

A Semliki Forest virus expression system as a model for investigating the nuclear import and export of hepatitis B virus nucleocapsid protein

A. ZAJAKINA, R. BRŪVERE, T. KOZLOVSKA

Latvian Biomedical Research and Study Centre, Ratsupites Str. 1, Riga, Latvia, LV-1067

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Summary. – HBV core protein (HBc), the major component of nucleocapsid is known to have an essential role in the virus life cycle as the transporter of virus genome to the host nucleus; however, molecular details of the intracellular transport of HBc remain unknown. In this study, we investigated the intracellular distribution of the HBc protein resulting from Semliki Forest virus (SFV)-driven HBc gene and HBV RNA pregenome (pgRNA) expression in BHK-21 cells. Fluorescent confocal microscopy revealed extensive HBc protein synthesis in the cytoplasm 12 hr post infection (p.i.) with recombinant pSFV1/HBc or pSFV1/HBVpgRNA viruses. Twenty-four hr p.i., the HBc protein showed asymmetric, predominantly nuclear localization in most cells. However, 42 hr p.i., the number of cells containing intranuclear HBc protein, dramatically decreased to ~5%, while cytoplasmic HBc protein was detected in all cells. Remarkably, 24 hr p.i. with pSFV1/HBVpgRNA virus, cytoplasmic HBc protein colocalized with HBs protein. We conclude that a HBs-HBc interaction partially prevents the transport of HBc from the cytoplasm to nucleus. The SFV-driven system can be used for identification of new factors involved in the HBV nuclear import and export.

Keywords: hepatitis B virus; nucleocapsid protein; surface protein; pregenomic RNA; Semliki Forest virus

Introduction

Hepatitis B virus (HBV) is a small double-stranded DNA virus (the *Hepadnaviridae* family, the *Orthohepadnavirus* genus), that causes both acute and chronic hepatitis in humans (Peppas and Maini, 2012). Many aspects of HBV biology remain unknown, hampering the development of effective antiviral therapy. Furthermore, the early events of HBV infection and genome transport to the nucleus are difficult to analyze because of the lack of a susceptible cell line model.

The nucleocapsid is thought to deliver the partially double-stranded HBV DNA genome to the nucleus, where the covalently closed circular HBV DNA genome (cccDNA)

is synthesized (Ganem and Prince, 2004). However, it is unknown whether the nucleocapsid particle is able to cross the nuclear membrane and enter the nucleus or disassembles prior to entry. Because HBV nucleocapsid particles were found in both the nucleus and cytoplasm of the hepatocytes from HBV-infected patients (Michalak and Nowoslawski, 1977; Petit and Pillot, 1985), it is possible that intact nucleocapsid particles are able to move in one or both directions across the nuclear membrane.

HBV replicates via pgRNA transcribed from cccDNA in the nucleus of an infected cell (Beck and Nassal, 2007). After export to the cytoplasm, pgRNA serves as a translational template for the synthesis of HBc and viral polymerase (Pol). HBc forms an isometric 27-nm capsid (core particle) together with encapsidated pgRNA and Pol. The shell of the capsid contains 180 or 240 identical capsid protein subunits of 21.5 kDa (Crowther *et al.*, 1994). Reverse transcription of pgRNA and the subsequent synthesis of an incomplete second strand of DNA generate the mature genome inside the newly formed cytoplasmic capsids. There is also evidence

E-mail: Anna@biomed.lu.lv; phone: +371-2808004.

Abbreviations: cccDNA = covalently closed circular DNA; HBc = core protein; HBs = surface protein; HBV = hepatitis B virus; pgRNA = RNA pregenome; SFV = Semliki Forest virus; ER = endoplasmic reticulum; p.i. = post infection; RT = room temperature

that mature capsids may transport the DNA genome back to the nucleus, thus enriching the pool of intranuclear cccDNA; some experimental data support this idea of retrograde transport of the HBV genome through HBc interaction with the nuclear pore complex (Schmitz *et al.*, 2010; Rabe *et al.*, 2009). Alternatively, in the presence of a sufficient amount of envelope proteins – HBV surface proteins (HBs), including small, middle, and large forms of the outer envelope components – translated from their own mRNAs, the nucleocapsids are segregated into the secretory pathway via the interaction with HBs proteins on the membrane of the endoplasmic reticulum (Bruss, 2007).

