Intracellular localization of the hepatitis B virus HBx protein

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The hepatitis B virus (HBV) X protein (HBx) was originally suggested to be a viral transcriptional activator, but its functional mechanisms are still unclear. In this study we have analysed the intracellular localization of HBx in transfected cells and demonstrate that its compartmentalization is dependent on overall expression levels. HBx was exclusively or predominantly localized in the nuclei in weakly expressing cells. However, elevated cellular levels correlated with its accumulation in the cytoplasm, suggesting that the capacity of HBx for nuclear compartmentalization might be limited. Cytoplasmic HBx was detected either as punctate granular staining or in dispersed, finely granular patterns. We have further analysed the detailed cytoplasmic compartmentalization, using confocal microscopy, and show no association with the endoplasmic reticulum, plasma membrane or lysosomes, but a substantial association with mitochondria. However, a major fraction of cytoplasmic HBx did not localize in mitochondria, indicating the presence of two distinctly compartmentalized cytoplasmic populations. Furthermore, high levels of HBx expression led to an abnormal mitochondrial distribution, involving clumping and organelle aggregation, which was not observed at lower expression levels. The data presented here provide novel insights into the compartmentalization of HBx and may prove important for future evaluations of its functions, both in the viral life-cycle and in the pathology of HBV-related liver disease.

Introduction

The hepatitis B virus (HBV) X protein (HBx) was originally proposed to be a transcriptional activator (Twu & Schloemer, 1987; Spandau & Lee, 1988; Zahm et al., 1988), and was shown to up-regulate expression of viral and cellular genes (for review see Henkler & Koshy, 1996). Since HBx activates a wide range of promoters and transcription factor binding sites, multiple functional pathways have been postulated. HBx activates Ras (Benn & Schneider, 1994) and induces Erk- and JNK-dependent signalling cascades (Benn et al., 1996; Henkler et al., 1998; Natoli et al., 1994). In addition, HBx was recently shown to activate Jak1-STAT signalling (Lee & Yun, 1998) and Src kinases (Klein & Schneider, 1997). HBx was further shown to transactivate via binding to several transcription factors in the nucleus (Maguire et al., 1991; Williams & Andrisani, 1995; Antunovic et al., 1993), including components of the basal transcription machinery (Qadri et al., 1995, 1996). HBx expression is often considered as an aetiopathological factor in HBV-related carcinogenesis and its proposed oncogenic risks are attributed to transcriptional activation of proto-oncogenes (Henkler & Koshy, 1996) and to an inactivation of the p53 tumour suppressor protein (Feitelson et al., 1993; Wang et al., 1994; Truant et al., 1995). However, more recently, HBx was suggested to induce or pre-dispose cells to apoptosis and to inhibit proliferation of transfected cells (Sirma et al., 1999; Su & Schneider, 1997; Terradillos et al., 1998; Schuster et al., 2000). In addition, inhibitory effects of HBx on DNA repair have also been reported (Lee et al., 1995; Becker et al., 1998; Groisman et al., 1999; Jia et al., 1999). These observations may explain both the anti-proliferative effects and the oncogenic potential of
HBx. Moreover, numerous alternative functions have been proposed, for example inhibition of the proteasome and protein degradation (Fischer et al., 1995), or interaction with subunits of RNA polymerases (Cheong et al., 1995). The precise functional evaluation of HBx at the molecular level is problematic, since very little is known about its expression in vivo and the intracellular distribution is not understood. Doria et al. (1995) demonstrated a major cytoplasmic fraction of HBx that accumulated as granules and activated Ras-dependent signalling. However, no hypothesis has yet been suggested to explain how granular HBx activates Ras and no molecular mechanism for HBx-related signalling has been established so far. Further, nuclear HBx was difficult to detect in cultured cells, but it is suggested to transactivate via binding to transcription factors (Doria et al., 1995). The intracellular distribution of HBx has not been considered adequately in explorations of its biological role and it is still difficult to relate its expression patterns with proposed functions. For example, various interactions have been suggested for HBx in the nucleus, but these are difficult to reconcile with the presence of a barely detectable nuclear fraction. The significance of its granular or perinuclear distribution has not been clarified and it is not known whether HBx exists in diverse cytoplasmic compartments.

