

Translation of hepatitis B virus (HBV) surface proteins from the HBV pregenome and precore RNAs in Semliki Forest virus-driven expression

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Hepatitis B virus (HBV) pregenome RNA (pgRNA) serves as a translation template for the HBV core (HBc) protein and viral polymerase (Pol). HBV precore RNA (pcRNA) directs the synthesis of the precore (preC) protein, a precursor of the hepatitis B e antigen (HBeAg). pgRNA and pcRNA were expressed in the Semliki Forest virus (SFV) expression system. Besides the HBc and preC proteins, there was revealed the synthesis of all three forms of HBV surface (HBs) proteins: long (LHBs), middle (MHBs) and short (SHBs), the start codons of which are located more than 1000 nt downstream of the HBc and preC start codons. Moreover, other HBV templates, such as 3'-truncated pgRNA lacking 3' direct repeat and Pol mRNA, both carrying internally the HBs sequences, provided the synthesis of three HBs protein forms in the SFV-driven expression system. Maximal production of the HBs was provided by Pol mRNA, while HBc- and preC-producing templates showed relatively low internal translation of the HBs. These data allow the proposal of a ribosome leaky scanning model of internal translation initiation for HBs proteins. The putative functional role of such exceptional synthesis of the HBs proteins from the pgRNA and pcRNA templates in the natural HBV infection process needs further evaluation.

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INTRODUCTION

Hepatitis B virus (HBV) is an enveloped DNA virus belonging to the family *Hepadnaviridae*. The spherical HBV virion consists of an icosahedral nucleocapsid (core) enclosing an open circular partially double-stranded, 3.2 kb DNA genome with viral DNA polymerase (Pol) (Summers & Mason, 1982). The HBV core shell is formed by the core protein (HBc) and surrounded by a lipid bilayer envelope containing three HBV surface (HBs) protein forms: the long (LHBs or L), the middle (MHBs or M) and the short (SHBs or S). Upon entry into a human hepatocyte, the viral genome is delivered to the nucleus and converted into a covalently closed circular DNA molecule, which is used as a template by the cellular transcriptional machinery for the synthesis of viral mRNAs. Transcription of each viral RNA is driven by specialized promoters in conjunction with shared enhancer elements (Antonucci & Rutter, 1989).

The main HBV mRNA (3.4 kb) or pregenome RNA (pgRNA), serves as a template for reverse transcription and genomic DNA formation inside the viral nucleocapsid, as well as for translation of the two proteins: HBc and Pol in the bicistronic way, where the Pol protein is synthesized via ribosome leaky scanning mechanism (Lin & Lo, 1992;

Fouillot *et al.*, 1993). Another genomic transcript – the precore RNA (pcRNA) – differs from that of the pgRNA by the presence of an additional initiation codon of the preC protein at its 5' end (Ou *et al.*, 1986; Yaginuma *et al.*, 1987). The three HBs proteins have two 3' co-terminal mRNAs (2.4 and 2.1 kb) (Heermann *et al.*, 1984). The 2.4 kb mRNA is a template for all three related HBs proteins, which are translated from a single open reading frame (ORF) by the use of three different translation start sites, dividing this ORF into three domains: the amino-terminal pre-S1 domain, which occurs exclusively in the L protein; pre-S2 domain, which is present in both L and M proteins and forms the amino-terminal end of the M protein; and the S domain, which is common to the S, M and L proteins (see Fig. 1a). The 2.1 kb mRNA is probably a template only for the S, or for both M and S protein synthesis. Efficient simultaneous synthesis of the three HBs variants from the same template was shown by transient expression of the L gene in different cell lines (Bruss & Vieluf, 1995; Bruss, 1997; Le Seyec *et al.*, 1999), where glycosylated and non-glycosylated forms of the L, M and S proteins were detected.

The molecular details of internal translation initiation on HBs mRNAs are not fully understood. It has been a dogma

for a long time that the first AUG on capped mRNA predominantly serves as a start codon for effective translation during ribosome scanning. However, HBV translates the S protein with the same efficiency as the L protein, which is located closer to the 5' end of the mRNA. There are many cellular and viral mRNAs avoiding the rule of the first AUG codon, where the following AUGs may initiate translation (Kozak, 1991, 2001). Recent studies suggest that specific sequences within some mRNAs are sites of direct binding to ribosomes and that these interactions affect initiation and efficiency of translation. Probably, the 18S rRNA plays a central role in this process, via binding to complementary sequences on mRNAs. Such a ribosome filter hypothesis arose recently after studies on viral and cellular internal ribosome entry sites (IRESs) (Mauro & Edelman, 1997, 2002; Hu *et al.*, 1999).