Although basic information on the role of the HBV capsid has been published, the signals and mechanism of such intracellular capsid segregation between nuclear and secretory pathways remain unknown. Recombinant plasmids based on the SFV genome have been introduced to express foreign genes in a broad range of eukaryotic cells (Liljestrom and Garoff, 1991; Zajakina *et al.*, 2008), and the universal nature of the system makes it ideal for the investigation of HBV protein interaction and transport. In this study, we attempted to determine the intracellular distribution of HBc protein expressed from recombinant SFV carrying HBc gene or HBVpgRNA. The used SFV system proved suitable for identification of viral and host factors involved in regulation of HBV life cycle.

Materials and Methods

Cells. BHK-21 cells (ATCC, Sweden) were grown in BHK medium (Gibco-BRL) containing 5% FCS, 10% tryptose phosphate broth, 20 mmol/l HEPES, and 2 mmol/l glutamine. The cells were incubated in a 5% CO₂ atmosphere at 37°C.

Vector constructs. The vector constructs used in this study were described previously (Zajakina *et al.*, 2004; Kozlovska *et al.*, 2004). Briefly, pSFV1/HBVpgRNA was generated by insertion of the full-length cDNA copy of HBV pgRNA (genotype D1) into the SmaI restriction site of the pSFV1 vector (Liljestrom and Garoff, 1991), whereas pSFV1/HBc was obtained by insertion of the HBc gene into the BamHI/SmaI restriction sites of the pSFV1 vector.

Recombinant SFVs. Recombinant alphaviruses were produced as previously described (Vasilevska *et al.*, 2012). Briefly, RNA transcripts were produced *in vitro* from NruI-linearized pSFV1/HBVpgRNA and SpeI-linearized pSFV1/HBc constructs. For the *in vivo* packaging of recombinant RNA into SFV particles, *in vitro* transcribed RNA was co-electroporated into BHK-21 cells together with SFV helper RNA (Liljestrom and Garoff, 1991). After 24 hr, the SFV particles from the culture medium were collected. The infection of BHK-21 cells was performed in a serum-free medium. The virus titers were determined by infecting BHK-21 cells with serial dilutions of the stocks, followed by an indirect immunocytochemistry assay for detection of HBc protein. The titers achieved

were from 1×10⁷ to 5×10⁷ viral particles per ml and were used at MOI = 10.

Immunofluorescent staining of HBc and HBs. BHK-21 cells grown on chamber slides (NUNC A/S) were infected with recombinant SFV particles as previously described (Vasilevska *et al.*, 2012) and incubated at 37°C (5% CO₂) for 12 hr, 24 hr, and 48 hr. The cells were then fixed in 4% paraformaldehyde solution (Sigma-Aldrich, USA) for 10 min at room temperature (RT), rinsed three times in PBS, immersed in PBS supplemented with 0.25% Triton X-100 for 10 min, and rinsed with PBS again. A blocking solution containing 3.5% of FCS diluted in PBS was added, and the cells were incubated for 30 min at RT. Cells were incubated with primary rabbit anti-HBc antibodies (Abcam, UK) at a dilution of 1:700 (in PBS with 3.5% FCS) overnight at 4°C. The cells were washed three times with PBS and incubated in the dark for 1 hr with an anti-rabbit IgG TRIC conjugate (Sigma-Aldrich, USA) diluted 1:350 in PBS supplemented with 3.5% FCS (RT). After rinsing with PBS, the cell nuclei were counterstained with DAPI solution (300 nmol/l in PBS), and the cells were mounted in Prolong Gold Antifade Reagent (Molecular Probes, USA). For double anti-HBc/anti-HBs staining, the cells were stained with rabbit anti-HBc antibodies and the anti-rabbit IgG TRIC conjugate as described above, washed with PBS, and stained with 1:1000-diluted monoclonal anti-HBs 1-9C1 antibodies (a gift from Dr. I. Sominska) and a 1:200-diluted anti-mouse IgG FITC conjugate (Dako, USA). The staining evaluation was performed under a confocal fluorescent microscope (Leica TCS Sp2 SE) using the appropriate filters. To determine the subcellular regions of protein co-localization and intranuclear distribution, individual red-, green-, and blue-stained images derived from the same field were merged using Photoshop 6.0. The areas of HBc/HBs co-localization appear yellow.