In this study we have analysed expression and intracellular localization of HBx in transfected cells, using a sensitive and highly specific monoclonal antibody (Henkler et al., 1995). Our data indicate that compartmentalization of HBx is more complex than thought previously, and we demonstrate that a significant proportion of cytoplasmic HBx is associated with mitochondria.

Methods

Antiseras, plasmids and reagents. The monoclonal antibody 16F1, the rabbit polyclonal antiserum pAB-X (Henkler et al., 1995) and the HBx expression vector pCMVX (Henkler et al., 1998) have been described before. MitoTracker (M-7512 and M-7513) and LysoTracker (L-7528) were purchased from Molecular Probes (Poot et al., 1996). Biotinylated Bovine serum albumin (BMA), which was used for staining the endoplasmic reticulum (Animashun & Hughes, 1989), was a generous gift from Theresa Animashun.

Tissue culture and transfection. A431 cells were cultured under 5% CO₂ in DMEM containing 10% FBS, 50 U/ml penicillin and 50 mg/ml streptomycin, split into six-well plates (5 x 10⁴ cells per well) and grown on 13 mm cover glasses. Transfections of subconfluent cells were carried out using Lipofectamine (1.5 µg pCMVX and 10 µl of Lipofectamine per 1 ml transfection mix), according to the manufacturer's (GIBCO-BRL) instructions. Cells were harvested 24 h post-transfection for analysis by immunofluorescence or Western blotting. Treatment of living cells with MitoTracker-Red (40–250 nM), MitoTracker Red CM-H₂XRos (80–300 nM) or LysoTracker (150 nM) was carried out for 30 min, followed by a thorough wash for 15 min in PBS prior to fixing.

Western blotting. Twenty µg total protein from cell lysates or 1 µg of GST-fusion proteins was separated by SDS–PAGE, blotted onto nitrocellulose and blocked in 5% non-fat milk. Blots were incubated with 16F1 hybridoma supernatant (diluted 1:10) or pAB-X (diluted 1:1000) for 1 h and for a further 1 h with HRP-labelled secondary antibodies. Immunocomplexes were visualized using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

Immunofluorescence and confocal microscopy. Cells were fixed in cold methanol–acetone (30:70, v/v), blocked for 20 min with PBS–1% BSA, incubated for 1 h with 16F1 hybridoma supernatant and washed in PBS–0.1% Tween 20. Cells were then incubated with a donkey anti-mouse FITC-labelled secondary antiserum (Jackson Immunoresearch Laboratories) for 45 min. Biotinylated BMA was diluted into 16F1 supernatant (1:50) and streptavidin–Texas Red (50 µg/ml) was applied with secondary antiserum for co-staining of HBx and the endoplasmic reticulum. Fluorescent specimens were analysed using an Olympus microscope with CCD camera, which was operated by Delta Vision computer software (Applied Precision Inc.). Alternatively, stained cells were examined with a Leica DM RXE confocal microscope.

Results

The epitope of MAb 16F1 is localized in the N-terminal domain of HBx

We have previously derived monoclonal and polyclonal antibodies directed against HBx (Henkler et al., 1995). One of these antibodies, named 16F1, is distinguished by its high sensitivity, which allows the specific detection of minute amounts of HBx by immunofluorescence (Fig. 1D). In this study, we analysed the epitope of 16F1 to clarify whether this antibody detects specifically full-length HBx, or also the shorter alternative translation products that were demonstrated in a previous report (Kwee et al., 1992). 16F1 reacted with full-length GST–HBx only, not with a mutant fusion protein that contained the 75 C-terminal amino acids of HBx that correspond to the proposed alternative gene product. In contrast, the polyclonal antiserum pAB-X recognized both full-length and truncated proteins by Western blotting (Fig. 1A). These observations suggest that the epitope recognized by 16F1 is located in the N-terminal domain of HBx, and indicate that the localization data described below apply specifically to full-length HBx. However, 16F1 only detected HBx protein that was derived from the HBV(adae2) genome (Fig. 1B), but not a closely related X-protein that was subcloned from an ayw genome (Renner et al., 1995). We cannot exclude therefore the possibility that variant HBx proteins might compartmentalize differently. The hbx gene sequence was verified in both constructs. The amino acid sequence of HBx, as expressed by pCMVX and recognized by 16F1, is given in Fig. 1(C).

