Alternate Translation Initiation on Hepatitis B Virus X mRNA Produces Multiple Polypeptides That Differentially Transactivate Class II and III Promoters

LIA KWEE,[†] ROBERT LUCITO, BARBARA AUFIERO,[‡] AND ROBERT J. SCHNEIDER*

*Department of Biochemistry and Kaplan Cancer Center, New York
University Medical Center, New York, New York 10016*

Received 4 March 1992/Accepted 20 April 1992

The hepatitis B virus X gene encodes a transcription activator which stimulates the synthesis of RNAs from a variety of class II and III promoter elements. In this report, we present a mutational analysis which genetically demonstrates that the X gene actually encodes two, and possibly three, related polypeptides from a single mRNA using alternate translation initiation from any of three in-frame AUG codons. Genetic analysis shows that translation initiates at the 5' proximal AUG of X mRNA and produces a full-length 17-kDa X protein but in addition also likely initiates at either of two conserved, in-frame AUG codons, producing two amino-terminally truncated X proteins presumably of 8 and 6.6 kDa. Expression of mRNAs capable of encoding only one of each X protein all individually transactivate class III (RNA polymerase III)-transcribed promoters. However, class II (RNA polymerase II)-transcribed promoters displayed various requirements for the different X proteins. Expression of two X proteins, the 17- and 6.6-kDa species, was required to activate transcription of the simian virus 40 enhancer/early promoter. In contrast, activation of an NF- κ B-dependent promoter was carried out only by mRNAs encoding the full-length 17-kDa X protein. These results indicate that the X gene encodes several related proteins that possess different transcriptional regulatory activities.

Persistent infection by hepatitis B virus (HBV) is one of the most prevalent human viral infections and is significantly correlated with the development of hepatocellular carcinoma (2). Despite the relationship between retroviruses and HBV (8), there is still no clear understanding regarding its role in carcinogenesis. In particular, it is widely believed that HBV does not encode an oncogene or activate cellular proto-oncogenes during viral DNA integration (8). Attention has therefore been directed to the viral X gene because it encodes a strong activator of genes transcribed by RNA polymerase (Pol) II (32-34, 37-39, 44) and RNA Pol III (1). Given the variety of regulatory sequences activated by the X protein, it is widely believed to act indirectly by associating with or modifying cellular transcription factors (1, 5).

The function of the X gene during viral infection is not clearly known, but it likely plays a significant role during the life cycle of HBV. It is expressed during at least certain stages of virus infection since some individuals possess a measurable anti-X antibody response (16, 28), particularly those with chronic infections or hepatocellular carcinoma (21). In this regard, it has been shown that the X gene is expressed and functions as an efficient transactivator during productive viral infection (5), implicating it in the enhancement of viral transcription and indirectly in viral replication.

Several transactivators have now been shown to stimulate transcription from both class II and III promoters, including the adenovirus (Ad) E1a protein (3, 10, 13), the simian virus 40 (SV40) t antigen (23), and the HBV X protein (1). It is not currently known whether these transactivators possess a common structural element involved in activation of class II and III promoters or whether distinct elements that may be

identified with different forms of the protein are involved. For instance, some transactivators, such as the E2 protein from bovine papillomavirus, are composed of full-length and amino-terminally truncated polypeptides which display different transcriptional regulatory activities through formation of hetero- and homodimers (19, 36). Accordingly, it was conceivable that transactivation of class II and III promoters by the HBV X gene product(s) might reside in different activation domains or encoded proteins. The X gene mRNA, for example, could potentially encode a full-length and two related amino-terminally truncated proteins by initiating translation at either of two internal AUG codons. This possibility is particularly attractive since the virus generates multiple proteins from the envelope, core, and polymerase open reading frames in such a manner (for a review, see reference 8).

We demonstrate by genetic and mutational studies that the X gene is similar to other HBV genes in encoding multiple products by alternate translation initiation on a single mRNA species. All three X polypeptides, when expressed individually, are sufficient for transactivation of class III promoters, but transactivation of several different class II elements displays various requirements for the different X proteins. Thus, our results suggest that transcriptional regulation of class II promoters by the HBV X gene involves several distinct activities contributed by the different X proteins.

