Hepatitis B Virus X Protein Colocalizes to Mitochondria with a Human Voltage-Dependent Anion Channel, HVDAC3, and Alters Its Transmembrane Potential

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Understanding the mechanism(s) of action of the hepatitis B virus (HBV)-encoded protein HBx is fundamental to elucidating the underlying mechanisms of chronic liver disease and hepatocellular carcinoma caused by HBV infection. In our continued attempts to identify cellular targets of HBx, we have previously reported the identification of a novel cellular protein with the aid of a yeast two-hybrid assay. This cellular gene was identified as a third member of the family of human genes that encode the voltage-dependent anion channel (HVDAC3). In the present study, physical interaction between HBx and HVDAC3 was established by standard in vitro and in vivo methods. Confocal laser microscopy of transfected cells with respective expression vectors colocalized HVDAC3 and HBx to mitochondria. This novel, heretofore unreported subcellular distribution of HBx in mitochondria implies a functional role of HBx in functions associated with mitochondria. Using a stable cationic fluorophore dye, CMXRos, we show that HBx expression in cultured human hepatoma cells leads to alteration of mitochondrial transmembrane potential. Such functional roles of HBx in affecting mitochondrial physiology have implications for HBV-induced liver injury and the development of hepatocellular carcinoma.

Human hepatitis B virus (HBV) is the leading causative agent of chronic hepatitis. HBV infection has been strongly associated with the development of hepatocellular carcinoma (HCC) (2), but the mechanism(s) by which HBV induces events leading to the genesis of HCC remains to be clearly understood. One of the open reading frames encoded by the HBV genome is a regulatory protein termed HBx, consisting of 154 amino acids (~16.5 kDa). HBx was first identified as a transcriptional activator (29, 32, 34), and the mechanism by which it accomplished that function was shown to occur via protein-protein interaction (19). HBx is predominantly localized in the cytoplasm with a low level of nuclear distribution (11, 30, 37). The activities of HBx which require its presence in the cytoplasm include its participation in a whole host of cellular signal transduction pathways, including Ras–Raf–mitogen-activated protein kinase, protein kinase C, and Src kinase (3, 9, 13, 15). A common theme that emerges from recent studies is the ability of HBx to deregulate cell growth (6, 16, 33). This property may be a two-edged sword; HBx's activities can either promote cellular proliferation or signal programmed cell death. In support of this view, two reports implied that HBx has an indirect role in apoptosis (6, 33). These and several other studies lend support to the notion that HBx may play a pivotal role in the pathways associated with liver oncogenesis.

We have recently identified a novel target of HBx during a yeast two-hybrid screening. Isolation of a full-length cDNA revealed a new member of the human voltage-dependent ion channel (VDAC) family, which was designated HVDAC3 (26). Multiple isoforms of VDAC-encoding genes have been identi-fied in mammals. In humans, two isoforms of VDAC (HVDAC1 and HVDAC2) genes have been cloned (4). VDAC proteins, also known as mitochondrial porins, are small (ranging from 30 to 34 kDa), abundant proteins that form pores in the outer membranes of mitochondria of all eukaryotic organisms (31). VDACs are presumed to act as pathways for ATP and metabolites across the mitochondrial membrane (8, 18, 27). Recent studies showed that VDAC is a part of the permeability transition pore complex in the mitochondrial membrane which regulates mitochondrial transmembrane potential and cytochrome *c* release (12, 21).

One of the novel aspects revealed during the course of this investigation is the subcellular distribution of HBx in mitochondria that has not been reported so far. We describe here an interaction between HBx and HVDAC3 using standard in vitro assays and cross-immunoprecipitation procedures. Double immunofluorescence by confocal laser microscopy colocalized HVDAC3 and HBx to mitochondria. We further show that mitochondrial association of HBx leads to alteration of mitochondrial transmembrane potential. This novel subcellular distribution of HBx and its association with VDAC provide clues to its possible functions in infected hepatocytes in liver disease pathogenesis associated with HBV infection.

MATERIALS AND METHODS

Plasmids. The full-length HVDAC3 cDNA was cloned into pCMV4HA to produce the plasmid pVDAC3 (pCMV4HA-VDAC3). The HBx-encoding gene was cloned into pCMV4 with a Flag tag cassette at the C terminus of the gene to produce the plasmid pCXF (pCMV4X-flag). The full-length HVDAC3 gene was cloned in a bacterial glutathione S-transferase (GST) vector (pGEX-5T-1) in frame with the GST-encoding gene to produce the plasmid pGVDAC3.

Purification of GST fusion proteins. The GST-VDAC3 fusion protein was purified by the following procedure. Briefly, a bacterial culture expressing the GST-VDAC3 fusion protein was centrifuged and the pellet was resuspended in STE buffer (10 mM Tris [pH 8.0], 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA) and treated with lysozyme (100 μg/ml) for 15 min on ice. Dithiothreitol and Sarkosyl were added to final concentrations of 5 mM and 1.5%, respectively. After sonication, the lysates were centrifuged and Triton X-100 was added to a final concentration of 4%. Supernatant was incubated with glutathione-Sepharose beads for 30 min at 4°C. The beads were washed several

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times with STE buffer containing decreasing concentrations of NaCl. Washed beads were equilibrated with buffer B (150 mM KCl, 6 mM MgCl$_2$, 25 mM HEPES [pH 7.9], 10% [vol/vol] glycerol, 0.1% NP-40, 1 mM ATP, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, aprotinin at 9 μg/ml) at 4°C. The purification of GST-X has been reported previously (24).

