Human hepatitis B virus X protein is detectable in nuclei of transfected cells, and is active for transactivation

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Abstract

Subcellular localization and transactivation of human hepatitis B virus X protein (HBx), a plausible causative factor for hepatocellular carcinogenesis, were studied in transiently transfected cells. The transactivation was detected not only by the cis-element driven chloramphenicol acetyltransferase (CAT) assay but also by immunostaining of CAT protein cotransfected into human hepatoma cell line HepG2. Scanning fluorescence microscopy showed the majority of immunological signals of HBx to be at the perinuclear region of transfected cytoplasm. HBx was also clearly detectable in the nucleus, though less intensely expressed. This was confirmed by Western analysis and immunoprecipitation of HBx with transcription factor IIIB (TFIIB) in subcellular fractionations. The percentage of HBx-positive cells coincided with that of CAT-positive cells, and confocal laser microscopy revealed the coexistence of CAT signals in GFP-HBx positive cells. The SV40 large T antigen nuclear localization signal (NLS) appended HBx, regardless of whether NLS was added to the N- or C-terminus, transactivated all the examined X-responsive elements (XRE) similarly as did wild-type HBx. Similar results were obtained in p53 negative Saos-2 cells. The detected nuclear HBx may be involved in modulating the transcription at the promoter level whereas the HBxs in cytoplasm may be working through signal transduction pathways. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hepatitis B virus X protein; Transactivation; Immunostaining; Subcellular localization; Transfection

1. Introduction

Human hepatitis B virus (HBV) induces acute and chronic hepatitis, and is closely associated with the incidence of human liver cancer [1]. Virally encoded X protein (HBx) has been thought to be a major risk factor for the development of human hepatocellular carcinoma. However, the exact function of HBx in the tumorigenic transformation of liver cells is still unclear [2–4]. Since a long latent period is necessary for the development of liver tumors after HBV infection, activation or inactivation of host cellular factors including proto-oncogenes and/or tumor suppresser genes has been proposed in relation to the HBx activity [5–9].

HBx protein, encoded in the smallest open reading frame (ORF) of the HBV genome, transcriptionally transactivates not only viral but also a wide variety of host cellular promoter elements known as X-re-
responsive elements (XRE) [10–15]. Many cellular factors have been proposed as possible targets for HBx transactivation [16–23] including human RPB5, a common subunit of RNA polymerase I, II and III [24,25]. We and others discovered the binding of HBx with transcription factor IIB (TFIIB), and suggested the direct modulation of transcriptional control by a trimeric interaction of RNA polymerase subunit, TFIIB and HBx [23–26]. Meanwhile, HBx is observable in association with p53 tumor suppressor in hepatocellular carcinoma [6,27], and has been discovered to bind directly with p53 [28,29]. HBx, however, has no obvious nuclear localization signal motif and has been detected preferentially in cytoplasm especially in the perinuclear region in experimental cells and in human specimens [30–32]. In other reports, however, HBx has been detected in both nuclear and cytoplasmic regions although the major signals were found in cytoplasm [9,33], or predominantly in nucleus [26]. Woodchuck hepatitis virus X protein (WHx), which is analogous to HBx, was also detected not only as soluble protein in cytoplasm but also as rather stable forms associated with nuclear matrix [34]. Thus, subcellular localization of HBx is still controversial.

In this study, we examined the subcellular localization of HBx in relation to its transactivation activity. HepG2, a human hepatoma cell line, and p53-negative Saos-2 cells were employed. After cotransfection with HBx expression vector and a chloramphenicol acetyltransferase (CAT) reporter, cells were stained immunologically with anti-HBx or with anti-CAT. Serial scanning fluorescence microscopy was applied for the detection of HBx. More than 80% of HBx-positive cells carried immunostained fluorescent signals in the nuclear region although these were weak in comparison with cytoplasmic signals. Confocal laser scanning observation was applied to examine the localization of HBx in cells cotransfected with GFP-HBx- and FLAG-tagged TFIIB, which is restricted in nucleus. Another immunological approach combined with cell fractionation and coimmunoprecipitation experiments was employed to examine the subcellular localization. The transacting activity of HBx was also examined by CAT assay. Colocalization of HBx and CAT protein in the same cell was confirmed by confocal observation using GFP-HBx and CAT staining.