Results

Intracellular distribution of HBc protein expressed by recombinant pSFV/HBc virus

To investigate the intracellular distribution of the HBc protein, BHK-21 cells were infected with recombinant pSFV1/HBc virus encoding the full-length HBc gene. For microscopy studies, the cells were immunostained with rabbit anti-HBc antibodies and TRIC-conjugated anti-rabbit serum at various time points after infection (12 hr, 24 hr, and 42 hr). The red staining of the infected cells was visualized using a Leica DM 6000 B microscope. As shown in Fig. 1a, a strong nuclear localization of HBc was observed 24 hrs p.i., whereas a preferentially cytoplasmic distribution of HBc was found at early and late p.i. times.

The microscopy results revealed nuclear HBc protein transport within the cell. Using the SFV expression system, recombinant protein synthesis began in the cell cytoplasm

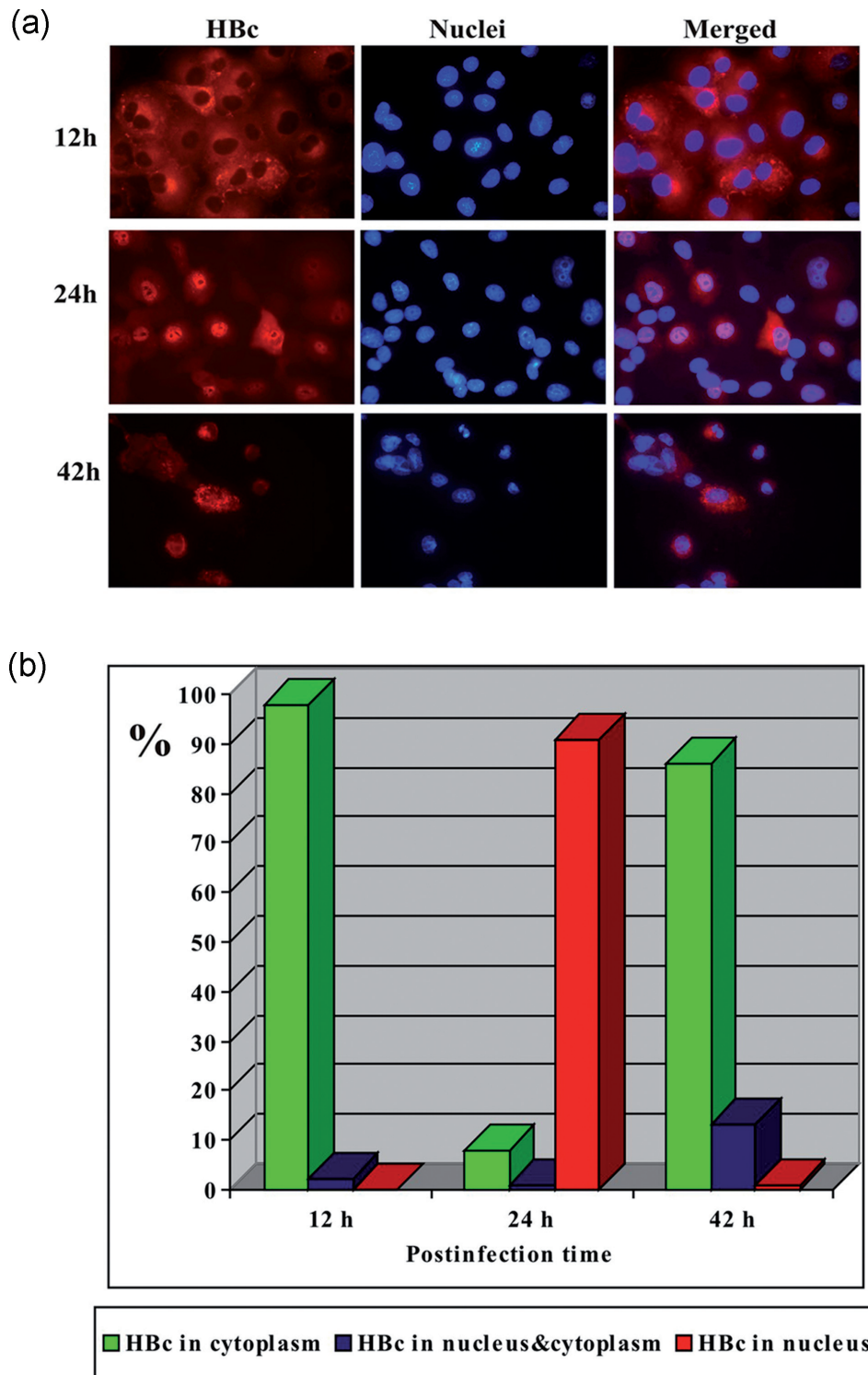


Fig. 1

Subcellular localization of HBc protein expressed by recombinant pSFV1/HBc virus

BHK-21 cells were infected with recombinant pSFV1/HBc virus and HBc was observed by immunofluorescent microscopy at different times after infection (for details see Materials and Methods). (a) Immunofluorescent microscopy. Staining: HBc (red), nuclei (blue). (b) Distribution of HBc between the cytoplasm and nucleus. The diagram shows the average % of HBc-positive cells in cytoplasm only, in nucleus and cytoplasm, in nucleus only. The experiments were performed in triplicate. The standard error of three independent experiments did not exceed 5%.

