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Subcellular localization of Hepatitis B virus core protein expressed from two different constructs in the Semliki Forest virus expression system

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Abstract

The subcellular localization of Hepatitis B virus (HBV) core protein in eukaryotic cells was studied by an immunocytochemical method following expression of the HBV core (HBc) gene in the Semliki Forest virus expression system from two different constructs. HBc protein showed cytoplasmic and nuclear localization when it was expressed from a construct that encoded the HBV pregenome, whereas HBc protein that was expressed from a construct encoding the HBc gene alone was found only in the cytoplasm. The possible reasons for this phenomenon are discussed.

Key words: HBV core protein, HBV pregenome, Hepatitis B virus, Semliki Forest virus, subcellular localization.

Introduction

Hepatitis B virus (HBV) core (HBc) protein is the structural material of viral capsids. HBV viral particles consist of an enveloped capsid that contains a 3.2 kb partially double stranded DNA genome and the viral polymerase. The viral relaxed circular DNA is completed to covalently closed circular DNA, which serves as the template for the transcription of the viral RNAs: pregenomic RNA (pgRNA) and three subgenomic RNAs that serve as mRNAs for the viral proteins; pgRNA also serves as a template for reverse transcription. Viral polymerase and pgRNA are packaged into nucleocapsids followed by synthesis of a DNA minus strand, which is converted into the rcDNA genome (reviewed in Seeger, Mason 2000).

HBc protein is composed of two domains, an amino-terminal self-assembly domain (amino acid residues 1 to 150) and a carboxy-terminal protamine-like arginine-rich domain, which contains a nuclear localization signal (Eckhardt et al. 1991). Three serine residues in the C terminal domain overlapping the nuclear localization signal may be phosphorylated and play a role in both regulation of HBV replication (Melegari et al.

2005) and in nuclear localization (Liao, Ou 1995), as only phosphorylated HBc protein binds to the nuclear pore complex (Kann et al. 1999).

In previous research the subcellular localization of internally deleted HBc gene variants was studied (Bruvere et al. 2004). These variants were isolated from renal transplant recipients, where their presence was associated with a severe course of illness leading to end stage liver disease (Guenther et al. 1996; Preikschat et al. 2002; Guenther et al. 2000). Variant HBc protein was localized in the cytoplasm and to a lower extent also in the nucleus of the infected cells. Wild type HBc protein however showed a strictly cytoplasmic localization when synthesized from a plasmid that encoded the HBc gene alone. Surprisingly, wild type protein was found in both nucleus and cytoplasm when expressed in the context of the HBV pregenome.

The strictly nuclear localization of wild type HBc protein could be a consequence of its assembly into stable core particles whereas unassembled HBc protein is transported to the host cell's nucleus. The present study further investigates the reasons for the nuclear and cytoplasmic localization of wild type HBc protein that is expressed in the context of the HBV pregenome.

Materials and methods

Plasmid construction

A wild type Hepatitis B virus core gene of genotype A was amplified by PCR using the following primers: 5'-GCGGATCCATGGACATTGACCCTTATA-3' and 5'-CGCC CGGGTAAAGTTTCCCACCTTATG-3' and ligated into restriction sites *Sma*I and *Bam*HI of vector pSFV1 (Liljestroem, Garoff 1991). A Hepatitis B virus pregenome (gene bank accession number: NCBI: 329640) of genotype D was cloned into the *Sma*I site of pSFVdelStNruI (pSFV1/pg) after PCR amplification with primers 3'-CCGGA AAGCTT GAGCTCTTC TTTTTCACCTCTGCCTAATCA-5', and 5'-CCGGA AAGCTT GAGCTCTTC AAAAAGTTGCATGGTGCTGG-3', restriction with *Hind*III and reconstruction of blunt ends by T4 polymerase.

Cell cultures, RNA transcription, generation of recombinant virus and cell infection

Cell cultures, RNA transcription, generation of recombinant virus and cell infection were performed as described previously (Zajakina et al. 2004).