Three ways of internal initiation might be considered – context-dependent leaky scanning, when the first AUG is sometimes ignored and the distantly located start codons became functional; translation reinitiation, when the already initiated protein translation is somehow aborted or terminated and a new protein with distantly located start codon is translated; and direct internal initiation allowing access to AUG codons through IRES elements. As a possible mechanism of internal translation initiation of HBs proteins and Pol from alternative start sites in HBV mRNAs, the context-dependent ribosome leaky scanning of the mRNA was proposed (Ou *et al.*, 1990; Fouillot *et al.*, 1993; Fouillot & Rossignol, 1996; Hwang & Su, 1999). However, this model was not studied in detail, especially for HBs initiation, where such a mechanism was suggested only by the analogy with Pol synthesis. The leaky scanning mechanism of translation provides inefficient synthesis of downstream proteins, which is true for Pol, but not for HBs proteins. The specific secondary structure of mRNA and/or the suboptimal context around the start codon, proposed by Kozak (1999), may be important in the case of HBs translation.

Therefore, it is important to investigate the possible leaky scanning model for internal translation of HBs proteins. In this study, we examined whether the other HBV templates, such as pgRNA and pcRNA, could promote an additional synthesis of HBs proteins, and how the length of the RNA's 5' end could interfere with translational efficiency. The expression system on the basis of Semliki Forest virus (SFV), providing effective cytoplasmic synthesis of the corresponding mRNAs (Liljestrom & Garoff, 1991), was used in these experiments.

METHODS

Cell culture. Baby hamster kidney (BHK) cells (ATCC) were grown in BHK medium (Gibco-BRL) containing 5% fetal calf serum, 10% tryptose phosphate broth, 20 mM HEPES and 2 mM glutamine. Cells were incubated in a 5% CO₂ atmosphere at 37 °C.

Plasmid construction. Constructs used in this work are represented in Fig. 1. All HBV sequences were amplified by PCR using

pHBT as a template plasmid harbouring a tandem repeat of HBV320 genomes, genotype D and subtype *ayw* (Bichko *et al.*, 1985). PCR fragments were cut out with *Sma*I and ligated into the pSFV1 vector (Liljestrom & Garoff, 1991), which had also been cleaved with *Sma*I. Oligonucleotides used for L gene amplification to create the pSFV1/L construct were: 5'-GCCCGGGGATGGGGCA-GAATCTTTCCA-3' and 5'-CGCCCGGGTTTAAATGTATACCCAA-AG-3' (here and below the *Sma*I site is underlined). Oligonucleotides used for HBV pgRNA amplification to create the pSFV1/pgRNA construct were: 5'-CCCGGGAACCTTTTTCACCTCTGCCTAATCA-TC-3' and 5'-CCCGGGTAACCTCCACAGTAGCTCCAAATCTTT-ATAAG-3'. Oligonucleotides used for 3'-truncated HBV pgRNA

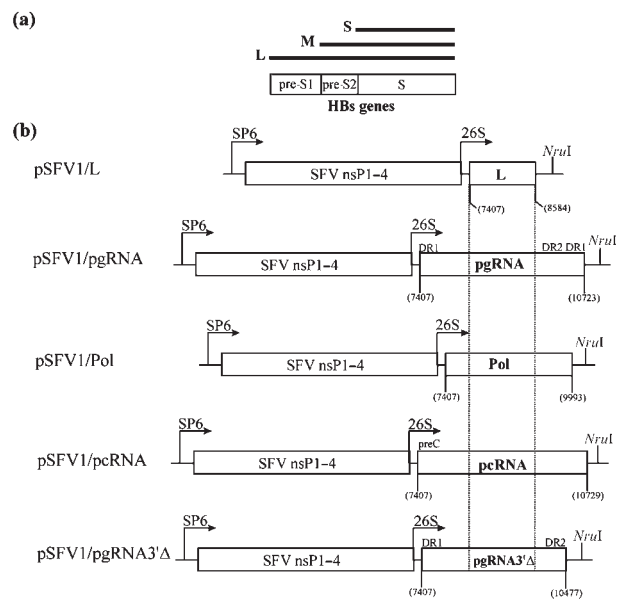


Fig. 1. Schematic diagram of recombinant SFV-driven constructs for analysis of HBs protein (L, M and S) translation. (a) HBs ORF and its products. The box representing ORF is divided into three domains (pre-S1, pre-S2 and S) by located in-frame AUG codons (vertical bars) for the translation of the L, M and S proteins (depicted above as horizontal bars), respectively. (b) Constructs used in this study. Only the SFV recombinant region of each construct is shown. This region extends from the SP6 promoter (SP6) to the *Nru*I site. All constructs contain, in the 5' to 3' directions, (i) the 5' replication signals of SFV RNA, (ii) genes encoding the SFV replication complex – non-structural proteins (SFV nsP1–4), (iii) the internal 26S subgenomic promoter of SFV (26S), (iv) the 3' replication signals of SFV RNA and (v) the poly(A) tract of the SFV genome. pSFV1/L, pSFV1/pgRNA and pSFV1/Pol contain the HBV sequences of L gene, pgRNA (with direct repeat elements DR1 and DR2) and Pol gene, respectively. pSFV1/pcRNA has preC region on the 5' end of pgRNA. pSFV1/pgRNA3'Δ represents the pgRNA with deleted 3' DR1. The vertical interrupted lines restrict the approximate position of HBs genes in all constructs. The recombinant RNAs for transfection were transcribed *in vitro* by SP6 RNA polymerase following linearization of the DNA construct with *Nru*I. The nucleotide positions of cloned HBV sequences are shown in parentheses. Note that coding regions indicated are not to scale.

