Human Hepatitis Virus X Gene Encodes a Regulatory Domain That Represses Transactivation of X Protein*

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Seishi Murakami‡, JaeHun Cheong‡, and Shuichi Kaneko†

From the ‡Department of Biophysics, Cancer Research Institute, and the †First Institute of Medicine, Medical School, Kanazawa University, Takara-Machi 19-1, Kanazawa 920, Japan

The human hepatitis B virus (HBV) X gene seems to be essential for establishment of viral infection, and the X gene product, HBx, transactivates virus and host genes through a wide variety of cis-elements, whereas regulation of HBx has not been fully understood. We found that transactivation-negative HBx mutants truncated at the C-terminal portion specifically repressed the HBx transactivation in trans. The ability to trans-repress the HBx transactivation is confined to the N-terminal third of HBx. Transactivation-positive constructs of HBx were divided into two groups by their sensitivity to trans-repression due to the presence of the N-terminal third. Thus the regulatory domain, the N-terminal third, is separated from the transacting domain and responsible for the negative regulations, the trans-repression and sensitivity to X trans-repression. A possible direct association between the HBx regulatory domain was tested by far-Western blotting using purified fused forms of HBx proteins. The regulatory domain was found to associate preferentially with the full HBx or the regulatory domain, but not with the transacting domain. Taken together, it is possible that HBx has a self-regulatory mechanism that avoids excessive HBx transactivation and is important for regulation of X gene expression.

*Plasmid Constructions—A plasmid, pSG5UTPL, was used as mammalian expression vector (9). Truncated versions of X were constructed by a polymerase chain reaction cloning method using a pair of primer oligonucleotides, GCGAATTCCATG, fused to the sequence of X ORF, generating an artificial EcoRI site and an initiation codon, and a primer oligonucleotide with a BglII site and a termination codon fused to the complementary sequence of X ORF, BSXH-1, a wild HBx expression plasmid, was used as a template for the polymerase chain reaction cloning (8). HX-3D36 and HX-3D39 were derived from a series of C-terminal truncated HBx. pSGHX-1 was digested with BglII, processed by a kilobase deletion kit according to the manufacturer’s instruction (Takara Shuzo Co. Ltd.), and finally digested with EcoRI. Fragments shorter than the intact size were collected, purified, and ligated to EcoRI and Smal digest of pSG5UTPL. The size of the inserted and the transacting ability of a series of mutants were characterized. Two clones, HX-3D36 and HX-3D39, represent transactivating and non-transactivating clones in the series, respectively. HX-3D36 and HX-3D39 have an extra 4 and 6 amino acid residues at the C-terminal, respectively, derived from the pSG5UTPL sequence, which has a universal terminator. All mutants described here were sequenced using a T7q sequence primer kit and a model 370A DNA Sequencer (Applied Biosystems). The chloramphenicol acetyltransferase (CAT) reporter plasmids were derived from pUAPI1CAT (31). A dimerized 23-bp sequence in the center of the 33-bp sequence strongly conserved among mammalian hepadnavirus genomes was inserted into the Smal 1 site of pUAPI1CAT (31).

We constructed two different expression vectors of glutathione S-transferase (GST) fusion protein, pGENT2 and pGENK1, derived from pGEX-1N (Amrad Co. Ltd., Kew Victoria, Australia) (32). The DNA sequence between the BamHI and EcoRI sites of pGEX-1N was replaced by oligonucleotide DNA fragments, so that pGENT2 has a thrombin site followed by EcoRI and BamHI sites and pGENK1 has an additional consensus sequence containing a phosphorylation site for the cAMP-dependent kinase between the thrombin site and the cloning sites of pGENT2 (33). The EcoRI and BglII fragment from the HBx construct derived from pSG5UTPL was inserted into the cloning sites of pGENT2.
and pGENK1 to purify fused forms of the full and truncated HBxs from E. coli transfectants.

