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Synthesis of all hepatitis B structural proteins in the Semliki Forest virus expression system

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Abstract

All structural genes of human hepatitis B virus (HBV), subtype *ayw*, have been expressed in the Semliki Forest virus (SFV) expression vectors pSFV1 and pSFV-C, allowing direct and SFV core-dependent expression of foreign genes, respectively. Three variants of HBV surface genes (large, middle and small), as well as the core gene were amplified by PCR technique as independent units and fusions with the SFV core protein gene, cloned in both SFV vectors and expressed in BHK-21 cell culture as single proteins or in different combinations. Maximal production of HBV proteins was achieved using pSFV/C vectors where target products were obtained after processing of SFV core-HBV protein fusions. After infection of the cultured BHK cells with recombinanat SFV-HBV viruses, the expression of all HBV structural genes was detected by immunocytochemistry. Electron microscopy revealed the formation of virion-like particles in the case of simultaneous expression of HBV surface and core genes. This efficient expression system of HBV subunits will be applied for the elucidation of molecular details of HBV assembly, structure and entry.

Key words: hepatitis B virus, Semliki Forest virus.

Introduction

Approximately 5 % of the world population is infected by the hepatitis B virus (HBV), which causes a necroinflammatory liver disease of variable duration and severity. Chronically infected patients with active liver disease carry a high risk of developing cirrhosis and hepatocellular carcinoma. HBV virions, or Dane particles, and attending macromolecular forms, the so-called 22-nm particles, are unique in their features, since the virions are formed by four structural proteins (for review, see Gerlich, Bruss 1993). One of them, the hepatitis B core antigen (HBc) encoded by viral gene C, forms an inner capsid packaging genomic partially double-stranded DNA, and three variants of the surface antigen (HBs) encoded by gene S form an outer envelope consisting of a phospholipid bilayer membrane with protein spikes. The major S gene product is the 226-amino acid-residue-long HBsAg, or S, or "small", or SHBs antigen; the two other HBsAg variants are N-terminally prolonged forms of the former, which contain an additional 55 amino acid residues (M, or "middle", or MHBs) and 119 or 108 amino acid residues for the two major HBV subtypes adw and ayw, respectively (L, or "large",

or LHBs). Ratios of superficially exposed epitopes of these three molecular variants of HBs determine in general the infectious capabilities of the virus and its extremely narrow host range (Berting et. al. 1995). The latter obstacle, together with the lack of efficient *in vitro* models for HBV replication, limit the possibilities for structural investigation of virus cell recognition, entry, self-assembly, and mutual protein-protein, protein-RNA and protein-DNA recognition. In the infected cell cytoplasm, virus assembly is directed by the structural proteins of virus in such a way that the capsid protein first complexes with the viral genome into a capsid structure. This then binds to viral spike proteins that have been inserted into the endoplasmic reticulum (ER) membrane, and buds into the ER lumen. The enveloped virus reaches the extracellular milieu via the secretory pathway. Virus entry into new host cells is directed by the spikes of the virus. These first bind to receptor structures on the host cell surface, and then subsequently mediate virus penetration, most likely via a process of virus membrane-host membrane fusion.

Direct analysis of the contribution of the HBV envelope proteins to virion assembly, by using infectious model and cultured hepatoma cells transfected with mutant HBV genomes bearing lesions in the envelope coding regions, was undertaken in the classical work of Bruss and Ganem (1991). Although the principal information about the role of S, M and L proteins in production of the virus was obtained, the low synthetic efficacy of the system, together with dependence on attendant viral processes, did not allow direct structural investigation of macromolecular protein structures. To reconstruct particles in high yields, simultaneous expression of S and C genes was undertaken in yeast (Shiosaki et. al. 1991) and in insect cells (Takehara et. al. 1988). Due to very long distances between the real HBV host and the used models, these attempts were not informative from the point of view of such intimate mechanisms as budding and self-assembly.

Recombinant plasmids based on the Semliki Forest virus (SFV) genome have been introduced to express foreign genes in a broad range of eukaryotic cells (Liljeström and Garoff 1991; Sjöberg et. al. 1994). The universal nature of the system made it ideal for accurate determination of HBV virion-like particles. The aim of this study was to adopt the SFV system for efficient expression of the above mentioned HBV structural genes, as well as to evaluate the possibility of synthesized recombinant proteins to form different kinds of HBV virion-like particles in transfected cells. This efficient expression system of HBV subunits could be very useful in studying the molecular details of HBV assembly, structure and entry.

Materials and methods

Construction of plasmids

To construct HBV-genes expressing plasmids, we used a plasmid pHB320 harbouring the complete genome of HBV, subtype *ayw* (Pumpen et. al. 1981) and two different variants of the SFV expression vectors – SFV1 (Liljeström, Garoff 1991) and SFVC-Bam HI (Sjöberg et. al. 1994). Restriction endonucleases, T4 DNA ligase and SP6 polymerase were purchased from Fermentas (Vilnius, Lithuania). DNA preparation, restriction enzyme cleavage, cloning of DNA fragments, bacterial transformation and growth were performed essentially as described by Sambrook et. al. (1989).

pSFV1/C. The oligonucleotides used for the HBc amplification were: 5'-GCGGATCCA-TGGACATTGATCCTTATA-3' and 5'-CGCCCGGGTAAAGTTTCCCACCTTATG-3'.