In vitro binding assay. In vitro synthesis of HBx and HVDAC3 was carried out by using the TNT coupled transcription-translation system (Promega). To eliminate nonspecific interactions, in vitro-translated proteins were precleared by incubation with GST protein bound to glutathione-Sepharose beads. Precleared proteins were allowed to bind GST or GST fusion proteins bound to glutathione-Sepharose beads in buffer B at 4°C for 2 h in a 300-μl volume. The reaction mixture was washed with buffer B several times, and bound proteins were eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

In vivo interactions. The coimmunoprecipitation procedure used here is a modified form of that of Xu and Reed (35). When pVDAC3 was transiently cotransfected with pCXF into COS cells using Superfect reagent (Qiagen), hemagglutinin (HA)-VDAC3 protein levels detected by enhanced chemiluminescence (ECL) were about twofold lower (at ~5 min of exposure) than those achieved by transfection with pCMV4 and pVDAC3 and detected by densitometric analysis (data not shown). Therefore, to compensate for differential levels of HA-VDAC3 protein, one plate (100-mm diameter) of COS cells transfected with pCMV4 and pVDAC3 and three plates of COS cells transfected with pCXF and pVDAC3 were used for coimmunoprecipitation experiments. In a reciprocal experiment, three plates of COS cells transfected with pCMV4HA and pCXF and three plates of cells transfected with pVDAC3 and pCXF were used. After 48 h of transfection, cells were washed with cold phosphate-buffered saline (PBS) and incubated with H buffer (142.5 mM KCl, 5 mM MgCl$_2$, 10 mM HEPES [pH 7.2], 1 mM EGTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, leupeptin at 10 μg/ml, aprotinin at 9 μg/ml) on ice for 10 min. Cells were collected by scraping and further incubated on ice for 20 min. Cell lysates were centrifuged for 10 min in an Eppendorf microcentrifuge. To eliminate nonspecific binding, the supernatant was preincubated with protein G-Sepharose for 30 min at 4°C. H buffer without NP-40 was added to the precleared lysates to a final concentration of 0.6 or 0.8%. The incubation was continued with the indicated antibodies (Abs) for 2 h and for an additional hour with protein G-Sepharose at 4°C. The immune complexes were washed three times with H buffer and analyzed by SDS-PAGE, followed by Western blotting. The signal was detected with the ECL reagent (Amersham).

Indirect immunofluorescence assay. Huh7 cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Indicated plasmids were transfected with Superfect reagent in accordance with the manufacturer's (Qiagen) instructions. Transfected cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with cold acetone (−20°C) for 7 min at −20°C, and incubated with 10% fetal bovine serum–0.1% Triton X-100 in PBS for 1 h at room temperature. For colocalization studies, cells were incubated with a polyclonal Ab against HBx and a monoclonal Ab against HA (12CA5). For localization of HBx to mitochondria, cells were incubated with a monoclonal Ab against cytochrome *c* oxidase subunit I (Molecular Probes) and a polyclonal Ab against HBx. For localization of the Golgi, cells transfected with an HBx-Flag expression vector containing a single Flag tag were incubated with a monoclonal Ab against Flag (Sigma) and tetramethyl rhodamine isocyanate (TRITC)-wheat germ agglutinin (WGA) (Molecular Probes). For localization of HA-VDAC3 to mitochondria, the monoclonal Ab against HA (12CA5) and a polyclonal Ab against mitochondrial electron transfer flavoprotein (ETF) were used. Fluorescein isothiocyanate (FITC)-coupled anti-mouse and TRITC-coupled anti-rabbit Abs were used as secondary Abs. Stained cells were analyzed by Olympus confocal microscopy and the Adobe Photoshop program.

Cell fractionation. The procedure used for cell fractionation was that of Yu et al. (36). Briefly, COS-1 cells transfected with pCXF and pVDAC3 were homogenized and fractionated into three different fractions by differential centrifugation. Equal amounts of protein from the low-speed pellet, high-speed pellet, and high-speed supernatant were analyzed by SDS-PAGE, followed by Western blotting. The signal was detected with the ECL reagent (Amersham).

CMXRos fluorescence. Huh7 cells were transfected with the indicated plasmids using Lipofectin (GIBCO Bethesda Research Laboratories). About 48 h posttransfection, cells were incubated with medium containing CMXRos (Molecular Probes) at a concentration of 250 to about 375 nM for 45 min and fixed for 30 min in 4% paraformaldehyde. Immunofluorescence assays of HBx and HVDAC3 were done as described earlier using anti-Flag and anti-HA Abs, respectively. Dye intensities were visually examined with Zeiss Axioplan 2 microscope within 12 to about 15 h. Cells expressing the indicated proteins were randomly chosen. Cells which showed substantially or relatively decreased CMXRos intensities compared to adjacent and/or surrounding cells were counted and converted to the percentage of cells with decreased CMXRos intensity. Cells with intensity too difficult to determine or in an area with very weak overall intensity were not counted.
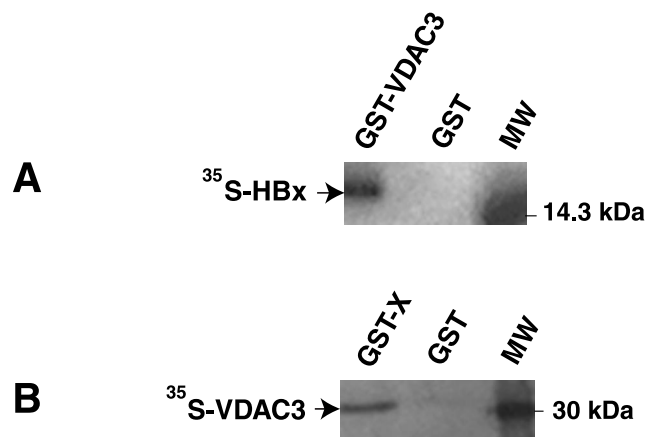

FIG. 1. HBx interacts with HVDAC3 in vitro. (A) HBx was translated in vitro with the TNT system (Promega) in the presence of [^{35}S]methionine. Precleared, ^{35}S-labeled HBx was added to GST or the GST-VDAC3 fusion protein. Bound proteins were washed with binding buffer (see Materials and Methods) eluted, resolved by SDS-PAGE, and exposed to X-ray film. (B) HVDAC3 was translated in vitro with the TNT system (Promega) in the presence of [^{35}S]methionine. The same amount of precleared, ^{35}S-labeled HVDAC3 was added to GST or the GST-X fusion protein. Bound proteins were washed with binding buffer, eluted, resolved by SDS-PAGE, and visualized by exposure to X-ray film. MW, ^{14}C-labeled molecular size marker.