2. Materials and methods

2.1. Cells, culture and transfection

One day before the transfection, 5–6×10⁵ HepG2, a human hepatoma cell line, were plated in 60-mm culture dishes. Transfection was performed for 20 h in 3% CO₂/air at 37°C followed by 24 h recovery in 5% CO₂/air at 37°C [35]. For the immunostaining, 1×10⁵ cells were plated on a slide glass, settled into a Quadriperm microscope slide culture well (Heraeus, Hanau, Germany). Saos-2, a p53 negative human osteosarcoma cell line, was also examined.

2.2. Plasmid constructions

The 154 amino acid full HBx coding sequence was inserted into mammalian expression vector pSG5UTPL (pSGHX-1) [12,36,37]. The polymerase chain reaction (PCR) cloning method was employed to add the SV40 nuclear localization signal (NLS) sequence to the 5’- or 3’- ends of HBx, giving NLS-X with MLPKKKRKV (pSGNLS-X), and X-NLS with LPKKKRKV (pSXL-NLS), respectively. By using the EcoRI and BamHI sites of a vector pNCFLAG, pNCFLAG-HBx or pNCFLAG-TFIIB encoding HBx or TFIIB with a Flag-tag both at the N- and C-termini were constructed. CAT reporter pHEC × 2CAT has a dimeric sequence of the 23 bp HBV Enh-1 core. Other CAT reporters were pNF-kB × 3CAT, pTRE × 3CAT and pSRECAT containing NF-kB binding site, AP-1 binding site of TPA responsive element and serum-responsive element of the regulatory sequence of c-fos gene, respectively [12,22,36,37]. All the constructed plasmids were confirmed after cloning with a DNA sequencer (370A; Applied Biosystems). An expression vector of green fluorescent protein (GFP)-tagged HBx, pGFP-HBx, was constructed by inserting the EcoRI and BglII fragment harboring the full size of HBx into the EcoRI and BamHI sites of pGFP [38]. pGFP-TFIIB was also constructed in a similar way.

2.3. Immunostaining and CAT assay

The IgG fractions of rabbit anti-HBx, prepared in our laboratory by using GST-fused proteins, were purified by a two-step affinity chromatography [25].
Properly diluted (1:10) anti-HBx IgG fraction (30 µg/ml) with 0.5% bovine serum albumin (BSA) was absorbed with acetone-powdered nontransfected control cells (1:1 volume, 4°C overnight) in phosphate-buffered saline (PBS). Commercially available rabbit anti-CAT IgG (5 Prime → 3 Prime, Boulder, CO) was also absorbed thoroughly.

Cells were fixed for 5 min with a freshly prepared cold (−25°C) one-to-one mixture of acetone and methanol, and then were stored at −80°C in the dried condition. Samples blocked with 1% BSA in PBS were stained with diluted antibody (at 1/100) for 2 h. Immunostaining was carried out by using biotinylated goat anti-rabbit IgG and streptavidin-FITC (Amersham Japan, Tokyo) following the maker’s manual with counterstaining by 0.0005% Evans Blue. For the observation, a superresolution scanning Cellsam system (Scanalytics, Billerica, MA) [39] mounted on a Zeiss Axioskop fluorescence microscope (Jena, Germany) equipped with a plan-neofluor 40× or plan-apochromat 63× lens and a narrow blue band pass filter set (BP485-20/FT510/BP515-565), or confocal laser scanning microscope (Zeiss Model LSM510 system), were used. An Olympus BX-50 fluorescence microscope (Tokyo, Japan) was also used with filters of NIBA and WIB. Scanned images were visualized by digital printing (Pictrography 3000, Fuji, Tokyo, Japan).

