Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 5647–5652, May 1996
Medical Sciences

A truncated mutant (residues 58–140) of the hepatitis B virus X protein retains transactivation function

(chloramphenicol acetyltransferase assay/Rouse sarcoma virus long terminal repeat/site-directed mutagenesis/transient transfection)

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Communicated by V. Ramalingaswami, All India Institute of Medical Sciences, New Delhi, India, January 22, 1996

ABSTRACT **The hepatitis B virus X protein (HBx) sequence (154 aa) has been divided into six regions (A–F) based on its sequence homology with X proteins of other mammalian hepadnaviruses. Regions A, C, and E are more conserved and include all the four conserved cysteines (C^7, C^{61}, C^{69}, and C^{137}). To localize the regions of HBx important for transactivation, a panel of 10 deletion mutants (X5–X14) and 4 single point mutants (X1–X4), each corresponding to a conserved cysteine residue, was constructed by site-directed mutagenesis. A HBx-specific monoclonal antibody was developed and used to confirm the expression of mutants by Western blot. Transactivation property of the HBx mutants was studied on Rous sarcoma virus–long terminal repeat (RSV–LTR) in transient transfection assays. We observed that deletion of the most conserved region A or substitution of the N-terminal cysteine (C^7) had no effect on transactivation. Deletion of the nonconserved regions B or F also had no deleterious effects. Deletions of regions C and D resulted in a significant loss of function. Substitution of both C^{61} and C^{69} present in region C, caused almost 90% loss of activity that could be partially overcome by transfecting more expression plasmid. The fully conserved 9 amino acid segment (residues 132 to 140) within region E including C^{137} appeared to be crucial for its activity. Finally, a truncated mutant X15 incorporating only regions C to E (amino acids 58–140) was able to stimulate the RSV–LTR quite efficiently, suggesting a crucial role played by this domain in transactivation function.**

Hepatitis B virus (HBV) is the infective agent for the widespread liver disease in humans known as hepatitis B. Chronic infection has been associated with a high risk for development of hepatocellular carcinoma (1, 2). Similar viruses are also found in several animal species such as woodchucks, ground squirrels, and ducks, and together they constitute the family Hepadnaviridae. The small 3.2-kb DNA genome of HBV has at least four open reading frames called S, C, P, and X. During the natural course of HBV infection, the X gene expresses a polypeptide (HBx) of 154 residues that is implicated in HBV-mediated hepatocellular carcinoma (3, 4).

HBx is a pleiotropic transactivator because it can stimulate the cis-elements of not only the HBV genome (5, 6) but also of a wide range of other viral promoters such as simian virus 40 early (5, 7, 8) and herpes simplex virus-tk (7, 8), and long terminal repeats (LTRs) of human immunodeficiency virus type 1 (9, 10), human immunodeficiency virus type 2 (11), human T-lymphotropic virus type I (7), mouse mammary tumor virus (5, 7), and Rous sarcoma virus (RSV) (7, 8). HBx can also upregulate the expression of some protooncogenes like c-*myc* (12) and c-*fos*/c-*jun* (13–15). Several cellular genes, such as α1-antitrypsin (16) and α-fetoprotein (15); metallothionein (7), epidermal growth factor receptor (17), and RNA polymerase II and III (18); and many genes encoding components of the immune system, such as major histocompatability complex I (19), major histocompatability complex II (20), intracellular adhesion molecule 1 (21), β-interferon (8), and interleukin 8 (22), can also be activated by HBx. As HBx does not bind to DNA, it may mediate transactivation through protein–protein interactions (23–25). Although there is no consensus among the regulatory regions of the HBx-responsive genes, most of them incorporate cis-elements for some common trans-factors like AP-1, AP-2, C/EBP, and NF-κB (22, 26). Interestingly, HBx interacts with several other host–cell transcription factors such as ATF-2 and CREB (23, 25), Oct-1 (27), RBP5 subunit of RNA polymerase (28), and the TATA binding protein (24). Besides, it can also bind to the tumor suppressor factor p53 (29), serine protease TL2 (30), cellular DNA repair protein (31), and the simian virus large tumor antigen (32). HBx shows ATPase (33) and protein kinase activities (34) and is able to modulate cellular signal-transduction pathways (35–37). Thus, HBx appears to use alternative pathways for its transactivation function.