RESULTS

HBx interacts with HVDAC3 in vitro. We have recently identified a human VDAC protein which specifically interacted with HBx using the yeast two-hybrid assay (26). Herein, we report further characterizations of that interaction using the GST pull-down assay. ^{35}S-labeled, in vitro-translated HBx was incubated with GST or a GST-VDAC3 fusion protein immobilized on glutathione-Sepharose beads. Bound proteins were washed with binding buffer, eluted, and analyzed by SDS-PAGE and autoradiography. The results of this analysis are shown in Fig. 1A. In vitro-translated HBx binds to GST-VDAC3 but not to GST. We also performed a reciprocal analysis in which HVDAC3 was in vitro translated in the presence of [^{35}S]methionine and incubated with GST and GST-X. The data shown in Fig. 1B demonstrate the specific interactions between HVDAC3 and full-length HBx (Fig. 1B). Together, these data demonstrate direct interaction between HBx and HVDAC3 in vitro.

HBx interacts with HVDAC3 in vivo. To determine whether HBx and HVDAC3 associate in vivo, epitope-tagged HBx-Flag and HA-VDAC3 expression vectors were developed and a transient-transfection scheme was employed. Previous studies with epitope tagging of HBx have demonstrated that epitope tagging does not interfere with the activities of HBx (11). COS cells were transiently cotransfected with the indicated vectors, and coimmunoprecipitation of transfected cell lysates, followed by Western blotting, was performed. Extracts prepared from transfected cells were immunoprecipitated with a monoclonal Ab against HBx, and the immunoprecipitates were subjected to SDS-PAGE and Western blot analysis using a monoclonal Ab against HA (12CA5). As shown in Fig. 2A, lanes 3 and 4, HVDAC3 is present in cell extracts prepared from either singly transfected or cotransfected COS cells. HVDAC3 was coimmunoprecipitated with HBx in cells expressing HBx (Fig. 2A, lane 2) but not in cells expressing HVDAC3 alone (Fig. 2A, lane 1). In a reciprocal experiment, COS cells were cotransfected with pCMV4HA (parental vector) and pCXF or pVDAC3 and pCXF. The transfected lysates were immu-
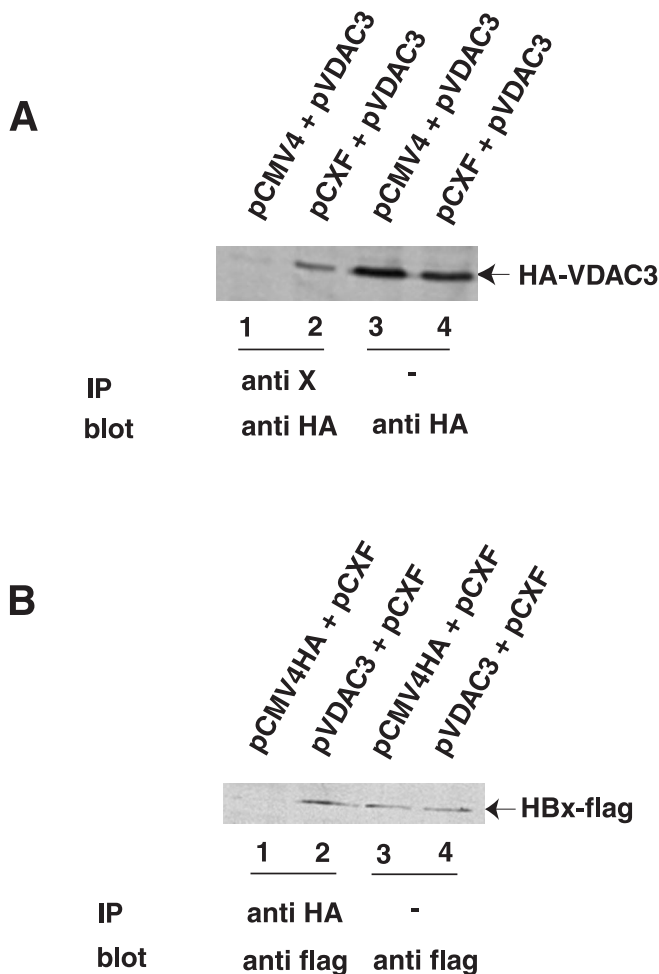
FIG. 2. HBx associates with HVDAC3 in vivo. (A) COS cells were transiently transfected with pVDAC3 and pCXF or with pVDAC3 and pCMV4. After 40 to 48 h of transfection, cell extracts were subjected to immunoprecipitation with a monoclonal Ab against HBx, followed by SDS-PAGE and Western blotting using a monoclonal Ab against HA (12CA5). The signal was detected by using the ECL reagent (Amersham). The immunoblot was exposed for ~10 min. Shown are immunoprecipitates (IP) from cells expressing HA-VDAC3 (lane 1), immuno-precipitates from cells coexpressing HA-VDAC3 and HBx-Flag (lane 2), total cell extracts (10 μg) from cells expressing HA-VDAC3 (lane 3), and total cell extracts (10 μg) from cells coexpressing HA-VDAC3 and HBx-Flag (lane 4). Signals from immunoglobulin G and two high-molecular-weight bands which are also seen in untransfected COS cell extracts are not shown. (B) COS cells were transfected with pCMV4HA and pCXF or with pVDAC3 and pCXF. After ~48 h of transfection, cell extracts were subjected to immunoprecipitation with a monoclonal Ab against HA (12CA5). The signal was detected by using the ECL reagent (Amersham). Shown are immunoprecipitates from cells expressing HBx-Flag (lane 1), immunoprecipitates from cells coexpressing HA-VDAC3 and HBx-Flag (lane 2), total cell extracts (10 μg) from cells expressing HA-VDAC3 (lane 3), and total cell extracts (10 μg) from cells coexpressing HA-VDAC3 and HBx-Flag (lane 4). Immunoglobulin G signals are not shown.

