Transcriptional trans-Activating Function of Hepatitis B Virus

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The ability of hepatitis B virus (HBV) to stimulate the expression of a cellular gene was investigated by using a transient-expression system. A plasmid in which the expression of the bacterial chloramphenicol acetyltransferase (cat) gene had been placed under the control of the DNA sequences that regulate the expression of the human beta-interferon gene was constructed. In Vero cells, cotransfection of the 2.7-kilobase BglII DNA fragment of HBV together with the test plasmid containing the cat gene resulted in stimulation of the expression of the cat gene. This HBV DNA fragment was specific in its trans-activation; no significant stimulation of CAT activity was observed in constructs when the promoter and enhancer elements were derived from the murine sarcoma viral long terminal repeat, Rous sarcoma virus, BK virus, or simian virus 40. Results of subcloning of the HBV DNA fragment indicate that the trans-activating function resides in a 944-base-pair EcoRV-BglIII DNA fragment of the HBV genome that contains the X structural gene and its promoter element. Removal of the promoter from the X structural gene resulted in loss of the trans-activating function. A frameshift mutation within the X gene region also eliminated the trans-activating activity. These results suggest that the X antigen could play a role in HBV infections by activating the expression of cellular genes.

Hepatitis B virus (HBV) is a small, enveloped virus that contains a partially double-stranded DNA genome (40). The DNA sequences of the HBV genomes isolated from various patients are heterogeneous (41), but the genomes have always been found to consist of four open reading frames (ORF). One of these regions codes for the HBV surface antigen (5, 43), and another encodes the HBV core antigen (32). The ORF that codes for the HBV core antigen also contains the information for the HBV e antigen (24). The largest ORF encompasses most of the genome and presumably encodes the DNA polymerase (41). The fourth and the smallest ORF, designated X, could specify a protein of approximately 154 amino acids (39), but to date, no HBV protein of this size has been identified in HBV-infected cells.

Inspection of the nucleotide sequence upstream from the 5' end of the ORF X revealed the absence of the typical consensus TATA box, which is usually found about 30 base pairs (bp) from the RNA cap site (4). However, an RNA species of approximately 900 nucleotides in length that hybridizes to the ORF X has been detected in rodent cells transfected with HBV DNA (15). Furthermore, a region that is 17 to 408 bp upstream from the ORF X region has been shown to contain a promoter element capable of regulating the expression of the bacterial chloramphenicol acetyltransferase (cat) gene (42).

A fusion protein containing 145 amino acids encoded by ORF X of HBV has been expressed in Escherichia coli, and this fusion protein reacts to human sera of individuals infected with HBV but not to normal human sera (28). Feitelson (9) demonstrated that antibodies raised against synthetic peptides representing domains of the putative X protein react with core particles isolated from the liver of an HBV-infected individual. Recently, Siddiqui et al. (37) detected, in mammalian cells transfected with the HBV ORF X, a protein that reacts with antiserum prepared against synthetic X peptides. These results suggest that the X gene region codes for a protein that is probably expressed during infection with HBV.

However, a biological function for the X antigen during infection with HBV is unknown. In this communication, we present evidence indicating that a fragment of the HBV genome trans-activates the regulatory region that governs the expression of a eucaryotic gene, the human beta-interferon gene. Results of subcloning and frameshift mutagenesis indicate that a trans-acting product of the X gene region is responsible for the activation.


MATERIALS AND METHODS

Plasmids. All constructs were made by using standard recombinant techniques (26), and pUC9 was used as the vector. The adw subtype of HBV was used in this study. The 2,755-bp BglII fragment of HBV DNA (map position [mp], 2429 to 1984) was cloned into the BamHI site of the vector to yield pTWU14. The 2,755-bp BglII segment of HBV DNA was subcloned by complete digestion with EcoRV; the resulting two fragments were separated by agarose gel electrophoresis. The 1,811-bp BglII-EcoRV fragment (mp, 2429 to 1040) and the 944-bp EcoRV-BglII fragment (mp, 1040 to 1984) were cloned into the HindII-BamHI region of pUC9, creating pTWU16 and pTWU17, respectively.