Nuclear–cytoplasmic compartmentalization is related to total cellular expression levels

Expression of HBx in cultured cells requires transient expression systems. The difficulties in establishing stable cell lines are widely acknowledged and attributed to the capacity of HBx to induce or to predispose cells to apoptosis. We have analysed here the intracellular compartmentalization in transfected Huh-7 cells. HBx-transfected and mock-transfected
Fig. 1. Mapping the epitope of 16F1. (A) Differential detection of GST-X fusion proteins by 16F1 and pAB-X. One µg of GST (lane 1), GST-X – 8 kDa [A aa1–78] (lane 2) and GST-X (lane 3) were separated by SDS–PAGE, blotted onto nitrocellulose and probed with 16F1 (left panel) or pAB-X. Migration of both full-length (43 kDa) and truncated (GST-X – 8 kDa) HBx fusion proteins is indicated. (B) Western blot analysis. A431 cells were transfected with pCMVX (lanes 1) or pCMVX ayw (lanes 2) (Renner et al., 1995) and probed either with 16F1 (left panel) or pAB-X (right panel). The position of the 17 kDa full-length HBx is indicated. (C) Amino acid sequence of HBx, as expressed by pCMVX and recognized by MAb 16F1. The epitope of 16F1 involves the region between the arrows (data not shown). N-terminal amino acids that diverge in both HBx proteins (Fig. 1B) and might be part of the epitope are indicated (*). (D) Specific detection of HBx by immunofluorescence using 16F1. Cells were transfected with pCMVX (top panels) or pcDNA3 (bottom panels). HBx was detected with 16F1 (left column) and nuclei were counterstained with DAPI (middle column); overlay staining is shown in the right column.
Fig. 2. HBx is predominately localized in the nuclei at low expression levels. (A)–(F) Huh-7 cells were transfected with 1 µg pCMVX and analysed for HBx expression 18 h post-transfection. HBx was visualized using 16F1 (green staining). Cells were counterstained with DAPI, thus visualizing cell nuclei (blue staining). Huh-7 cells transfected with 'empty' pcDNA3 were used as a negative control (F). The detection patterns of HBx (upper panels) and overlays of HBx staining and counter-stained nuclei (lower panels) are shown. The scale bar indicates 5 µm.

control cells were stained with 16F1 and counter-stained with DAPI, a dye that intercalates into DNA and stains nuclei (Fig. 2). In these experiments HBx was expressed in up to 10% of transfected cells. In contrast, no specific fluorescent signal was observed in mock-transfected cells that were used as a negative control (Fig. 2. In these experiments, we observed significant
differences in the HBx expression levels between individual cells. Such differences are frequently observed in transient transfections (F. Henkler & I. A. King, unpublished observations) and may be related to a differential uptake of plasmid DNA. Single positively stained cells were analysed using a CCD camera, as described in Methods. Fluorescence intensities were quantitatively compared between selected, equally sized, fields using the program softwoRX (Applied Precision Inc.). These fields were arranged around single HBx-expressing cells and the fluorescence intensities of these cells were converted into digital signals (Inoue & Spring, 1997). This approach facilitated a quantitative estimate of relative expression levels between cells on the same slide (see Fig. 4). HBx was exclusively or predominantly found in nuclei in 30–40% of positively stained Huh-7 cells (Fig. 2A). The staining intensities of individual cells showing either exclusive nuclear or dual nuclear–cytoplasmic detection were compared for 50 single cells from each population (Fig. 4). This experiment was repeated in two independent transfections. Notably, the detection of cytoplasmic HBx was strongly related to an increase in its overall cellular expression (Fig. 4). Cells in which HBx was detectable in the cytoplasm always showed higher expression levels compared to cells where HBx was detected only in the nucleus. Our observations indicate that low amounts of HBx are predominantly compartmentalized in the nuclei in Huh-7 cells. These results are in conflict with the current concept of a predominantly cytoplasmic distribution, and might suggest that newly synthesized HBx is primarily engaged in nuclear functions during the initial stages of HBV infection.