noprecipitated with a monoclonal Ab against HA (12CA5) and subjected to SDS-PAGE, followed by Western blot analysis using a monoclonal Ab against Flag. As shown in Fig. 2B, lanes 3 and 4, transfected lysates contained HBx. In the experiment in which lysates were immunoprecipitated with an Ab against HA and Western blotted with an Ab against Flag, HBx and HVDAC3 were coimmunoprecipitated, suggesting that these proteins are in association in vivo (Fig. 2B, lane 2).

HBx and HVDAC3 colocalize to mitochondria. Based on the observation that HBx and HVDAC3 interact with each other in vitro and in vivo, cellular localization was investigated by indirect double immunofluorescence assay. To study the sub-cellular localizations of HVDAC3 and HBx, Huh7 (human hepatoma) cells were transiently transfected with HBx and HVDAC3 expression vectors and analyzed by confocal micros-copy. As shown in Fig. 3A, image 2, immunostaining of a cell expressing HBx with a polyclonal Ab against HBx, followed by a TRITC-conjugated secondary Ab, showed punctate cytoplas-mic staining. Only transfected cells showed specific staining in the cytoplasm with the Ab against HBx. When HVDAC3 ex-pression was studied using the monoclonal Ab against HA (12CA5), followed by an FITC-conjugated secondary Ab (Fig. 3A, image 1), the pattern was again punctate staining in the cytoplasm. Overlaying of two images indicates that HBx and HVDAC3 colocalize in the cytoplasm of transfected Huh7 cells (Fig. 3A, image 3). Similar results were obtained with transfected COS cells (data not shown).

The cellular compartment where HBx and HVDAC3 colo-calize was investigated using antibodies against mitochondrial marker proteins and double immunofluorescence analysis. To examine whether HBx localizes to mitochondria, Huh7 cells transfected with an HBx expression vector were immuno-stained with a polyclonal HBx Ab (Fig. 3B, image 2) and a monoclonal Ab against cytochrome c oxidase subunit I (Fig. 3B, image 1), a mitochondrial inner membrane protein. As shown in Fig. 3B, image 3, HBx colocalized with cytochrome c oxidase subunit I. To examine whether HVDAC3 localizes to mitochondria, cells expressing HVDAC3 were stained with the monoclonal Ab against HA (12CA5) (Fig. 3B, image 4) and a polyclonal Ab against ETF, a mitochondrial matrix protein (Fig. 3B, image 5). Overlaying of two images indicates that HVDAC3 colocalizes with ETF (Fig. 3B, image 6). Similar results were obtained with transiently transfected COS cells (data not shown). The localization of HVDAC3 or HBx to mitochondria did not require coexpression of either protein. Next, we employed a nonmitochondrial marker, WGA, to ex-amine HBx's localization to a nonmitochondrial organelle, the Golgi. Huh7 cells transfected with an HBx expression vector containing a single Flag were immunostained with monoclonal Abs against Flag (Fig. 3B, image 7) and TRITC-WGA (Fig. 3B, image 8). Overlaying of two images (Fig. 3B, images 7 and 8) shows an absence of colocalization of HBx to Golgi (Fig. 3B, image 9). Thus, these results clearly demonstrate the colocal-ization of HBx to mitochondria.

Cellular localization of HBx and HVDAC3 was further stud-ied by subcellular fractionation. COS cells transfected with HBx and HVDAC3 expression vectors were lysed and fraction-ated into mitochondrial, membrane, and supernatant fractions by differential centrifugation using the procedure previously described (36). Western blot analysis of different fractions in-dicates that HBx and HVDAC3 are present mainly in a frac-tion enriched in mitochondria (Fig. 4A and B). ETF was ob-served in the supernatant, as well as in the mitochondrial fraction (Fig. 4C), because the fractionation procedure used here does permit modest leakage of soluble mitochondrial proteins. Based on the analysis presented here, it appears that HBx has a primary mitochondrial subcellular distribution and that it colocalizes with HVDAC3.