Immunocytochemical detection of intracellular Hepatitis B virus core protein by monoclonal antibodies

Immunocytochemical detection of intracellular Hepatitis B virus core protein by monoclonal antibodies was performed as described in Zajakina et al. (2004). Anti-HBc monoclonal antibody 10C-6 (epitope aa 134 to 140; Bichko et al. 1993) was used at a dilution of 1 : 200 (in PBS + 0.25 % Triton X-100 + 0.25 % BSA).

Cell lysis

Cell monolayers were lysed in buffer containing 1 % Nonidet P-40 (NP-40), 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 μg mL^{-1} PMSF. After 10 min on ice cell nuclei were removed by centrifugation. Supernatants were used for the isolation of DNA.

Isolation of DNA

DNase I was added to cell lysates at a final concentration of 200 μg mL^{-1} and $MgCl_2$ at a final concentration of 20 mM and incubated at 37 °C for 30 min. DNase was subsequently inactivated by adding EDTA at a final concentration of 50 mM.

To disintegrate capsids, proteinase K (final concentration 1 mg mL^{-1}) and SDS (final concentration 2 %) were added and incubation was performed for 2 h at 37 °C. The suspension was cleared from proteins by adding an equal volume of phenol; after centrifugation DNA was precipitated in the supernatant by adding an equal volume of isopropanol and NaCl to a final concentration of 0.3 M. Precipitation was performed at –20 °C overnight. After centrifugation at 11 000 rpm at 4 °C the pellets were washed in 75 % ethanol, dried and resuspended in water.

Southern blot

The samples were run on a 1 % agarose gel and transferred overnight to a nylon membrane (Boehringer) by capillary blot. After transfer the DNA was immobilized on the membrane by exposing it to ultra violet light for 3 min in a transilluminator.

Detection

The immobilized DNA was hybridised with a Dig labelled probe (random primed with pregenome of genotype A as a template, Roche) overnight at 58 °C. The DIG luminescent detection kit (Roche) and CDP (Tropix) were used for the detection as described by the manufacturers. The blot was developed by exposition to X-ray film.

Analysis of intracellular RNA

The *in vitro* transcribed RNA was transfected into BHK cells by lipofection with Oligofectamine (Invitrogen) according to the manufacturer's instruction. At 24-h post-transfection the total cellular RNA was isolated from cells by TRIzol RNA isolation method (Sigma). Then RNA samples were separated in denaturing conditions on a 1.2 % agarose gel containing 0.65 % formaldehyde and transferred to a positively charged Nylon membrane (Boehringer) by capillary transfer. A digoxigenin-labelled probe (random primed, Roche) directed against the HBV genome, genotype A, was used to detect specific RNAs. For detection the membrane was incubated with anti-digoxigenin alkaline phosphatase (Roche). The CDP – chemiluminescence substrate (Tropix) was used to develop the signal that was detected by a CCD camera.

Results and discussion

The subcellular localization of HBV core protein (HBc) was analyzed by an immuno-cytochemical method following infection of mammalian cells. Intracellular localization of wild type HBc protein expressed from a construct that encoded the HBc gene alone was compared to the localization of HBc protein expressed from a construct that encoded the entire HBV pregenome. To this purpose a wild type (wt) HBc gene of genotype A was cloned into the Semliki forest virus (SFV) derived expression vector pSFV1 (Liljestroem, Garoff 1991); a complete HBV pregenome of genotype D (HBVpg) was cloned into vector pSFV1delStNruI. Baby hamster kidney cells (BHK-21) were transfected with recombinant RNA, which was transcribed *in vitro* from the SP6 promoter or alternatively, infected

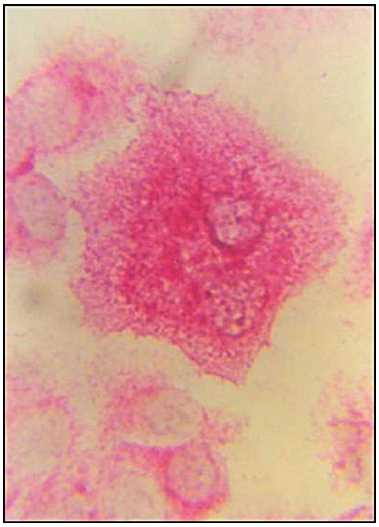

Fig. 1. Immunocytochemical analysis of HBc protein in BHK-21 cells. Cells were infected with recombinant pSFV1/HBVpg particles expressing the HBV pregenome. HBc protein (red staining) was detected by monoclonal antibody 10c/6 in both cytoplasm and nucleus of the cells (magnification × 270).