MATERIALS AND METHODS

Cell culture and DNA transfections. Cells were propagated in Dulbecco's modified minimal essential medium supplemented with 10% calf serum, 50 μ g of gentamycin sulfate per ml, and L-glutamine. DNA transfections were performed by the calcium phosphate precipitation technique (12) by using 10-cm plates of cells at 60 to 70% confluency. Total DNA concentrations of 20 μ g per plate were maintained with

* Corresponding author.

[†] Present address: Hoffman LaRoche Institute, Nutley, NJ 07110.

[‡] Present address: Howard Hughes Medical Institute, Harvard University, Boston, MA 02115.

salmon sperm DNA. Unless otherwise specified, transfections employed 10 μ g of X plasmid and 5 μ g of reporter plasmids. Cells were incubated with DNA precipitates for 16 to 24 h, washed, and harvested 24 to 36 h later. Cells from three plates were pooled for each experimental sample. Transfection efficiencies were routinely monitored by immunofluorescence staining of a marker protein (Ad DBP) produced from a cotransfected, nonstimulated plasmid. Cells were grown on coverslips in plates, removed prior to cell harvest, and stained with fluorescein isothiocyanate-conjugated antibodies. This method provides direct visualization of transfection efficiencies.

Analysis of RNAs and CAT assays. Preparation of cell lysates and analysis of chloramphenicol acetyltransferase (CAT) activity were performed as described previously (11) by using equal amounts of protein and ensuring that enzyme activity remained within the linear (<50% conversion) range. Quantitation was achieved by liquid scintillation counting of spots on thin-layer chromatography plates, and the percent conversion to acetylated chloramphenicol was calculated; data are presented below as the fold activation with respect to basal CAT activity levels. All data for CAT assays are the averages of at least three independent determinations using pools of two plates per sample. Stimulation of VA1 RNA synthesis was determined by ribonuclease protection analysis. Whole cytoplasmic RNA was purified by Nonidet P-40 lysis of cells and phenol-chloroform extraction, which were followed by ethanol precipitation. A complementary VA1 RNA riboprobe was derived by cloning the Ad type 2 *Xba*I-*Bam*HI VA1 gene fragment into vector pGem4; cloning was followed by transcription with SP6 RNA Pol in the presence of [α - 32 P]GTP (18). Transcription, purification, and hybridization of labeled antisense RNA to whole cytoplasmic RNA were carried out as described previously (31). An excess of riboprobe (~200,000 cpm) was hybridized to 5 μ g of cytoplasmic RNA. The riboprobe specifically protects a 90-nucleotide VA1 RNA fragment, although stuttering occurs because of the high degree of secondary structure present in VA1 RNA. Nuclease protection products were resolved in denaturing 8 M urea-8% acrylamide gels. Quantitation was achieved by scanning densitometry of original autoradiograms.

Construction of HBV X gene variants. All X gene variants were constructed into vector pNL3C (6), which contains the Ad E1A enhancer, major late promoter, tripartite leader 5' noncoding region, and E1B polyadenylation signal. The HBV_{ayw} X gene was digested with *Xmn*I (nucleotide 1351), *Sal*I linkers were added and then digested with *Sal*I and *Bgl*II (nucleotide 1990), and the DNA fragment was inserted between the *Sal*I site at the immediate 3' end of the tripartite leader and the *Bgl*II site upstream of the E1B polyadenylation signal. To create variant pX8, the 5' proximal AUG (which is located within an *Nco*I site) was digested with *Nco*I and then nuclease S1 to remove the overhanging nucleotides containing the initiation codon as described previously (31) and religated. Variant pX9 contains a frameshift mutation inserted after amino acid 10 and was derived from the HBV_{adw} X gene containing plasmid pEN4- κ B (37). pX9 was created by inserting the *Xmn*I-*Bgl*II X gene fragment from pEN4- κ B into pNL3C as described above. Variant pX7 expresses X proteins truncated after the C terminus at position 118. pX7 was created by insertion at the *Dra*I site (position 1740) of an *Nhe*I nonsense linker (no. 1060; New England Biolabs) which contains a termination codon in all three reading frames. All remaining variants were created by oligonucleotide site-directed mutagenesis by using the in