pSFV1/S. The oligonucleotides used for *S* gene amplification were: 5'-ATTGGGGTC-CTGCGCTGAACATGGAG-3' and 5'CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV1/M. The oligonucleotides used for the *M* gene amplification: 5'-ACGGATC-CTCAGGCCATGCAG-3' and 5'CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV1/L. The oligonucleotides used for the *L* gene amplification: 5'-GCCCCGGGAT-GGGGCAGAATCTTTCCA-3' and 5'-CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV-C/C. The terminal primers used for the amplification of the fused DNA were: 5'-G-ATGTATCTTCGAAGTCAAACACG-3' (the 5'-end primer) and 5'-CGCCCGG-GTAAAGTTTCCCACCTTAGT-3' (the 3'-end primer). The fusion primers used were: 5'-GAGGGGTCCGAAGAGTGGATGGAGAACA-TCACATCAGG-3' (HBV C-gene fragment primer) and 5'-CCTGATGTGATGCTTCTCCATCCACTCTTCGGACCCCT-CGG-3' (SFV C-gene fragment primer).

pSFV-C/S, pSFV-C/M and pSFV-C/L. The terminal primers used for the amplification of fused DNA fragments were the same for all three constructs: 5'-GACTGTATCTTCGAAGT-CAAACACG-3' (the 5' end primer) and 5'-CGCC-CGGGTTTAAATGTAGACCCAAAG-3' (the 3' end primer). The fusion primers used were: 5'-AGGGGTCCGAAGAGTGGA TGGAGAACATCACATC-3' (HBV S-gene fragment primer) and 5'-GATGTGATGTT CTCCATCCACTCTTCGGACCCCT-3' (SFV C-gene fragment primer) 5'-AGGGGTC CGAAGAGTGGATGCAGTGGAACTCCAC-3' (HBV M-gene fragment) and 5'-GTG GAGTTCCACTGCATCCACTCTTCGGACC-CT-3' (SFV C-gene fragment primer); 5'-CCGAGGGGTCCGAAGAGTGGATGGGGCAGAATCTTTCCACC-3' (HBV L-gene fragment primer) and 5'-GGTGGAAAGATTCTGCCCCATCCACTCTTCGGACCCCT CGG-3' (SFV C-gene fragment primer).

RNA transcription, electroporation and metabolic labeling of transfected cells

RNA transcripts were synthesized *in vitro* by SP6 RNA polymerase using SpeI or NruI-digested plasmids as templates. Reaction conditions for *in vitro* RNA transcription have been described previously (Liljeström, Garoff 1991). *In vitro* made RNA was transfected into BHK (Baby Hamster Kidney) cells (obtained from American Type Culture Collection) by electroporation. Confluent cell monolayers were trypsinized, washed once with complete BHK medium (GIBCO) supplemented with 5 % fetal calf serum, 20 mM HEPES (pH 7.3), once with ice-cold phosphate-buffered saline (PBS, without $MgCl_2$ and $CaCl_2$) and resuspended in 0.8 ml PBS. The cell suspension was mixed with *in vitro*-made RNA (20 ml of the reaction mix) and transferred to a 0.4-cm electroporation cuvette (Bio-Rad). In cotransfection experiments equal amounts (20 ml) of both RNA were used, except in the SFVC/C, SFVC/S, SFVC/M and SFVC/L cotransfection where half the SFVC/L RNA (10 ml) amount was employed.

Electroporation was carried out at room temperature by two consecutive pulses at 0.85 kV and 25 mF, using a Bio-Rad Gene Pulser apparatus (without the pulse controller unit). These conditions yielded virtually 100 % transfection efficiency. Electroporated cells were diluted into 18 ml complete BHK medium, transferred onto tissue culture plates and incubated at 37 °C. At 4 - 16 h post-electroporation, the culture medium was replaced with methionine-free minimal essential medium (GIBCO) supplemented with 10 mM HEPES. After 30 min at 37 °C, the medium was replaced with the same methionine-free medium containing 100 mCi of [^{35}S]methionine per ml and the cells were incubated at 37 °C for 30 min to 4 h (pulse). After an appropriate pulse time, the culture medium was collected and

clarified by centrifugation twice in an Eppendorf cenrifuge (5 min at 5,000 rpm at 4 °C). Cell monolayers were washed with PBS and solubilized with 1 % sodium dodecyl sulfate (SDS) or 1 % Nonident P-40 (NP-40) lysis buffers containing 10 mM iodoacetamide (Peränen et. al. 1988; Suomalainen et. al. 1990). Nuclei were removed from cell lysates by centrifugation in an Eppendorf centrifuge (5 min at 5,000 rpm at 4 °C).

Immunoprecipitation of proteins from cell lysates

HBV core antigen was immunoprecipitated from NP-40 cell lysate (in the case of pSFV1/C), or from SDS-solubilized cell lysate (in the case of pSFV-C/C) with a polyclonal rabbit anti-HBc antiserum (a kind gift of V. Tsibinogin, Rīga). HBVS, HBVM and HBVL proteins were immunoprecipitated from NP-40-solubilized cell lysates with polyclonal goat anti-HBs antiserum (V. Tsibinogin). Immunocomplexes were brought down with protein A-Sepharose (Pharmacia; 1:1 (v/v) slurry in 10 mM Tris-HCl, pH 7.5) using rabbit anti-mouse immunoglobulins (Dakopatts a/s, Denmark) as linking antibodies when necessary. Immunuprecipitates were solubilised in SDS-sample buffer (200 mM Tris-HCl, pH 8.8, 20 % glycerol, 5 mM EDTA, 0.02 % bromphenol blue, 4 % SDS, 50 mM ditiothreitol) by heating at 95 °C for 5 min. The samples were alkylated with an excess of iodoacetamide and spun for 2 min in an Eppendorf microcentrifuge at full speed before being analysed by SDS-PAGE. SDS-PAGE was performed in 12 % gels (Laemmli 1970). The gels were treated with 1 M sodium salicylate for 30 min at room temperature, then dried and exposed to Kodak XAR-5 film at -70 °C.