amplification to create the pSFV1/pgRNA3' Δ construct were: 5'-CCCGGGAACTTTTTCACCTCTGCCTAATCATC-3' and 5'-CCCGGGTCAAGGTCGGTCG-3'. Oligonucleotides used for preC containing pgRNA amplification to create the pSFV1/pcRNA construct were: 5'-CCCGGGCCATGCAACTTTTTCAC-3' and 5'-CCCGGGTAACTCCACAGTAGCTCCAAATCTTTATAAG-3'. The preC ATG codon is marked with bold letters. Oligonucleotides used for HBV polymerase gene amplification to create the pSFV1/Pol construct were: 5'-CCCGGGCCACCAAATGCCCTATC-3' and 5'-CCCGGGTCAAGGTCGGTCG-3'. The Pol ATG codon is marked with bold letters.

RNA transcription and transfection. RNA transcripts were produced *in vitro* from 3 μ g *Nru*I-linearized pSFV1/L, pSFV1/HBVpgRNA, pSFV1/pgRNA3' Δ , pSFV1/pcRNA and pSFV1/Pol plasmids using SP6 RNA polymerase (Fermentas). RNA (3–5 μ g) was transfected into $\sim 1 \times 10^7$ BHK cells by electroporation at 850 V (2125 V cm⁻¹), 25 μ F, pulsed twice, using Bio-Rad Gene Pulser apparatus without the pulse controller unit. Electroporated cells were diluted into 15 ml complete BHK medium, transferred into tissue culture plates and incubated at 37 °C (5% CO₂). About 70% of the cells survived the transfection step.

Generation 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 under above-mentioned conditions. After 20 h, SFV particles in the culture medium were collected and frozen rapidly to be stored as a virus stock. Titres of stocks were determined by infecting cells with serial dilutions of the stocks followed by indirect immunocytochemistry assay for the expressed HBs proteins. The achieved titres were 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 virus stocks, which infect 100% of cells.

Analysis of cellular RNA. At 18 h after infection, total cellular RNA (from cells grown on 3 cm diameter plates) was isolated by Tri Reagent (Sigma) as described by the manufacturer. RNA was dissolved in 30 μ l DEPC-treated water and immediately frozen at -70 °C. RNA (5 μ l) was analysed by ECL direct nucleic acid labelling and detection system (Amersham Biosciences) using Northern blotting protocol as described by the manufacturer. RNA was immobilized on a Hybond-N⁺ membrane (Amersham Biosciences). The PCR fragment of pgRNA isolated from the agarose gel (the same as used for construction of pSFV1/pgRNA) was used for the anti-HBV probe preparation as described in ECL direct nucleic acid labelling and detection system.

Metabolic labelling of infected cells. At 16–20 h after infection, cell monolayers (on 3 cm diameter plates) were washed with PBS, overlaid with starvation medium (methionine-free MEM, 2 mM glutamine, 20 mM HEPES) and incubated at 37 °C in 5% CO₂ for 30 min. The starvation media was then replaced with the same media containing 100 μ Ci ml⁻¹ (3.7 MBq) of [³⁵S]methionine (Amersham Biosciences) and incubated at 37 °C (5% CO₂) for 2 h. Cells were lysed with 300 μ l lysis buffer [1% Nonidet P-40 (NP-40), 50 mM Tris/HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 μ g PMSF ml⁻¹] and left to stand on ice for 10 min. Lysates were transferred to microcentrifuge tubes for cell nuclei centrifugation at 3000 g for 5 min. Supernatants were used for specific protein immunoprecipitation (IP).