vitro mutagenesis kit from Amersham, as described by the manufacturer. Oligonucleotides were synthesized to be complementary to amino acid position 79 or 103 with flanking sequences and containing a single base-pair mismatch. Variants X79 and X103 converted the AUG at position 79 or 103 to GUG (Met to Val). Variant X79/103 lacks both internal Met residues. In variants X9/79 and X9/103, the Met to Val conversion was carried out by using the X9 variant, which contains a frameshift mutation after amino acid 10. All mutations were verified by DNA sequence analysis.

RESULTS

Genetic analysis of X protein transcriptional activities. Previous analyses have shown that transactivation of a number of class II transcriptional elements requires synthesis of the full-length 17-kDa X protein, as demonstrated by mutations which shifted the X reading frame after the 10th amino acid or deleted the 5' proximal AUG codon (5, 37, 39, 44). Similar mutations (Fig. 1) were employed in our study and tested for the ability to transactivate promoters transcribed by RNA Pol II and Pol III. All constructs contained the full-length X open reading frame controlled by the Ad major late promoter, which is not responsive to the X protein, thereby eliminating complications due to autoregulation. The 5' proximal AUG codon was deleted in variant pX8, eliminating the ability to encode the full-length 17-kDa X protein. All variants except pX9 were derived from the *ayw* strain of HBV. Variant pX9 was derived from strain *adw* (37) and contains a frameshift mutation introduced after amino acid 10, resulting in termination of translation 37 amino acids downstream. This variant correctly initiates translation at the 5' proximal AUG but synthesizes a short nonsense polypeptide. Variant pX7 terminates translation of X protein at position 118, deleting the C-terminal region which is presumably required for X protein transactivating activity (7, 20). The remaining variants contain single nucleotide mutations which convert the in-frame AUG (Met) codons at positions 79 and 103 to GUG (Val), to test for the synthesis of additional X polypeptides. It should be noted that there are no potential AUG codons in other reading frames of the X gene which might also serve to initiate translation (unpublished observations). Assuming that the two in-frame AUG codons are utilized in the X mRNA, variant pX79 could potentially encode a variant full-length 17-kDa X protein and a 6.6-kDa species, whereas pX103 could encode variant forms of the 17- and 18-kDa polypeptides. Mutant pX79/103 possesses a double Met to Val mutation and potentially encodes only a variant 17-kDa X protein. Variants pX9/79 and pX9/103 contain the AUG to GUG conversion in the pX9 frameshift background. The last three variants therefore make up a set of mutations that permit the exclusive synthesis of only one form of X protein, either a variant 17-kDa form (pX79/103) or an 8-kDa form (pX9/103), and a wild-type 6.6-kDa protein (pX9/79). X gene constructs were transfected at low effector-to-target ratios to ensure that subsaturating amounts of X polypeptides were tested. Transfection efficiencies were routinely monitored as described in Materials and Methods.

X gene variants were tested for the ability to transactivate class II transcriptional elements by cotransfection with plasmid pSV2-CAT (11), which expresses the CAT gene linked to the SV40 enhancer/early promoter (Fig. 2). The X protein has been previously shown to act predominantly on the SV40 enhancer rather than promoter element (1, 32, 37). Both variants pX8 and pX9 failed to stimulate transcription