To better understand the pleiotropic behavior of HBx, we need to identify the protein domains that are important for its various activities. We therefore constructed 15 HBx mutants carrying either single amino acid substitutions or inframe deletions. The transactivation properties of these mutants were analyzed in transient transfection assays, and the results identified an internal segment of HBx that is essential for its transactivation function.

MATERIALS AND METHODS

HBx Expression Vector and HBx Mutants. The HBx gene was amplified as a 481-bp DNA fragment by PCR using the full-length HBV template (*adw* subtype) (38) and the following oligonucleotide primers: forward, 5′-CGGAATTCATGGCTGCTAGGCTGT-3′ and reverse, 5′-CGGAATTCTTAGGCAGAGGTGAAAAAG-3′. Finally a 471-bp *Eco*RI fragment of the HBx gene (hereafter called X0) was cloned into the eukaryotic expression vector, pSG5 (Stratagene) and the DNA sequence was verified. The cysteine mutants of HBx (X1–X4) were constructed by site-directed mutagenesis using the single-stranded X0 template and the following oligonucleotide primers:

$C^7 \rightarrow T$
X1:5′-GCT AGG CTC TAT **ACA** CAG CTG GAT GCT TCG-3′

$C^{61} \rightarrow T$
X2:5′-GGT CTC CCA GTG **ACA** GCA TTC TCA TCT-3′

$C^{69} \rightarrow T$
X3:5′-TCT GCC GGC CCA **ACA** GCT CTT CGC TTC-3′

$C^{137} \rightarrow T$
X4:5′-GTA TTA GGT GGT **ACC** CGT CAC AAA TTG-3′

Abbreviations: HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; HBx, hepatitis B virus X protein; RSV, Rous sarcoma virus; LTR, long terminal repeat; X0, wild-type HBx expression vector; WHVx, woodchuck hepatitis virus; GSHVx, ground squarrel hepatitis virus.

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The 10 deletion mutants of HBx (X5–X14) were also constructed by site-directed mutagenesis by using the single-stranded X0 template according a published procedure (39). The truncated form X15 was constructed by PCR using the following primers: forward, 5′-CGGAATTCCATATGCTC-CCCGTCTGTGCCTTC-3′ and reverse, 5′-CGGAATTCG-GATCCTTATTTGTGCCTACAGCCTCCTAA-3′. Finally, a 270-bp *Eco*RI fragment of X15 was cloned into pSG5.

Monoclonal Antibody, Western Blot Analysis, and Immunoprecipitation. A monoclonal antibody was developed against HBx by immunizing BALB/c mice with the purified antigen expressed in *Escherichia coli* (40). Spleen cells from the immunized animals were fused with myeloma cells (P3x63Ag8.653; ATCC no. CRL 1580) by using polyethylene glycol (41), and the anti-HBx secreting hybridomas were screened by ELISA on wells coated with purified HBx. Culture supernatant of one of the positive clones, B-8/2/8, was used to analyze the expression of HBx mutants.

For expression analysis, the wild-type and the mutant HBx expression vectors were transfected (1 μg DNA per 60-mm culture dish at 30% confluence) in COS 1 cells (ATCC no. CRL 1650) by using the lipofectin reagent (GIBCO/BRL). Cells were harvested 48 h after transfection, and the cell extracts were processed for Western blot analysis (40). For immunoprecipitation, the 48-h posttransfected COS 1 cells were metabolically radiolabeled in the presence of [^{35}S]methionine (Amersham) for 5 h, processed as described (39), and autoradiographed. B-8/2/8 is specific for HBx and does not recognize any other cellular protein (see Fig. 3*A* lane 4 and 3*B* lane 3).

DNA Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. Functional analysis of the HBx mutants was performed on RSV–LTR in transient transfection assays. The human hepatoma Hep G2 cells (ATCC no. HB 8065) at 50% confluence in a 60-mm culture dish were cotransfected with the wild-type or mutant HBx expression vectors, and the RSV–CAT reporter plasmid was cotransfected by either the calcium phosphate method (42) or by using lipofectin reagent (GIBCO/BRL). Cell extracts were prepared 48 h posttransfection, and CAT activity was measured as described by Gorman *et al.* (43) by using equal amounts of protein. After thin layer chromatography and autoradiography, radioactivity of the substrate and product spots was measured by liquid scintillation counting.