The CAT assays were processed for 60 min at 37°C using 20 µg protein of cell lysates [12,36,37]. Activities of the CAT were determined as the percentage of acetylated conversion of [14C]chloramphenicol (Amersham) using a bioimage analyzer (BAS1000: Fuji). Three independent assays were performed for each experiment.

3. Results

3.1. Transactivation by wild and modified HBx

Transactivation activity of HBx was examined by CAT assay in a human hepatoma cell line, HepG2 (Fig. 1). Reportedly, HBx transactivated all of the viral and cellular cis-elements including NF-kB-binding site, TPA-responsive element and the serum-responsive element. The classical nuclear localization signal (NLS) of SV40 T antigen was introduced into the HBx sequence [41]. Regardless of whether the NLS was added to the N- or C-terminus of HBx, both NLS-X and X-NLS transactivated all the XRE including the NF-kB binding site and AP-1 binding site of TPA responsive element (Fig. 1). No significant difference in transactivation activity was observed between wild-type and NLS appended HBx. Thus the transactivation activity of HBx was not altered by the addition of the classical NLS motif.

For cis-element specificity of HBx, mutated CAT reporters having two-basepair substitutions in the
promoter region of Enh1 core were provided. Neither the HBx nor NLS appended HBx transactivated the mutated reporters (not shown). We then chose the HBV Enh1 core sequence as an example for the later experiments.

3.2. Immunological detection of HBx in transfected cells

Subcellular localization of HBx was examined by immunostaining of cells after the appropriate transfection. As described in Section 2, all the antibody was absorbed thoroughly with nontransfected cells to avoid nonspecific reactions. No positive cells were detected with preimmune serum in any samples (not shown).

By superresolution scanning fluorescence microscopy, immunologically HBx-positive cells were obvious in HBx-transfected samples and no signal was detected in the control transfection (Fig. 2a,c). FITC signals by immunostaining were clearly distinguished from counterstaining with Evans Blue, by which cellular or nuclear profiles were visualized in an integrated image picture. Signals with HBx were much more intense at the perinuclear region of cytoplasm than the nucleus. In some cases, granular signals scattered throughout the cytoplasm and/or organized signals along the periphery of the cells were observed (Fig. 2b). Granular or dotted HBx stainings at the nuclear region were difficult to define whether they were in the nuclei or at the apical periphery of the nuclei. Thus a serial scanning with a horizontal optical cut at 0.82-μm intervals was applied to determine the localization of the signals on the same region of Fig. 2a. Signal granules in the nuclear region in Fig. 2a were confirmed to be inside the nuclei (Fig. 3). A vertical section analysis also revealed the exact position of HBx in the nuclei (not shown). HBx-positive cells in NLS-appended pSGNLS-X or pSGX-NLS transfection bore the signals preferentially in their nuclei (Fig. 2e). Few cytoplasmic signals, however, were also detected in some cells (Fig. 2d). Signals with NLS-X or X-NLS transfection were weaker than that with wild-type HBx; presumably the appended NLS sequence affected the antigen–antibody recognition, since transactivation of CAT reporter was not altered with NLS-X or X-NLS (Fig. 1) and CAT protein was detectable in staining (Fig. 6).
3.3. Detection of HBx in fractions of transfected cell lysates

In these experiments, we used anti-FLAG monoclonal antibody because of its high sensitivity in Western analysis. HepG2 cells were transfected with pNCFLAG-HBx or with empty vector. Cell extracts were fractionated into cytosol and nuclear fractions as described in Section 2. HBx localization was examined by Western blotting with anti-FLAG and anti-actin for detection of cytosolic contamination on the same filter (Fig. 4a). FLAG-HBx was detected in both cytosol and nuclear fractions (lanes 1 and 3) but in neither fraction from the control transfection (lanes 2 and 4). Approximately, one-tenth of FLAG-HBx was detected in nuclear extract. Since the sole cytosol protein, actin, could not be detected in the nuclear extracts (lanes 3 and 4) even with long exposure, the possibility of contamination by cytosolic HBx of the nuclear fraction could be excluded.