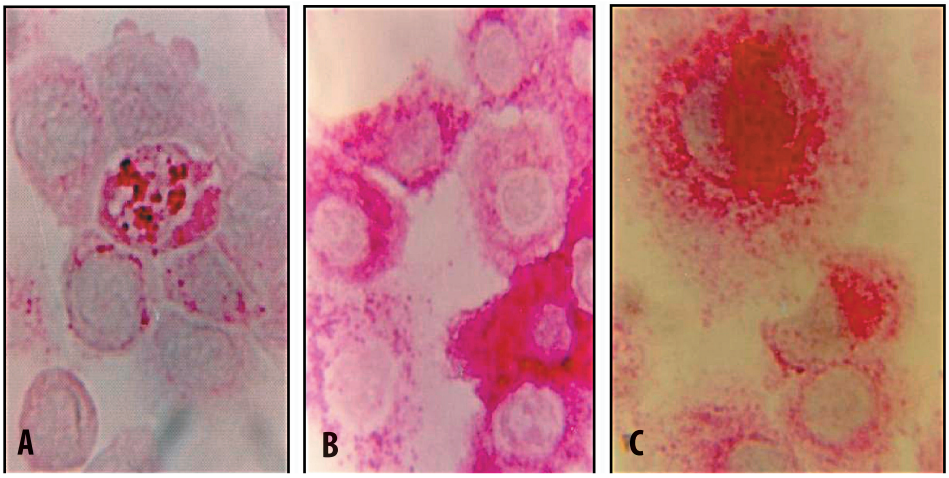

Fig. 2. Intracellular localization of HBc protein in BHK-21 cells. Cells were infected with recombinant SFV particles expressing different HBc genes. HBc protein was detected by immunostaining after reaction with monoclonal antibody 10c/6 (magnification × 108). A, the picture shows HBc protein expressed by HBc variant deleted from aminoacids 86 to 93 (described in Bruvere et al., 2004) in both nucleus and cytoplasm of the cells. B, HBc protein that is expressed from construct pSFV1/wt core is purely cytoplasmic. C, HBc protein that is expressed from construct pSFV1/HBVpg is found in both cytoplasm and nucleus of the cells.

with recombinant SFV viral particles (recSFV), which were generated by packaging the recombinant RNA replicons into the SFV capsid. In both cases the expression of HBc genes is subject to transcriptional regulation by the SFV subgenomic promoter. Synthesis of HBc protein was monitored by light microscopy with an immunocytochemical method after reaction of HBc antigen with a monoclonal antibody whose epitope comprises aminoacids 134 to 140. As the percentage of cells that stained positive increased during the first 24 h post infection and in most cases reached a maximum at this time, HBc synthesis was detected. For both constructs the numbers of cells that stained positive reached values of up to 80 % (but generally above 60 %). Cytoplasmic staining was strong in both cases. However, a striking difference regarding the presence of HBc protein in the nucleus was observed. No HBc protein was found in the nucleus when the HBc gene of the construct that encoded the HBc gene alone was expressed (Fig. 2B). A small amount of HBc protein was detected in the nucleus of BHK cells when the HBc gene was expressed from the construct that encoded the HBV pregenome (Fig. 1, Fig. 2C). The same expression pattern – cytoplasmic and nuclear staining – had been observed for internally deleted HBc proteins (Bruvere et al. 2004; Fig. 2A). We supposed that this staining pattern was explained by the stability of the core particles.