FIG. 1. Schematic representation of wild-type and variant HBV X gene constructs and the polypeptides potentially encoded. The full-length X open reading frame can maximally encode a full-length 154-amino-acid polypeptide (8). Solid triangles refer to the three conserved in-frame AUG codons with numbers (1, 79, and 103) denoting amino acid positions. Other numbers refer to the boundaries employed in mutational analyses. Open arrows represent potential X proteins initiating from the three AUG codons corresponding to the full-length 17-kDa (p17), amino-terminally truncated 8-kDa (p8), and 6.6-kDa (p6.6) species. Thick lines denote deleted regions. Open triangles denote positions of mutagenesis containing insertions (frameshift, fs in pX9) or single-base substitutions (AUG Met to GUG Val). Truncated proteins are marked by a T, and conversion of Met to Val is marked by an asterisk. MLP refers to the Ad major late promoter, 3LDR refers to the Ad tripartite leader 5' noncoding region, and E1B refers to the Ad E1B polyadenylation signal.

of pSV2-CAT (Fig. 2, lanes 4 and 5; see also Fig. 4), in agreement with results published previously (5, 37). Thus, transactivation of the SV40 enhancer/early promoter apparently requires synthesis of the full-length 17-kDa X protein. In contrast, both variants pX8 and pX9 were found to efficiently stimulate transcription from class III promoters. The Ad VA1 RNA gene, which is transcribed by RNA Pol III, was cotransfected with mutant pX8 or pX9. VA1 RNA levels were then measured by RNase protection analysis (Fig. 3A). Variant pX9 stimulated class III promoter activity as strongly as wild-type X protein (about 30-fold; lanes 2 and 5), whereas activation by variant pX8 was slightly reduced, averaging about 50% that of the wild type (Fig. 3A, lane 4, and Fig. 4). A slight reduction was also observed in the amount of 6.6-kDa X protein synthesized in vitro (data not

shown), suggesting that the mutation introduced a small translational impairment for unknown reasons. Activation by pX8 and pX9 occurs independently of the class III promoter employed since similar results were also obtained by using a tRNA^{Ala} gene (data not shown). The pX9 mutation permits ribosomes to bind at the 5' proximal AUG but shifts the X protein reading frame after the 10th amino acid, suggesting that a second translation product must be produced in vivo, perhaps by initiating at an internal AUG codon. Synthesis of the additional transactivator cannot initiate upstream of the 5' proximal AUG codon because it would also be shifted out of frame by the pX9 mutation. Northern (RNA) analysis revealed that only a single X mRNA species was detectable in transfected cells (data not shown), eliminating the possibility that variant X proteins are produced from alternatively spliced transcripts.

A comparison of human HBV sequences revealed the presence of two fully conserved internal AUG codons in all isolates of the four viral serotypes (22) (Fig. 5). Furthermore, AUG-1 is conserved in a rather poor codon context (17) (UccAUGG) and therefore should not fully impede the initiation of translation downstream at internal AUG codon 79 or 103. AUG-79 is conserved in an equally poor codon context as AUG-1, whereas AUG-103 is conserved in a slightly better recognition context (GCAAUGU). Therefore, the X gene in all human HBVs may potentially encode up to two additional in-frame, amino-terminally truncated polypeptides. Additional X gene variants were constructed to determine whether the X gene synthesizes several polypeptides from a single transcript and whether the X protein regions required for transactivation of class II and III promoters segregate into these additional X polypeptides.

Variant pX7 was designed to terminate translation of all forms of X protein at position 118, thereby deleting the carboxy-terminal region which is presumably required for stimulation of RNA Pol II (Fig. 1). This variant failed to transactivate the SV40 enhancer/early promoter (Fig. 2, lane 3) and largely failed to stimulate RNA Pol III transcription (Fig. 3B, lane 3), as demonstrated by cotransfection with the Ad VA1 RNA gene. Thus, the C terminus of X protein is required for activity by all potential forms of X protein in the transactivation of class II and III promoters (Fig. 4).

