INTRACELLULAR LOCALIZATION OF HBV CAPSID IN HEPATOCYTE LINE AFTER TRANSFECTED BY THE ENTIRE HBV GENOME

LOKALISASI INTRASELULER KAPSID HBV PADA SEL LINE HEPATOSIT SETELAH DITRANSFEKSI DENGAN GENOM UTUH HBV

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ABSTRACT

HBV replicates within the nucleus of hepatocyte using cellular transport machinery for the import of their genomes into the nucleus. Genome of HBV has to transported through the cytoplasm towards the nuclear pore complex (NPC) followed by subsequent passage through the pore. HBV capsid is involved in a number of important functions in the replication cycle of HBV. It can be detected in the nucleus, cytoplasm or both within infected hepatocytes. Nuclear localization of HBV capsid protein, which is karyophilic, depends on the cell cycle. The objective of the present study was to analyzes the intracellular localization of HBV capsid protein after transfected by entire HBV genome into hepatocyte cell lines (HuH-7) and to determine the predominantly localization of the capsid into cell compartment. In this work we analysed the intracellular localization of the HBV capsid in human hepatocyte cell lines HuH-7 by transfection using entire HBV genome and transient expression. The transfected cells were fixed and an indirect immune staining against the HBV capsid was performed to detect the capsid. To verify the location within the cell, an additional co-staining against the nuclear pore complexes was performed. The Intracellular localization of the HBV capsid and NPC were analyzed by a confocal laser scan microscope. The observed of localizations into the transfected cells were classified to be predominantly as nuclear localization, cytoplasmic localization or distributed within both of these compartments. Result of this study indicated that Staining of HBV capsid found predominantly within the nucleus (71%). Less frequently, the HBV capsid localized within the cytoplasm (26%). Only in a minority of cases, the capsids were localized within cytoplasm and nucleus (3%). This low frequency indicate that the capsids were not diffusing within the cells being in accordance to the *in vivo* situation in which the nuclear membrane was impermeable for the capsid.

Key words: HBV capsid, hepatocyte, NPC, HuH-7, confocal laser scan microscope

ABSTRAK

Replikasi HBV terjadi di dalam nukleus hepatosit dengan mempergunakan mekanisme transport seluler untuk mengirimkan genomnya ke dalam nukleus. Genom HBV harus ditransportasikan ke dalam nukleus melalui *nuclear pore complex* (NPC). Kapsid HBV terlibat dalam sejumlah fungsi penting dalam siklus replikasi HBV. Protein kapsid HBV dapat dideteksi didalam nukleus, sitoplasm ataupun di kedua kompartemen tersebut di dalam sel hepatosit yang terinfeksi HBV. Lokalisasi protein kapsid HBV yang bersifat karyophilik tergantung pada siklus sel. Adapun tujuan dari penelitian ini adalah untuk menganalisis lokalisasi intraseluler protein kapsid HBV pada sel line hepatosit HuH-7 pasca ditransfeksi dengan genom HBV utuh serta untuk menentukan lokalisasi kapsid HBV yang dominan dalam kompartemen sel. Pada penelitian telah dilakukan analisis lokalisasi intraseluler kapsid HBV dengan mentransfeksikan genome HBV utuh dan mengekspresikannya di dalam sel line HuH-7. Sel yang sudah ditransfeksi selanjutnya difiksasi dan dilakukan penwarnaan secara tidak langsung terhadap kapsid HBV. Untuk menentukan lokalisasi dalam sel, pewarnaan lanjutan dilakukan terhadap NPC. Selanjutnya, dengan menggunakan mikroskop scan laser konfokal dianalisis lokalisasi intraseluler kapsid HBV dan NPC. Hasil pengamatan menunjukan bahwa distribusi HBV di dalam nukleus lebih dominan dibandingkan dengan distribusi di dalam sitoplasma dan distribusi pada kedua kompartemen tersebut. Secara kuantitatif menunjukkan bahwa distribusi protein kapsid HBV dalam nukleus adalah dominan dengan 71%, sedangkan dalam sitoplasma adalah 26%, sangat minoritas dijumpai distribusi protein kapsid HBV pada kedua kompartemen sel, yaitu hanya 3%. Hal ini menunjukkan bahwa masuknya kapsid HBV kedalam nukleus tidak terdifusi secara pasif, namun secara aktif melalui mekanisme protein transport melalui NPC. Membran nukleus bersifat permeable terhadap protein HBV kapsid.