HBc protein contains a nuclear localization signal in the carboxy-terminus. In phosphorylated form core particles bind to the nuclear pore complex (Kann et al. 1999), probably by inducing a conformational change through which the otherwise hidden nuclear localization signal becomes exposed. Phosphorylation is thought to act as a maturation

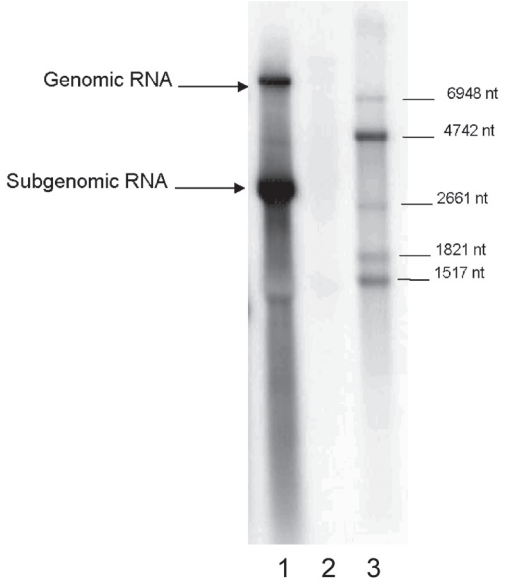

Fig. 3. Analysis of HBV transcripts. Total RNA was isolated from the cells lipofected with *in vitro* transcribed recombinant SFV/HBVpg RNA. The RNA was separated by agarose gel electrophoresis in denaturing conditions and blotted on a nylon membrane where specific RNA was visualized by a CCD camera after reaction with a DIG labeled probe directed against the HBV genome. Lane 1: isolation of RNA from BHK cells transfected with the HBV pregenome as described above, lane 2: negative control (uninfected cells), lane 3: RNA marker.

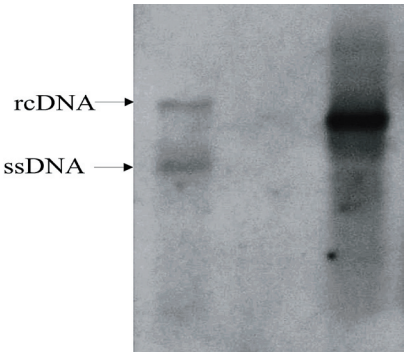

Fig. 4. Analysis of HBV replicative intermediates. BHK-21 cells were infected with recombinant pSFV1/HBVpg particles of the HBV pregenome. Total DNA was isolated from the cells, separated by agarose gel electrophoresis, and blotted on a nylon membrane where specific DNA was visualized after reaction with a DIG labeled probe directed against the HBV genome. DNA isolated from infected BHK cells shows two HBV specific bands, the upper band corresponds to relaxed circular DNA, the lower band is ssDNA. Lane 1: DNA isolated from cells infected with pSFV1/HBVpg, lane 2: negative control (DNA isolated from uninfected cells), lane 3: PCR fragment of HBV pregenome.

signal when RNA is degraded during reverse transcription of the HBV genome (Kann et al. 1997; Kann et al. 1999). Loss of nucleic acid binding renders the core particles less stable (Birnbaum, Nassal 1990). In the transgenic mouse model intact core particles do not pass the nuclear membrane (Guidotti et al. 1994) and disintegration of capsids is thought to be a prerequisite for HBc transport into the host cell's nucleus. This view has been challenged by recent research. It was found that the diameter of the nuclear pore is wider (Pante, Kann 2002) than originally thought (Dworetzki, Feldherr 1988), such that HBV capsids could pass it in intact form (Rabe et al. 2003). In any case, some kind of rearrangement of the capsids occurs as C-terminal sequences of HBc protein that contain the nuclear localization signal become exposed upon phosphorylation (Rabe et al. 2003).

We suppose that a replication cycle is initiated by the transfected pregenome and that HBc protein is imported concomitantly to the nucleus of BHK cells. Experimental evidence are two HBV specific DNA species that were detected at low frequency (Fig. 4) upon infection of BHK cells with viral particles expressing the HBV pregenome. It has been reported previously that the expression of the pregenome is enough to drive HBV replication in an alpha-viral system for duck HBV expression in Sindbis virus (Huang, Summers 1991). Also, a model of HBV replication in an alphaviral system has been proposed (Huang, Summers 1991). In the alphaviral system the transfected or infected (+)-RNA is transcribed *in vivo* into (-)-RNA from which (+)-genomic RNA and subgenomic RNA are transcribed. These RNA species were detected by Northern blot (Fig. 3). In pSFV1/HBVpg the alphaviral structural genes in the region of the subgenomic RNA are replaced by the HBV pregenome sequence. From this RNA the HBV proteins are translated. Upon translation of the HBV polymerase protein, reverse transcription of the HBV pregenome into (-)-DNA occurs followed by usually incomplete synthesis of (+)-DNA. The appearance of two DNA species probably marks these first steps of the replication cycle (Fig. 4). The larger DNA species, which migrates slower than a PCR fragment of the HBV genome, might correspond to double-stranded relaxed circular DNA whereas the smaller fragment may constitute single-stranded DNA. Although a full