To remove the region of HBV DNA containing the promoter for the X gene, a pUC9 plasmid derivative containing the 2,755-bp BglII HBV DNA insert was digested with HindIII and NcoI. This digestion resulted in the removal of both the EcoRV-NcoI fragment (mp, 1040 to 1372) of HBV DNA that contains the promoter for the X gene and the HindIII-HindII fragment of the pUC9 vector. The resulting linearized plasmid was blunt ended, and the ends were converted to HindIII sites by the addition of HindIII linkers. The plasmid was then ligated and termed pTWU18. To place the HBV ORF X under the control of the simian virus 40 (SV40) early promoter and enhancer elements, a 352-bp HindIII fragment containing the SV40 early promoter and enhancer elements was inserted at the HindIII site of pTWU18. The resulting plasmid was designated pTWU19.

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The HBV DNA inserts in the various plasmids are diagrammed in Fig. 1.

The construction of the beta-interferon cat hybrid gene is shown in Fig. 2. The 1.6-kbp HindIII DNA fragment that contains the human beta-interferon-coding sequence and the regulatory DNA sequence was constructed from pIFR (46). The 353-bp HindIII-HincII fragment corresponding to positions -286 to +67 (the mRNA cap site being designated as +1) was isolated, blunt ended with the Klenow fragment of E. coli DNA polymerase I, and ligated with HindIII linkers. The resulting HindIII fragment was inserted into the HindIII site of pTWU53, a pUC9 derivative containing the cat structural gene as a 1,632-bp HindIII-BamHI DNA insert. The resulting plasmid that contains the cat structural gene and the regulatory elements involved in the expression of the human beta-interferon gene (β-ifNh-cell) is referred to as pTWU54.

To assay for promoter activity on the 5′-flanking sequence of the HBV X gene, pTWU17, which contains the EcoRV-BgIII HBV DNA fragment, was linearized with NcoI, and the ends were converted to HindIII sites. The plasmid then was digested with HindIII, and the fragment containing the HBV enhancer and promoter for the X gene (mp, 1040 to 1372) was isolated. This fragment was then cloned into the HindIII site of pTWU53, creating the plasmid pExpCat. The HBV enhancer (mp, 1040 to 1236) was removed from pExpCat by digestion with PsiI and SphI. The resulting linearized plasmid was blunt ended and then ligated to form pExpCAT.

Other plasmids used in this study were pSV2-CAT and pA14CAT, in which the cat gene is under the control of the early regulatory region of SV40, either with the 72-bp enhancer element (pSV2-CAT) or without the enhancer region (pA14CAT) (14, 21); pRSVCAT and pMS.LTR2, in which the cat gene is controlled by the long terminal repeat of Rous sarcoma virus (pRSVCAT) or of murine sarcoma virus (pMS.LTR2) (13, 21); pBKs5'CAT, in which the enhancer element of BK virus and the early promoter of SV40 virus control the expression of the cat gene (33); pEC113, in which the promoter of the E2 gene of adenovirus type 5 drives the expression of the cat gene (23); and pELA1, which encodes the adenoviral E1A antigen (23). All of these plasmids were kind gifts of G. Khoury, National Institutes of Health, Bethesda, Md. PhD1, in which the cat gene is driven by the early promoter of SV40 and the HBV enhancer, was a gift from D. Spandau and C.-H. Lee of Indiana University, Indianapolis.