Additional X proteins are synthesized by using alternate in-frame AUG codons. The synthesis and function of additional X polypeptides were directly investigated by converting the in-frame AUG codons (methionine) to GUG (valine). Variant pX79, which may synthesize a full-length variant 17-kDa X protein and a 6.6-kDa species, stimulated transcription of the SV40 enhancer/early promoter (RNA Pol II; Fig. 2, lane 7) and VA1 RNA gene (RNA Pol III; Fig. 3A, lane 7). These results indicate that the methionine at position 79 is not required for activity of the full-length 17-kDa protein. These results also show that if the 8-kDa X protein is synthesized in vivo, it is not essential for transactivation of either class II or III promoters tested (Fig. 4). However, variant pX103, which can synthesize a variant 17-kDa X protein, and potentially a variant 8-kDa species, failed to stimulate transcription by either RNA Pol II or III (Fig. 2, lane 8, and Fig. 3, lane 6). Two interpretations can be drawn from these data: (i) the Met at position 103 is required for activity of the 17-kDa X protein, (ii) both the 17- and 6.6-kDa X polypeptides are required for transactivation of the SV40 enhancer/early promoter. Results presented below resolve this issue and demonstrate that the 6.6-kDa protein is in fact also required for transactivation of the SV40 enhancer/early promoter.

FIG. 2. Effect of X gene mutations on stimulation of transcription of the SV40 enhancer/early promoter by RNA Pol II. Chang liver cells were cotransfected with the indicated X gene variants and the reporter plasmid pSV2-CAT (11), which is under the control of the SV40 enhancer and early promoter, shown previously to be transactivated by X protein (37). Plates of cells were transfected in triplicate with plasmids and pooled, and extracts were prepared 36 to 48 h later for analysis of CAT enzyme activity (11). Transfection efficiencies were monitored by immunofluorescent staining of a marker protein transcribed from cotransfected, nonstimulated samples as described in Materials and Methods. Quantitation of CAT assays was performed by liquid scintillation counting of spots in thin-layer chromatography plates. Data were averaged from at least three experiments and calculated as the percent chloramphenicol acetylated. Data presented below lanes represent the fold activation of CAT activity above basal levels (pSV2-CAT alone). T and *, same as for Fig. 1.

To further explore the potential synthesis and function of multiple X polypeptides, three additional variants which can individually produce the 17-, 8-, or 6.6-kDa form of X protein were constructed. Variant pX79/103 contains a double Met to Val mutation which synthesizes only a variant form of the 17-kDa X protein. As expected, this variant failed to stimulate transcription of the SV40 enhancer/early promoter by RNA Pol II (Fig. 2, lane 9, and Fig. 4) because it possesses the conversion of Met-103 to Val. However, this variant efficiently stimulated transcription by RNA Pol III (Fig. 3A, lane 8). Thus, the full-length 17-kDa X protein is sufficient to activate class III promoters. Although the failure of mutant pX103 to transactivate transcription of VA1 RNA is not currently understood, it appears to be related to the cosynthesis of the variant 17- and 8-kDa polypeptides. This was tested directly (Fig. 3B) by cotransfection of pX9/103 (synthesizes only the 8-kDa protein) and pX79/103 (synthesizes only the 17-kDa protein). It is clear that cosynthesis of the variant 17- and 8-kDa X polypeptides interferes with transactivation of class III promoters. Nevertheless, the exclusive synthesis of only the 6.6-kDa X protein (pX9/79) and the 8-kDa species (pX9/103) was sufficient to stimulate transcription from the VA1 RNA promoter (Fig. 3A, lanes 9 and 10) but not from the SV40 enhancer/early promoter (Fig. 2, lanes 10 and 11). We can therefore conclude that the 17-, 8-, and 6.6-kDa X proteins can each independently stimulate transcription by RNA Pol III (Fig. 4). Furthermore, these results also indicate that at least one additional X polypeptide, either the 8- or 6.6-kDa species, is normally synthesized from the X mRNA *in vivo*. The compelling evidence for this derives from the observation that mutants which shift the translation reading frame shortly after AUG-1 synthesize a protein which activates transcription of class III promoters.