IP of proteins from cell lysates. For IP of Hbc and preC proteins, rabbit polyclonal anti-Hbc antibodies (gift from I. Sominskaya, Biomedical Research and Study Center, University of Latvia, Riga) were used. IP of HBs proteins was performed with goat polyclonal anti-HBs antibodies (gift from V. V. Tsinbinogin,

Biomedical Research and Study Center, University of Latvia, Riga). Pol protein IP was done by rabbit anti-Pol Ab (gift from M. Seifer, Idenix Pharmaceuticals, MA). Cell lysates in amounts of 300 μ l (pSFV1/L and pSFV1/Pol) or 600 μ l (the remaining constructs) were incubated for 2 h with appropriate antibodies (1–2 μ l) on ice. After incubation, 80 μ l protein A Sepharose (Amersham) soaked in lysis buffer was added, and incubated with rotation overnight at 4 °C. Protein A Sepharose was washed twice with buffer containing 0.2% NP-40, 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA; twice with buffer containing 0.2% NP-40, 10 mM Tris/HCl pH 7.5, 500 mM NaCl, 2 mM EDTA; and twice with 10 mM Tris/HCl pH 7.5. Protein A Sepharose pellet was resuspended in 30 μ l Laemmli buffer (Laemmli, 1970) and analysed by 12% SDS-PAGE. Gels were dried and exposed to autoradiography film (Amersham) at -70 °C for overnight or longer.

Immunocytochemical detection of intracellular HBV antigens by mAb. BHK cells grown on sterile tissue culture chamber slides (NUNC A/S) were infected with recombinant SFV and incubated at 37 °C (5% CO₂) for 20 h. After drying the slides at room temperature, cells were fixed with ethanol/acetic acid (3:1) for 20 min and rinsed thoroughly (three times) in distilled water. Slides were immersed in PBS for 10 min, rinsed with PBS supplied with 0.25% Triton X-100, incubated for 24 h in a humidity chamber at 4 °C with the anti-HBs mAb (gift from I. Sominskaya) and then rinsed in PBS with 0.25% Triton X-100. Cells were then incubated with anti-mouse IgG conjugated with alkaline phosphatase (Sigma) at room temperature, in the dark for 1 h, then rinsed with PBS, and alkaline phosphatase activity was developed by Sigma FAST reagent, which produces an intense red stain. Finally, cells were rinsed in deionized water; counterstained with haematoxylin and mounted in glycerol gelatin (Sigma). The evaluation was done using a light microscope.

RESULTS

Generation of recombinant SFV plasmids carrying HBV templates, and production of corresponding recombinant SFV viruses

The synthesis of HBs proteins is directed by two 3' co-terminal transcripts of 2.4 and 2.1 kb. In order to test whether the other HBV templates, such as pgRNA and pcRNA (both about 3.4 kb), could provide translation of the HBs proteins, we generated four constructs expressing pgRNA, pcRNA, 3'-truncated pgRNA lacking 3' direct repeat (pgRNA3' Δ) and full-length polymerase gene (Pol). The positive control for production of HBs proteins represented the L gene (Fig. 1b). DNA copies of pgRNA, pgRNA3' Δ , pcRNA and Pol mRNA, as well as of the L gene, were PCR-amplified and cloned into the pSFV1 expression vector under the control of the SFV subgenomic promoter (26S). The resulting plasmids pSFV1/pgRNA, pSFV1/pgRNA3' Δ , pSFV1/pcRNA, pSFV1/Pol and pSFV1/L were used as templates for *in vitro* transcription of the recombinant SFV region by SP6 RNA polymerase. Translation of this RNA molecule, in the cytoplasm of transfected BHK cells, yields the non-structural SFV proteins (replicase complex), which are responsible for both RNA replication and subgenomic RNA transcription, the latter representing the 3' part of *in vitro*-transcribed RNA. The subgenomic RNA, carrying in our case different HBV sequences

(depending on the construct), serves as a template for translation of HBV proteins.

In order to enhance efficiency of the expression system, infection of cells was used instead of transfection. Therefore, recombinant SFV particles were produced for each HBV construct by the simultaneous infection of cells with RNA of an appropriate construct and SFV helper RNA.

Expression of HBV L gene and pgRNA in BHK cells

The concomitant synthesis of HBV M and S proteins during the transient L gene expression have been described previously (Stibbe & Gerlich, 1983; Bruss, 1997; Le Seyec *et al.*, 1998). For pgRNA, translation was shown for HBC and Pol proteins, but not for HBs proteins (L, M, and S) that have the start codons located more downstream. We proposed that translation of HBs proteins were also from the HBV pgRNA.

To test this idea, BHK cells infected with pSFV1/L and pSFV1/pgRNA constructs were metabolically labelled with [³⁵S]methionine and lysed with NP-40 containing lysis buffer. The analysis of anti-HBs and -HBc immunoprecipitates in SDS-PAGE is demonstrated in Fig. 2. The L gene mRNA, efficiently synthesized in cytoplasm by SFV replicases (pSFV1/L), served as a template for translation of all three variants of HBs proteins (Fig. 2a, lane 1), which were found in glycosylated and non-glycosylated forms corresponding to earlier findings (Stibbe & Gerlich, 1983; Bruss, 1997). Thus, the S products existed as non-glycosylated p24 and mono-glycosylated gp27 molecules. The M products revealed two bands: mono- and di-glycosylated gp33 and gp36 forms. The non-glycosylated form of the M protein was not identified as p31 protein. However, a protein of lower molecular mass of about 29 kDa was found. Taking into consideration that the electrophoretic mobility of the MW protein marker could vary in different gel systems, we suppose that this protein represents the non-glycosylated form of the M protein (p31). The products of the L protein were found in non-glycosylated form p39 and mono-glycosylated form gp42.

