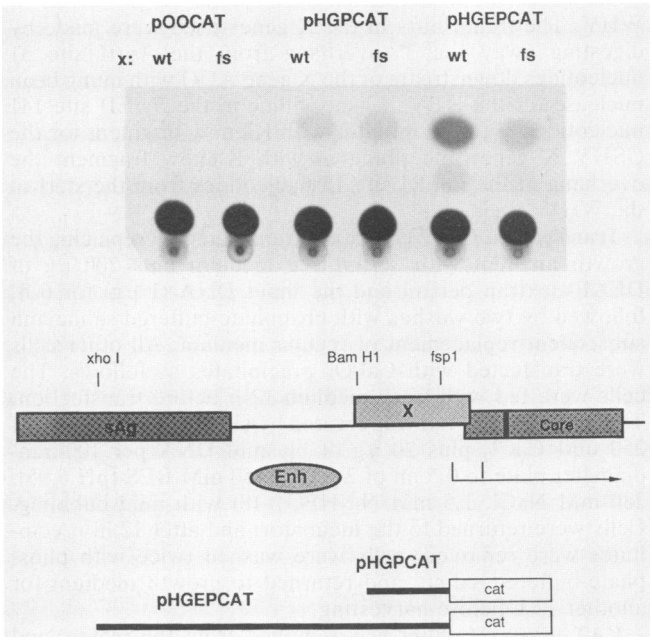

FIG. 2. (Top) CAT assays following transfection into Huh7 cells of CAT plasmidswith no promoter (pOOCAT), the HBV genomic promoter alone (pHGPCAT), or the genomic promoter plus the HBV enhancer (pHGEPCAT). CAT plasmids were cotransfected with either the wild-type HBV X gene driven by the RSV LTR (wt) or a frameshifted derivative of this plasmid (fs). (Bottom) Schematic diagram of the CAT constructions used, showing the relationship of the sequences included to important HBV landmarks. sAg, Surface antigen.

described above. A subset of these results obtained by using an SV40-driven GSHV X gene in mouse L cells and human PLC/PRF/5 (Alexander) cells is shown in Table 2. In nearly all cases we examined, cotransfection with the wild-type X gene conferred severalfold greater CAT activity than did the frameshift control. These effects ranged from 2- to 10-fold, with most in the range of 4- to 5-fold (Table 2). Transactivation occurred in cells from several different species (including mouse, monkey, and human) and tissues (including epithelial and fibroblastic cell types) and with promoters of diverse origin (results not shown). Similar experiments with HBV and WHV X genes revealed a comparable spectrum of transactivation for all three X gene products (data not shown).

Transactivation of hepadnavirus promoters in permissive cells. All of the above experiments were carried out in cell lines that are nonpermissive for hepadnavirus replication. We next tested whether HBV sequences themselves were effective targets for X activity, using Huh7 hepatoma cells in which HBV can replicate (28). We cotransfected pHBVx or its frameshift mutant derivative with CAT plasmids bearing HBV sequences immediately 5′ to the cap site of the genomic transcript (Fig. 2). The plasmid bearing the smaller insert (pHGPCAT), containing only the putative promoter, showed a basal level of activity much higher in these cells than in the nonpermissive hepatoma line, PLC/PRF/5, (data not shown) but consistently showed only a small transactivation by X (1.5-fold). The larger insert (pHGEPCAT) also contained the region known to act as an enhancer in heterologous contexts (18) and conferred upon its CAT plasmid a higher constitutive level of activity as well as an increased level of transactivation by X protein (four- to sixfold).

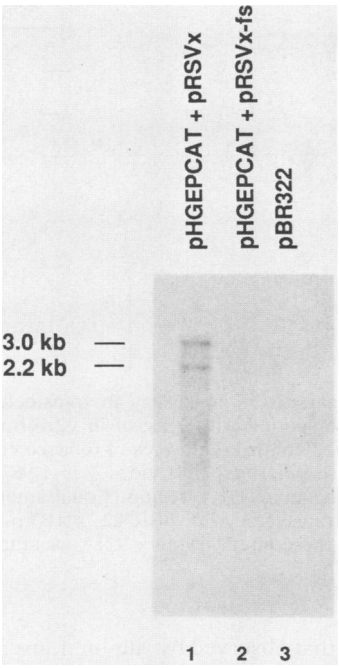

FIG. 3. Northern blot analysis of poly(A)$^+$ RNA isolated from Huh7 cells transfected with pHGEPCAT plus either pRSVx or pRSVx-fs. A lane of RNA from cells transfected with pBR322 is included as a control. Filter was probed with nick-translated pSV2CAT.