Construction of recombinant viruses and cell infection

For *in vivo* packaging of recombinant RNA into SFV particles, *in vitro*-transcribed RNA was electroporated into BHK cells together with SFV helper RNA (Fig. 1; Berglund et al. 1993) under the conditions described above. After 20 h, SFV particles were collected from the culture medium and frozen rapidly to be stored as virus stocks. The titres of SFV stocks were determined by infecting cells with serial dilutions of the stocks followed by indirect immunochemistry assay for the expressed proteins. The estimated titres varied from 1×10^7 to 5×10^7 viral particles per ml. The infection of BHK cells was carried out in serum-free medium with appropriate dilution of the virus stock to achieve 100 % cell infection.

Immunocytochemical detection of intracellular HBV antigens by monoclonal antibodies

BHK cells grown on sterile tissue culture chamber slides (Nagle Nunc International) were infected with recombinant SFV, and incubated at 37 °C (5 % CO_2) for 20 h. After drying the slides at room temperature, the cells were fixed with ethanol/acetic acid (3:1) for 20 min, and rinsed thoroughly (3 ×) in distilled water. Then the slides were immersed in PBS for 10 min, then rinsed with PBS supplied with 0.25 % Triton X-100. Then slides were incubated for 24 h in a humidity chamber at 4 °C with the anti-HBs monoclonal antibody, or with anti-HBc monoclonal antibody (gift of Dr. I. Sominskaya, BMC, Riga) at a dilution 1:200 (in PBS + 0.25 %Triton X-100 + 0.25 % BSA), and after that rinsed in PBS + 0.25 % Triton X-100. Then cells were incubated with anti-mouse IgG conjugated with Alkaline Phosphatase (Sigma) at a dilution 1:200 in PBS + 0.25 % Triton X-100 + 0.25 % BSA at room temperature, in dark for 1 h, and subsequently rinsed with PBS.

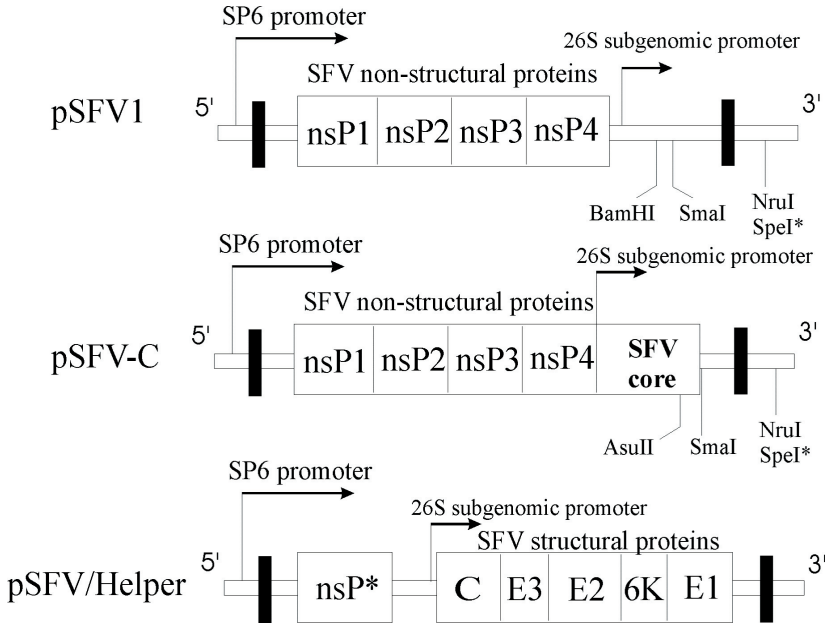

Fig. 1. General scheme of SFV vectors. Only SFV recombinant cassettes are shown. nsP, SFV non-structural proteins encoding replicases for transcription of subgenomic RNA from subgenomic 26S promoter. The pairs of cloning sites BamHI/SmaI (for pSFV1 vector) and AsuII/SmaI (for pSFV-C vector) were used for introduction of HBV structural genes (C, S, M, L). NruI, or SpeI is a plasmid linearization site. pSFV/Helper construct was used for production of recombinant SFV particles. The nsP* region, including the packaging signal, is deleted from the helper plasmid, which provides therefore the synthesis of SFV structural proteins only. The SP6 RNA polymerase promoter for *in vitro* transcription of recombinant RNA is indicated. Black boxes along the edges of the SFV cassettes denote sequences used by the SFV replicase for amplification of the SFV replicons.

Alkaline Phosphatase activity was developed by Sigma FAST reagent for 30 min at room temperature, where the Fast Red TR/Napthol AS-MX is the immunohistology substrate of choice for alkaline phosphatase conjugated antibodies, as it produces an intense red stain. The cells were then rinsed in deionized water; counterstained with hematoxylin; and mounted in glycerol gelatin (Sigma). The evaluation was conducted under a light microscope.