replication cycle of the HBV pregenome in the SFV system cannot be proven, as it was not possible to detect covalently closed circular HBV DNA, which may be due to the fact that this is an inefficient process, we suppose that DNA is transferred to the nucleus. We assume that concomitantly HBc protein is imported to the host cells's nucleus either by disintegration of the capsids or in assembled form. As a consequence, wild type HBc protein is detected in the nucleus when expressed in the context of the HBV pregenome.

In contrast to the nuclear and cytoplasmic subcellular distribution of HBc protein in BHK cells expressing the HBV pregenome, only cytoplasmic location of HBc protein was detected in cells that were infected with recSFV expressing the wild type HBc gene. It has been suggested that empty capsids in the nucleus are formed from HBc protein, which is over-expressed and transported to the nucleus in unassembled form or as assembly intermediates, that is, in a form in which the nuclear localization signal is not hidden (Kann et al. 2007). In the SFV system however, no HBc protein is imported to the nucleus, probably as it quickly assembles to stable core particles. Contrary to this, internally deleted HBc protein had shown in previous research a staining pattern in which both nuclear and cytoplasmic location occur (Bruvere et al. 2004). As the deletions mapped in a region which is mostly α-helical (Wynne et al. 1999) and where mutations are hardly tolerated (Koschel et al. 1999), even small deletions lead to a deformation and partial unfolding of the protein. Internally deleted HBc protein is therefore unstable and quickly degraded by the proteasomal pathway (Braun et al. 2007). Due to the unfolding the nuclear localization signal becomes exposed and variant HBc protein is imported to the nucleus.

In summary, it seems that an at least partial unfolding of the HBc protein and the subsequent exposition of the nuclear localization signal is a precondition for nuclear import. It occurs during the replication of the genome. At least in the SFV system it does not occur when wild type HBc protein is over expressed, probably as it assembles and the nuclear localization signal is hidden.

Furthermore a replicating HBV pregenome in the SFV system may serve – after optimization – as a useful tool for the study of HBV biology.

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References

Bichko V., Schodel F., Nassal M., Gren E., Berzinsh I., Borisova G., Miska S., Peterson D.L., Gren E., Pushko P., Will H. 1993. Epitopes recognized by antibodies to denatured core protein of hepatitis B virus. *Mol. Immun*ol. 30: 221–231.

Birnbaum F., Nassal M. 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. *J. Virol.* 64: 3319–3330.

Braun S., Zajakina A., Aleksejeva J., Sharipo A., Bruvere R., Ose V., Pumpens P., Garoff H., Meisel H., Kozlovska T. 2007. Proteasomal degradation of core protein variants from chronic hepatitis B patients. *J. Med. Virol*. 79: 1312–1321.

Bruvere R., Braun S., Zajakina A., Alekseeva E., Sharipo A., Meisel H., Pumpens P., Kozlovska T. 2004. Subcellular localisation of internally deleted HBV core proteins. *Acta Univ. Latv.* 676: 53–64.

Dworetzky S.I., Feldherr C.M. 1988. Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* 106: 575–584.

Eckhardt S.G., Milich D.R., McLachlan A. 1991. Hepatitis B virus core antigen has two nuclear localization sequences in the arginine-rich carboxyl terminus. *J. Virol.* 65: 575–582.

Günther S., Baginski S., Kissel H., Reinke P., Krüger D.H., Will H., Meisel H. 1996. Accumulation and persistence of Hepatitis B Virus core gene deletion mutants in renal transplant patients are associated with end-stage liver disease. *Hepatology* 24: 751–758.