To further elucidate the nature of this phenomenon, we examined the levels of CAT mRNA produced in cotransfected cells. Huh7 cells were transfected with the same constructs and conditions that gave the best transactivation by CAT assay (pHGEPCAT plus pRSVx versus pRSVx-fs); poly(A)$^+$ RNA was isolated and subjected to Northern blot analysis, using nick-translated pSV2CAT as the probe (Fig. 3). A 2.2-kb RNA is produced by pHGEPCAT and is the product expected from transcription initiation directed by the genomic promoter of HBV. The origin of the larger (3-kb) transcript is uncertain but may result from initiation at the X promoter described by Treinen and Laub (25). Importantly, the levels of both RNAs were increased six- to eightfold in the presence of the HBV X product. Thus, the transactivation appears to operate primarily by the elevation of the steady-state concentrations of CAT mRNA, and both mRNAs are augmented to approximately the same extent. (Although the 3-kb RNA could potentially encode X product, it is clear from Fig. 2 and 3 that if it were to do so, the active levels of such a product would be well below those required for maximal transactivation.)

HBV genome as a source of transactivating functions. To determine whether transactivation would still occur with the X gene product as produced during a productive HBV infection, we repeated our cotransfections using recircularized monomers of the HBV genome as the source of X protein, pRSVCAT as the target, and the permissive hepatoma line Huh7 as the recipient cells (Fig. 4). When wild-type HBV DNA was used to donate X protein (Fig. 4, lane 5), a degree of transactivation comparable with that produced by pRSVx was observed (compare with lanes 3 and 4). For controls, we constructed a pair of HBV mutants in which the 5′ four-nucleotide overhang of the *Nco*I site present at the first AUG of the X open reading frame was

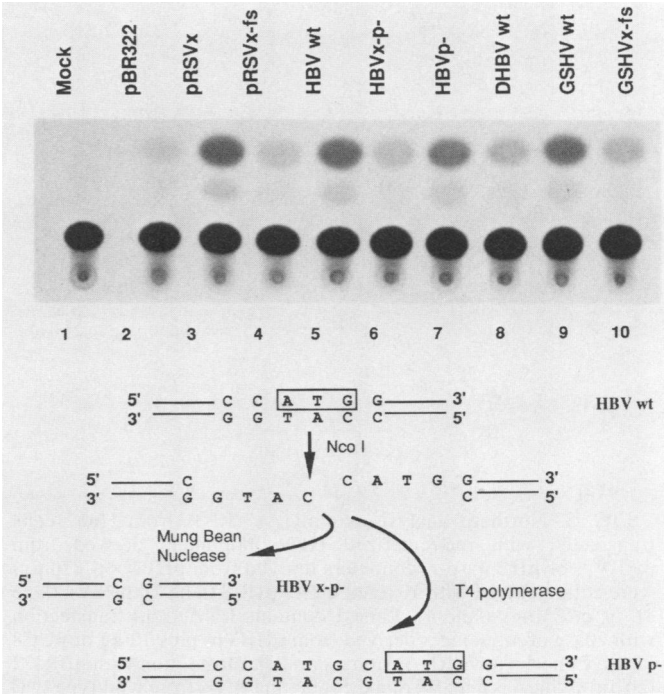

FIG. 4. (Top) CAT assay of cell extracts prepared from Huh7 cells cotransfected with recircularized monomers of the HBVgenome. Lane 1 was mock transfection (no DNA). Cell extracts were cotranfected with pRSVCAT plus the following cotransfectants: pBR322 (lane 2), RSV LTR-driven wild-type X gene (lane 3), pRSV-X frameshift (lane 4), wild-type (wt) HBV genome excised from plasmid and recircularized with DNA ligase (lane 5), recircularized mutant HBVx-p- (lane 6), recircularized mutant HBVp- (lane 7), wild-type DHBV genome (lane 8), wild-type GSHV genome (lane 9), and monomer GSHV genome with X frameshift (lane 10). (Bottom) Schematic of the strategy for constructing HBV mutants, pHBVx-p- and pHBVp-. The *Nco*I site, located at position 1370 relative to the *Eco*RI site in HBV, is at the first AUG in the X open reading frame.

either filled in with T4 DNA polymerase (restoring the X gene AUG but creating a frameshift in the overlying *pol* frame) or removed by digestion with mung bean nuclease (destroying both X and *pol* frames) (Fig. 4). Examination of CAT transactivation by these mutants proves that the X gene is the source of the activating activity: X^+P^- genomes augmented CAT expression (lane 7), while X^-P^- genomes did not (lane 6). Additional evidence that the X gene product is responsible for this effect is provided in the remaining lanes of Fig. 4. DHBV genomes (lacking the X gene) do not transactivate even though they can replicate well under these conditions (8, 16). By contrast, a GSHV genome (which in parallel studies did not replicate at detectable levels [R. Colgrove, unpublished results]), could transactivate pRSVCAT, while a mutant X^- derivative did not (Fig. 4, lanes 9 and 10).