Cells and transfections. Vero cells were cultured at 37°C in Eagle minimum essential medium supplemented with 10% fetal calf serum. Vero cells were plated at a concentration of 10⁶ cells per 100-mm plate. After 24 h, the medium was replaced for 2 to 4 h prior to the addition of the DNA. DNA transfections were performed by the calcium phosphate method according to a modified procedure of Wigler et al. (45). After incubation with the added DNA for 4 to 8 h, the cell monolayer was rinsed with serum-free medium and treated with 15% glycerol for 1.5 min at room temperature; 10 ml of fresh medium was then added. In all transfections, 10 µg of various plasmids containing HBV DNA and 10 µg of the plasmid containing the cat gene were used.

Treatment of transfected cells with poly(I) · poly(C). Transfected Vero cells were treated with poly(I) · poly(C) as described by Havell and Vilcek (17). After 48 h of transfection, cell monolayers were washed with phosphate-buffered saline and treated with 5 ml of serum-free Eagle minimum essential medium containing 50 µg of poly(I) · poly(C) per ml and 600 µg of DEAE-dextran per ml. After 2 h, cycloheximide (50 µg/ml) was added, and incubation was continued for 3.5 h. Actinomycin D was then added at a concentration of 1 µg/ml for 30 min. The treated cells were then washed twice with phosphate-buffered saline and incubated for 12 to 16 h in Eagle minimum essential medium containing 2% fetal calf serum. Control cells were treated in the same manner, except that poly(I) · poly(C) was omitted.

CAT assays. Cells were harvested and CAT assays were performed essentially as described by Gorman et al. (14). Untreated cells or cells treated with poly(I) · poly(C) were washed with phosphate-buffered saline, and cells were removed by scraping. Cells were pelleted and suspended in 0.25 M Tris hydrochloride (pH 7.8) and disrupted by two cycles of freeze-thawing. The protein concentration of cell lysates was determined, and CAT activity was assayed by using the same amount of total protein for all samples in an individual experiment. The activity of the CAT enzyme was...
determined quantitatively by measuring the amount of radioactivity associated with the various acetylated forms of chloramphenicol and by expressing this value as the percentage of the total radioactivity associated with the [14C]chloramphenicol substrate. All experiments were performed on multiple occasions and with independent DNA preparations. In general, because of variations in transfection efficiencies, comparisons between experiments should be considered primarily qualitative.

RESULTS

Detection of an HBV DNA fragment functioning as a trans-activator. The region that flanks the 5′ end of the human beta-interferon-coding sequence is responsible for the induction of beta-interferon in response to either viral infection of cells or treatment of cells with the synthetic, double-stranded RNA, poly(I)·poly(C) (47). To determine whether a trans-acting HBV moiety might be involved in the control of the expression of this gene, we used a readily assayable target gene, the bacterial cat gene, which had been placed under the control of the regulatory DNA region that flanks the 5′ end of the human beta-interferon-coding sequence.

To ascertain the effect of the 2,755-bp BglII HBV DNA fragment on the expression of the β-ifnR-cat construct, Vero cells were transfected with pTWU54 and either pUC9 DNA or pUC9 DNA containing the 2,755-bp BglII HBV DNA insert (pTWU14). Transfected cells were either mock treated or treated with poly(I)·poly(C), and the amount of CAT activity was determined (Fig. 3). The basal CAT activity from the β-ifnR-cat construct was very low (lane 1). Upon treatment of cells with poly(I)·poly(C), the amount of CAT activity in these transfected cells increased by fivefold (lane 2).

In other experiments, the increase in CAT activity after the treatment of cells with poly(I)·poly(C) ranged from 4- to 10-fold. Although increased CAT activity occurred upon treatment with poly(I)·poly(C), the strength of the inducible beta-interferon-regulatory elements was clearly less than that of the SV40 early promoter and enhancer elements (lanes 5 and 6). Extracts of poly(I)·poly(C)-treated Vero cells that received the β-ifnR-cat construct together with the 2,755-bp BglII HBV DNA that contains the genes for the surface antigen and for the X antigen showed approximately a 400% increase in the expression of the cat gene (lanes 3 and 4). In other experiments, the extent of the activation of CAT activity in the cotransfected Vero cells was proportional to increasing amounts of HBV DNA in the transfection mixture; optimal activation was obtained when 10 μg of plasmid containing HBV DNA was used (data not shown).