Kata kunci: kapsid HBV, hepatosit, NPC, HuH-7, mikroskop scan laser konfocal

INTRODUCTION

Many viruses depend on nuclear host factor for genome replication. At some stages, the viral genome must enter the nucleus (Whittaker and Helenius, 1998). In dividing cells, the genome could be trapped during the reconstitution of the nucleus in the late telophase of mitosis (Roe *et al.*, 1993). For a successful infection of non dividing cells, viruses must have to develop a strategy that mediates active transport of their genome through the nuclear pore. In general, the strategies imply the multiple steps after the viruses have entered their target cells. The genome containing subviral structure (capsid or protein-genome complexes) must move to the nucleus, then bind to the NPC and are transported through the central channel of the NPC. Due to the size of most viral capsids, which exceed the maximal functional diameter of the nuclear pore, release of the viral genome from the capsid must be assumed prior to nuclear import. All viruses use a variety of host proteins for the delivery of their genomes into the nucleus.

Hepatitis B virus (HBV) is a prototype member of the hepadnavirus family that belongs to the pararetroviruses. It is a small enveloped DNA virus that is composed of an outer envelope and an inner nucleocapsid. The nucleocapsid is also termed core particle or capsid consists of a single protein species called core protein. The majority of capsids have a T=4 symmetry and a diameter of 36 nm, while the minority has a T=3 symmetry with diameter of 32 nm (Crowther *et al.*, 1994; Kenney *et al.*, 1995). The HBV encodes four partially overlapping open reading frames (ORF). By negative staining and electro microscopy visualization, HBV appears as double-shelled particle of 42 nm in diameter. The surface of the HBV virions consist of around 240 surface proteins of 3 different membrane-spanning polypeptides: The large (L), middle (M) and small (S) surface (HBs) proteins. The HBs proteins are co-terminally with their carboxy end and differ in additional amino terminal domains. The LHBs consist of an S domain and a pre S1 plus pre S2 domains. The preS1 domain of LHBs can be localized externally or internally. The MHBs contains only the preS2 and S domains, and the SHBs consist of only of the S domain (Heermann *et al.*, 1984; Bruss *et al.*, 1994; Lambert and Prange, 2003).

Hepatitis B virus replicates within the nucleus of nondividing cells such as hepatocyte, uses the cellular transport machinery for the import of their genomes into the the nucleus. Nucleic acids are not karyophilic, thus their nuclear import is mediated by karyophilic proteins, to which they are attached. In a consistent manner, viral genomes are imported as a complex with karyophilic viral proteins using the same recognition motifs and regulation mechanisms as cellular proteins (Whittaker *et al.*, 2000). In addition to the generally smaller cellular proteins, they might only bind to the NPC without being imported if their size exceeds the maximal functional diameter of the nuclear pore of 39 nm (Pante and Kann, 2002).

The HBV genome has to be transported through the cytoplasm towards the nuclear pores followed by subsequent passage through the pore. The conversion of the viral relaxed circular (rc) DNA to the covalently closed circular (ccc) DNA by cellular enzymes requires its release from the capsid (Köck *et al.*, 2003). Then the viral DNA is delivered to the nucleus where the RNA template for reverse transcription (the pregenome) is synthesized by cellular RNA polymerase II (Rall *et al.*, 1983). After export to the cytoplasm, this viral RNA is used for translation of core protein and polymerase (pol) and for encapsidation into progeny core particles (Hirsch *et al.*, 1990). Then the viral components, such as core, polymerase and pre genomic RNA, as well as cellular proteins are packaged into the core particle (Kann *et al.*, 1993; Kann and Gerlich, 1994).