HBx alters $\Delta\Psi_m$. To investigate the functional relevance of the observed association of HBx with mitochondrial VDAC, we utilized a membrane potential-sensitive dye, CMXRos (Molecular Probes), and examined the mitochondrial uptake of the dye. CMXRos is a cationic fluorophore, a relatively stable dye that is preserved during formaldehyde fixation (23) and therefore facilitates efficient monitoring of the changes in mitochondrial function(s). To examine the effects of HBx on mitochondrial transmembrane potential ($\Delta\Psi_m$), transiently
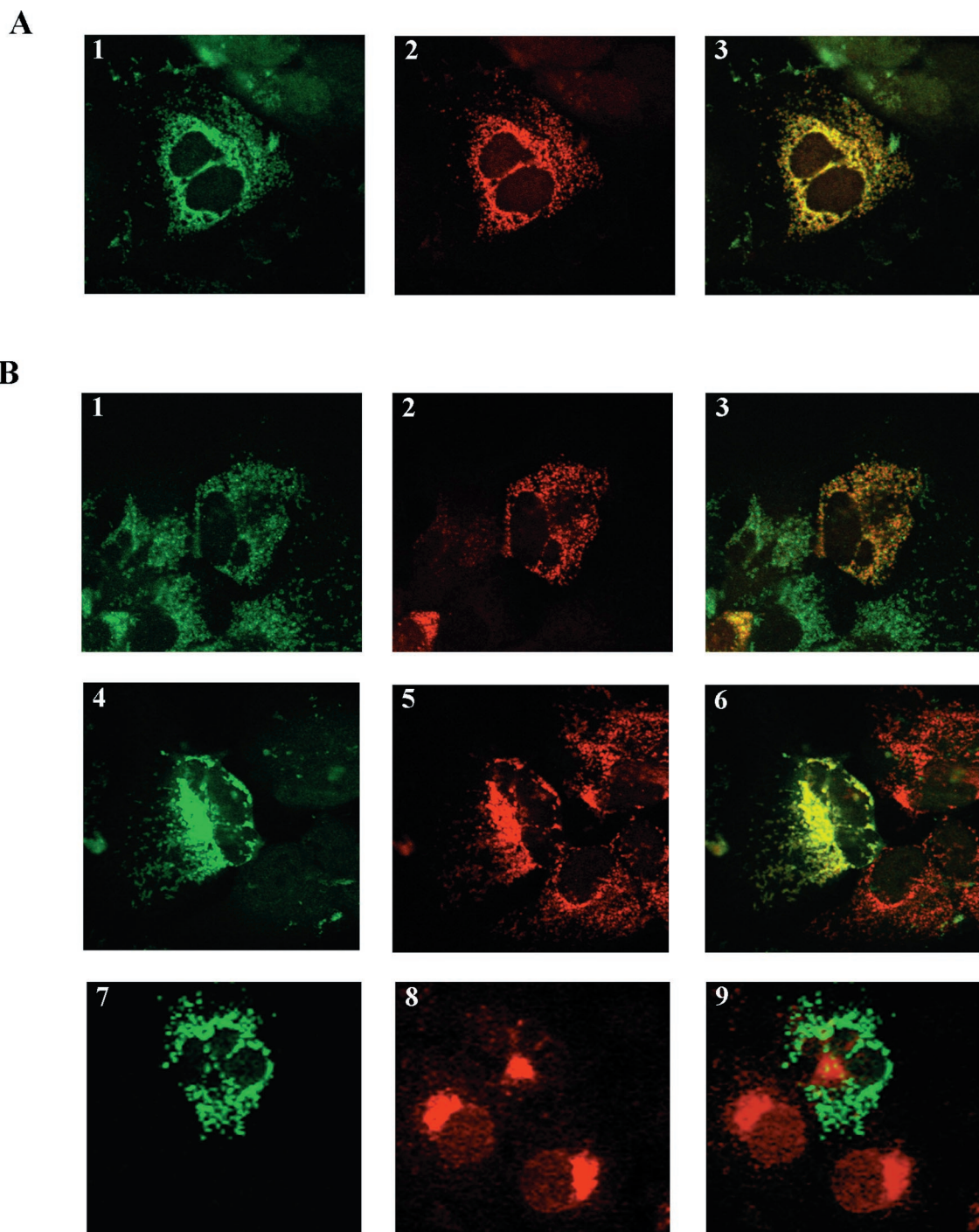
FIG. 3. (A) HBx and HVDAC3 colocalize in vivo. Huh7 cells were transfected with pVDAC3 and pCXF and doubly immunostained with a polyclonal Ab against HBx (image 2) and a monoclonal Ab against HA (12CA5) (image 1). Primary Abs were detected by using a TRITC-conjugated secondary Ab for HBx-Flag detection and an FITC-conjugated secondary Ab for HA-VDAC3 detection. Image 3 is overlapping composite images of HBx-Flag (red) and HA-VDAC3 (green). The overlapping regions appear orange or yellow. (B) HBx and HVDAC3 localize to mitochondria. Huh7 cells were transfected with either pCXF or pVDAC3. Cells expressing HBx-Flag were doubly labeled with a polyclonal Ab against HBx, followed by a TRITC-conjugated secondary Ab (image 2) and a monoclonal Ab against cytochrome *c* oxidase subunit I, followed by an FITC-conjugated secondary Ab (image 1). Overlapping composite images of HBx-Flag (red) and cytochrome *c* oxidase subunit I (green) appear orange or yellow (image 3). Cells expressing HA-VDAC3 were doubly immunostained with a monoclonal Ab against HA (image 4) and a polyclonal Ab against ETF (image 5). Two overlaid images appear orange or yellow (image 6). A cell expressing HBx-Flag was doubly labeled with a monoclonal Ab against Flag (image 7) and WGA (image 8). Two overlaid images show an absence of colocalization (image 9).