Subcloning of the HBV DNA fragment containing the trans-activating function. Since the 2,755-bp BglII fragment of HBV DNA contains the genes for the surface antigen and for the X antigen, various subclones of this fragment were constructed in order to identify the biologically active DNA region. The plasmids containing the various HBV DNA fragments that are diagrammatically represented in Fig. 1 were used to cotransfect Vero cells with pTWU54 to assess their ability to trans-activate the cat gene. As controls, pUC9 and pTWU14 were used as cotransferring DNA.

Cotransfection with the BglII-EcoRV HBV DNA fragment (mp, 2429 to 1040) that contains the gene for the surface antigen had no effect on the expression of the cat gene (Fig. 4). However, the presence of pTWU17 (EcoRV-BglII HBV DNA fragment that contains the X gene with its 5′- and 3′-flanking sequences) resulted in the activation of CAT activity. The level of trans-activation caused by pTWU17 was comparable with that caused by pTWU14. Of interest is the observation that trans-activation of the expression of the β-ifnR-cat construct by pTWU17 occurred in the absence of treatment of transfected cells with poly(I)·poly(C); in this case, a 13-fold increase in CAT activity in these transfected cells occurred.
activity was detected (Fig. 3, lane 8). A similar trans-
activation by the 2,755-bp BglII fragment (pTWU14) was
seen in the absence of poly(I)·poly(C) treatment but not
consistently (see Fig. 5, lane 3). These results suggest that
the trans-activating activity is due to a product of the HBV
X region.

Confirmation of these results was obtained by using an
HBV DNA sequence that contains the structural X gene but
lacks the native promoter. Since the putative promoter lies
within the EcoRV-Ncol fragment of HBV DNA (mp, 1372
to 1372; unpublished data), the Ncol-BglII HBV DNA frag-
ment (mp, 1372-1984) was used as the source of the struc-
tural X gene (pTWU18). Cotransfection of Vero cells with
pTWU54 and pTWU18 did not result in a significant change
in the expression of the cat gene in cells that were mock
treated or induced with poly(I)·poly(C) (Fig. 4, lanes 10 and
11). When the structural X gene was placed behind a strong
heterologous promoter, such as the SV40 early promoter and
enhancer (pTWU19), the trans-activating activity was re-
stored (Fig. 4, lanes 12 and 13).

Frameshift mutation within the X gene abolished trans-
activating activity. If, as the above results suggest, a product
of the X gene of HBV is responsible for trans-activation of
the expression of the cat gene that is under the control of
the regulatory region of the human beta-interferon gene, then
disruption of the ORF X by mutation ought to abolish the
trans-activating function. To test this possibility, an inser-
tion mutation at the unique BamHI site (mp, 1400) that lies
within the X gene of HBV was created. The plasmid
pTWU17 that contains the EcoRV-BglII fragment of HBV
DNA was linearized at the unique BamHI site, and the
linearized plasmid was blunt ended by using the Klenow
fragment. The plasmid was then ligated and designated
pTWU17ΔBamHI. The insertion of four bases at the BamHI
site caused a frameshift in the ORF X.

The plasmid containing the frameshift mutation was used
to cotransfect Vero cells with pTWU54. Whereas the pres-
ence of plasmids containing the normal ORF X (pTWU17
and pTWU14) trans-activated the expression of the cat gene
on pTWU54, the plasmid containing the mutated X gene did
not affect the expression of the cat gene (Fig. 5).