Transfection and CAT assay—Transient transfection and CAT assay were carried out as described previously according to the method of Chen and Okayama (9, 34). A human hepatoma cell line, HepG2, was used to provide recipient cells for transfection. The cells were cultured in modified Dulbecco’s medium supplemented with 10% fetal calf serum in a 35-mm cell culture dish (Corning Co., Ltd.) as reported previously (9). Reporter plasmid (6 ng) and varying amounts of transactivators and pSGUTPL (total 5 ng) were added to the cultured cells. Cell lysates were harvested 48 h after DNA transfection. The CAT assay was carried out according to methods described previously (9) and incubated for 30 min at 37°C using approximately 20 μg of transfected cell lysate protein. CAT activities were measured as the conversion rate of 14C-chloramphenicol (Amersham Corp.) into acetylated forms, using a BA100 Bioimage Analyzer (Fuji Co., Ltd.).

Western Blotting with Lysates of Transfected HepG2—HepG2 cells were transfected with varying amounts of HBx expression plasmids and 5 μg of the reporter DNA under the same conditions as transfection for CAT assays. Cells were harvested 2 days after transfection, and washed with phosphate-buffered saline. Cell pellets in microcentrifuge tubes were reuspended in 150 μl of LAC buffer, consisting of 50 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 9 mM CHAPS, 10% glycerol. After sonication, lysates were kept on ice for 5 min, and subjected to 15% SDS-PAGE electrophoresis. Samples were precipitated with 5% trichloroacetic acid (final concentration) on ice. Precipitates were collected by centrifugation, washed with phosphate-buffered saline and dried, dissolved with sample-loading buffer, heated for 3 min, and subjected to 15% SDS-PAGE. Supernatants were collected and stored at -80°C. The gels were transferred to nitrocellulose filters with 0.22-pm pore size (Schleicher & Schuell) using the transfer buffer consisting of 25 mM Tris-HCl (pH 8.3), 190 mM glycine, 0.1% methanol, for 2-4 h. Proteins were electrophoretically transferred to nitrocellulose filters with 0.22-μm pore size (Schleicher & Schuell) in TBST buffer consisting of 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.05% Tween 20. Anti-HBx IgG and anti-HBx-5D1 IgG had been purified from serum of rabbits immunized with purified recombinant GST-HBx and GST-HBx-5D1 (51-154) fusion proteins, respectively.2 The filters were sequentially treated with anti-HBx IgG (12 ng/ml final concentration) or anti-HBx-5D1 IgG (10 ng/ml final concentration) for 1 h, protein A-conjugated horseradish peroxidase (1/15,000 dilution) and finally subjected to chemiluminescence reaction, according to the manufacturer’s instructions (Amersham Corp.). Western Blotting with Lysates of Transfected HepG2 was performed with a labeled probe (100 ng/ml protein, 1 x 10^6 cpm/μg protein) in modified GBT buffer (10% glycerol, 50 mM Hepes-NaOH (pH 7.5), 7.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1% Triton X-100) supplemented with 1% of bovine serum albumin and 1 mg/ml sonication supernatant from E. coli transfected with pGENK1. The filters were then washed and equilibrated with GBT buffer. Purified fusion proteins containing the phosphorylation site were labeled by γ-32P]ATP using the catalytic subunit of cAMP dependent protein kinase (Sigma) according to the method reported previously (32). Protein-protein binding reaction (far-Western) was performed with a labeled probe (100 ng/ml protein, 1 x 10^6 cpm/μg protein) in modified GBT buffer (10% glycerol, 50 mM Hepes-NaOH (pH 7.5), 150 mM KCl, 7.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1% Triton X-100). Filters were rotated in the binding mixture for 30 min at room temperature, washed three times with GBT buffer, and exposed to imaging plates (Fuji Co.) or x-ray films (XAR-Omat, Kodak).