RESULTS

Sequence Homology of HBx. Fig. 1 shows the homology in amino acid sequence of X proteins of the human hepatitis B virus (HBx), the ground squirrel hepatitis virus (GSHVx), and the woodchuck hepatitis virus (WHVx). The overall homology among the three viral proteins is about 35% with nine conserved basic amino acids and four conserved cysteines (C^7, C^{61}, C^{69}, and C^{137}). Further, HBx can be subdivided into at least six regions (A–F) of which the more conserved regions, A, C, and E, are interspersed with relatively variable regions B, D, and F.

Region A, containing the N-terminal 20 aa, is the most conserved segment of HBx (80% identity) including three arginines and one cysteine (C^7). Rich in serine and glycine residues, region B (21–57) is poorly conserved (only 5.4% identity). With about 55% homology and rich in hydrophobic amino acids, region C spans residues 58–84 and is characterized by two conserved cysteines, C^{61} and C^{69}. Located between amino acids 85 and 119, region D shows poor homology (17%), with two conserved basic amino acids. Region E, between residues 120 and 140, is well conserved (≈67%) and is characterized by a stretch of nine fully conserved amino acids including a cysteine (C^{137}) and three basic amino acids. With

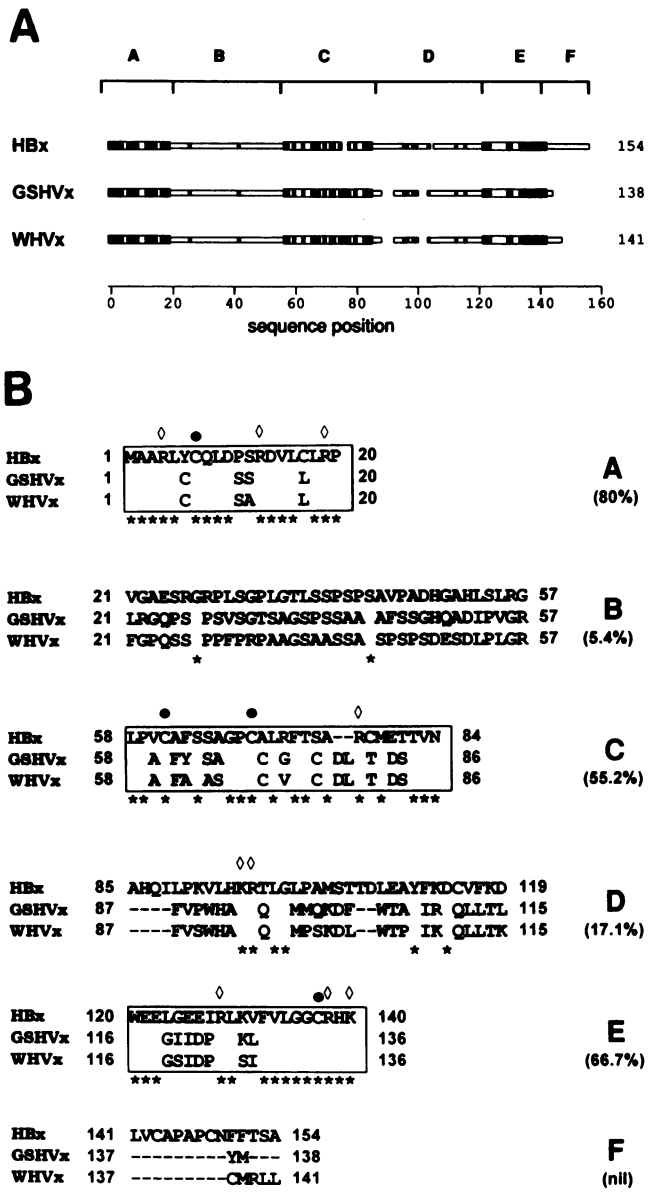

FIG. 1. Sequence homology of HBx (38), GSHVx (44), and WHVx (45). (*A*) Schematic alignment of the three X proteins were made by using MACAW software (46). Identical residues are represented as dark bands. Segments of higher homology—i.e., A, C, and E—are prominently shown while gaps in C and D regions are introduced to have maximal alignment. (*B*) Alignment of amino acid sequences of the three X proteins using the CLUSTAL program of PC/GENE. Only the nonidentical amino acids are shown in the GSHVx and WHVx sequences. Gaps (−) are introduced in order have maximum alignment of the identical amino acid residues (*). The HBx sequence (154 amino acids) was divided into six regions (A–F) based on its homology with GSHVx and WHVx and the percent homology of each region is shown in parenthesis on the right. The three most conserved regions (A, amino acids 1–20; C, 58–84 and E, 120–140) are boxed, and the four conserved cysteines (●) and the nine conserved basic amino acids (◇) are indicated.

no apparent homology, region F is highly variable in length and is longest (14 aa) in HBV.