Capsid of HBV (nucleocapsid) is involved in a number of important functions in the replication cycle of HBV. It can be detected

in the nucleus, cytoplasm or both within infected hepatocytes (Mondelli *et al.*, 1986). Yeh *et al.*, (1993) stated that nuclear localization of HBV core protein, which is karyophilic, depends on the cell cycle. It enhanced during the G0/G phase but suppressed during the S phase. In cultured hepatocytes, internalized capsids accumulate in association with the nuclear membrane (Qiao *et al.*, 1994). In transgenic mice in which core proteins are expressed from the integrated HBV gene, core particles are found in the nucleus of hepatocytes (Farza *et al.*, 1988; Guidotti, *et al.*, 1994).

Study of the nuclear import of cellular protein showed that HBV capsids bind to the nuclear pore complex, following the classical pathway of karyophilic proteins, which was mediated by the cellular protein importin α and β (karyopherin α and β). The exposure of a corresponding NLS on the surface of the nucleocapsids, which was bound by importin α appeared to be dependent upon genome maturation and or phosphorylation of the core subunits (Kann et al, 1999). The objectives of the present study was to analyze the intracellular localization of HBV capsid proteins after transfected by entire HBV genome into hepatocyte cell lines (HuH-7) and to determine the predominantly localization of the capsid in cell compartment.

MATERIAL AND METHOD

To prepare the entire HBV genome, plasmid vector pUC-991 which derived from pUC-19 and the entire HBV genome from isolate 991, was digested by Eco RI. One µl DNA pUC-991 (1 µg/µl) was digested by 1 µl Eco RI enzyme (2 U/µl) in total volume 10 µl in the buffer recommended by the manufacturer. After incubated over night at 37°C, the digestion product was separated on 1% gel agarose electrophoresis. The gel was stained by ethidium bromide (SERVA) and visualized using UV light. The expected bands appear at a size of 2.686 bp (vector pUC-19) and 3.199 bp (entire HBV genome). The 3.199 bp band was extracted from the agarose gel (SEAKEM) and purified using a gel extraction purification kit (QIAGEN) according to the manufacturer recommendation. The DNA was then transfected into HuH-7 cells.

HuH-7 cells were seeded on the culture dish (FALCON) and incubated over night in the incubator CO_2 (HERAEUS) at 37°C. The growing cells were harvested and washed with PBS. The PBS was replaced by 1x trypsin (GIBCO-BRL) in PBS, which has been preincubated at 37°C. While the dish with the cells was incubated at 37°C for 2-5 minutes, 0.5 ml 10% Fetal Calf Serum (FCS) containing medium (GIBCO-BRL) were added to the each well of 24 well dish containing collagen-treated cover slips (NUNC INC). The trypsin in PBS was removed and the cells was washed with PBS. The PBS was changed with fresh 10% FCS containing medium. Each cover slip (MAGV) was filled with 2-5 drops cell suspension. Then the cells were allowed to grow over night at 37°C in humidified CO_2 incubator.

Tfx-20 (PROMEGA) was used as transfection agent. For each well, 1 µl DNA (1µg/µl) was mixed gently with 3 µl Tfx-20, then 300 µl FCS free DMEM (GIBCO-BRL) medium was added and immediately mixed again. The mixture was incubated at room temperature for 5-10 minutes. During the incubation, the DMEM medium in 24 well plate were removed and replaced with FCS free DMEM medium. The DNA/Tfx-20 reagent/FCS free DMEM medium mixture were vortexed briefly, then added the mixture to the cells (304 µl per well) and the plate was incubated in the CO_2 incubator at 37 °C for 1 hour. During incubation the 10% FCS containing DMEM medium was warmed at 37°C in a waterbath. After 1 hour FCS free medium was replaced with the 10% FCS containing medium and the plate was returned into the CO_2 incubator for 24-48 hours.