Specificity of the trans-activating activity of the HBV X
gene. The HBV DNA fragment that contained the X gene
activated CAT activity from the beta-interferon-regulatory
region. To investigate whether the HBV X gene product can
trans-activate a homologous promoter element, plasmids in
which the cat gene was driven either by the promoter for the
HBV X gene (pXpCAT) or by both the HBV enhancer
and X gene promoter (pExpCAT) were used in cotrans-
fection experiments with the 2,755-bp BglII fragment of
HBV DNA (pTWU14). The promoter for the HBV X gene
was functional in Vero cells; the presence of the HBV
enhancer did not significantly affect the activity of the
promoter (Table 1). The presence of the HBV X gene in
cotransfection experiments did not result in any activation of
CAT activity.

The ability of the HBV X antigen gene to trans-activate a
series of plasmids containing the cat gene driven by different
regulatory elements, namely, (i) the early region of SV40
that retained the 72-bp enhancer sequences (pSV2CAT) or
that lacked the enhancer sequences (pA1oCAT), (ii) the SV40
early promoter and the HBV enhancer (pHBExE1), (iii) the
long terminal repeat of murine sarcoma virus (pMS.LTR) or
of Rous sarcoma virus (pRSVCA), (iv) the SV40 early
promoter and the enhancer of BK virus (pBKs5'CAT), and
(v) the regulatory region of the adenoavirus type 5 E2 gene
(pEC113), was investigated. In addition, attempts were
made to assess the strength of trans-activation by the HBV
X gene by comparison with a known trans-activator, the
adenoviral EIA protein (23). The results of a typical ex-
periment are shown in Table 2. The expression of only the cat

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<tr>
<th>Test plasmid</th>
<th>CAT activity in cells cotransfected with test plasmid and*</th>
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| pUC9         | 0.8
| pTWU14       | 0.8
| pExpCAT      | 16.2
| pXpCAT       | 13.6
| pRSVCAT      | 92.0

* Vero cells were cotransfected with equal amounts (10 μg each) of the indicated test plasmid and one of pUC9 or pTWU14. After 48 h of
transfection, cell extracts were assayed for CAT activity, which is expressed as the percentage of substrate that was acetylated. ND, Not determined.
trans-activating function. Dissociation of the promoter from the coding sequence for the X antigen abolished the trans-activating activity; replacement of the HBV native promoter with a strong heterologous promoter (SV40 early promoter and enhancer) restored trans-activation. Finally, a frameshift mutation within the X gene inhibited the trans-activating function. These results suggest that the trans-activating motifs is the X antigen.

The product of the HBV ORF X appears to be quite specific in the activation of regulatory elements; none of the hybrid genes in which the cat gene was under the control of the different viral promoters and enhancers was significantly activated by the HBV X gene product. Of interest is the observation that the HBV X promoter with or without the HBV enhancer was not affected by the presence of the HBV X gene in Vero cells. Analysis of the ability of a known viral trans-activator to stimulate the beta-interferon-regulatory region reveals that the adenoviral EIA protein suppressed the activity of the beta-interferon enhancer and promoter. A similar suppression by EIA has been demonstrated previously with the SV40 early promoter (44).

The significance of the stimulation of the expression of the regulatory elements of the human beta-interferon gene by the HBV X gene product is unclear. Miller and Robinson (29) suggested that the X gene of HBV may be of cellular origin, since the X antigen has the same preference for codon usage as do genes of eucaryotic cells. If the HBV X gene was originally a cellular gene that functioned as a regulatory protein in the expression of cellular genes, then it is not unexpected that the HBV X gene region encodes an antigen that displays trans-activating function. In this context, it is intriguing to speculate that the development of hepatocellular carcinoma may arise as a consequence of the trans-activating function of the X gene product. It is unlikely that the X gene of HBV is an oncogene since (i) no homology exists between the predicted amino acid sequence of the X antigen and any of the retroviral oncoproteins, (ii) no cellular analog for the X gene has been identified, and (iii) hepatocellular carcinoma develops over a long period of time (29).