Electron microscopy

Electron microscopic analysis was performed on a JEM100C electron microscope at 80 kV accelerating voltage and screen magnification of × 100,000. The specimens were negatively stained in neutral 2 % phosphotungstic acid.

Results and discussion

Construction of expression plasmids

As a basis for the construction of HBV-genes expressing plasmids, we used a plasmid pHB320 (Pumpen et. al. 1981) that spans the complete genome of HBV (Bichko et. al.

1985). The HBV C-gene and three variants of HBV S-genes were amplified by PCR and, after appropriate treatment with DNA-modifying enzymes, introduced into two different variants of the SFV expression vectors: SFV1 (Liljeström, Garoff 1991) for direct expression of HBV genes and SFV-C (Sjöberg et al. 1994) to create fused chimeric proteins with the capsid protein of SFV (Fig. 1).

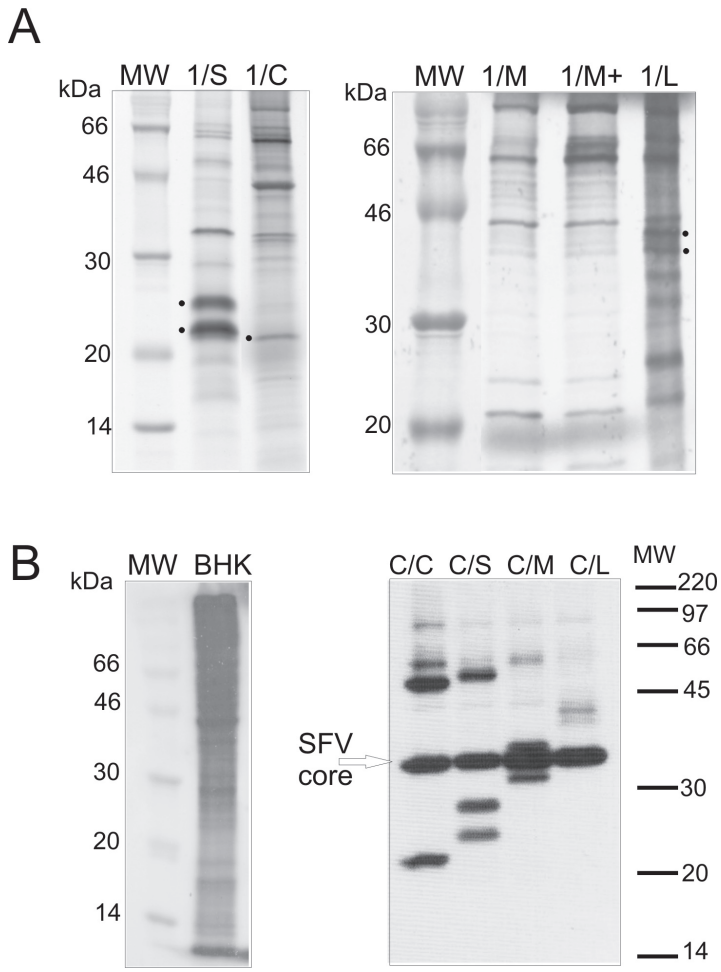

Fig. 2. Expression of HBV structural proteins in BHK cells upon RNA transfection. (A) Cells were transfected with RNAs of SFV1/S (1/S), SFV1/C (1/C), SFV1/M (1/M), SFV1/L (1/L), and pulse-labelled 2 h at 16 h post electroporation. In 1/M+ two times more of the RNA was used for electroporation. Equal amounts of NP-40 lysates were run on a 12 % gel. The bands of corresponding HBV proteins are marked with dots, except 1/M and 1/M+, where HBV specific bands were not identified. MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated on the left. (B) Cells were transfected with RNAs of SFV-C/C (C/C), SFV-C/S (C/S), SFV-C/M (C/M), SFV-C/L (C/L) and pulse-labelled 30 min at 6 h post electroporation. Equal amounts of NP-40 or SDS-lysates (C/C) were run on a 12 % gel. MW, High-Range Rainbow Molecular Weight Marker (Amersham) Non-transfected BHK cells were used as a negative control (BHK).

The genes for the HBV core (C) and three variants of HBV surface (S, M and L) proteins were engineered into the pSFV1 vector under the SFV subgenomic promoter (26S). For construction of pSFV1/C plasmid, the amplified HBV DNA fragment (602 bp) was cleaved with restriction enzymes BamHI and SmaI, and the 594bp-fragment was ligated with the SFV1 vector processed in the same way. To construct pSFV1/S and pSFV1/M plasmids, HBV DNA fragments corresponding to the HBV S (711 bp) and HBV M (875 bp) genes were cleaved with BamHI and SmaI restriction enzymes, and the appropriate fragments (700 bp and 862 bp) were inserted into the BamH1/SmaI-treated pSFV1 vector.

To construct the pSFV1/L plasmid, the HBV DNA-fragment coding for the HBV L gene (1187 bp) was inserted into the SFV1 expression vector using SmaI restriction sites.

The precise fusion of SFV C and HBV structural protein genes was carried out by PCR. The plasmid pSFV-C/C was constructed by ligating the 1026-bp AsuII-SmaI SFVC-HBVC-fusion fragment to pSFV-C vector treated by the same restriction enzymes. The amplified fused DNA fragments for HBV S (1140 bp), HBV M (1350 bp), and HBV L (1624 bp) were cloned in the SFV-C vector after cleaveage with AsuII and SmaI. The plasmid for analysis of HBV S protein synthesis was named pSFV-C/S, the plasmid for HBV M protein production was named pSFV-C/M and the plasmid for HBV L gene expression was named pSFV-C/L.