As we anticipated, the expression of pgRNA (pSFV1/pgRNA) in BHK cells led to a translation pattern of HBs proteins similar to those observed from the pSFV1/L construct, although the level of synthesis was reduced (Fig. 2c, lane 2). The highest level of production was achieved for S and L proteins, whereas the M protein translation was lower. To visualize the HBs proteins translated from pgRNA, double the number of cells were lysed and used for anti-HBs IP, than used for HBs proteins translated from the L construct.

Beside this, the anti-HBc IP of SFV1/pgRNA-infected cell lysate revealed a clear p21 band, which corresponded to the HBc protein (Fig. 2c, lane 3). An additional upper band of

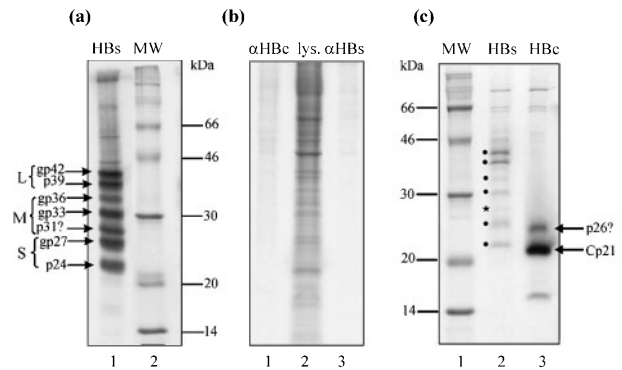


Fig. 2. Analysis of HBV proteins produced in BHK cells infected with recombinant SFV1/L and SFV1/pgRNA viruses. Infected cells were pulse-labelled with [³⁵S]methionine and cell lysates were used for specific HBV protein IP with goat anti-HBs antibodies (α HBs) or with rabbit anti-HBc antibodies (α HBc). The IP was analysed by SDS-PAGE. (a) SFV1/L. The positions of various forms of the S, M and L proteins are given on the left (lane 1). The band indicated as p31? could represent a non-glycosylated form of the M protein, although its molecular mass on the gel is smaller (about 29 kDa) than reported for the non-glycosylated M protein. (b) Negative controls. The IP of uninfected BHK cells incubated with rabbit anti-HBc antibodies and goat anti-HBs antibodies (lanes 1, 3). The lysate of uninfected BHK cells – lys. (lane 2). (c) SFV1/pgRNA. HBs proteins marked with dots from bottom to the top: S (p24 and gp27), M (gp33 and gp36), L (p39 and gp42) (lane 2). The possible band corresponding to a non-glycosylated form of the M protein (p31?) is very weak on this gel (marked with a star). Double the number of cells were used for the lysis and IP of HBs proteins produced by SFV1/pgRNA than in the case of SFV1/L-infected cells. HBc protein – Cp21 (lane 3). The origin of an additional band of about 26 kDa (p26?) in anti-HBc IP is unclear (see Discussion). MW – rainbow [¹⁴C]methylated protein marker (Amersham). The positions of protein size markers (in kDa) are indicated on the sides.

about 26 kDa (p26), the origin of which is still unclear (see Discussion), was also seen.

Unfortunately, we could not detect Pol protein translation in SFV1/pgRNA-infected cell lysate. The IP with anti-Pol Ab demonstrated strong non-specificity (data not shown). Moreover, the very low level of production of this protein caused by (i) translation via the ribosome leaky scanning model (Lin & Lo, 1992; Fouillot *et al.*, 1993) and (ii) unfavourable sequence context around the Pol start codon (Kozak, 1987) established additional difficulties for the detection of the HBV Pol protein.

RNA analysis of infected cells

Although proteins with the molecular mass corresponding to the appropriate HBs proteins have been detected in pSFV1/L- and pSFV1/pgRNA-driven expression, the question remained whether such translation is directed only

by the full-length L transcript and pgRNA as subgenomic RNAs of SFV constructs or whether other smaller mRNAs appeared during synthesis, which were used by translational machinery for production of HBs proteins.

The SFV subgenomic promoter (26S) recognized by SFV replicases (nsP1–4) is responsible for the recombinant mRNA production (Liljestrom & Garoff, 1991). The comparative analysis of the sequence of SFV 26S subgenomic promoter with the sequence of the HBV genome did not display any similarity (not shown), allowing us to exclude the SFV-driven synthesis of additional RNAs from HBV genome sequence.