transfected Huh7 cells with various expression vectors were incubated with CMXRos. The fluorescence intensities of the dye in the mitochondria correlate with the changes in $\Delta\Psi_m$ (21, 23). The expression of HBx and HVDAC3 was detected by immunofluorescence using Flag and HA Abs, respectively. Since certain cells did not express HBx but showed relatively weak CMXRos intensities, US9GFP-expressing cells were included as a negative control. US9GFP is a pseudorabies virus
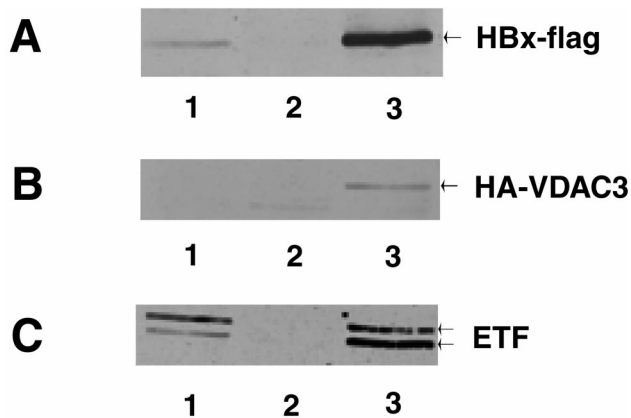
FIG. 4. Subcellular fractionation of HBx and HVDAC3. Subcellular fractionation of COS cells transfected with pCXF and pVDAC3 was used to detect the distribution of HBx-Flag and HA-VDAC3 in the cytoplasm. The cytoplasm was separated into three different fractions by differential centrifugation. Equal amounts of proteins (5 µg) from each fraction were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and incubated with Abs against Flag (A), HA (12CA5) (B), or ETF (two subunits of 26 and 32 kDa) (C). The signal was detected by using the ECL reagent (Amersham). Lanes: 1, high-speed supernatant; 2, high-speed pellet; 3, low-speed pellet enriched in mitochondria.

protein fused to green fluorescent protein, which localizes to the Golgi apparatus (5). The CMXRos intensities of HBx-, HVDAC3-, or US9GFP-expressing cells were visually examined, and cells with relatively weak dye intensities in comparison to the surrounding or adjacent cells were counted and expressed as the percentage of cells with decreased fluorescence (Fig. 5A and B). A higher percentage of HBx-expressing cells exhibited decreased fluorescence intensities than those expressing either HVDAC3 or US9GFP (Fig. 5A). This decreased fluorescence intensity caused by HBx correlates with a decrease of mitochondrial $\Delta\Psi_m$ (Fig. 5B). Although we were not able to examine a cumulative increase of the loss of mitochondrial $\Delta\Psi_m$ in cells coexpressing HBx and HVDAC3, our data clearly demonstrate that HBx, by itself, is capable of altering mitochondrial $\Delta\Psi_m$.

DISCUSSION

HBx is a pleiotropic viral regulatory protein which has been the subject of intense investigation since its identification as an HBV-encoded gene product. The functional role(s) of this rather elusive protein has been shown to occur via a wide variety of cellular targets in both the nucleus and cytoplasm. To better understand the role and mechanism(s) of action of HBx, we have carried out yeast two-hybrid screening to identify the relevant cellular target(s) of HBx. A partial cDNA encoding an open reading frame showing homology to the human VDAC family was identified as an HBx-interacting protein during this search (26). The subsequent analysis to confirm the binding affinity of HBx for HVDAC3 obtained via the yeast two-hybrid expression system is presented in this communication. This analysis first revealed a heretofore unreported subcellular distribution of HBx in mitochondria. Further, the data clearly indicate that HBx and HVDAC3 colocalize to mitochondria and that the association of HBx with mitochondria functionally leads to alteration of $\Delta\Psi_m$.

The subcellular localization of HBx has been previously addressed by several investigators, including ourselves, with the conclusion that it is localized predominantly in the cytoplasm with a low level of nuclear distribution (11, 30, 37). Although punctate cytoplasmic staining of HBx has been previously reported (11), none of these studies characterized the organellar distribution of HBx. Using double immunofluorescence and confocal microscopic analysis, we demonstrate here that HBx and HVDAC3 colocalize mainly to mitochondria in transfected Huh7 cells. This result is further supported by subcellular fractionation results. The localization of HVDAC3 to mitochondria is correlated with a previous report that isoforms of the human VDAC family, HVDAC1, HVDAC2, and HVDAC2′, localized exclusively to mitochondria in transfected COS cells and rat astrocytes (36). The association of HBx with mitochondria seems to be an intrinsic property of the viral protein rather than an artifact of overexpression. It is believed that the level of HBx expression achieved by using the cytomegalovirus promoter-enhancer in Huh7 cells is within the range found in HBV- and woodchuck hepatitis virus-infected cells (10, 33). Analysis of the HBx amino acid sequence using the PSORT program, an algorithm that recognizes mitochondrial targeting signals, predicts mitochondrial localization of the protein. This suggests that a mitochondrial targeting signal may reside within the primary structure of HBx.

The mitochondrion is a key organelle which generates cellular energy and controls apoptosis by releasing death-promoting proteins into the cytoplasm. The association of HBx with mitochondria and HVDAC3 suggests that HBx is capable of
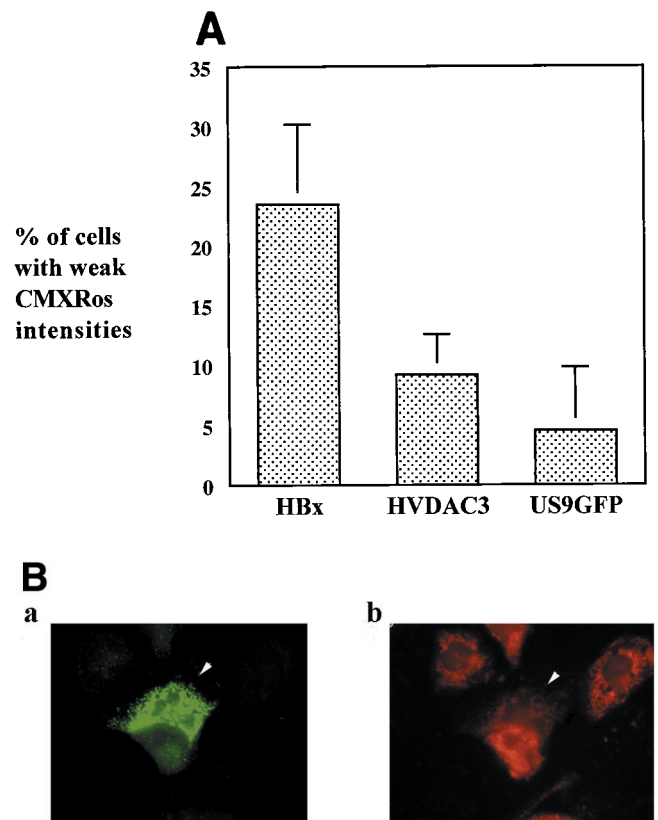