All synthesized constructs were confirmed by sequencing.

Expression of HBV proteins in SFV1 and SFV-C vectors

The resulting plasmids on the basis of the SFV1 vector (pSFV1/C, pSFV1/S, pSFV1/M and pSFV1/L) and SFV-C vector (pSFV-C/C, pSFV-C/S, pSFV-C/M and pSFV-C/L) were used as templates for *in vitro* transcription of the corresponding recombinant SFV genomes. The RNA of each construct was separately transfected into BHK cells. Fig. 2A, B shows an analysis of transfected BHK cell lysates. At 6 to 16 h post-infection cells were metabolically labeled with [^{35}S]-methionine for 2 h (SFV1 constructs), or for 30 min (SFV-C constructs), and lysed with NP-40 containing lysis buffer.

In contrast to the relatively inefficient synthesis of HBV proteins in the SFV1 system, especially in the cases of SFV1/M, the SFV-C system assured high level expression of all structural HBV proteins (Fig. 2B), where only a short pulse-labeling time was necessary. The expression of fused SFVC-HBV genes shut off completely the synthesis of cellular proteins, in contrast to the BHK control. All fused SFVC-HBV proteins were split correctly, although, in the case of C and S, traces of unsplit precursors were seen. Probably, processing was dependent on specific N-terminal amino acid sequences of added HBV protein. In contrast to the S, M and L products, the C product appeared as nuclear associated protein, since it was soluble only in SDS lysis buffer, but not in NP-40 lysis buffer. Products of S, M, and L genes appeared also in glycosylated forms in the same way as during HBV infection in men (Stibbe, Gerlich 1983). S products existed as non-glycosylated p24 and mono-glycosylated gp27 molecules. The M product showed three bands: non-glycosylated p31 (not detected during viral infection), mono- and double glycosylated forms gp33 (co-migrating with SFV core on the gel), and gp36. The L product showed non-glycosylated form p39 and mono-glycosylated form gp42.

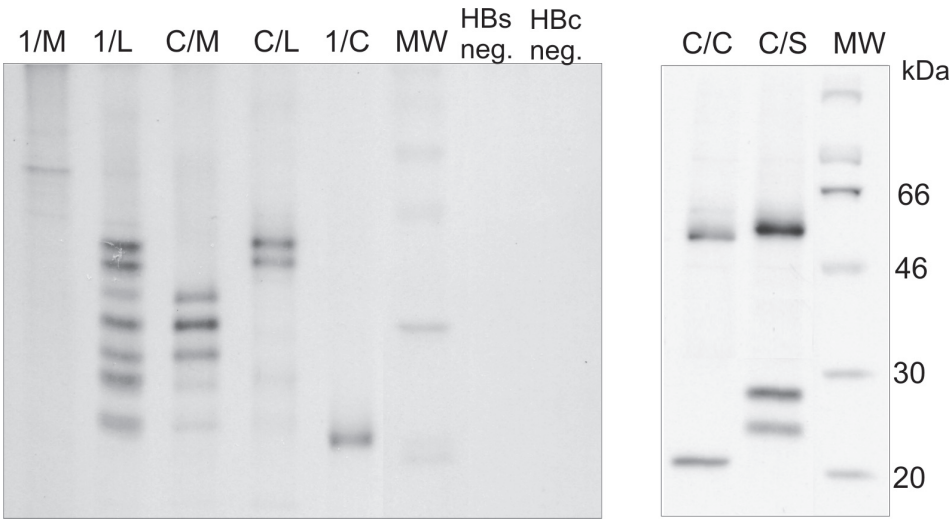

Fig. 3. Immunoprecipitation of intracellular products of BHK cells transfected with SFV1/M (1/M), SFV1/L (1/L), SFV-C/M (C/M), SFV-C/L (C/L), SFV1/C (1/C), SFV-C/C (C/C), SFV-C/S (C/S). Rabbit polyclonal anti-HBc antibodies were used for HBc protein immunoprecipitation, and goat polyclonal anti-HBs – for HBs immunoprecipitation. As negative controls were used the immunoprecipitations of the lysate of untransfected BHK cells incubated with the same rabbit anti-HBc antibodies (HBc neg.) and goat anti-HBs antibodies (HBs neg.). MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated on the right.

Immunological properties

Immunoprecipitation of the expression products with the appropriate antibodies demonstrated their immunological specificity (Fig. 3). Immuno-precipitation of the C product revealed a clear p22 band and an additional band which could be explained as SFV-C-HBc fusion protein. The same situation was observed in the case of HBV S gene expression, where additionally to p24 and gp27 bands, the possible SFV-C-HBs fusion protein was visible. Unfortunately, we did not detect M protein synthesis by the pSFV1/M construct. The reason of this is unclear, despite the strong confirmation of the nucleotide structure of this construct by sequencing.

Surprisingly, the pSFV1/L construct provided the synthesis of all variants of HBs proteins (L, M, S) in almost equal amounts, while the SFV-C dependent expression of L demonstrated only trases of M and S protein synthesis. We suppose that the reason for this phenomenon is the preferable translation of fusion protein while the distantly located AUGs of M and S proteins can not be reached by ribosomes.