RESULTS

The N-terminal Third of HBx Is dispensable for Transactivation—HBx expression vectors in mammalian cells were constructed in order to delineate the transacting domain of HBx in a transient expression system (Fig. 1, left). A hepatoma cell line, HepG2, was co-transfected with a HBx expression plasmid and a CAT reporter, pHEC2CAT, which harbors the core of HBV enhancer 1 and the SV40 early promoter upstream of the CAT gene of E. coli. The plasmid pSGHX-1, expressing the full HBx, transactivates the CAT activity by more than 15-fold (Fig. 1, right). The HBx mutants truncated either at the N-terminal or the C-terminal portion were examined for their transacting ability (Fig. 1). The result showed that HBx mutants truncated for transacting function is between 136 and 148, since HX-D12 (51-154) had transactivating function, whereas HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function as summarized in Fig. 1. The two margins of the C terminus necessary for transacting function is between 136 and 148, since HX-D12 (51-154) had transactivating function, whereas HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function. The margin of the C terminus necessary for transacting function is between 136 and 148, since HX-D12 (51-154) had transactivating function, whereas HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function. The result showed that HX-5D1 (51-154) had transactivating function, and HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function. The result showed that HX-5D1 (51-154) had transactivating function, whereas HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function. The margin of the C terminus necessary for transacting function is between 136 and 148, since HX-5D1 (51-154) had transactivating function, whereas HX-5D4 (72-154) had none. HX-D12 (51-154) was less efficient, but exhibited transacting function.

The N-terminal Third of HBx Represses the HBx Transactivation—During delineation of the HBx transacting domain, we noticed that large amounts of pSGHX-1 DNA decreased the

Fig. 2. The N-terminal third of HBx has a trans-repressing activity. HepG2 cells were transfected with varying amounts of transactivator plasmid(s) and 5 μg of a CAT reporter, pHECxxCAT. Total amount of transactivator DNA was adjusted to 5 μg with pSG5UTPL. Squelching effects are observed with excess amounts of HX-1 but not with HX-5D1. Lanes 1-5 contain varying amounts of HX-1 (0, 1, 2, 3, and 5 μg, respectively), lanes 6-10 contain varying amounts of HX-5D1 (0, 1, 2, 3, and 5 μg, respectively), and lanes 11 and 12 contain HX-5D3 (3 and 5 μg, respectively). Lanes 13-15 and lanes 16 and 17 are 1 μg of HX-1 and HX-5D1, respectively, in addition to varying amounts of HX-3D5 (1, 2, and 4 for lanes 13-15, and 2 and 4 μg for lanes 16 and 17, respectively). Transfection and CAT assay were as in Fig. 1. Conversion rates of acetylated chloramphenicol are shown beneath the lane numbers.

Transactivation (Fig. 2, lanes 2-5). This squelching effect was not observed with HX-5D1, in which the N-terminal third was deleted (Fig. 2, lanes 7-10). This result suggested that the N-terminal third had some negative effects. To test this possibility, HepG2 cells were transfected with constant amounts of the CAT reporter plus pSGHX-1 and varying amounts of HX-3D5 (1-50), which encodes the N-terminal third only. As shown in Fig. 2, HX-3D5 had no transactivating activity but repressed the transactivation with the full HBx in trans (lanes 11-15). In contrast, HX-3D5 did not repress the transactivation by HX-5D1 (51-154) (Fig. 2, lanes 16 and 17). Therefore, transactivation-positive HBx constructs were tested for sensitivity to the trans-repression by HX-3D5 (Fig. 3c). The result indicates that the constructs, HX-5D1 and 5D12 (72-154), missing the N-terminal third are resistant to trans-repression. Thus, transactivation-positive constructs may be divided into two groups according to sensitivity toward trans-repression (summarized in Fig. 7).

To delineate the trans-repressing function, transactivation-negative constructs were examined for their ability to repress the HBx transactivation (Fig. 3, a and b). Constructs missing the N-terminal third of HBx exhibited no trans-repression. This result indicates that the trans-repression activity is not a general property of transactivation-negative HBx mutants, but a unique property of constructs containing the N-terminal third of HBx. The results shown in Figs. 2 and 3 indicate the presence of a regulatory domain at the N-terminal third of HBx, which is separated from the transactivation domain. The essential region for the trans-repressing function coincides with the domain necessary for the sensitivity to the trans-repression. The squelching effect observed with the full HBx is also dependent upon the presence of the regulatory domain (data not shown). The full activity of the negative regulations seems to require the regulatory domain of HBx, since the transactivation of HX-5D3 (21-154) is partially sensitive to trans-repression (see Fig. 3c and “Discussion”).