We also studied transactivation with intact HBV genomes as both the source of and target for X activity in cells permissive for viral replication. Huh7 cells were transfected with recircularized monomers derived from either of the *Nco*I mutants shown in Fig. 4, and poly(A)$^+$ RNA from these cells was analyzed by Northern blotting. The genomic (3.4-kb) and subgenomic (2.3-kb) transcripts appeared at ca. 10-fold-higher levels when the X gene AUG was intact (pHBVp-) than when it was ablated (pHBVx-p-). Transacti-

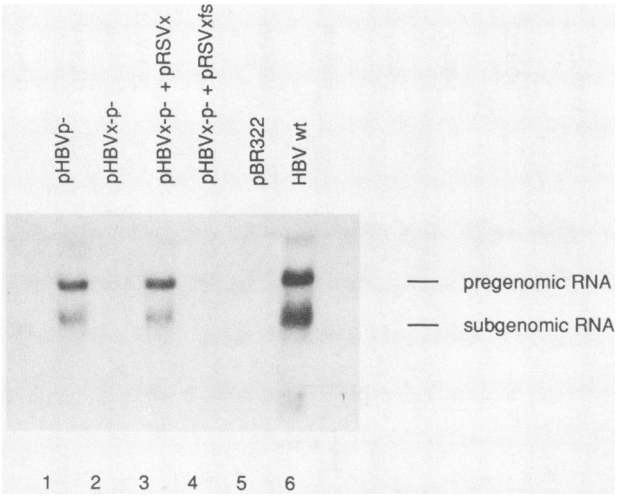

FIG. 5. Northern analysis of poly(A)$^+$ RNA from Huh7 cells transfected with recircularized HBV monomers derived from pHBVp- or pHBVx-p-. Monomers derived from pHBVx-p- (20 μg) were cotransfected with 10 μg of either pUC8 (lane 2), pRSVx (lane 3), or pRSVx-fs (lane 4). Lane 1 contains RNA from transfection with 20 μg of monomers derived from pHBVp- plus 10 μg of pUC8 DNA. Lane 5 shows RNA from a parallel transfection with pBR322 (20 μg). Lane 6 is a marker lane containing RNA from wild-type (wt) HBV-transfected Huh7 cells from an unrelated experiment to show the expected size of the major genomic and subgenomic transcripts. After electrophoresis and blotting the samples were annealed to ^{32}P-DNA homologous to the S region of the HBV genome (nucleotides 1 to 1,375). (This probe does not hybridize to the X region of pRSVx.)

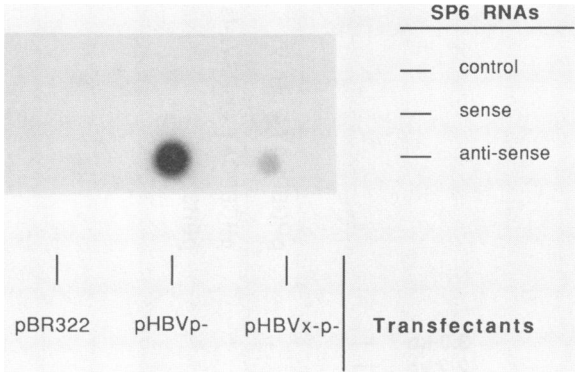

FIG. 6. Run-on transcription assay in transfected Huh7 cells. Nylon filters were spotted with 1 μg of in vitro-transcribed RNA coding for unrelated control sequences of tobacco etch virus, HBV sequences of sense polarity (nucleotides 1 to 1,987), or antisense sequences from the same HBV region. Equal amounts of labeled RNA from cells transfected with pBR322, pHBVp-, or pHBVx-p- were annealed to these filters. Duplex RNA was then identified by autoradiography.

vation activity could be restored to the X$^-$P$^-$ genome by cotransfecting an exogenous source of X, pRSVx (Fig. 5, lanes 3 and 4). This plasmid, but not its frameshifted derivative, restored normal levels of HBV mRNAs, indicating complementation of the defect. The levels of transactivation of these mRNAs were comparable with those seen by CAT assay in the previous experiment, suggesting that the effects of X gene product act at the level of RNA synthesis or stability.