The glycosylated nature of the three variants of HBV surface proteins was revealed by treatment with endoglycosydase H. After this treatment, all glycosylated forms were transferred to positions of the appropriate non-glycosylated forms on the gel (not shown).

Secretion of HBV gene S products

Analysis of intracellular and extracellular fractions revealed the successful secretion of

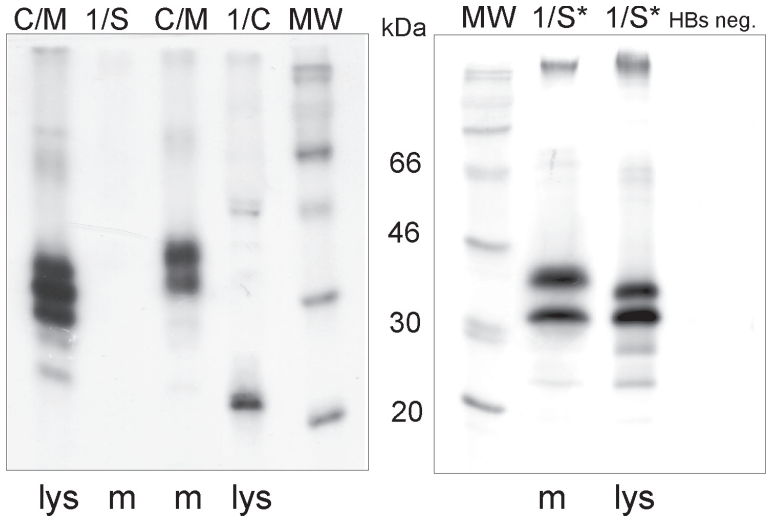

Fig. 4. Analysis of intracellular (lys) and extracellular (m) HBV proteins synthesized in RNA-transfected BHK cells. Cells were transfected with RNAs of SFV-C/M (C/M), SFV1/S (1/S), SFV1/C (1/C), and SFV1/S adw subtype (1/S*). Appropriate proteins were immunoprecipitated with goat polyclonal anti-HBs, or rabbit polyclonal anti-HBc antibodies. HBs neg., untransfected BHK cells incubated with goat polyclonal anti-HBs antibodies. MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated in the middle.

the M, but not S product (Fig. 4). However, later we found the S product secretion of *adw* subtype of HBV. These results are the focus for further investigation. As expected, the products L and C were not secretable. As seen on the figure, the secreted forms of HBs proteins are different from that of the intracellular forms, because of specific posttranslational modifications during transport.

Immunocytochemical analysis of BHK cells producing HBV proteins

As was shown above, we revealed the production of HBV structural proteins in SFV-driven expression by SDS-PAGE. More obviously these results were observed by immunocytochemical analysis of BHK cells infected with the appropriate recombinant SFV-HBV virus (Fig. 5). We showed that more than 90 % of tested cells were positive for HBV proteins. However, the intensity of staining was different and correlated with the levels of proteins detected by ELISA (not shown) and SDS/PAGE. The most intensive staining was found in all SFV core fused variants. Staining patterns showed a cytoplasmic, granular distribution of the HBV proteins in the cells. At the same time we observed mainly unipolar, perinuclear localization of HBc proteins in the case of SFV1/HBVC expression, and more homogeneous cytoplasmic distribution in the case of the SFV1/HBVS expression. All SFV core-dependent constructs demonstrated similar staining of cell cytoplasm with over-expressed HBV proteins.

The BHK cells, which were used in these experiments, are not natural host cells for HBV. However, they are optimal for infection with and production of recombinant SFV particles, allowing the highest yields of recombinant proteins. Beside the BHK cells, we established similar expression patterns of the HBV proteins for all studied constructs in