Both the 17- and 6.6-kDa X proteins are required to stimulate transcription by the SV40 enhancer/early promoter. The results obtained from transfection of mutant pX9/79 suggested either that the transactivation of the SV40 enhancer/early promoter by X protein requires the full-length 17-kDa species and the amino-terminally truncated 6.6-kDa polypeptide or that the Met at position 103 is critical for activity of the 17-kDa protein. To resolve this issue, experiments were performed to determine whether the variant 17-kDa X protein could be complemented *in trans* by the 6.6- or 8-kDa polypeptide. Cells were cotransfected with plasmid pX79/103, which synthesizes only the variant 17-kDa X protein, and pX9/79, which synthesizes only the 6.6-kDa species, or pX9/103, which produces only the variant 8-kDa form (Fig. 6). As observed previously, neither the 8- nor the 6.6-kDa X protein could individually stimulate transcription from the SV40 enhancer/early promoter (Fig. 6, lanes 3 and 4). However, when provided *in trans*, the 6.6-kDa X protein fully restored transactivation activity to the variant 17-kDa X protein, whereas the 8-kDa species did not (lanes 5 and 6). These results demonstrate that X protein stimulation of the SV40 enhancer/early promoter, which is dependent on activation of transcription factors AP1 and AP2 (32), requires cosynthesis of both the full-length 17-kDa and the amino-terminally truncated 6.6-kDa X proteins.

The 17-kDa X protein is sufficient for activation of NF- κ B-dependent promoters. Experiments were performed to determine whether two X proteins, the full-length 17-kDa and the amino-terminally truncated 6.6-kDa species, are required for activation of class II promoters other than the SV40 enhancer/early promoter. It was previously shown that X protein stimulation of the SV40 enhancer is mediated through activation of factors AP1 and AP2 (32). We therefore investi-

FIG. 3. Effect of X gene mutations on stimulation of transcription by RNA Pol III. Chang liver cells were cotransfected with X gene variants and reporter plasmid pVA1 (1), which encodes the class III gene, Ad type 5 VA1 RNA. Transfections were performed and efficiencies were monitored as described in the legend to Fig. 2. Quantitation was achieved by densitometry of original autoradiograms. Data presented below lanes are averaged from at least three experiments and represent the fold transactivation of VA1 RNA synthesis above basal levels (pVA1 alone). T and *, same as for Fig. 1.

type X gene and 20-fold by pX79/103. However, mutant pX9, which does not synthesize the 17-kDa form of X protein, failed to stimulate NF- κ B-dependent transcription. These results are also supported by a study in which it was shown that the full-length 17-kDa X protein produced in

gated the requirements for multiple X proteins in the activation of a promoter element dependent on a different transcription factor, NF- κ B. X protein has been shown to stimulate transcription of the human immunodeficiency virus (HIV) long terminal repeat (LTR) through activation of NF- κ B (7, 24, 34, 38). Transcriptional activation studies used plasmid p κ B-CAT, which contains four tandem copies of the NF- κ B binding sequence inserted upstream of the rat minimal angiotensinogen promoter and CAT gene (24). This transcription unit was shown to be activated only by binding of inducible NF- κ B (24). For Fig. 7, cells were transfected with either p κ B-CAT alone or with pwtX (lane 2), pX9 (p8 and p6.6; lane 3), or pX79/103 (only p17; lane 4). NF- κ B-dependent transcription was activated approximately 12-fold by the wild-

FIG. 4. Quantitation and summary of variant X gene transactivation activities. The results of at least three independent analyses were averaged. CAT assays (Pol II transcription of SV40 enhancer/early promoter) were quantitated by liquid scintillation counting of regions of thin-layer chromatography plates corresponding to unacetylated and acetylated chloramphenicol. Nuclease protection analyses (Pol III transcription of VA1 RNA gene) were quantitated by densitometry of autoradiographs. Values are expressed relative to wild-type X gene controls and varied among experiments by less than 10%.