HBx Expression Vector, HBx Mutagenesis, and Expression of the Mutants. The HBx gene (*adw* subtype) was PCR amplified and cloned into the pSG5 expression vector to get the X0 construct. All 15 mutants were constructed by using the X0 template. The four point mutants (X1–X4) and the 10 inframe deletion mutants (X5–X14) were constructed by site-directed mutagenesis (Fig. 2). The four conserved cys-

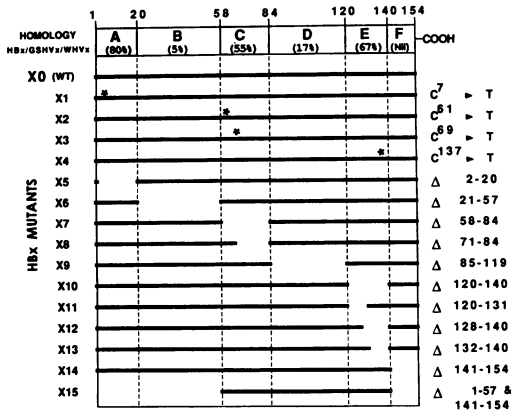

FIG. 2. Scheme of point and deletion mutants of HBx. The 154-aa-long HBx sequence has been divided into six regions (A–F) based on its homology with GSHVx and WHVx. Figures in parentheses represent extent of homology among X proteins of the three viral strains in individual regions (for details see Fig. 1). The point (X1–X4) and deletion (Δ) mutants (X5–X15) of HBx are shown schematically. The positions of point mutations in X1–X4 are shown by asterisks. In deletion mutants, a solid bar represents the portion of HBx present in the mutant, with the deleted portion represented as a gap. The broken vertical lines represent the boundaries of the six regions of homology (A–F).

teines (C^7, C^{61}, C^{69}, and C^{137}) were individually replaced by threonine residue in mutants X1–X4, respectively. Similarly, the six regions of HBx (i.e., A-F) were deleted in mutants X5, X6, X7, X9, X10, and X14, respectively. For a finer mapping of the transactivation domain of HBx, additional mutants were constructed for regions C (X8) and E (X11–X13). In mutant X8, the segment 71–84 of region C was deleted without affecting the two conserved cysteines C^{61} and C^{69}. In mutant X11, all the acidic amino acids of region E were deleted, whereas in X12 the three conserved basic amino acids along with C^{137} were deleted. The fully conserved stretch of nine amino acids in region E encompassing C^{137} was deleted in mutant X13. Finally, to explore if the regions of HBx identified by the above mutational study alone were sufficient for transactivation, a truncated mutant with only regions C, D, and E (X15) was constructed by PCR amplification.

Expression of the HBx mutants X1–X14 in transiently transfected COS 1 cells was verified by Western blot analysis by using the HBx-specific monoclonal antibody B-8/2/8 (Fig. 3*A*). All the mutants except X9 (lane 13) expressed a specific band of the expected size. The wild-type HBx (X0) construct expressed a protein of 16.5 kDa (lane 4) with a mobility similar to the X protein expressed in *E. coli* (lane 2). As expected, all the point mutants (X1–X4) showed a size similar to X0 (lanes 5–8) while all the deletion mutants (X5–X14) had smaller gene products (lanes 9–12 and 14–18). Mutant protein X6 with the largest deletion of 37 aa, showed the highest mobility (lane 10). No band was observed for X9 in lane 13 probably due to deletion of the epitope for the monoclonal antibody. However, expression of X9 was confirmed using an anti-X polyclonal rabbit serum (data not shown). Expression of mutant X15 could be verified only after metabolically labeling the transfected cells with [^{35}S]methionine followed by immunoprecipitation (Fig. 3*B*, lane 4). Higher steady-state level of X0 under identical experimental conditions (compare lanes 3 and 4) suggests the labile nature of X15 when expressed in mammalian cells. No such problem was observed when this mutant was expressed in *E. coli* (data not shown).