FIG. 5. (A) Analysis of CMXRos fluorescence. HBx-, HVDAC3-, and US9GFP-positive cells were scored for decreased CMXRos fluorescence, and the data are presented as the percentage of cells with weak intensities (number of cells with weak intensities/total number of indicated protein-positive cells × 100). The data are averages of values from five different experiments. (B) Decreased CMXRos fluorescence in HBx-expressing cells. Huh7 cells transfected with an HBx expression vector were stained with CMXRos (b), and HBx expression was detected by using an Ab against Flag, followed by an FITC-conjugated secondary Ab (a). The arrowhead indicates an HBx-expressing cell showing relatively weaker CMXRos staining than adjacent cells.

influencing mitochondrial functions. Some of the bcl2 family members localize in mitochondria or translocate from the cytoplasm to mitochondria and regulate key events of apoptosis, such as cytochrome c release and alteration of $\Delta\Psi_m$ (22, 28). A recent study suggests that regulation of cytochrome c release and alteration of $\Delta\Psi_m$ are due to interaction of these proteins with VDAC and to modulation of the opening state of VDAC (28). Due to the nature of the assay employed, we were not able to examine whether cells coexpressing HBx and HVDAC3 have a synergistic effect on the decrease in $\Delta\Psi_m$. We examined the HBx-expressing transfected cells by double immunofluorescence assay and failed to observe an immediate effect of HBx expression causing cytochrome c release (data not shown). This result indicates that while HBx expression can lead to alteration of $\Delta\Psi_m$, it is unable to induce cytochrome c release, unlike proapoptotic proteins such as Bax. It remains to be determined whether HBx association with mitochondria alters other mitochondrial functions in conjunction with VDAC or other mitochondrial proteins.

While HBx may not be directly apoptotic or antiapoptotic, it certainly can participate in these pathways, as evidenced by previous reports (6, 14, 33), including the fact that apoptotic death of HBV-infected hepatocytes driven by the cell-mediated immune response occurs during viral infection (7). HBx can certainly be a significant player in this process. In light of these observations, it must be pointed out that the fate of HBx-expressing hepatocytes is determined by the predominance of either apoptotic or antiapoptotic stimuli within the cellular environment, which may reflect the state of chronicity, the state of cell growth, and perhaps the status of HBV gene expression. Inappropriate apoptosis has been implicated in many human diseases, including several forms of cancer (25). In principle, the expression of an oncogene that deregulates the cell cycle can either induce apoptosis or sensitize cells to proapoptotic stimuli. Therefore, in the final analysis, it is the balance between proapoptotic activities and antiapoptotic survival signals that dictates whether a cell proliferates or dies.

In summary, we present evidence that a member of the human VDAC family, HVDAC3, identified in a yeast two-hybrid search interacts with HBx in vitro and associates with it in vivo. Further, these proteins localize to mitochondria and HBx expression is associated with decreased $\Delta\Psi_m$. Based on our data, we hypothesize that HBx alters mitochondrial function by association with VDAC. Mitochondrial dysfunction and structural changes have been associated with chronic liver disease and oncogenic processes (1, 17). Future investigations will focus on the identification of HVDAC3 properties and functional consequences of HBx's interaction with HVDAC and probably with other mitochondrial proteins. Future inquiries in this area may pave the way for investigation of possible mechanisms leading to chronic infection and subsequent progression to HCC in the context of mitochondrial association of HBx.