Diverse staining patterns of cytoplasmic HBx in Huh-7 and A431 cells

Compartmentalization of HBx was observed in the cytoplasm of cells that expressed elevated amounts of protein. We analysed the cytoplasmic compartmentalization of HBx in two different cell lines, the human hepatoma cell line Huh-7, and A431, a squamous carcinoma line. In Huh-7 cells, cytoplasmic HBx was typically detected in distinctively granular patterns (Fig. 2). Similar distribution patterns have been described
Fig. 5. Intracellular localization of HBx in transfected A431 cells. (A)–(C) HBx was detected by 16F1 and visualized as green staining using an FITC-labelled secondary antibody. Sections were scanned with a laser beam (488 nm) and analysed for green light emission (~ 528 nm) using a Leica DM RXE confocal microscope. Column 2: mitochondria (A), lysosomes (B) and the endoplasmic reticulum (C) were detected using Texas Red-labelled reagents, as described in Methods. Sections were scanned with a laser beam (568 nm) and analysed for red light emission (~ 617 nm). Column 3 represents an overlay of columns 1 and 2 to visualize co-localization. Associations between HBx and mitochondria are indicated by arrowheads in (A). The nuclei...
before in cultured cells (Doria et al., 1995), but it is still not known whether these structures have any functional significance. Notably, such granular distribution has not been reported in the previous studies of HBx expression in liver biopsies of chronically infected HBV carriers (Katayama et al., 1989; Haruna et al., 1991; Su et al., 1998; J. Hoare and others, unpublished). We have further analysed the cytoplasmic compartmentalization of HBx in A431 cells. Notably, in this cell line cytoplasmic HBx is frequently detected in a dispersed, non-granular distribution (in up to 40% of positively stained cells) and the HBx staining is more consistent with patterns that have been previously described in vivo. As in Huh-7 cells, a range of HBx staining patterns was observed (Fig. 3 and Fig. 4). A major and sometimes predominant fraction of HBx was nuclear (Fig. 3 A, B). Again, these cells expressed apparently small amounts of protein compared with cells that showed predominantly cytoplasmic staining. However, in contrast to Huh-7 cells, an exclusively nuclear staining pattern was very rare. Cytoplasmic HBx was distributed either homogeneously (Fig. 3 C) or finely granular (Fig. 3 D). Nuclear X-protein became less apparent at elevated expression levels, suggesting that the relative proportion of nuclear HBx was decreased. In strongly expressing cells, cytoplasmic HBx frequently accumulated in large granules (Fig. 3 E) or strongly stained aggregates (Fig. 3 F). The nature of these granules is not clear. Formation of these structures could be linked to precipitation of abundant HBx in highly expressing cells, because granular staining normally coincided with detection of high levels of HBx.

**Cytoplasmic compartmentalization of HBx in transfected cells**

We investigated possible associations of HBx with the endoplasmic reticulum, lysosomes and mitochondria by using confocal microscopy. HBx was detected by MAb 16E1 and visualized as green fluorescent staining (Fig. 5 A–C). The distribution of HBx in various cytoplasmic compartments was examined using Texas Red-labelled reagents that specifically identify distinct organelles. Lysosomes were detected using LysoTracker, a dye that selectively distributes into acidic organelles (Fig. 5 B). Staining of the endoplasmic reticulum was done with BMA (Fig. 5 C), a legume lectin that interacts only with glycoproteins in the endoplasmic reticulum (Animashaun & Hughes, 1989). In these experiments, no apparent associations were found between HBx and either the endoplasmic reticulum (Fig. 5 C) or lysosomes (Fig. 5 B). Furthermore, the finely granular cytoplasmic distribution of HBx was interrupted by lysosomal staining, suggesting that it is excluded from these organelles. This observation confirms a previous report, which demonstrated that HBx and lysosomes are not co-purified from lysates of HBx-expressing cells (Doria et al., 1995). In contrast, association between HBx and mitochondria was suggested by co-staining experiments using two mitochondrion-selective probes [Fig. 5 A, MitoTracker CMXRos; Fig. 6 A–E, MitoTracker CM-H2XRos (Poot et al., 1996)]. Mitochondria were detected as elongated structures with a length of up to 2 µm. A condensed distribution of organelles was observed in the perinuclear region and extending into the cytoplasm. Immunofluorescent detection of HBx was concentrated in areas with a high density of mitochondria and it was found in distinct association with these organelles (Fig. 5 A; Fig. 6 A, B; localization of HBx in mitochondria is indicated by arrowheads). However, this co-localization was only partial and a significant fraction of cytoplasmic HBx was not associated with mitochondria. Furthermore, very few granules were observed in these cells and a substantial amount of HBx was also detectable in the nuclei (Fig. 5 A; Fig. 6 A). We did not detect significant differences in the staining intensity of mitochondria in HBx-expressing and negative cells using a variable concentration range of CMXRos (40, 100 and 250 nM). This suggests that the staining of mitochondria was not affected by the expression of HBx.