Günther S., Piwon N., Jung A., Iwanska A., Schmitz H., Will H. 2000. Enhanced replication contributes to enrichment of Hepatitis B virus with a deletion in the core gene. *Virology* 273: 286–299.

Guidotti L.G., Martinez V., Loh Y.T., Rogler C.E., Chisari F.V. 1994. Hepatitis B virus nucleocapsid particles do not cross the hepatocyte nuclear membrane in transgenic mice. *J. Virol.* 68: 5469–5475.

Huang M., Summers J. 1991. Infection initiated by the RNA pregenome of a DNA virus. *J. Virol.* 65: 5435–5439.

Kann M., Bischof A., Gerlich W.H. 1997. In vitro model for the nuclear transport of the hepadnavirus genome. *J. Virol.* 71: 1310–1316.

Kann M., Sodeik B., Vlachou A., Gerlich W.H., Helenius A. 1999. Phosphorylation-dependant binding of Hepatitis B virus core particles to the nuclear pore complex. *J. Cell Biol.* 145: 45–55.

Kann M., Schmitz A., Rabe B. 2007. Intracellular transport of hepatitis B virus. *World J. Gastroenterol.* 13: 39–47.

Koschel M., Thomssen R., Bruss V. 1999. Expensive mutagenesis of the Hepatitis B virus core gene and mapping of mutations that allow capsid formation. *J. Virol.* 73: 2153–2160.

Liao W., Ou J.H. 1995. Phosphorylation and nuclear localization of the Hepatitis B virus core protein: significance of serine in the three repeated SPRRR motifs. *J. Virol.* 69: 1025–1029.

Liljeström P., Garoff H. 1991. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology*. 9: 1356–1361.

Melegari M., Wolf S.K., Schneider R.J. 2005. Hepatitis B virus replication is coordinated by core protein serine phosphorylation and HBx expression. *J. Virol.* 79: 9810–9820.

Pante N., Kann M. 2002. Nuclear Pore Complex is able to transport macromolecules with diameters of ~ 39 nm. *Mol. Biol. Cell* 13: 425–434.

Preikschat P., Guenther S., Reinhold S., Will H., Budde K., Neumayer H.H. et al. 2002. Complex HBV populations with mutations in core promoter, C gene, and pre-S region are associated with development of cirrhosis in long-term renal transplant recipients. *Hepatology* 35: 466-477.

Rabe B., Vlachou A., Pante N., Helenius A., Kann M. 2003. Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc. Natl. Acad. Sci. USA* 100: 9849–9854.

Seeger C., Mason W.S. 2002. Hepatitis B virus biology. *Microbiol. Mol. Biol. Rev*. 64: 51–68.

Wynne S.A., Crowther R.A., Leslie A.G.W. 1999. The crystal structure of the human Hepatitis B Virus capsid. *Mol. Cell* 3: 771–780.

Zajakina A., Kozlovska T., Bruvere R., Aleksejeva J., Pumpens P., Garoff H. 2004. Translation of hepatitis B virus (HBV) surface proteins from the HBV pregenome and precore RNAs in Semliki Forest virus-driven expression. *J. Gen. Virol*. 85: 3343–3351.

Semliki Meža vīrusa ekspresijas sistēmā ar diviem atšķirīgiem konstruktiem ekspresēta hepatīta B vīrusa serdes proteīna lokalizācija šūnā

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Kopsavilkums

Hepatīta B vīrusa (HBV) serdes proteīna lokalizāciju eikariotu šūnās pētīja ar imunocitoķīmijas metodi, izsekojot HBV serdes gēna (HBc) ekspresiju Semliki Meža vīrusa ekspresijas sistēmā ar divām atšķirīgām konstrukcijām. Izmantojot konstrukciju, kura kodēja HBV pregenomu, HBc proteīns bija lokalizēts citoplazmā un kodolā, kamēr, izmantojot konstrukciju, kura kodēja tikai pašu HBc gēnu, HBc proteīns bja atrodams tikai citoplazmā. Rakstā apspriesti šīs parādības iespējamie iemesli.