Further, nuclear localization of HBx was confirmed by coimmunoprecipitation of FLAG-HBx with endogenous TFIIB (Fig. 4b). Nuclear extracts were at first immunoprecipitated by rabbit anti-TFIIB antibody (lane 3), anti-GST antibody (lane 2) and anti-FLAG (lane 4). Lane 1, 25% of the nuclear extract used in immunoprecipitation. The eluted proteins were fractionated by SDS-PAGE and Western blotted by anti-FLAG. The asterisks indicate the mouse IgG heavy and light chains recognized by the second antibody.
3.4. Colocalization of HBx and TFIIB in nucleus

To confirm the nuclear localization of HBx, we examined the colocalization of HBx and the nuclear protein, TFIIB, by cotransfection of pNKFLAG-TFIIB and pGFP-HBx. Immunostaining of FLAG by Texas Red (TexRd) was clearly distinguished from green fluorescence of GFP-HBx by using a confocal microscope equipped with a Nomarski differential-interference-contrast apparatus.

Almost all FLAG-TFIIB was detected in nuclei of the transfected cells (Fig. 5a) whereas the GFP-HBx was in the nuclei and/or cytoplasm (Fig. 5b,c). In some cases, GFP-HBx colocalized with FLAG-TFIIB was observed in the nuclei of the cotransfected cells (Fig. 5d–f). Overlaying the images of FLAG-TFIIB (red) and GFP-HBx (green) and the Nomarski image provided overlapped signals (yellow) in the nuclear region. In other cases, however, HBx was detected in nuclei and/or cytoplasm independently from TFIIB (Fig. 5g–i). In order to confirm these results, GFP-TFIIB was applied for the cotransfection with HBx (Fig. 5j–l). Perinuclear accumulation of HBx was clear in this picture. The detected nuclear HBx may correlate with the interaction of HBx and transcription components recruited to promoters, whereas the HBx in cytoplasm may be working to modulate signal transduction pathways.

3.5. Detection of induced CAT protein by immunostaining

Transactivation of HBx was also detected by the immunostaining of resultant CAT protein using anti-CAT serum. CAT-positive cells were observable in the samples cotransfected with HBx constructs (Fig. 6), and only a few cells with extremely weak staining were detected with control plasmid (Table 1a). There was no difference in the appearance of CAT protein among the cotransfected HBx constructs.

3.6. Relationship between subcellular localization of HBx and transactivation

Results of HBx immunostaining in HepG2 cells are summarized in Table 1a. To monitor the transfection efficiency, pUC-LacZ was transfected and...
examined by staining with X-gal. Although transfection efficiency fluctuates among experiments, X-gal staining in this series of experiments was 3.3%. The percentage of HBx-positive cells was calculated by counting the signal-positive and negative cells (total of 4000–10 000 cells) in randomly picked areas. FITC-positive cells were categorized into three groups by the signal localization in the cell. Regardless of the constructs used, more than 80% of HBx-positive cells bore the signals in their nuclei with or without cytoplasmic signals. Not only was the percentage of HBx-positive cells lower in the NLS-appended HBx, especially in NLS-HBx, but the signals were weaker than those with wild-type HBx. Transfection of HBx-NLS resulted in greater nuclear localization and decreased cytoplasmic signals of HBx, so that 94% of the positive cells exhibited nuclear signals. The percentage of CAT-positive cells in samples prepared in parallel with NLS-HBx or HBx-NLS at the same time was similar to or a little more than that of wild-type HBx. The intensity of CAT signals was also similar or a little increased in NLS appended HBx cotransfections.