Localization of Region(s) Essential for Transactivation. Functional analysis of the HBx mutants was done by transiently transfecting them in Hep G2 cells together with the reporter construct, RSV–CAT that contains the LTR of RSV (43). The sensitivity of the assay was determined by transfecting increasing amounts of the reporter in the absence or presence of a fixed amount (1 μg) of X0 plasmid. X-mediated stimulation of CAT activity could be detected even when 0.1 μg of the reporter construct was used (Fig. 4*A*). Maximum stimulation (≈3-to 6-fold) of RSV–LTR by X0 was seen with 0.5 μg of RSV–CAT, which plateaued at higher concentrations of the reporter. Therefore, in all subsequent experiments, only 0.5 μg of RSV–CAT plasmid was used. Further, we also determined the activation curve for X0 and worked out the conditions under which functional analysis of the HBx mutants could be done without having a squelching effect. It was observed that with 0.5 μg of the transfecting RSV–CAT plasmid, the enzyme

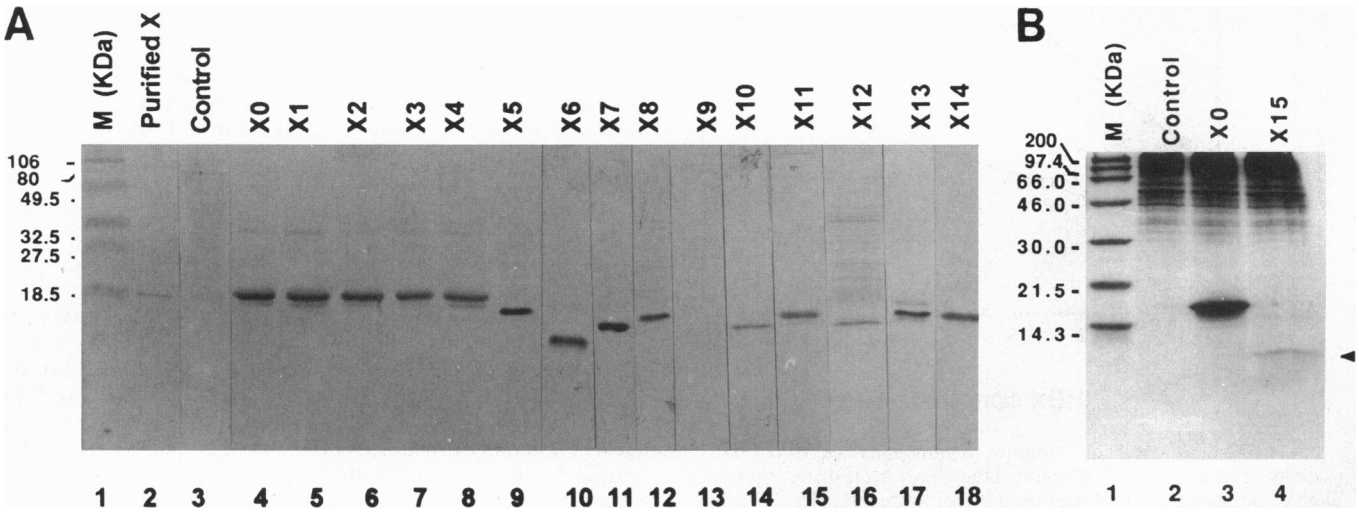

FIG. 3. Western blot analysis of the HBx mutants. (*A*) COS 1 cells were transfected (1 μgper 60-mm culture dish) with the wild-type (X0) and mutant HBx expression vectors (X1–X14) and total protein extracts were resolved by SDS/15% PAGE. Samples were electrotransferred onto nitrocellulose membrane and western blot analysis was performed using a monoclonal antibody. Purified X protein (expressed in *E. coli*) is shown in lane 2. Lane 3 (control) shows extract prepared from the pSG5-transfected cells. Cell extracts of the HBx constructs are run in lanes 4–18. Lane 1 shows Bio-Rad prestained markers (lane M). (*B*) COS 1 cells transfected with pSG5, X0 or X15 constructs were metabolically labeled with [^{35}S]methionine. Cell extracts were immunoprecipitated, electrophoresed in a 15% polyacrylamide/SDS gel, and autoradiographed as described. Lane 2 (control) represents cells that were transfected with pSG5. Position of the X15 gene product is shown by an arrowhead. ^{14}C-Labeled methylated markers (lane M; from Amersham) are shown in lane 1.

activity peaked (≈11-fold stimulation) in the presence of 5 μg of the cotransfecting X0 plasmid, but declined when 10 μg of the plasmid was used (Fig. 4*B*). Therefore, functional analysis of all the HBx mutants was carried out at three submaximal plasmid concentrations of 0.5, 1, and 2.5 μg.