Nevertheless, the potential capability of pgRNA to be transported into the cell nucleus (Kann *et al.*, 1999) and to initiate production of all HBV mRNAs through the replication of the genome, prompted us to analyse HBV-specific mRNAs in infected BHK cells. Total RNA from cells producing pgRNA (pSFV1/pgRNA) and L (pSFV1/L) transcripts was isolated, and HBV-specific sequences were detected by Northern blot technique (Fig. 3). As expected, the recombinant SFV provided synthesis of two types of

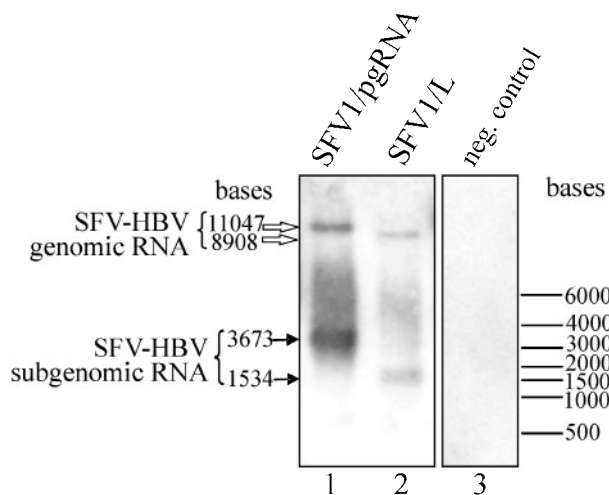


Fig. 3. Northern blot of SFV-derived HBV RNAs synthesized in BHK cells infected with SFV1/pgRNA and SFV1/L recombinant viruses. Cells were infected with appropriate virus and at 18 h post-infection total RNA was extracted, loaded on the denaturing 1% agarose gel and analysed by Northern blot technique as described in Methods. HBV sequence-specific RNAs produced by the SFV1/pgRNA (lane 1) and SFV1/L (lane 2) were developed by ECL reagents (Amersham) and identified as recombinant SFV-HBV genomic (indicated by open arrows) and subgenomic (marked with filled arrows) RNAs. The sizes of the detected RNAs are shown in bases on the left. The negative control represents the anti-HBV Northern blot of total RNA isolated from uninfected BHK cells (lane 3). The positions of High Range RNA ladder (Fermentas), visualized before blotting, are depicted on the right.

mRNAs: genomic and subgenomic. In the pSFV1/L construct, genomic RNA carries 8908 bases, the subgenomic – 1534 bases. The length of pSFV1/pgRNA genomic and subgenomic transcripts are: 11 047 and 3673 bases, respectively (Fig. 3). No other mRNAs, which could be considered as natural HBs transcripts for translation of HBs proteins, were found. These results confirm the hypothesis that all HBV proteins synthesized by our constructs are translated from recombinant SFV subgenomic RNA.

Expression of HBV pgRNA-derived templates in BHK cells

To evaluate, whether such internal translation of HBs proteins is only prerogative of HBV pgRNA or whether other HBV pgRNA-like templates could provide this synthesis, we expressed the 3'-truncated pgRNA (pSFV1/pgRNA3' Δ) lacking 3' direct repeat of HBV genome. This construct completely excludes the possible replication of HBV and production of HBs-specific mRNAs during expression. Fig. 4(b) shows anti-HBs (lane 1) and -HBc (lane 2) IPs of lysates of BHK cells infected with pSFV1/pgRNA3' Δ construct. Beside the expected HBc protein and unknown anti-HBc-specific p26 protein synthesis, we observed translation of all variants of HBs proteins. Thus, production of HBs protein was similar to that directed by pgRNA (pSFV1/pgRNA). However, the level of protein production, especially for M protein, was reduced. We do not know, whether the deleted 3' part of pgRNA may affect its stability or translational properties.

The same situation was observed by analysis of BHK cells infected with pcRNA (pSFV1/pcRNA), which served as a template not only for preC protein (preCp25) and its processed forms (preCp22, HBe p17) synthesis (Fig. 4a, lane 3), but also for L, M and S protein translation (Fig. 4a, lane 2). Interestingly, HBc (p21) protein and the unknown anti-HBc-specific p26 protein expressed by pgRNA were also observed (lane 3).

Pol gene expression provoked efficient synthesis of HBs proteins

For further investigation of internal translation initiation of HBs proteins, we shortened the 5' end of pgRNA. The construct used for this experiment represented the full-length Pol gene (pSFV1/Pol), carrying internally intact sequences of HBs proteins. Our attention was focused on the possible enhancement of the L, M and S synthesis from Pol template in the absence of preferable HBc and preC protein synthesis, which was inevitable for pgRNA-derived translation. As we supposed, the infection of BHK cells with recombinant SFV1/Pol virus resulted in the successful production of all HBs proteins (Fig. 4c), with significantly higher yield than in the case of SFV1/pgRNA, SFV1/pcRNA and SFV1/pgRNA3' Δ constructs.