subtype	AUG 1	AUG 79	AUG 103
	-3 -2 -1 +1 +2 +3 +4	-3 -2 -1 +1 +2 +3 +4	-3 -2 -1 +1 +2 +3 +4
ayw	UCCAUGG	UCCAUGG	GCAAUGU
adw	UCCAUGG	UGCAUGG	GCAAUGU
adr	UCCAUGG CCCAUGG	UCCAUGG UCCAUGG	GCCAUGU GCAAUGU
ayr	UCCAUGG	UACAUGG	GCAAUGU

FIG. 5. Comparison of nucleotide sequences for X gene AUG codons in HBV subtypes. Positions of nucleotides (−3 to +4) which comprise the extended AUG codon are shown according to the outline of Kozak (17). Also shown, in boldface, are the nucleotides found to influence codon context. Data were generated by analysis of human HBV sequences in GenBank by using Genetics Computer Group (University of Wisconsin) software.

bacteria could stimulate in vitro transcription by the HIV LTR (15). Thus, activation of NF- κ B-dependent transcription is carried out only by the full-length 17-kDa X protein.

DISCUSSION

In this article, we have provided evidence that the HBV X gene encodes several transcriptional transactivating proteins: a full-length 17-kDa protein, an amino-terminally truncated 6.6-kDa polypeptide, and possibly an 8-kDa amino-terminally truncated species. In addition, we demonstrated that different class II transcription elements display various requirements for the multiple X proteins. Previous studies showed that mutations which interfered with translation from the 5' proximal AUG and prevented synthesis of the full-length 17-kDa X protein inactivated the ability to stimulate several class II transcriptional elements that were

FIG. 6. Requirement for different X proteins in activation of the SV40 enhancer/early promoter. Chang liver cells were cotransfected with equal amounts of X gene variants and reporter plasmid pSV2-CAT. Transfections were performed and efficiencies were monitored as described in the legend to Fig. 2. *, same as for Fig. 1.

FIG. 7. X protein requirement in activation of NF- κ B-dependent promoters. Plasmid p κ B-CAT contains four NF- κ B-binding sites and the rat minimal angiotensinogen promoter upstream of the CAT coding region. Chang liver cells were transfected with p κ B-CAT alone or in combination with pwtX, pX9, or pX79/103. Transfections and CAT assays were performed as described in the legend to Fig. 3. *, same as for Fig. 1.

tested, including the SV40 enhancer/early promoter and the HIV LTR (20, 37, 44). Our results confirm these reports and demonstrate that transactivation of the SV40 enhancer/early promoter required the synthesis of an additional 6.6-kDa amino-terminally truncated X protein. We are therefore investigating the requirements for multiple X proteins in transactivation of a number of class II transcriptional elements. Nevertheless, from the two examples described here it is clear that different types of class II promoters will require different combinations of X proteins for activation. This is in agreement with other reports indicating that bacterially derived 17-kDa X protein was found to be sufficient for activation of the HIV LTR in an in vitro assay (15, 43). Thus, it might be expected that different promoters will demonstrate different patterns of regulation by the various X proteins.

In contrast to the results obtained with the SV40 enhancer/early promoter and NF- κ B-dependent promoters, all three X proteins were found to be capable of independently activating class III promoters. Activation of both class II and III promoters was also found to require a common region of X protein downstream of position 118. It is conceivable that a common transactivation domain between amino acids 118 to 154 is required for both classes of promoters, which would be present in the putative smaller polypeptides as well as in the full-length X protein. In this case, a class II-specific element could be located upstream of position 118 and involved, for instance, in promoter association, as suggested for Ad E1a protein (26) and recently for HBV X protein (25). Finer mutational analysis should provide a more detailed characterization of X protein activity and accurately localize the functional domains.