The reduced MitoTracker variant CM-H2XRos was used as an alternative probe. CM-H2XRos is a non-fluorescent dye which is converted by intracellular oxidation into a mitochondrion-selective probe and allows very specific detection of mitochondria. As observed before, a major proportion of cytoplasmic HBx was clearly associated with mitochondria (Fig. 6 A, B), but again the translocation of HBx into the organelles was apparently inefficient. The protein was only concentrated in mitochondria by an estimated 2 to 4-fold, as compared with its dispersed distribution in the cytoplasm or nucleus (Fig. 6 A, B; Fig. 5 A). Notably, extramitochondrial HBx was frequently detected as the predominant cytoplasmic fraction (Fig. 6 B). This observation suggests the presence of two cytoplasmic fractions of HBx protein. It is possible that these distinct populations are functionally diverse, since they are associated with different compartments.

Using high resolution confocal microscopy at high magnification, mitochondrion-like structures were visualized that showed a strong peripheral staining of HBx (Fig. 5 D). In contrast, no or very little X-protein was detectable in the centres. This may indicate that HBx is associated either with the organelle surface, or is localized within the membrane system, but not in the mitochondrial matrix. This hypothesis requires further corroboration and the precise suborganelar distribution remains to be established by electron microscopy.
Fig. 6. For legend see facing page.
Fig. 7. Detection of HBx in strongly expressing A431 (A) and Huh-7 (B) cells. Staining patterns of HBx (column 1), mitochondria (column 2) and an overlay (column 3) are shown. Organelle clumping and abnormal mitochondrial distribution patterns were observed in conjunction with granular or aggregated HBx. This may indicate that co-aggregations of HBx and mitochondria occur in overexpressing cells. The scale bars indicate 10 µm (A) and 5 µm (B).

Associations between HBx and mitochondria were further confirmed in Huh-7 cells (Fig. 6C–E). In agreement with our observations in A431 cells, a substantial fraction of cytoplasmic HBx was associated with mitochondria in cultured liver cells. Our data support a very recent report which suggested that associations occur between HBx and mitochondria in these cells (Rahmani et al., 2000). However, our experiments do not support the concept that the X-protein is primarily a mitochondrial protein. Firstly, HBx maintained a predominantly nuclear distribution in moderately expressing Huh-7 cells where its cytoplasmic distribution was dispersed. Secondly, a major extramitochondrial fraction of HBx was also found in the cytoplasm, suggesting a differential compartmentalization of cytoplasmic HBx.

High-level expression of HBx leads to aggregation and to an abnormal distribution of mitochondria

Detection of HBx in punctate or aggregated structures raised the question of whether these patterns were related to the proposed compartmentalization of HBx in mitochondria. Although granular HBx was rarely observed at moderate expression levels, these structures were occasionally found, but neither cytoplasmic nor nuclear granules were co-stained by MitoTracker dyes. These observations suggest that the granular staining pattern of HBx is initially not related to an association with mitochondria (examples of granular HBx are indicated with blue arrowheads in Fig. 6). In contrast, a partial co-aggregation of HBx and mitochondria was often observed in highly expressing cells, which showed a granular and predominantly cytoplasmic staining pattern (Fig. 7). However, the distribution of mitochondria was not altered in cytoplasmic areas where no granular or aggregated HBx was detected (Fig. 7B). Again, this observation indicates a distinction between granular punctate detection of HBx and its localization in mitochondria. In a very recent report, HBx was shown to cause aggregation of mitochondria at the nuclear periphery, which led to induction of apoptosis (Takada et al., 1999). However, our experiments suggest that co-aggregation of X-protein and mitochondria requires high expression levels and is unlikely to occur when the protein is expressed at the low levels expected in HBV infection.
Discussion

The functional mechanisms of the hepatitis B virus HBx protein are not very well understood. It has been classified as a transcriptional activator, although its capacity to activate viral and cellular promoters is rather weak in cultured cells (Nakatake et al., 1993), and numerous alternative functions have been suggested (Henkler & Koshy, 1996).