The results of mutational analyses are presented in Table 1. We observed that deletion of the most conserved N-terminal region A (X5) had no effect on the HBx-mediated transactivation. Interestingly, deletion of the nonconserved regions B and F in mutants X6 and X14, respectively, also did not alter the levels of transactivation. However, a significant decrease in transactivation of about 50–80% was seen when the entire region C (X7) or its portion (X8) were deleted. Similarly, deletion of the poorly conserved region D (X9) also showed nearly a 50% drop in the transactivation level. The fully conserved nine amino acid segment (residues 132–140) of region E appeared crucial for the activity because all deletions involving this segment (X10, X12, and X13) resulted in a 5- to 10-fold decrease in activity. Surprisingly, deletion of the segment 120–128, which is rich in acidic residues (X11), resulted in only a marginal decline (up to 35%) in transactivation. These results suggest that the HBx segment spanning regions C to E (i.e., residues 58–140) is important for its transactivation function. Interestingly, in cotransfection experiments none of the inactive or poorly active mutants was able to suppress the X0 activity (data not shown).

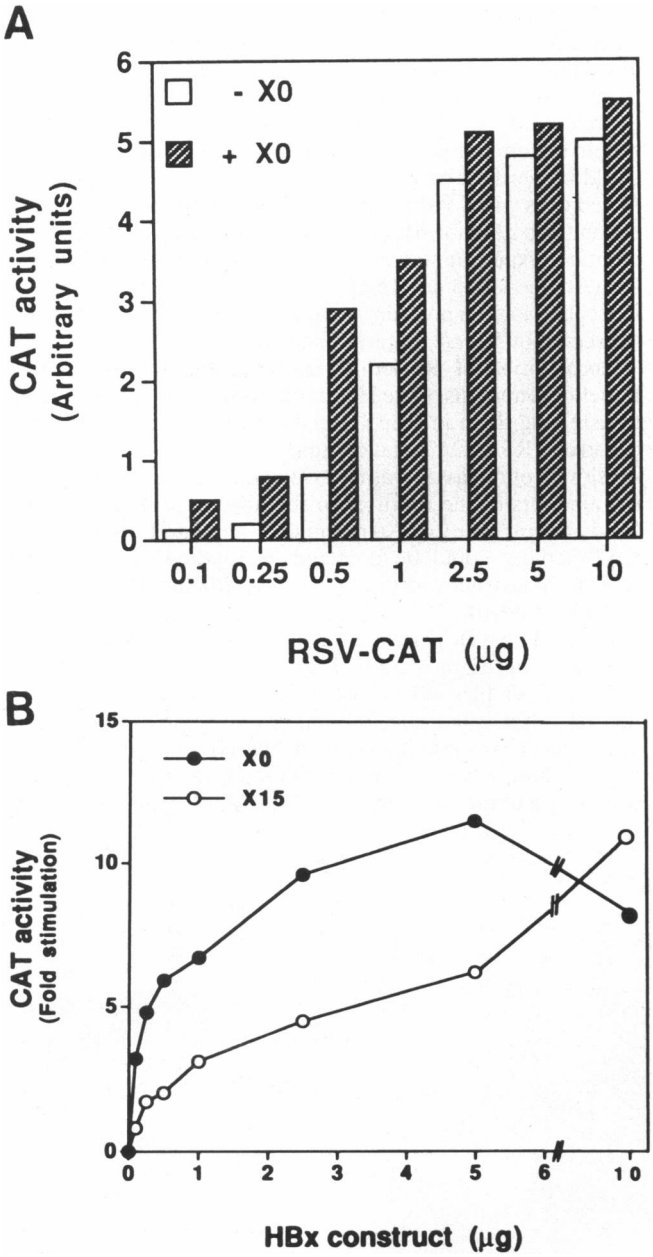

FIG. 4. Experimental conditions for the functional analysis of HBx mutants on RSV–LTR. (*A*) Amount of the reporter construct required to give optimum signal was measured by cotransfecting Hep-G2 cells with X0 (1 μg) and increasing amounts (0.1–10 μg) of the RSV–CAT plasmid. CAT assay was performed using cell extracts and the radioactivity was measured. Mean value of three observations is shown as bars. (*B*) Activation curves for X0 and X15 on RSV–LTR was determined by cotransfecting Hep G2 cells with RSV–CAT (0.5 μg) and increasing amounts (0.1–10 μg as indicated in *A*) of either X0 or X15 plasmids. After CAT assay, radioactivity was measured and plotted. Each point in the figure represents mean of two independent observations.