To examine the relationship between CAT and HBx, GFP-HBx and immunostaining of CAT by TexRd were employed in cotransfections (Table 2). Almost all CAT-positive cells (about 95%) carried GFP-HBx, suggesting detected CAT signals are products operated by the expressed GFP-HBx, which is transacting active. No CAT-positive cells were observed in control experiments without HBx and/or without CAT transfection. Subcellular localization of GFP signals in GFP/CAT-positive cells in Table 2 were nucleus, 16%; cytoplasm, 17.8%; both, 66.2%. Fig. 7 shows the integrated confocal image of anti-CAT staining (red), GFP signals (green) and Nomarski differential-interference-contrast. GFP signals are

Table 1
Summary of immunostaining

<table>
<thead>
<tr>
<th>Transfection</th>
<th>% X-positive(^a)</th>
<th>Location(^c)</th>
<th>% CAT-positive(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% nucleus</td>
<td>% cytoplasm</td>
</tr>
<tr>
<td>(a) HepG2 cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSGHX-1 and pHEC(x2)CAT</td>
<td>2.6</td>
<td>13.7</td>
<td>11.6</td>
</tr>
<tr>
<td>pSGNLS-X and pHEC(x2)CAT</td>
<td>0.15</td>
<td>28.5</td>
<td>14.0</td>
</tr>
<tr>
<td>pSGX-NLS and pHEC(x2)CAT</td>
<td>0.84</td>
<td>61.0</td>
<td>5.7</td>
</tr>
<tr>
<td>pSGHX-1 and pSG5UTPL</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pHEC(x2)CAT and pSG5UTPL</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(b) Saos-2 cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSGHX-1 and pHEC(x2)CAT</td>
<td>2.8</td>
<td>13.2</td>
<td>36.1</td>
</tr>
<tr>
<td>pSGNLS-X and pHEC(x2)CAT</td>
<td>0.01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pSGX-NLS and pHEC(x2)CAT</td>
<td>1.7</td>
<td>37.0</td>
<td>20.6</td>
</tr>
<tr>
<td>pHEC(x2)CAT and pSG5UTPL</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\)Percentage of HBx-immunopositive cells over total cells scored in an area. 4–10\(\times\)\(10^3\) cells were counted in a dark field for an independently chosen area.

\(^b\)Percentage of CAT-immunopositive cells over total cells in an area. 4–10\(\times\)\(10^3\) cells were counted in a dark field for an independently chosen area.

\(^c\)Detailed localization of HBx signals in the immunopositive cells.
detectable in the nuclei (Fig. 7a) or perinuclear (Fig. 7b) whereas the expressed CAT protein distributes throughout the cell. Regardless of the subcellular localization in nuclei or in cytoplasm, GFP-HBx trans-activated the cotransfected CAT reporter. As mentioned previously, CAT staining was not detected in cells transfected with CAT vector alone (data not shown).

Nuclear localization of HBx protein and transactivation were also examined in Saos-2 osteosarcoma cells. Results are summarized in Table 1b. Fundamentally, no significant difference in the distribution of HBx was observed between HepG2 and Saos-2 cells. Reduction of HBx-positive cells, as in the case of HepG2, was also observed on addition of NLS to HBx, so that staining signals were rarely detected in these transfections. The percentage of CAT-positive cells did not differ between cotransfection with X-NLS and HBx.

In relation to the transactivation, we examined the subcellular localization of HBx in transfected cells. The profile of our immunostaining showed HBx both in nucleus and cytoplasm. Serial scanning analysis confirmed the granular HBx signals in nucleus although the majority of them were detected in the perinuclear region of cytoplasm. Since very similar results were obtained in samples fixed with 1.5% paraformaldehyde and treated with 0.2% Triton X-100, the immunological signal profile or distribution was not influenced by the fixation treatments. In addition to those cells having HBx signals exclusively in nuclei or cytoplasm, cells carrying HBx both in nuclear and cytoplasm were observed (classified as both). About one-third of these cells have HBx signals mainly in the nucleus. Western blotting analysis of the cell fractionations revealed that the majority of expressed HBx was recovered in the cytoplasmic fraction with around 10% recovered in the nuclear fraction. Efficient recovery of HBx in the coimmunoprecipitates with TFIIB, a sole nuclear protein, clearly supports