A possible mechanism for the development of hepatocellular carcinoma could be that the trans-activating properties of the HBV X antigen can result in cell transformation by activation of a cellular gene(s), resulting in tumor formation after a long latent period. Since the HBV genome has been found to be integrated into the cellular genome within the HBV X gene region (7, 20, 35), it is possible that a fusion protein consisting of part of the X antigen and part of a cellular protein could possess trans-activating activity.

The HBV X gene has many features in common with the pX genes of human T cell lymphotropic virus type I (HTLV-I), HTLV-II, and HTLV-III/human immunodeficiency virus. Both genes are located at the 3' ends of the linearized genomes, share the codon preference of eucaryotic genes, and encode proteins that display trans-activating activity (6, 27, 29). Furthermore, like the HBV X gene, the pX gene is not an oncogene, yet HTLV-I is clearly oncogenic. Moreover, HBV has been implicated as a potential cofactor in the establishment of the acquired immunodeficiency syndrome (31). Epidemiological studies have indicated that a majority of patients with acquired immunodeficiency syndrome have prior or concurrent infections with HBV. Since HBV DNA has been detected in peripheral blood mononuclear cells (36), an intriguing possibility is that the HBV X antigen is involved in the expression of human immunodeficiency virus by functioning as a transcriptional activator.

Other viral proteins that have been shown to trans-

| TABLE 2. Target specificity for trans-activation by HBV DNA |
|---------------------------------|-----------------|-----------------|
| Test plasmid                   | CAT activity in cells cotransfected with test plasmid and‡ |       |
| pUC9                          | pTWU14          | pE1A           |
| pTWU54 mock treated           | 1.8             | 2.9             | 0.7            |
| pTWU54 poly(I)·poly(C) treated| 8.7             | 53.7           | 1.1            |
| pA9CAT                        | 2.2             | 2.9             | 3.1            |
| pSV-CAT                       | 63.8            | 62.0           | 31.9           |
| pBE1                          | 2.3             | 3.4             | 2.8            |
| pRSV-CAT                      | 54.1            | 54.2           | 48.9           |
| pBrk5CAT                      | 1.3             | 3.6             | 2.8            |
| pMS-LTR2                      | 2.7             | 2.4             | 30.1           |
| pEC113                        | 2.2             | 4.9             | 21.9           |

‡ Vero cells were cotransfected with equal amounts of the indicated test plasmid and of pUC9, pTWU14, or pE1A as described in Table 1, footnote a. The cells that received pTWU54 were either treated with poly(I)·poly(C) or mock treated prior to CAT assays. CAT activity is expressed as the percentage of substrate that was acetylated.

activate homologous target promoters in transient-expression assays include the SV40 large T antigen (2, 3), the MS-EA protein of Epstein-Barr virus (22), the IE proteins of herpes simplex virus (8, 12), and the Tat protein of human immunodeficiency virus (6). Adenovirus EIA (1, 30), pseudorabies IE regulatory proteins (16, 18), and p40 of HTLV-I (27) are viral regulatory proteins that are also capable of trans-activating the expression of some cellular genes. All these viral trans-acting proteins function by increasing transcription from the target promoters. These observations are consistent with the supposition that the activation of transcription by viral proteins involves the better use of cellular transcriptional factors.

Since other viral proteins known to have trans-activating properties perform key regulatory roles in infection, it is possible that the HBV X protein has a regulatory role in HBV infections. Such a role would be consistent with the observation that the X ORF exhibits a high degree of conservation among the HBV DNAs isolated from different patients; less than a 4% divergence among the encoded amino acid sequences was detected (39). Cloning and sequencing of other HBV subtypes also indicated the conservation of the X gene region (10, 19, 38). Other members of the hepadnavirus group, such as ground squirrel hepatitis virus and woodchuck hepatitis virus but not duck hepatitis virus, contain regions of the genome that correspond to the ORF X of HBV (11, 25, 34).

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LITERATURE CITED