The C-Terminal Cysteine (C^{137}) Is Important for Transactivation Function. Involvement of the four conserved cysteines in the HBx-mediated transactivation was studied by using the point mutants X1–X4. We observed that substitution of the N-terminal conserved cysteine C^7 (X1) had no effect on the ability of the mutant to transactivate RSV–LTR (Table 1). Point mutations of the other three conserved cysteines—i.e., C^{61}, C^{69} and C^{137} (in X2–X4, respectively)—however, resulted in a dramatic loss (≈10-fold) in the transactivation property of HBx when 0.5 μg of the expression vectors were transfected. For X2 and X3, this effect was, however, partially overcome by transfecting more (2.5 μg) mutant plasmid. Mutant X4 remained inactive under similar conditions (see Table 1). Thus, the integrity of the conserved C-terminal cysteine C^{137} of HBx was essential for its transactivation function.

A Mutant with Only C, D, and E Regions Is an Efficient Transactivator. After identifying the regions of HBx that were involved in transactivation, we planned to explore the possibility of having a functional mutant with just the minimal activation domain of HBx. Thus, using PCR, we constructed a truncated mutant X15 with only regions C, D, and E (residues 58–140) and determined a transactivation curve for X15 by using the experimental protocols described for X0. Analysis of the curves (Fig. 4*B*) as well as subsequent comparative mutational analyses (Table 1) showed that X15 was able to stimulate the RSV–LTR quite efficiently. Although the level of stimulation was about 60% of the wild type when 0.5 μg of the X15 plasmid was used, the stimulation values were at par with each other when plasmid dosages were 1 and 2.5 μg, respectively. Cotransfection of X0 and X15 showed an additive effect on transactivation (data not shown). Apparently, the target interaction and the transactivation functions of HBx converge in the same domain represented by X15.

DISCUSSION

We have investigated the regions of HBx that are important for its transactivation function. Our mutational analysis has identified a segment in the C terminus of HBx corresponding to regions C, D, and E that is essential for its activity. We found that deletions involving either the entire E region (X10) or its fully conserved segment of nine amino acids (X13) as well as substitution of the conserved cysteine (C^{137}) of this region (X4) resulted in a drastic loss of function. The difference in the transactivation property is unlikely to be related to the expression levels because all the mutants appeared to be expressed with equal efficiency. Thus, HBx region E is crucial for its transactivation property. Likewise, deletion of the entire C region (X7) or its portions (X8) as well as substitution of the conserved cysteines C^{61} and C^{69} of this region (X2 and X3) were equally deleterious. There was, however, one important difference about the C-region mutants in that their effect could be partially overcome by transfecting more expression plasmids. Therefore it is unlikely that region C with two

Table 1. Relative ability of the HBx mutants to stimulate expression of RSV–CAT

HBx construct	% CAT activity by the amount of HBx constructs transfected, μg		
	0.5 (n = 5)	1.0 (n = 3)	2.5 (n = 4)
X0 (wt)	100	100	100
X1 ($C^7 \rightarrow T$)	101.3 ± 8.4	102.0 ± 3.8	110.1 ± 9.0
X2 ($C^{61} \rightarrow T$)	12.6 ± 3.7[a]	5.5 ± 2.6[a]	48.6 ± 6.1[a]
X3 ($C^{69} \rightarrow T$)	5.4 ± 2.0[a]	8.0 ± 3.5[a]	33.4 ± 9.1[a]
X4 ($C^{137} \rightarrow T$)	6.7 ± 3.7[a]	10.3 ± 3.2[a]	14.6 ± 6.9[a]
X5 (Δ2–20)	105.1 ± 10.6	105.5 ± 3.9	98.8 ± 12.7
X6 (Δ21–57)	94.8 ± 8.9	100.2 ± 17.3	88.0 ± 9.2
X7 (Δ58–84)	56.2 ± 16.1[d]	18.3 ± 8.4[b]	37.8 ± 11.1[b]
X8 (Δ71–84)	19.5 ± 8.3[a]	21.7 ± 8.3[b]	26.3 ± 8.7[a]
X9 (Δ85–119)	51.2 ± 12.8[b]	11.7 ± 3.8[a]	38.3 ± 16.8[c]
X10 (Δ120–140)	9.6 ± 4.0[a]	13.7 ± 4.7[a]	20.8 ± 10.4[a]
X11 (Δ120–131)	76.7 ± 27.1	66.3 ± 3.5[a]	91.3 ± 8.9
X12 (Δ128–140)	8.3 ± 3.6[a]	10.1 ± 5.5[a]	12.6 ± 4.7[a]
X13 (Δ132–140)	9.9 ± 2.9[a]	9.6 ± 2.9[a]	15.9 ± 4.9[a]
X14 (Δ141–154)	103.6 ± 7.7	106 ± 13.9	104.5 ± 9.4
X15 (Δ1–57 and 141–154)	57.2 ± 9.2[b]	85.0 ± 6.7	94.7 ± 3.5