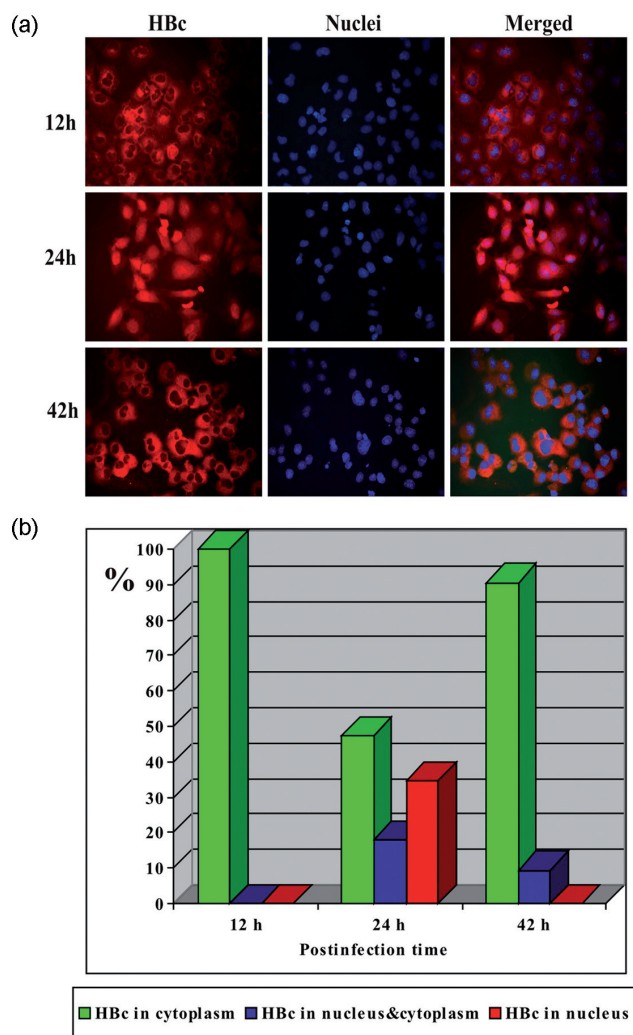


Fig. 2

Subcellular localization of HBc protein expressed by recombinant pSFV1/HBVpgRNA virus

BHK-21 cells were infected with recombinant pSFV1/HBVpgRNA virus and HBc was observed by immunofluorescent microscopy at different times after infection (for details see Materials and Methods). (a) Immunofluorescent microscopy. Staining: HBc (red), nuclei (blue). (b) Distribution of HBc between the cytoplasm and nucleus. The diagram shows the average % of HBc-positive cells in cytoplasm only, in nucleus and cytoplasm, in nucleus only. The experiments were performed in triplicate. The standard error of three independent experiments did not exceed 5%.

6 hr p.i., with maximum synthesis 20–30 hr p.i. Our observations show that approximately 98% of the cells contained cytoplasmic HBc 12 hr p.i. (Fig. 1b). Twenty-four hours p.i., most cells (91%) contained HBc exclusively in the nucleus, indicating the efficient intracellular transport and nuclear accumulation of HBc. However, at a late p.i. time (42 hr and thereafter), most of the nuclei were again negative, whereas the cytoplasm (80% of cells) was HBc protein positive.

A small number of cells contained HBc both in the nucleus and cytoplasm (Fig. 1b). Therefore, the HBc protein was found to have strong nuclear tropism 24 hr p.i.

Intracellular distribution of HBc protein expressed by recombinant pSFV1/HBVpgRNA virus

In native HBV infection, pgRNA serves as a template for HBc protein expression with further encapsidation of pgRNA into the core particles. The reverse transcription of pgRNA to a DNA genome and its transport to the nucleus is a key step in the HBV infection cycle. In contrast, the mature core particles are subjected to segregation to the ER following HBs protein interaction for viral particle formation. To investigate the intracellular localization of HBc protein expressed from the entire pgRNA molecule, BHK-21 cells were infected with recombinant pSFV1/HBVpgRNA virus.

Immunofluorescence microscopy was performed using rabbit anti-HBc antibodies and an anti-rabbit-TRIC conjugate. The HBc protein distribution between the nucleus and cytoplasm was evaluated at various time points after infection (12 hr, 24 hr, and 42 hr). As shown in Fig. 2a, most cells contained HBc only in the cytoplasm at the early and late p.i. times. Conversely, as was observed for the pSFV1/HBc virus, HBc was found in the nucleus 24 hr p.i., indicating strong nuclear tropism of HBc at this time point.

There was no major difference with regard to the localization of HBc expressed by the pSFV1/HBc and pSFV1/HBVpgRNA viruses. Indeed, the nuclear localization of HBc was detected at the middle (24 hr) p.i. time for both recombinant viruses. However, in the case of pgRNA, an important percentage of the cells accumulated HBc both in the nucleus and in the cytoplasm (12%) or only in the cytoplasm (43%) 24 hr p.i. (Fig. 2b), compared to the preferential nuclear localization (91%) of HBc expressed by the pSFV1/HBc virus. These observations indicate that a portion of the cells expressing pgRNA retained HBc in the cytoplasm, thus preventing its nuclear translocation.