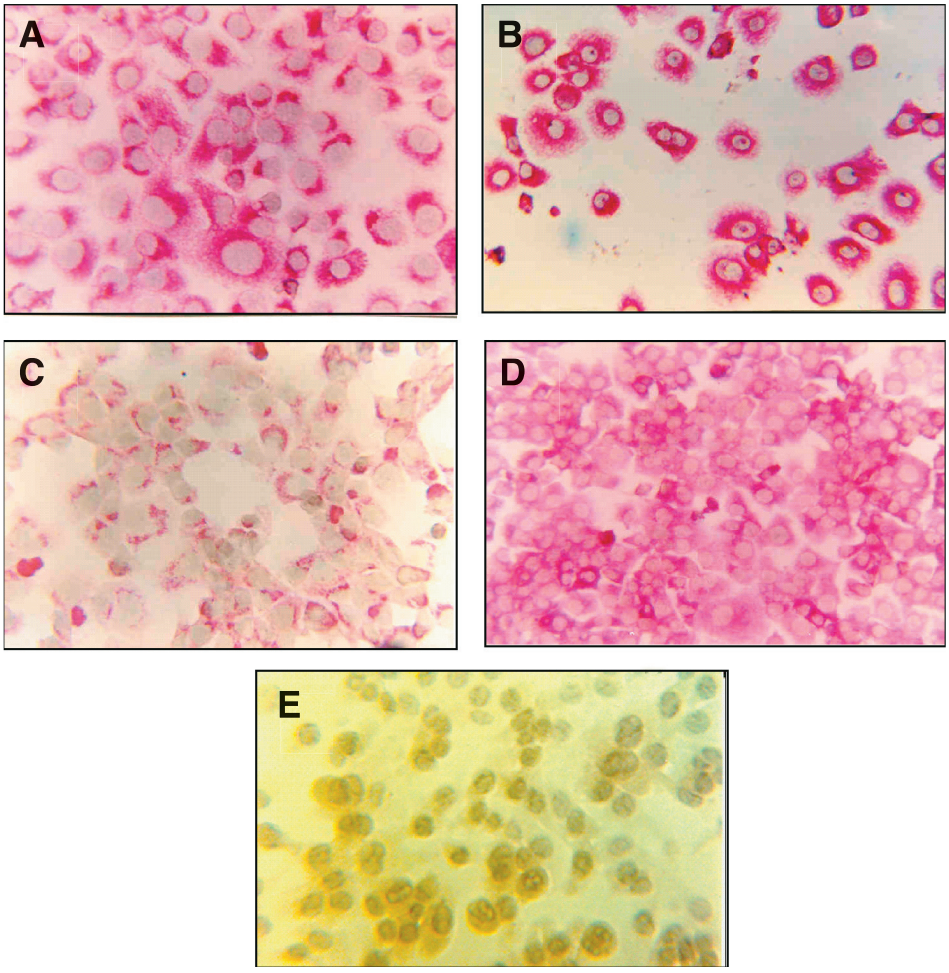

Fig. 5. Immunocytochemical detection of HBV proteins in BHK cells. A, SFV1/L antiHBs; B, SFV1/S anti HBs; C, SFV1/C antiHBc; D, SFV-C/M antiHBs; E, antiHBs/antiHBc neg. Cells were infected with the appropriate recombinant virus (SFV1/L; SFV1/S; SFV1/C; pSFV-C/M). At 20 h postinfection, the cells were fixed and processed as described in the Materials and Methods section. Proteins were detected with monoclonal anti-SHBs, or anti-HBc antibodies. Neg. control – uninfected BHK cells incubated with the same antibodies.

other cell lines (HuH-7, HepG2, COS-7), only the levels of production were lower (not shown).

Co-expression of HBV structural genes and secretion of HBV virion-like particles

Co-transfection of BHK cells with different combinations of RNAs led to efficient equimolar production of HBV structural proteins, also when all four genes were expressed simultaneously (not shown). Since L product was considered to be an inhibiting

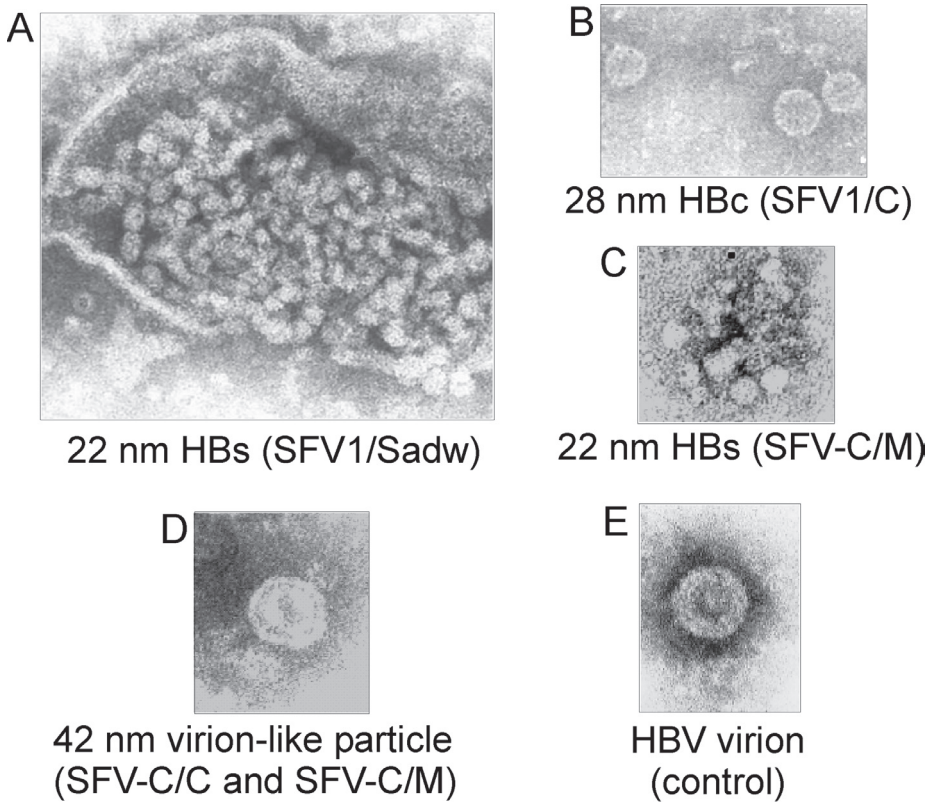

Fig. 6. Electron micrographs of HBV particles. A, HBsAg-22-nm-like particles in cryo-lysate of pSFV1/S-transfected (subtype *adw*) BHK cells. B, HBV core particles (HBc) in the NP-40 containing lysate of BHK cells transfected with pSFV1/C. C, Secreted HBsAg-22-nm-like particles in the medium of pSFV-C/M-transfected BHK cells. D, HBV virion-like particles in medium of BHK cells co-transfected with SFV-C/C and SFV-C/M. E, the native HBV virion.

secretion agent, we limited its production level by a two-fold decrease of its RNA for cell transfection. In the media, we found M products in the case of simultaneous expression of M and C genes. Unfortunately, we found no trace of C protein in the medium, probably due to insufficient sensitivity of method used (SDS-PAGE). Electron microscopy, as a more sensitive method, showed the presence of not only 22 nm HBsAg particles (Fig. 6C), but also virion-like particles similar to native Dane particles from human blood (Fig. 6 D, E). The separate expression of the Core particle formation was revealed by electron microscopy of the lysate of pSFV1/C transfected cells (Fig. 6B). The 22 nm HBsAg particles were seen in the cryo-lysates of pSFV1/S transfected cells (Fig. 6A). To enhance the productivity of the system and to broaden the possible host cell range, virus stocks for all four producers will be used to infect model cell cultures.