The table is a compilation of data from 12 independent experiments. In each experiment, a subset of mutants was compared to X0. Hep G2 cells were cotransfected with 0.5 μg RSV–CAT and three different amounts of the HBx mutant expression plasmids. Cells were cultured for 48 h, and CAT activity was measured. The values are the percentage CAT activity obtained against X0 and are expressed as mean ± SEM. The number of independent observations is shown in parenthesis against each concentration of the mutant plasmids tested. The levels of significance were determined by using the unpaired t test: a, $P < 0.001$; b, $P < 0.005$; c, $P < 0.01$; and d, $P < 0.025$.

conserved cysteines plays a crucial role in transactivation. Using the *E. coli*-expressed X protein, a disulfide linkage has been assigned between residues C^{61} of region C and C^{137} of region E (47). The structural and functional relevance of this intramolecular linkage remains to be established. The poorly conserved region D may not play an important role in transactivation because deletion of this region (X9) did not obliterate the activity. Although, the ATPase activity of HBx maps to this region (33), its relevance in transcriptional regulation is not known. Nevertheless, the epitope for the monoclonal antibody used in this study appears to be localized in region D because it failed to recognize mutant X9. It was puzzling, however, to observe that deletion of the most conserved region A (X5) or mutation of the N-terminal conserved cysteine (X1) had no bearing on the transactivation function of HBx. Recent reports suggest that region A may have a role in transrepression (48–50). The poorly conserved region B (X6) and nonconserved region F (X14) are unlikely to have a major role in transactivation.

Mutational analyses of HBx using different rationales and approaches have been attempted by several groups. Using linker insertion mutagenesis, Runkel *et al.* (51) were able to localize two separate regions of HBx that were important for transactivation (one around amino acid 68 and the other between segments 110 and 139). Other reports on the mutational studies of HBx (13, 48, 49, 52) suggest that its transactivation region is localized toward the C-terminal region. However most of these analyses are based primarily on the loss of reporter function. In the present study, we have mapped the "minimum functional domain" of HBx that could show transactivation function. On the basis of our results of deletion and point mutagenesis, a highly truncated mutant X15 (with only regions C, D, and E) was constructed. Although the gene product was relatively unstable in mammalian cell lines, it was able to show a transactivation property like the wild-type HBx. Thus, segments 58–140 or regions C–E of HBx is the minimum domain involved in transactivation function. Interestingly, two important sequence regions of HBx (i.e., C and E, identified by us) also map to the regions that interact the RPB5 subunit of RNA polymerase (28) as well as a serine protease (30). These interactions have been proposed to influence the transactivation function of HBx. The role of region E in transactivation is further substantiated by the fact that the TATA-binding protein, which is considered central to transcription regulation, also interacts with HBx through this region (24).

Further, we find an interesting correlation of our work with the naturally occurring variants of HBV (usually sero-negative by ELISA). DNA sequence analysis of some of these variants has shown that very often their genomes have deletions or premature "stop" codons around region E of the X gene (6, 53–55). The presence of a mutated X protein in those cases might be responsible for a subdued expression of the HBV genes. Yet another interesting possibility could be the integration of a minimum transactivation region (similar to X15) of the HBx gene in HBV carriers. Analysis of patient sera using a sensitive ELISA based on this region and/or PCR amplification of this region of the X gene should provide interesting results and more insight into HBV-related pathogenesis.

We thank D. Sahal and V. L. Kumar for helpful discussions, S. Jameel for plasmids pET-X and HBV (*adw* subtype) and critical reading of the manuscript, and J. A. Wolff for the RSV–CAT construct. Technical assistance was provided by Honey V. Reddi, and A. Bhardwaj synthesized the oligonucleotides. This work was supported by an internal fund of the International Centre for Genetic Engineering and Biotechnology.

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