In order to fix on the cover slips during washing, the cells were fixed with 3% paraformaldehyde (PFA) in PBS at room temperature for 30 minutes. The immunofluorescent staining was performed to prove the intracellular localization of entire

HBV genome within the HuH-7 cells using confocal microscope. A mouse monoclonal antibody 414 (mAb 414, BABCO) anti nuclear core complex and a rabbit anti core antibody (DAKO, Dako Cytomation) were used as primary antibodies, and a Texas Red marked anti mouse antibody (DIANOVA) and Alexa 488 marked anti rabbit antibody (DIANOVA) were used as secondary antibodies.

After fixed with 3% PFA, the cells were washed with PBS three times and in each well filled with 500 µl 0,1% Triton x-100. The plate was incubated at room temperature for 10 minutes in order to destroy the cell membranes and cell membranes of core. The wells were then washed PBS three times. In the primary antibody mixture, mAb 414 was diluted in 1 to 300 in antibody solution (PBS/5% BSA/5% goat serum), whereas Dako antibody was diluted 1 to 200 in antibody solution. For each cover slip 40 µl antibody mixture was pippeted and dropped on the parafilm, that laid in a humidified chamber. The up side of cover slips were laid on the drop of antibody mixture. The humidified chamber was incubated at 37°C for 1 hour in the CO_2 incubator. After 1 hour, the up side of cover slips were rinsed with 100 µl PBS, then put back into the well plate. The 24 well plate was washed three times with PBS. The cells were labelled with a secondary antibody from mouse that were marked Texas Red dye. The Texas Red marked anti mouse antibody was diluted 1 to100 in antibody solution. Antibody from rabbit which marked Alexa 488 dye was diluted 1 to 200 in antibody solution. The 40 µl antibody mixture was dropped on the parafilm in the humidified chamber. The up side of cover slips were put on the drops. The chamber was incubated at 37°C for 45 minutes in the CO_2 incubator. After 45 minutes, the cover slips were rinsed with 100 µl PBS and put back again into 24 well plate. The cells were washed with PBS three times. On the clean glass slide, Dabco-Moviol (HOECHST) was dripped and the up side of cover slips were put on the drops. The glass slides were kept over night in the darkness at room temperature.

A drop of immersion oil was dripped on each of cover slips in the slide. The slide was put under a microscope. The cells were microscopied with a 63x apochromat objective. Then the picture screen of transfected cells were observed, in which by their green fluorescence were detected. Because of the fluorescence fades fast by the laser light, a picture screen should be regarded no longer than 1-2 minutes. For the confocal representation, cells were focused in the point of view and at laser achievement of 30% of maximal value, signal amplification of 85%, a sealing thickness of 0,9 and double enlargement scanned. For the representation Alexa 488, the FITC suggestion and filter adjustment were selected, whereas for the representation of Texas Red staining, the TRITC suggestion and filter adjustment were adjusted. The height of the cutting level was adjusted, in order that the nucleus cell appear as a red sharp rim. At the end of this work, all pictures were regarded and saved in the microscope software. The picture results were worked and arranged in the ADOBE-PHOTOSHOP software program.

The HBV capsid protein which found localized in the compartment of HuH-7 cells, were quantified manually using confocal laser microscope. The amount of HBV capsid that found in the cytoplasm, nucleus or both of cell compartments were quantified in the absolute and relative values.

RESULT AND DISCUSSION

Immunohistochemical detection of the HBV capsid proteins in human liver cells from HBV infected individuals showed that the capsid localized predominantly within the nucleus (Akiba *et al.*, 1987; Serinoz *et al.*, 2003). Nuclear localization was also observed in mice transgenic for HBV (Guidotti *et al.*, 2002). Using this technique it was shown that the nuclear membrane was impermeable for the entire HBV capsid. Since liver cells divide only rarely, the nuclear capsids might be thus derived from capsid protein that either diffuses through the nuclear pore complex or from