Therefore, high potential of simultaneous expression of all four HBV structural genes within SFV vectors in tissue culture cells was shown conceptually, in contrast to earlier attempts (Takehara et. al. 1988; Shiosaki et. al. 1991). The SFV system presents a unique capabilities to efficient model intimate mechanisms of HBV virion self-assembly and

turnover. The possible application of SFV-derived HBV virion-like structures as gene therapy tools and/or RNA vaccines is planned to be investigated fully in the future.

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References

Berglund P., Sjoberg M., Garoff H., Atkins G., Sheahan B., Liljeström P. 1993. Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *BioTechnology* 11: 916–920.

Berting A., Hahnen J., Kroger M., Gerlich W.H. 1995. Computer-aided studies on the spatial structure of the small hepatitis B surface protein. *Intervirology* 38: 8–15.

Bichko V., Pushko P., Dreilina D., Pumpen P., Gren E. 1985. Subtype *ayw* variant of hepatitis B virus. DNA primary structure analysis. *FEBS Lett*. 185: 208–212.

Bruss V., Ganem D. 1991. The role of envelope proteins in hepatitis B virus assembly. *Proc. Natl. Acad. Sci. USA* 88: 1059–1063.

Gerlich W.H., V. Bruss. 1993. Functions of hepatitis B virus proteins and molecular targets for protective immunity. In: Ellis R.W. (eds): *Hepatitis B Vaccines in Clinical Practice*. Marcel Dekker, NY, Basel, Hong-Kong, pp. 41–82.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

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

Peränen J., Takkinen K., Kalkkinen N., Kääriäinen L. 1988. Semliki Forest virus-specific non-structiral protein nsP3 is a phosphoprotein. *J. Gen. Virol*. 69: 2165–2178.

Pumpen P.P., Dishler A.V., Kozlovskaia T.M., Bychko V.V., Gren E.J. 1981. Cloning of hepatitis B virus DNA in *Escherichia coli*. *Dokl. Akad. Nauk SSSR* 260: 1022–1024.

Sambrook J., Fritsch E. F., Maniatis T. 1989. *Molecular Cloning*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Shiosaki K., Takata K., Nishimura S., Mizokami H., Matsubara K. 1991. Production of hepatitis B virion-like particles in yeast. *Gene* 106: 143–149.

Sjöberg E.M., Suomalainen M., Garoff H. 1994. The Semliki Forest virus expression system – capsid gene segment enhances translation activity. *BioTechnology* 12: 1227–1131.

Stibbe W., Gerlich W. H. 1983. Structural relationships between minor and major proteins of hepatitis B surface antigen. *J. Virol*. 46: 626–628.

Suomalainen M., Baron M., Garoff H. 1990. The E2 signal sequence of Rubella virus remains part of the capsid protein and confers membrane association *in vitro*. *J. Virol*. 64: 5500–5509.

Takehara K., Ireland D., Bishop D.H.L. 1988. Co-expression of the hepatitis B surface and core antigens using baculovirus multiple expression vectors. *J. Gen. Virol*. 69: 143–149.

Hepatīta B strukturālo proteīnu sintēze, izmantojot Semliki Meža vīrusa ekspresijas sistēmu

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Kopsavilkums

Visi Hepatīta B vīrusa (HBV) *ayw* subtipa strukturālie gēni tika ekspresēti Semliki Meža vīrusā (SFV) pSFV1 un pSFV-C vektoros. Labākais ekspresijas veids bija saistīts ar SFV *core* gēnu saturošo vektoru (pSFV-C sērijas) pielietošanu, kur ekspresējamos gēnus sapludināja ar SFV *core* gēnu un svešas daļas atbrīvošanās notika specifiskā procesinga rezultātā. Izmantojot endoglikozidāzes H apstrādi, parādīja glikozilēšanu visiem trim HBV virsmas proteīniem (LHBs, MHBs, SHBs). Intracelulārās un ekstracelulārās frakcijas analīze parādīja veiksmīgu M produktu sekrēciju, kas nebija novērota S produktam. Kā varēja paredzēt, L un C produkti nesekrētējas. BHK šūnu kotransfekcija ar dažādiem HBV gēniem ļauj efektīvi producēties HBV strukturāliem proteīniem arī tad, ja visi četri gēni ekspresējas vienlaicīgi. Izmantojot elektronmikroskopijas analīzi, parādīja 42nm HBV daļiņu veidošanos, kuras ir līdzīgas natīvam HBV virionam. Principā tika noskaidrota gēnu koekspresijas iespēja un jaukto vīrusveidīgo daļiņu veidošanās, tomēr pēdējās parādības izpratnei nepieciešami tālāki padziļināti pētījumi.