Effects of X expression on the rates of hepadnavirus mRNA synthesis. Run-on transcription assays were performed to determine whether X protein-mediated transactivation acts at a transcriptional or posttranscriptional level. Huh7 cells were transfected with either pHBVp-, pHBVx-p-, or pBR322. One day after transfection, cells were washed, permeabilized with digitonin, and pulse-labeled with α-^{32}P-labeled UTP, as described in Materials and Methods. Total labeled RNA from these samples was extracted and hybridized to nylon filters bearing single-stranded HBV RNA sequences of either plus or minus polarity. After hybridization, the filters were washed and subjected to autoradiography. Results of this experiment are shown in Fig. 6. As expected, all primary transcripts were of the correct polarity; when the transfected genome carried a mutant X gene, an approximately eightfold decrease in the rate of transcription of HBV mRNAs relative to an X$^+$ genome was observed. Since the in vitro-transcribed RNAs spotted onto the filters contained the surface antigen and X regions of the HBV genome, both pregenomic and subgenomic messages are being detected in this analysis. Within the level of precision of these experiments, the amount of transactivation seen by Northern analysis was approximately equal to that observed by run-on transcription. These data indicate that the enhanced levels of RNA produced in the presence of the X gene product (Fig. 5) can be entirely accounted for by effects of this protein on the rate of transcription.

DISCUSSION

The primary aims of this study have been to determine the generality of X protein-mediated transactivation among the hepadnaviruses and to examine this activity in the context of authentic viral replication. The data presented here contribute to a greater understanding of the role of X gene product in several ways.

First, the data of Fig. 4 and 5 clearly establish that transactivation of gene expression by the HBV genome occurs in productive infection and is referable to the product of the X coding region. Second, transactivation activity appears to be a general property of all hepadnavirus X gene products (Tables 1 and 2). The absence of this activity in DHBV-infected cells (Fig. 4) is evidence against the notion that the C gene product of this virus has assumed this function as a result of fusion with an ancestral X gene during evolution (5). Transcriptional control in DHBV appears to differ importantly from that in the mammalian viruses in this respect.

Nuclear run-on transcription assays (Fig. 6) establish that activation of gene expression by the X protein occurs at the level of primary transcription. Our survey of X activity in many heterologous settings indicates that the X product can activate a considerable array of promoters in a wide variety of cellular contexts. In this regard, the X protein recalls the activity of the adenovirus transactivator E1A, which is likewise able to activate many heterologous constructs and does not display sequence-specific DNA-binding activity (1, 12). The details of the molecular mechanism by which X protein acts to enhance transcription remain unknown. Although a direct action of X protein on target DNA is not excluded, we favor the notion that (like E1A) the X product acts indirectly, by influencing components of the gene expression machinery common to the expression of many genes. For example, it could increase the production or

activity of one or a few broadly acting transcription factors or it could activate multiple activator proteins whose target sequences are less widely distributed. The data of Fig. 2 showing increased X protein-transactivation when the HBV enhancer sequences are present suggest that factor(s) recognizing this element may be among such targets of X activity. Whatever the targets of X action are, most are present in a wide variety of mammalian cells from several species and tissues and are not restricted to well-differentiated hepatocytes.

The use of the HBV genome as the source of X proteins allays concerns that transactivation might be influenced by expression of the X protein at unnaturally high levels or in a form different from that of the natural protein product (e.g., due to posttranslational modifications unique to nonpermissive cells). Our data clearly show that this is not the case. X protein expressed in its most native context is as competent to transactivate as X protein expressed from powerful recombinant vectors. Conversely, the fact that the magnitude of transactivation directed by the intact HBV genome is no greater than that produced by the isolated X gene in transfected cells (Fig. 4) may indicate that other viral genes do not significantly modulate the effects of the X gene product.

Our data also bear on the question of the biosynthesis of the X protein. Formally speaking, the protein could be made conventionally from a minor transcript beginning at the X promoter (25) or by translation of the X sequences positioned internally within the known 2.1- or 3.5-kb mRNAs. Translation initiation might begin at the first AUG of the X open reading frame or at a downstream initiator; alternatively, by analogy with retroviruses, the X protein might even be synthesized by a ribosomal frameshift from the overlying P frame. The simplest model consistent with the data of Fig. 4 and 5 is that the first AUG codon is itself required for the initiation of X translation. However, these data are also consistent with other models. For instance, we point out that our *Nco*I fill-in mutant (pHBVp-), which is phenotypically X^+, can also generate an in-frame *pol*-X fusion protein which might supply X activity. Clearly, further mutational dissection of the X region will be required to definitively determine the strategy of X gene expression.

Our results indicate that the HBV promoters themselves are good targets for transactivation in permissive cells. Whether direct or indirect, the transactivation seems to operate more or less equally on pregenomic and subgenomic promoters, a pattern that is not surprising given the broad range of X transactivation of heterologous promoters. Thus, during productive infection, X protein supplied from and acting upon the HBV genome boosts the levels the HBV mRNAs required for viral replication. This augmentation of viral transcription is clearly a major effect of X protein in the infected hepatocyte, although these findings do not exclude additional roles for this gene product in the life cycle. The broad spectrum of X gene product activity on transcription also raises the provocative possibility that its expression during viral replication may influence the activity of other important cellular genes. If so, then X protein could play a role in other, more poorly understood aspects of hepadnavirus biology and pathogenesis.

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