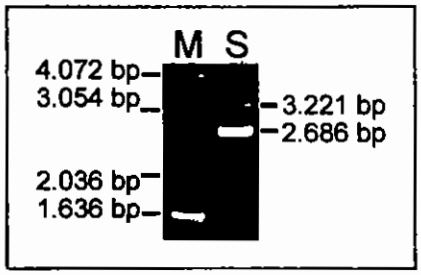

Figure 1. Digestion products of pUC-991 by Eco RI.
Ethidium bromide staining was perform after agarose gel electrophoresis. Plasmid UC-991 contains a monomer of the HBV genome (isolate 991) cloned via the single Eco RI restriction site within the vector pUC-19. S shows the reaction products of an Eco RI digest. The vector backbone migrates as a linear DNA fragment of 2.686 bp, the entire HBV genome migrated slower representing 3.221 bp. M: DNA molecular weight marker (1 kb plus DNA ladder). Electrophoresis was done on a 1% agarose gel in TAE buffer.

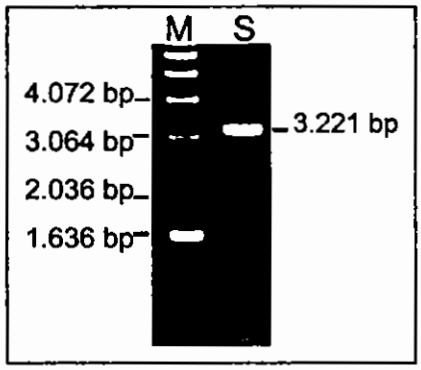

Figure 2. Electrophoresis of the linear purified entire HBV-991 genome.
S shows the HBV-991 genome after purification. No contamination to the vector could be observed. M indicates the molecular weight marker. Electrophoresis and staining were done as in Figure 1.

capsid protein that is actively transported through the pores. In both hypotheses capsid formation occurs within the nucleus when the capsid protein concentration exceeds the minimal assembly concentration, as it was determined by others (Seifer and Standring, 1995).

To study the nuclear localization of capsid protein more detail, we analysed the localization of the HBV capsid in human hepatocyte cell lines by transfection. The HBV capsid was transiently expressed in the hepatoma cell line (HuH-7 cell) to validate whether transfection of entire HBV genome into cell line resulted in the same intracellular localization of HBV capsid as in human liver cell. *In vivo,* transcription of HBV genome are synthesized from a circular HBV DNA genome with overlapping promoters and open reading frames (ORF). Consequently, it cannot be excluded that a promoter is down-regulated by transcription of an overlapping ORF. In order to prevent artefacts by overexpression the capsid was thus expressed in the context of the

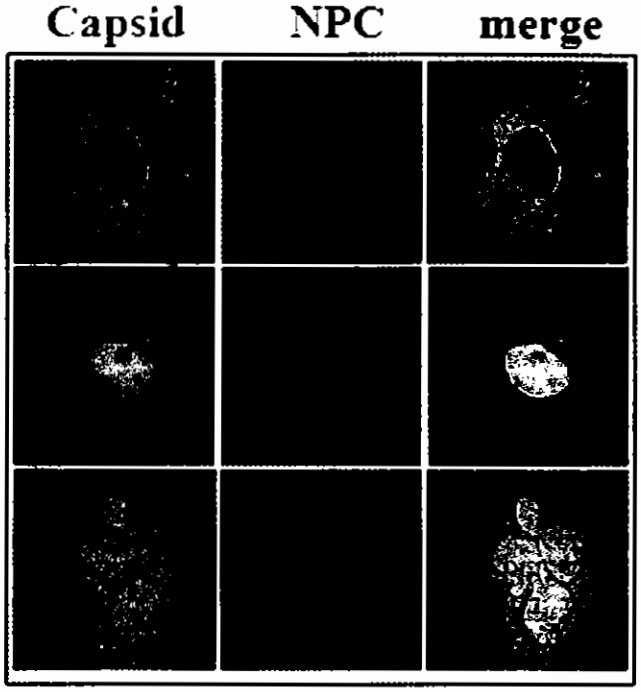

Figure 3. Indirect immune fluorescence staining of the HBV capsid in HuH-7 cells.
HBV capsids were stained using an anti-HBV capsid primary antibody and a FITC-labeled secondary antibody. The staining patterns are depicted in green (left column). The staining control of the NPCs was performed using a monoclonal antibody (mab) 414 as primary antibody and a Texas Red-labeled secondary antibody, which is shown in red (middle column). The overlay of both staining are shown in the right column. The panels show examples of cytoplasmic localization (upper row), nuclear localization (middle row) and localization into nucleus and cytoplasm (lower row).