We believe that our results are most consistent with the

FIG. 8. Predicted secondary structure and conserved sequence elements of human HBV X protein. Structural features were predicted by the algorithms of Garnier et al. (9) and Chou and Fasman (4). Conservation of regions was determined by inspection of published sequence data (22) for numerous isolates of human HBV serotypes (unpublished results). The boundaries of the three potential X proteins are aligned with predicted structural features.

synthesis of additional, amino-terminally truncated X polypeptides. First, the frameshift mutation (pX9) prevented synthesis of the full-length 17-kDa X protein without deleting the 5' proximal AUG. Thus, *in vivo* ribosomes must normally bypass the first AUG of the X mRNA with a reasonably high frequency and initiate at one or both of the downstream AUGs which occur in frame. The other two potential reading frames of the X mRNA do not contain AUG codons and therefore do not give rise to alternate protein products. Second, activation of class III promoters by X gene variants which do not synthesize the 17-kDa protein cannot be explained as a fortuitous result of promoter competition or production of aberrantly spliced X mRNAs. Promoter competition effects were excluded by expression of the X gene under the control of different promoters and in a variety of different vector backgrounds, which did not alter the observed results (data not shown). The possibility of alternate X mRNAs is unlikely because only a single species of mRNA was observed in transfected cells (data not shown). Third, it was shown that the AUG-103 (Met) to GUG (Val) mutation, which eliminates the potential synthesis of the 6.6-kDa X protein, prevented activation of the SV40 enhancer/early promoter but was complemented *in trans* by transfection of a plasmid that exclusively synthesized the 6.6-kDa form of the X protein. It now remains to be determined whether the 6.6-kDa X protein is the only polypeptide synthesized in addition to the full-length 17-kDa polypeptide or whether the 8-kDa form is made as well. The experiments presented here cannot distinguish between these two possibilities because class III promoters, which were used to assay for the production of additional X proteins, are transactivated equally well by all three forms of X protein. *In vitro* translation of X mRNA suggests that the 17- and 6.6-kDa forms of X protein are predominantly synthesized (unpublished results), but this remains to be examined *in vivo*. We suspect that the additional X polypeptide(s) was not observed by previous mutational analysis of the X gene because the activation of class III promoters was not investigated. Furthermore, we have found by *in vitro* translation analysis that the 6.6-kDa X protein migrates in a section of sodium dodecyl sulfate-polyacrylamide gels that is often masked by radioactive label and that the protein is not efficiently immunoprecipitated by antisera raised against the full-length 17-kDa X protein (unpublished results). By using an Ad recombinant which expresses the X gene in place of Ad E1a (24), we have obtained preliminary results demonstrating the synthesis of 17- and 6.6-kDa polypeptides and minor amounts of an

8-kDa species which are immunoprecipitable by X protein antisera. However, quantitative *in vivo* detection of the additional X proteins must await the production of antisera specific for the amino-terminally truncated forms and should be performed with a replicating HBV system.

The amino acid sequence of the X protein reveals relatively few structural motifs suggestive of a possible mechanism for transactivation. Computer modeling studies predict that the C terminus of X protein may contain the only moderately acidic α -helix in the protein (Fig. 8). This structure is of some interest given that acidic α -helices are important functional elements for many transactivators which bind DNA directly (30) and possibly for those that may associate through DNA binding proteins (26, 29). However, no DNA binding property has been detected in the X protein (35, 43), and the X protein lacks obvious structural features, such as zinc finger motifs, generally associated with this activity (22). Some support for the notion that the C terminus of the X protein may act as an acidic activator was provided by fusing the X protein to a DNA binding protein (32, 40). Nevertheless, the biological significance of the moderately acidic C terminus is not clear because all X proteins contain this region, although the smaller proteins were unable to independently activate transcription of class II promoters.

Several transactivating proteins have now been shown to be synthesized in full-length and amino-terminally truncated forms. The bovine papillomavirus E2 transactivator is composed of two such polypeptides (19) that assemble into hetero- and homodimers with repressor and activator activities, respectively (27). The pituitary-specific transcription factor Pit-1 has also been shown to be composed of two related proteins (14) by alternate translation initiation at either of two in-frame AUG codons on a single mRNA species (41). It is not yet known whether the different proteins possess distinct activities in the case of Pit-1, although it is clear that this is the case for bovine papillomavirus E2 and HBV X proteins. Future studies should now be directed toward understanding the multiple roles of the X proteins in gene regulation by HBV during infection and transformation.

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