### Discussion

HBx is a multifunctional regulatory protein and has been reported to be responsible for hepatocellular carcinogenesis. HBx transactivates not only viral but also a variety of host cellular elements related to cell proliferation and acute inflammatory response [10–15]. Several mechanisms of the transactivation have been proposed, and HBx interacts with multiple factors in nucleus and cytoplasm [16–26]. Subcellular localization of HBx is still controversial. Woodchuck WHx, which is analogous to HBx, has been observed in cytosol and in nuclear matrix fractions [34]. HBx in human specimens and in transient transfection experiments has been reported to be localized in both nucleus and cytoplasm [9,33] or in nucleus [26] or cytoplasm [30–32].
the notion that a minor but distinct portion of HBx localizes in nucleus.

We addressed the effect of NLS-tagging on subcellular localization in transfected cells by examining a large number of cells with several different methods. The NLS-taggings had little effect on delocalization from cytoplasm and transactivation in HepG2, and similar results were obtained in p53-negative Saos-2 cells. Since the HBx protein has no nuclear localization signal motif, which is essential for translocation of proteins through nuclear pores [41,44], HBx might be introduced into nuclei by diffusion or by protein–protein interaction. This may explain the slight effect of the NLS tagging in our experiments. These results, however, were different from others; NLS addition severely impaired or abolished ability to transactivate the NF-kB and AP-1 driven CAT reporters [31,33]. The reason for this discrepancy is unclear; however, differences in experimental conditions including subtype of HBx (ayw and adr), flanking amino acid sequence of NLS, and recipient cells for transfection may be responsible.

Our results with the HBx signal profile and subcellular localization are consistent with previous reports [33] but different from results of exclusively nuclear [26] or cytoplasmic localization [30–32]. A possible transactivation mechanism involving cytoplasmic signal transduction pathways with protein kinase C, Ras–Raf membrane activated protein kinase and/or Src family of tyrosine kinases have been proposed to be consistent with the exclusive cytoplasmic localization of HBx, and with the reduced transactivation by NLS-tagged HBx [7,33,42,43,45]. The perinuclear or scattered signals of HBx in cytoplasm in our study may relate to these possibilities or to the colocalization of HBx with proteasome[31].

On the other hand, HBx has been discovered to interact directly with several components of transcription machinery including RPB5 of RNA polymerase, TATA box binding protein, and a general transcription factor IIB [18–26]. These studies may correlate with our biochemical and morphological finding that some HBx signals are present in the nucleus or nuclear fraction. It may be that parts of the HBx enter the nucleus along with cellular factors. We have compared the localization of HBx in co-transfection with or without TFIIB. However, the ratio of the cells with nuclear HBx was little affected by cotransfection with TFIIB (not shown). By the confocal analysis of the cotransfected cells with the HBx expression vector and a CAT reporter, the activated expression of CAT was detected by immunostaining in HBx expressing cells. Regardless of nuclear or cytoplasmic localization of HBx, CAT signals were obvious in cells with HBx but not in those without HBx, and were not detected in those transfected with the reporter alone. Since, however, optical observation has limitations in the detection of specific protein, we cannot rule out the possibility that a small amount of specifically localized HBx may be enough to make active host cellular elements.

Thus, HBx may have a dual role in transcriptional activation. The cytoplasmic HBx may influence the regulation of gene expression through signal transduction pathways or through processing of other proteins while the nuclear one may directly modulate transcriptional machinery. In the latter case, HBx interacts with polII complex through binding to RPB5, recruiting TFIIB for transactivation, and at the same time HBx antagonizes the repressor function of RMP, which is a novel regulator protein interacting with RPB5 [46].

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References