However, we were unable to detect the synthesis of the Pol protein, as in the case of the pgRNA expression, probably

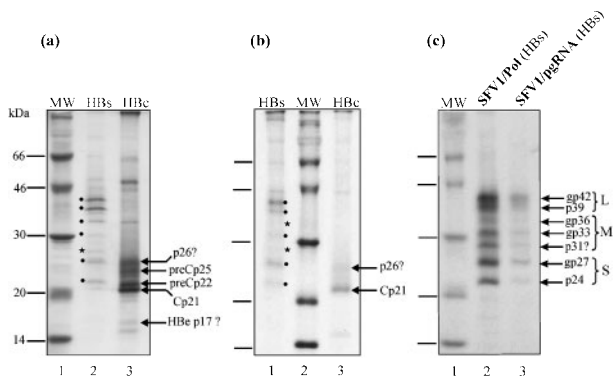


Fig. 4. Analysis of HBV proteins produced in BHK cells infected with recombinant SFV1/pcRNA, SFV1/pgRNA3'Δ and SFV1/Pol viruses. Infected cells were processed as described for Fig. 2 and corresponding immunoprecipitates were analysed by SDS-PAGE. (a) SFV1/pcRNA. HBs proteins marked with dots from bottom to the top: S (p24 and gp27), M (gp33 and gp36), L (p39 and gp42) (lane 2). A possible band corresponding to the non-glycosylated M protein is marked with a star. preC protein (preCp25), its processed forms preCp22 and HBe p17, as well as an unknown protein of about 26 kDa (p26?), are shown with arrows (lane 3). (b) SFV1/pgRNA3'Δ. HBs proteins marked with dots from bottom to the top: S (p24 and gp27), M (gp33), L (p39 and gp42) (lane 1). The non-glycosylated and di-glycosylated forms of the M protein were not found. However, their expected positions are indicated with stars. HBc protein (Cp21) and unknown HBc-specific protein (p26?) are marked with arrows (lane 3). (c) SFV1/Pol (lane 2). The anti-HBs immunoprecipitate of SFV1/pgRNA-infected cells was analysed in lane 3, allowing us to compare its production level with the pSFV1/Pol construct. The positions of various forms of the S, M and L proteins are given on the right. Double the number of cells were used for the lysis and IP of HBs proteins produced by SFV1/pgRNA than in the case of SFV1/Pol. MW – rainbow [¹⁴C]methylated protein marker (Amersham). The positions of protein size marker (in kDa) indicated on the left of (a) correspond to the bars depicted on the left sides of both (b) and (c). Overexposure of the film was necessary to visualize the very low level of translation of the HBs proteins.

because of its low production and limitations in the detection method (see above).

Immunocytochemical analysis of BHK cells producing HBs proteins

As was shown above, the level of production of HBs proteins by pgRNA-like templates was low. The detection of anti-HBs immunoprecipitates in SDS-PAGE requires overexposure of the film for an indefinite time. Therefore, it is difficult to compare different gels. Moreover, the analysis of cell lysates did not allow us to evaluate the distribution of the product over the cells. To compare different constructs and estimate the level of HBs translation, we used an immunocytochemical method, allowing us to show the

expressed product in infected BHK cells more obviously (Fig. 5).

We observed that more than 90% of tested cells were positive for HBs proteins in all constructs. However, the intensity of staining was different and correlated with the levels of HBs proteins as a preliminary detected by SDS-PAGE. Staining patterns revealed a cytoplasmic, granular distribution of HBs proteins in the cells. At the same time we revealed mainly unipolar, perinuclear localization of HBs proteins in the case of Pol and pgRNA expression, and more homogeneous cytoplasmic distribution in the case of L gene expression, where the highest HBs production level was detected. The anti-HBs immunostaining of BHK cells expressing pcRNA and pgRNA3'Δ was identical to pgRNA expressing cells (data not shown).

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. Besides the BHK cells, we established similar expression patterns of HBs proteins for all studied constructs in other cell lines (HuH-7, HepG2 and COS-7), only the level of production, even for HBc and preC proteins, were lower (data not shown).

DISCUSSION

In our study, we have demonstrated that HBV pgRNA and pcRNA may serve as templates for the translation of all three variants of HBs proteins. This represents a new example of internal translation initiation for three proteins in the same ORF, the AUG start codons of which are located more than 1000 nt downstream from the 5' end of the template.

It is difficult to evaluate the influence of SFV to such internal translation of HBs proteins. The SFV replication within the cell shuts down the host-cell protein synthesis. The mechanism of this regulation is not understood. Probably, the strong cytopathic effect of SFV is caused by the efficiency of its structural protein production, which is regulated by a specific translational enhancer (Sjoberg *et al.*, 1994). However, our observations suggest that pSFV1-vector-driven expression, where no SFV structural proteins are presented, does not affect the cell protein synthesis dramatically. Specifically, the analysis of the lysates of HBV-producing cells and non-treated cells did not display a significant difference in the level of cellular protein synthesis (results not shown). Nevertheless, the question about the possible affect of the SFV replication on recombinant protein production remains open.