Next, we tested whether the regulatory domain of HBx specifically represses the HBx transactivation or has a more general inhibitory effect. Human T cell leukemia virus 1 (HTLV1) Tax protein is known to transactivate both HBV Enh1 and the enhancer in HTLV1 LTR, which is also responsive to HBx (6, 10, 15) (Fig. 4, a and b). Co-transfection of increasing amounts of HBX-3D5 repressed the HBx transactivation of HBV Enh1 and also that of HTLV1 LTR in a dose-dependent manner. The transactivation by Tax was unaffected by excess amounts of HBX-3D5 (Fig. 4, a and b). The endogenous CAT activity of pSV2CAT was insensitive to HBX-3D5 (Fig. 4c). These results indicate that the regulatory domain of HBx specifically represses the transactivation of HBx but has no inhibitory effect on the Tax transactivation or the endogenous enhancer activity.

Expression Levels of HBx Proteins in Transiently Transfected Cells—The construct expressing the regulatory domain, HX-3D5, represses transactivation of HBx when examined by CAT activity of the reporter in a co-transfection assay. We addressed the question whether the observed trans-repression occurs at the level of HBx function or at the level of HBx expression. Therefore HBx proteins were measured in lysates of HepG2 cells transiently transfected under the same experimental conditions as used in the CAT assay. As shown in Fig. 5a, proteins of the full HBx, the transacting domain (51-154, derived from HX-5D1), and the regulatory domain (1-50, derived from HX-3D5) were detected by Western blotting using anti-HBx rabbit IgG. The amounts of each HBx protein expressed depended on the amounts of DNA added. Approximately similar levels of HBx protein bands were detected in cells transfected with varying amounts of HBx-5D1 or HBx-3D5, along with a constant amount of pSG5HX-1. Since the polyclonal anti-HBx IgG has different immunoreactivities against the regulatory domain and the transacting domain (Fig. 5b), the HBx 5D1 (51-154) bands were stained faintly with anti-HBx IgG (Fig. 5a). Western blotting using anti-HBx-5D1 IgG showed that similar amounts of HBx and HBx-5D1 were present in the lysates of co-transfected HepG2 cells (Fig. 5c). The presence of equal levels of the three HBx proteins in the lysates of transfected cells eliminates the possibility that the regulatory domain affects expression or stability of the transacting proteins in the transfected cells.

Specific Binding of the Regulatory Domain of HBx—Since the HBx regulatory domain is crucial, not only for the trans-repression but also for the sensitivity toward trans-repression, the regulatory domains may interact for the negative function. To test this possibility, fused forms of GST and the full or truncated HBx proteins were expressed in E. coli. We constructed two types of GST-HBx expression plasmids to purify fused forms of HBx proteins. One of these contains the consensus sequence of a phosphorylation site for cyclic AMP-depend--
FIG. 3. Delineation of trans-repression activity and sensitivity to the trans-repression. Trans-repressing activities of HBx constructs on the transactivation by HX-1 (the full-size HBx) are shown in a and b. HepG2 cells were transfected with pHECx2CAT (5 μg), HX-1 (1 μg), and 4 μg of truncated HBx construct or vector (pSG5), as indicated. The results of the constructs without and with the N-terminal third of HBx are shown in a and b, respectively. All constructs in a and b are transactivation-negative, as shown in Fig. 1. Sensitivity to the trans-repression by HX-3D5 was examined with transactivation-positive constructs, as shown in c. HepG2 cells were transfected with the CAT reporter (5 μg), HX-3D5 (4 μg), and 1 μg of truncated plasmid as indicated. Transfection and CAT assay were carried out as in Fig. 1.

FIG. 4. Specificity of the trans-repression activity of the N-terminal third of HBx. HepG2 cells were cotransfected with the reporter indicated (5 μg), the transactivator (pSGHX-1 or pSgtax), and varying amounts of HX-3D5 as indicated. The reporters used were pHECx2CAT, HTLV1LTRCAT, and pSV2CAT in a, b, and c, respectively. Transfection and CAT assay were performed as in Fig. 1.