HBV genome. Although the HBV genome has to be circular to express all viral gene products, a linear genome was used since circularization occurs after transfection, as it was determined by others (Sterneck *et al.*, 1998).

Restriction digestion of plasmid DNA pUC-991 that contains a full-length linear HBV genome of genotype D (EMBL accession no. X51970) by Eco RI resulted linear entire HBV genome and linear vector backbone pUC-19. The ORFs of polymerase and surface protein were discontinuous but the overlapping X protein transcription and core protein transcription units were unaffected. Elektroforesis of digestion products on an 1% agarose gel and subsequent ethidium bromide staining resulted two cleavage products of 3.221 bp depicting the HBV genome and 2.686 bp depicting the pUC-19 vector (Fig. 1). Then the 3.221 bp band was isolated, purified (Fig. 2) and transfected into HuH-7 cells.

The observed of localizations were classified in a number of cells as to be predominantly intranuclear, cytoplasmic localization or distributed within both of these compartments. Representative fluorescence results are depicted in Fig. 3, the frequency of the distribution are shown in Tabel 4.

As in human HBV-infected livers, the HBV capsid staining was predominantly found within the nucleus (71%). Less frequently, the capsid localized within the cytoplasm (26%). Since the used anti capsid antibody recognizes capsid protein monomers and dimers at least 240 fold less than assembled capsids (Kann *et al.*, 1999), it must be concluded that the stain reflects assembled capsids instead of unassembled capsid protein. Only in a minority of cases, the capsids were found to show a more or less equal distribution between cytoplasm and nucleus (3%). This low frequency indicates that the capsids are not

Tabel 4. Distribution of HBV capsids after transient transfection of the HBV genome into HuH-7 cells.

Absolute and relative numbers of cells showing an equal distribution or a predominantly nuclear or cytoplasmic localization of HBV capsids.

Cytoplasm	Nucleus	Both	Total
26	71	3	100
26%	71%	3%	100%

diffusing within the cells being in accordance to the *in vivo* situation in which the nuclear membrane is impermeable for the capsid. However the significant number of cells that show different localizations indicates that the cells differ either in their nuclear transport capacity or in that they modify the core protein in a way that affects its nuclear import.

It was previously reported that the HBV capsid protein contained a nuclear localization signal (NLS) within amino acid 157-168 (PRRRTPSPRRR) (Kann *et al.*, 1999) that interacted with the nuclear transport receptor importin. This complex bound to importin ß that facilitates the nuclear transport of the capsid (Kann *et al.*, 1999, Rabe *et al.*, 2003). The consensus motif for monopartite NLS is K-(K/R)-x-(K/R). Based on co-crystallisation of importin □ with SV40 Tag and Lamin B2-NLS, it was proposed that NLS must contain a Lysine residue within the NLS interacted with the binding pocket P2 of the importin □ molecule (Fontes *et al.*, 2000). The larger side chain of arginine probably according to this study, prevent efficient interaction between NLS and importin α.

The HBV capsid NLS should thus show only a weak interaction importin □while a similar NLS was described for the HIV Vpr protein (Sherman *et al.*, 2001). However, Vpr is part of the HIV preintegration complex that contains multiple NLS on different proteins (Vpr, MA, integrase) while nuclear transport of the HBV capsid protein is not supported by other karyophilic proteins. On the other hand, on capsids or assembly intermediates the HBV NLS is exposed in redundancy. Such a multiple exposure of an NLS increases the probability of interactions with importin α, assumed to enhance transport competence. Alternatively, additional interactions between the capsid protein and importin α outside the NLS-binding pocket may stabilize the importin α-NLS interaction, as it was shown previously for the BDV protein p10 (Wolff *et al.* 2000).

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