ACKNOWLEDGMENTS

REFERENCES

1. **Bannasch, P., F. Klimek, and D. Mayer.** 1997. Early bioenergetic changes in hepatocarcinogenesis: preneoplastic phenotypes mimic responses to insulin and thyroid hormone. J. Bioenerg. Biomembr. **29:**303–313.
2. **Beasley, R. P., C. C. Liu, L. Y. Huang, and C. S. Chien.** 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet **ii:**1129–1133.
3. **Benn, J., and R. J. Schneider.** 1994. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. Proc. Natl. Acad. Sci. USA **91:**10350–10354.
4. **Blachly-Dyson, E., A. Baldini, M. Litt, E. R. McCabe, and M. Forte.** 1994. Human genes encoding the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane: mapping and identification of two new isoforms. Genomics **20:**62–67.
5. **Brideau, A. D., B. W. Banfield, and L. W. Enquist.** 1998. The US9 gene product of pseudorabies virus, an alphaherpesvirus is a phosphorylated, tail-anchored type II membrane protein. J. Virol. **72:**4560–4570.
6. **Chirillo, P., S. Pagano, G. Natoli, P. L. Puri, V. L. Burgio, C. Balsano, and M. Levrero.** 1997. The hepatitis B virus X gene induces p53-mediated programmed cell death. Proc. Natl. Acad. Sci. USA **94:**8162–8167.
7. **Chisari, F. V.** 1996. Hepatitis B virus transgenic mice: models of viral immunobiology and pathogenesis. Curr. Top. Microbiol. Immunol. **206:**149–173.
8. **Colombini, M.** 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. Nature **279:**643–645.
9. **Cross, J. C., P. Wen, and W. J. Rutter.** 1993. Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen activated cellular serine/threonine kinases. Proc. Natl. Acad. Sci. USA **90:**8078–8082.
10. **Dandri, M., P. Schirmacher, and C. E. Rogler.** 1996. Woodchuck hepatitis virus X protein is present in chronically infected woodchuck liver and woodchuck hepatocellular carcinomas which are permissive for viral replication. J. Virol. **70:**5246–5254.
11. **Doria, M., N. Klein, R. Lucito, and R. J. Schneider.** 1995. The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activators of transcription factors. EMBO J. **14:**4747–4757.
12. **Green, D. R., and J. C. Reed.** 1998. Mitochondria and apoptosis. Science **281:**1309–1312.
13. **Kekule, A. S., U. Lauer, L. Weiss, B. Luber, and P. H. Hofschneider.** 1993. Hepatitis B virus transactivator HBx uses a tumor promoter signalling pathway. Nature **361:**742–745.
14. **Kim, H., H. Lee, and Y. Yun.** 1998. X-gene product of Hepatitis B virus induces apoptosis in liver cells. J. Biol. Chem. **273:**381–385.
15. **Klein, N. P., and R. J. Schneider.** 1997. Activation of Src family kinases by hepatitis B virus HBx protein and coupled signaling to Ras. Mol. Cell. Biol. **17:**6427–6436.
16. **Koike, K., K. Moriya, H. Yotsuyanagi, S. Lino, and K. Kurokawa.** 1994. Induction of cell cycle progression by hepatitis B virus HBx gene expression in quiescent mouse fibroblast. J. Clin. Investig. **94:**1107–1114.
17. **Krahenbuhl, S.** 1993. Alterations in mitochondrial function and morphology in chronic liver disease: pathogenesis and potential for therapeutic intervention. Pharmacol. Ther. **60:**1–38.
18. **Liu, M. Y., and M. Colombini.** 1992. Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC. Biochim. Biophys. Acta **1098:**255–260.
19. **Maguire, H. F., J. P. Hoeffler, and A. Siddiqui.** 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science **252:**842–844.
20. **Marzo, I., C. Brenner, N. Zamzami, J. M. Jurgensmeier, S. A. Susin, H. L. A. Vieira, M.-C. Prevost, Z. Xie, S. Matsuyama, J. C. Reed, and G. Kroemer.** 1998. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science **281:**2027–2031.
21. **Marzo, I., C. Brenner, N. Zazami, S. A. Susin, G. Beutner, D. Brdiczka, R. Remy, Z. Xie, J. C. Reed, and G. Kroemer.** 1998. The permeability transition complex: a target for apoptosis regulation by caspase and Bcl2-related proteins. J. Exp. Med. **187:**1261–1271.
22. **Narita, M., S. Shigeomi, T. Ito, T. Chittenden, R. J. Lutz, H. Matsuda, and Y. Tsujimoto.** 1998. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc. Natl. Acad. Sci. USA **95:**14681–14686.
23. **Poot, M., Y.-Z. Zhang, J. A. Kramer, S. Wells, L. J. Jones, D. K. Hanzel, A. G. Lugade, V. L. Singer, and R. P. Haugland.** 1996. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J. Histochem. Cytochem. **44:**1363–1372.
24. **Qadri, I., J. W. Conaway, R. C. Conaway, J. Schaack, and A. Siddiqui.** 1996. Hepatitis B virus transactivator protein, HBx, associates with the components of TFIIH and stimulates the DNA helicase activity of TFIIH and stimulates the DNA helicase activity of TFIIH. Proc. Natl. Acad. Sci. USA **93:**10578–10583.
25. **Raff, M.** 1998. Cell suicide for beginners. Nature **396:**119–122.
26. **Rahmani, Z., C. Maunoury, and A. Siddiqui.** 1998. Isolation of a novel human voltage-dependent anion channel gene. Eur. J. Hum. Genet. **6:**337–340.
27. **Rostovtseva, T., and M. Colombini.** 1996. ATP flux is controlled by a voltage-

gated channel from the mitochondrial outer membrane. J. Biol. Chem. **271:** 28006–28008.

28. **Shimizu, S., M. Narita, and Y. Tsujimoto.** 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399:**483–412.

29. **Siddiqui, A., R. Gaynor, A. Srinivasan, J. Mapoles, and R. W. Farr.** 1989. Trans-activation of viral enhancers including the long terminal repeat of the human immunodeficiency virus by the hepatitis B virus X protein. Virology **169:**479–484.

30. **Siddiqui, A., S. Jameel, and J. Mapoles.** 1987. Expression of the hepatitis B virus X gene in mammalian cells. Proc. Natl. Acad. Sci. USA **84:**2513–2517.

31. **Sorgato, M., and O. Moran.** 1993. Channels in mitochondrial membranes: knowns, unknowns, and prospects for the future. Crit. Rev. Biochem. Mol. Biol. **18:**127–171.

32. **Spandau, D. F., and C.-H. Lee.** 1988. *trans*-activation of viral enhancers by the hepatitis B virus X protein. J. Virol. **62:**427–434.

33. **Su, F., and R. J. Schneider.** 1997. Hepatitis B virus HBx protein sensitizes cells to apoptotic killing by tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **94:**8744–8749.

34. **Twu, J. S., and R. H. Schloemer.** 1989. Transcription of the human beta interferon gene is inhibited by hepatitis B virus. J. Virol. **63:**3065–3071.

35. **Xu, Q., and J. C. Reed.** 1998. Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol. Cell **1:**337–346.

36. **Yu, W. H., W. Wolfgang, and M. Forte.** 1995. Subcellular localization of human voltage-dependent anion channel isoforms. J. Biol. Chem. **270:** 13998–14006.

37. **Zentgraf, H. G., R. Herrmann, P. Klein, I. Schranz, D. Loncarevic, K. Herrmann, H. Hubnerand, and C. Schroder.** 1990. Mouse monoclonal antibody directed against hepatitis B virus X protein synthesized in *Escherichia coli*: detection of reactive antigen in liver cell carcinoma and chronic hepatitis. Oncology **47:**143–148.