In this study, we analysed the intracellular localization of HBx and showed that the protein is primarily localized in the nucleus at low expression levels. In contrast, at elevated levels HBx accumulated in the cytoplasm, leading to a proportional decrease of its nuclear fraction. This indicates further that the capacity for nuclear import or retention of HBx is limited. Our data are consistent with a recently proposed model which suggested that the nuclear import of HBx is dependent on cellular proteins, such as kBx (Weil et al., 1999), or on other viral or host cell factors that have not yet been identified. Our finding that HBxs is preferentially compartmentalized in the nucleus at low expression levels has wide implications. These experiments indicate a much higher significance of nuclear HBx than suggested in previous localization studies, where the protein was only or predominantly found in the cytoplasm. These localization data support the concept that trans-activation by HBx involves binding to transcription factors and nuclear proteins. Further, associations between HBx and DNA repair enzymes have been described (Lee et al., 1995; Becker et al., 1998; Groisman et al., 1999; Jia et al., 1999). These interactions could also be important, since the HBV replication cycle involves the conversion of its incompletely synthesized viral genome into a covalently closed circular DNA. In addition, nuclear HBx could also interfere with the function of the p53 tumour suppressor protein.

We have further demonstrated that the levels of nuclear HBx remain low, while elevated expression correlated with its accumulation in the cytoplasm. This may further explain the difficulties in detecting nuclear HBx, both in vivo and in cultured cells. In addition, there is evidence that the X-protein functions at a very low expression level. HBx was recently shown to activate the HBV-core promoter in transgenic mice. Interestingly, the magnitude of this effect was much higher than previously reported in transfected cells, but HBx was not detectable in these mice using standard detection methods (Reifenberg et al., 1999).

Moderately expressing A431 cells were used as a model to analyse the cytoplasmic compartmentalization of HBx using confocal microscopy. We demonstrated by immunofluorescence staining that a substantial fraction of cytoplasmic HBx is associated with mitochondria. Computer analysis of the HBx amino acid sequence, using PSORT software (University of Tokyo), suggested that the N-terminal sequence of HBx may have features of a putative mitochondrial targeting signal (data not shown). However, Western blotting detected only the wild-type full-length protein when both this and truncated variant proteins were synthesized using expression vectors (Fig. 1). Consistent with this, no cleavable signal vector was predicted in the primary structure of HBx using the SignalP server (Technical University of Denmark). These observations suggest that the association of HBx with mitochondria, or any other organelle, does not involve processing of the protein. Furthermore, putative mitochondrial targeting sequences in HBx must be weak, since a substantial amount of cytoplasmic X-protein does not accumulate in mitochondria.

Attempts to analyse the subcellular distribution of HBx by differential centifugation were inconclusive, because the protein co-sedimented with a mitochondrial marker both in the nuclear (1000 g) and mitochondrial (10000 g) fractions. The value of cell fractionation experiments is further limited, because these experiments do not allow a differential analysis of individual cells according to expression levels. This leads to a disproportionate representation of aggregated HBx in strongly expressing cells. Aggregation of HBx has been recently described by Takada et al. (1999). In this report, HBx and p53 were co-detected in abnormally aggregated mitochondrial structures and it was further suggested that HBx could induce mitochondria-related cell death. Our data do not support all of these conclusions. We have demonstrated here a differential compartmentalization of HBx in both nucleus and cytoplasm, with a fraction associated with mitochondria. However, alterations to the mitochondrial staining or organelle distribution were not apparent in moderately expressing cells.

The functions of HBx in mitochondria are not known. HBx was previously shown to induce or to predispose cells to apoptosis, although there is also evidence for an inhibition of p53-dependent apoptosis (Elmore et al., 1997). These proposed apoptotic or cytotoxic effects of HBx could be related to its mitochondrial localization, since this organelle has a major role in regulation of programmed cell death (Martinou, 1999). However, it is not yet clear whether HBx-mediated cytotoxic effects are significant in the immunopathology of hepatitis B infection and there is no apparent linkage between its pro-apoptotic activities and virus replication. Alternatively, HBx could affect different mitochondrial functions, such as calcium storage, which may be directly related to cytoplasmic signalling events. Very recently, HBx was shown to bind HVDAC3, a mitochondrial ion-channel protein (Rahmani et al., 2000). Although the functional significance of this interaction is not yet clear, this observation could suggest that HBx may affect the permeability of the mitochondrial membrane for metabolites or ions. The precise functions of mitochondrial HBx remain to be established. However, we speculate here that the demonstrated association of HBx with mitochondria may have a central significance in its biological role and functions.

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