FIG. 5. Detection of transiently expressed HBx proteins in transfected HepG2 cells. a, HepG2 cells were transfected as in Fig. 1 with 5 μg of pHECx2CAT and varying amounts of HBx constructs as indicated. Total amount of DNA was adjusted to 10 μg by pSG5UTPL. Proteins in the cell lysates were prepared and fractionated by 15% SDS-PAGE. HBx proteins were detected by Western blotting using polyclonal anti-HBx IgG, as described under “Materials and Methods.” Positions of the full HBx, the transacting domain (HX-5D1 protein), and molecular markers are indicated. b, varying amounts of GST-fused forms of HBx proteins (lanes 1–10) were fractionated by 12.5% SDS-PAGE. Western blotting was carried out using anti-HBx IgG (lanes 1–5) and anti-HBx-D1 IgG (lanes 6–10) as in a. Approximately 100, 50, 20, 10, and 5 ng of each protein were mixed and loaded in lanes 1 and 6, lanes 2 and 7, lanes 3 and 8, and lanes 4 and 10, respectively. The positions of fused forms of HBx proteins and GST are indicated. c, Western blotting of the HepG2 transfected cell lysates detected by anti-HBx-5D1 IgG. The HepG2 lysates were fractionated by 15% SDS-PAGE, and Western blotting was carried out using anti-HX-5D1 IgG. The lysates, identical to those used in Fig. 6a, applied in lanes 1–3 are from the cells transfected with 1 μg of pSGHX-1, 1 μg of pSGHX-5D1, and 1 μg of pSGHX-1 plus 4 μg of pSGHX-5D5, respectively. Amounts of HBx and HBx-D1 in lanes 1–3 are approximately 5, 7, and 5 ng, respectively.
Forms of HBx from E. coli, as indicated at the bottom of the figure, were applied and stained with Coomassie Brilliant Blue (a), or proteins were electrically transferred to nitrocellulose membranes and subjected to far-Western blotting (b-d). The labeled probes are indicated at the top of the figure. Expression, purification, and phosphorylation of GST-fused forms of HBxs are described under "Materials and Methods."

**FIG. 6. Association of the regulatory domain of HBxs.** Purified GST-fused forms of HBx from *E. coli*, as indicated at the bottom of the figure, were applied and fractionated by 15% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (a), or proteins were electrically transferred to nitrocellulose membranes and subjected to far-Western blotting (b-d). The labeled probes are indicated at the top of the figure. Expression, purification, and phosphorylation of GST-fused forms of HBxs are described under "Materials and Methods."

**FIG. 7. Summary of the domain analysis and far-Western blotting.** a, transactivation, trans-repression, sensitivity to trans-repression, and relative strength of binding activity to HBx are summarized from the results of Figs. 1-4 and 6 (nd, not determined). b, the domain structure of HBx. The Ser/Pro-rich region (21–50) is the minimal region necessary for the strong homologous association (see "Discussion").

The regulatory domain of human hepatitis B virus X protein (GSTHX-3D5:1–50) but bound very weakly to the trans-acting domain (GSTHX-5D1:51–154). Since no binding was detected with GSTHX-5D4 (72–154) or GST protein (Fig. 6b), specific interactions could be demonstrated under the binding conditions used here. The regulatory domain probe bound to the full HBx, HX-5D3, and HX-5D1 fusion proteins, but not to the transacting domain (HX-5D1 fusion protein) as shown in Fig. 6c. In the binding assays HX-5D3 protein (21–154) was detected by the full HBx, as well as by the regulatory domain. This result indicates, that the essential region for the interaction of the regulatory domains (GSTHX-3D5:1–50) is within the Ser/Pro-rich region spanning residues 21–50 of HBx. The labeled full HBx, but not the regulatory domain, could associate weakly with the transacting domain (HX-5D1), demonstrating that the transacting domain may have a weak binding activity to the homologous domain. This weak association was proved by the binding of the labeled transacting domain (Fig. 6d). The results of the far-Western assay indicate that HBx association consists of two homologous interactions between the regulatory domains and the transacting domains. The former interaction is a major contribution to the HBx association, since the HBx band obtained using the full HBx probe and that obtained with the regulatory domain have similar intensity, while the transacting domain showed only a faint band with the full HBx probe. Taken together, these results suggest that the negative regulation operates through interaction between the regulatory domains of X proteins.