Intracellular distribution of HBc and HBs proteins expressed by recombinant pSFV1/HBVpgRNA virus

As was shown previously, the expression of HBV pgRNA by the pSFV1 expression vector allows the synthesis of all HBV structural proteins (Zajakina *et al.*, 2004), including HBc and three variants of envelope (HBs) proteins: S, M, and L. As the HBc capsid interacts with HBs protein residing on the membrane of the ER to form the HBV virion (Patient *et al.*, 2009), it is possible that HBc retention in the cell cytoplasm at the middle p.i. time was influenced by capsid interaction with envelope proteins in this experimental system.

To confirm this idea, double immunostaining in BHK-21 cells infected with pSFV1/HBVpgRNA was performed using

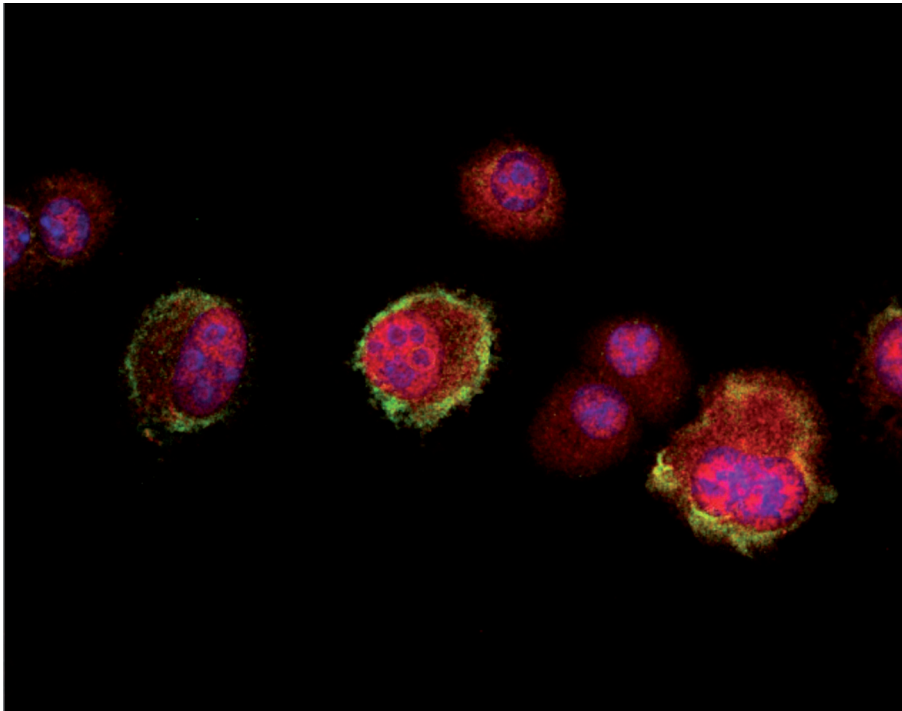


Fig. 3

Colocalization of HBc and HBs proteins expressed by recombinant pSFV1/HBVpgRNA virus

BHK-21 cells were infected with recombinant pSFV1/HBVpgRNA virus and HBc and HBs were observed by immunofluorescent microscopy 24 hr p.i. (for details see Materials and Methods). Staining: HBc (red), HBs (green), HBc + HBs colocalized (yellow), and nuclei (blue).

anti-HBc and anti-HBs antibodies, followed by fluorescence microscopy. The analysis revealed the prevalence of HBc expression in most cells 24 hr p.i. However, some cells were positive for both HBc and HBs proteins, and these cells were subjected to HBc/HBs colocalization studies under confocal microscopy. As shown in Fig. 3, the HBs protein was found in the cytoplasm in the perinuclear area and/or distributed asymmetrically in the cytoplasm. The confocal microscopy revealed the colocalization of HBs (green staining) and HBc (red staining) in the cytoplasm. The colocalization of both proteins confirms their interaction in the cell cytoplasm, which may prevent HBc transport into the nucleus. Therefore, some cells expressing HBs partially retain HBc in the cytoplasm.