**DISCUSSION**

The X gene is conserved among mammalian hepadnaviruses and has been shown to be essential for viral multiplication in the woodchuck (2, 3). HBx transactivates not only virus genes but also host genes that are essential for cell proliferation and inflammatory responses (4–17). Therefore it is believed that the oncogenic role of HBx may be associated with the transacting function of X protein (11, 21–23). However, the X expression is very low or barely detectable in the livers of chronic HBV carriers, both in man and in the woodchuck. This is in contrast to the high expression levels in liver tumors and hepatoma cell lines (24–27). These discrepancies suggest the presence of a regulatory mechanism preventing overproduction of HBx in livers of chronic HBV carriers.

The squelching effect using HBx has been reported (6), but the presence of the regulatory domain in HBx was not previously recognized. Our results clearly show that the N-terminal third of X gene has a distinct function. The evidence that the N-terminal third of the X gene is well conserved among mammalian hepadnavirus genomes (2) implies a biological significance for the region. The nature of this biological function has not previously been elucidated. The N-terminal one third is necessary for the negative regulations detected by the trans-repression experiments, as well as the sensitivity to trans-repression, as summarized in Fig. 7a. Thus HBx encodes two separate functional domains, the regulatory domain and the transacting domain (Fig. 7b). The regulatory domain of HBx did not repress the CAT activities of the HBV Enh1 core sequence or HTLV1 LTR transactivated by HTLV1 Tax protein. Similarly, the regulatory domain of HBx failed to affect the endogenous enhancer activity of pSV2CAT. Thus, the negative effect of the regulatory domain is not a general inhibition or cell toxicity, but specifically interferes with the HBx transactivation. Since similar protein levels of the full and truncated HBx were detected by Western blotting in lysates of cells co-transfected under conditions similar to those used in the CAT assay, the presence of excess amounts of the N-terminal third of HBx had no effect on expression or stability of the full or truncated HBx proteins. Therefore, the regulatory domain seems to inhibit the HBx function, specifically transactivation.
The Ser/Pro-rich region in the regulatory domain (21–50) is indispensable for the negative regulations and for the specific association of the regulatory domains. This region is less conserved than amino acid sequence 1–20 of HBx, but most amino acid substitutions in this region are among Ser, Pro, and Thr residues. As HX-5D3, lacking the first 20 amino acids, exhibits an intermediate property with respect to sensitivity to trans-repression, the first 20 amino acids in the sequence might contribute to the negative regulation, although it is dispensable for dimerization of the regulatory domain as detected by far-Western blotting. Further analysis is necessary to understand why the negative regulation requires not only the association of the regulatory domain but also the N-terminal 20 amino acids of HBx. Clear evidence for the biological role of X protein has been obtained in an experimental animal model (3) and in transgenic mice (21). However, only the full HBx or X proteins mutated in the C-terminal region were examined (3, 21). Therefore the biological role and the mechanism of the negative regulation of the N-terminal third of HBx still remain to be addressed.

We used the CAT reporter, pHBCx2CAT, which has a dimerized 23-bp sequence in the Enhl core (31). Within the core there is a 33-bp sequence that is highly conserved among mammalian hepatadnavirus genomes and contains an AP-1 related site (31). The cis-element, TGACGCAA, is not a typical AP-1 site, but it is the same as that flanking the serum responsive element in the Fos gene (32). The AP-1 site is crucial for the enhancer activity and the HBx transactivation (6, 29). Therefore, the core of Enh1 harbors an X-responsive element and Enh1 controls HBx expression (5, 13, 27–30, 37). In this context, the X gene seems to be under autoregulation (38, 39). The regulatory domain of HBx may be crucial for the negative regulation that avoids overexpression of HBx.

Several stimuli, such as phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, and retinoic acid, augmented the enhancer 1 activity (29, 37, 40). HBx transactivation could synergize with some of these stimuli (37, 40). In hepatic tumors or hepatoma-derived cells, regulation of X expression may be altered so that high level of X protein ensues. The mechanism and modulation of X gene regulation must be better elucidated to understand the role of HBx in inflammation and oncogenesis. To this end, cloning of host proteins interacting with the transacting domain or the regulatory domain of HBx would yield substantial information.

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