Discussion

Herein, we describe the intracellular distribution of HBc protein expressed by the pSFV1 vector. HBc protein translocation to the nucleus within a short period of time (24 hr) was detected for both the pSFV1/HBVpgRNA and pSFV1/HBc recombinant viruses. The strong nuclear tropism and accumulation of HBc in the nucleus correlates well with

previous ultrastructural and immunohistochemical studies of HBV-infected liver tissues. In chronically infected patients with mild hepatitis, the core protein accumulates preferentially in the nucleus, whereas it is mainly detected in the cytoplasm of hepatocytes in patients with active disease (Chu and Liaw, 1995; Gowans *et al.*, 1985; Naoumov *et al.* 1990; Serinoz *et al.*, 2003) and may be associated with inflammation and immune hepatocytolysis.

Despite numerous investigations, the mechanism of HBc translocation and the role of its nuclear accumulation remain unclear. According to the cell division concept, during the premitotic phase, HBc is transported across the nuclear membrane as monomers or dimers, accumulating and forming capsids in the nucleus. During mitosis, when the nuclear membrane dissolves, the core particles are released into the cytoplasm and cannot reenter the nucleus, most likely because of their size. Our results also indicate the disappearance of HBc from the nucleus at a late p.i. time. However, this return of HBc does not appear to be affected by cell division, because nondividing BHK-21 cells pre-treated with mitomycin C did not differ in HBc localization compared to untreated cells (data not shown). Moreover, alphaviral vectors, including pSFV1, are known to be cytostatic; thus, no cell division should occur during HBc protein synthesis and transportation. Ad-

ditionally, SFV induces apoptosis at a late p.i. time (2–3 days). The presence of nuclear export signals in the HBc protein was recently proposed by Li and co-authors (Li *et al.*, 2010). Although the mechanism of HBc translocation is unknown, we can assume that the induction of apoptosis at a late p.i. time may lead to the activation of cellular transporters to accomplish HBc retrotransport to the cytoplasm.

No major difference was found with regard to HBc localization when expressed by recombinant pSFV1/HBVpgRNA and pSFV1/HBc viruses. However, in the case of pgRNA, the number of cells with cytoplasmic plus cytoplasmic/nuclear localization (55%) was significantly higher compared to HBc gene expression alone (6%) 24 hr p.i. These results indicate the retention of HBc in the cytoplasm of pgRNA-expressing cells. We have previously shown that pgRNA expression from the pSFV1 vector achieves the synthesis of the HBc protein and also all three variants of envelope proteins, possibly through a ribosome leaky scanning mechanism (Zajakina *et al.*, 2004). In the present study, we performed double anti-HBc and anti-HBs immunostaining and found a colocalization of these proteins in the cytoplasm. Thus, we may conclude that HBc/HBs interaction partially prevents HBc nuclear transport. Surprisingly, not all HBc-expressing cells demonstrated anti-HBs staining, which was possibly associated with a low HBs protein expression level.

Therefore, our experimental model based on a replication-defective pSFV1 vector is a new tool for the investigation of HBc intracellular transport. The most interesting observation described herein is HBc retrotransport from the nucleus to the cytoplasm. Until now, HBc nuclear translocation has been studied using an artificial model based on digitonin-permeabilized mammalian cells, which does not allow the investigation of HBc export from the nucleus. In the system proposed here, the nuclear export signals recently identified in HBc could be tested in a broad range of eukaryotic cells, including human hepatoma cells, which can be efficiently infected with recombinant alphaviruses. Similar HBc localization results were observed in the human hepatoma cell lines Huh7 and HepG2 (data not shown) in addition to BHK-21 cells. However, as this cell line is most convenient for propagation and alphaviral infection, we propose the use of BHK-21 cells for HBc transport studies. Additionally, the proposed system is independent of the nuclear delivery of HBV genetic material and can be used in non-dividing cells. Accordingly, resting cells can be analyzed for the intracellular distribution of HBV proteins in order to elucidate the molecular and functional roles of these proteins.

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