It is not typical for eukaryotic cells or animal viruses to use polycistronic templates, when more than one protein is initiated from the same mRNA. The common way, especially for viruses, is the expression of a polyprotein from the single mRNA with further post-translational cleavage of the polyprotein into separate proteins. HBV is a unique

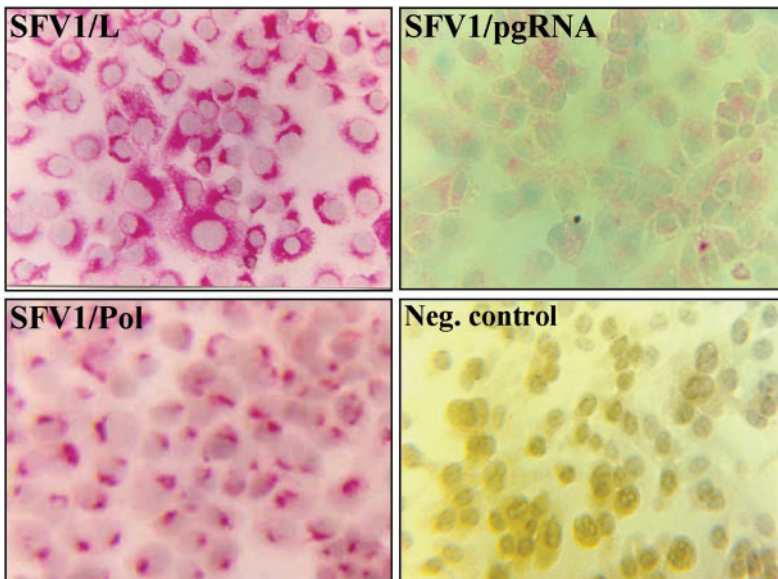


Fig. 5. Immunocytochemical detection of HBs proteins in BHK cells. Cells were infected with appropriate recombinant virus (SFV1/L, SFV1/pgRNA or SFV1/Pol). At 20 h post-infection, the cells were fixed and processed as described in Methods. Proteins were detected with monoclonal anti-SHBs antibodies. Neg. control – uninfected BHK cells incubated with the same antibodies.

object in this context, which is able to express HBc and Pol in different ORFs, and three HBs variants in the one ORF by initiation of them from the same mRNA. It ensures an evolutionary advantage in terms of restricted genome size, when the virus needs to express sufficient quantity of specific proteins, but in different amounts, which is difficult in the case of the polyprotein model.

Probably, all HBV mRNAs express the maximum number of proteins by individual translation of them. We found that the largest HBV template – pcRNA – provided translation of preC, HBc and all variants of HBs proteins. The synthesis of Pol protein from pcRNA was described earlier (Lin & Lo, 1992). Moreover, we did not exclude the possible translation of HBx (*X* gene) protein from the pcRNA, as well as from the pgRNA templates. A weak anti-HBx immunostaining was revealed in BHK cells infected with pcRNA and pgRNA viruses (data not shown).

Besides the proteins mentioned above, we observed the appearance of an additional high-molecular mass protein (p26) of unknown nature in anti-HBc immunoprecipitates of pcRNA and pgRNA-infected cells. There were no additional AUG codons found upstream and located in-frame of the authentic HBc AUG as a possible consequence of cloning. This was confirmed by sequencing of the corresponding region around the SFV 26S subgenomic promoter in both (pSFV1/pcRNA and pSFV1/pgRNA) constructs. Therefore, the p26 protein could represent (i) a host protein co-precipitated together with the HBc or (ii) a post-translationally modified form of HBc. However, this protein was also revealed by the anti-HBc immunoblot in the total SDS cell lysate (not shown). This result excludes the possibility of co-precipitation. There were several studies devoted to the post-translational modification of the core protein, such as phosphorylation (Yeh & Ou, 1991; Duclos-Vallee *et al.*, 1998; Kann *et al.*, 1999). However, the

appearance of a 26 kDa band as HBc protein post-translational modification product was not described before and is the subject of further investigation.

Does the HBV genome carry IRESs? IRES cannot yet be predicted by the presence of characteristic RNA sequence or structural motifs (Vagner *et al.*, 2001). As a rule, there are no significant similarities among individual IRES elements (unless they are from related viruses). Nevertheless, probably all IRES elements are also functional when they are positioned at the midpoint (intercistronic gap) of dicistronic artificial mRNAs and their activity does not depend on the length and the properties of the first 5' cistron (Sachs, 2000). In contrast, our results show the extreme correlation between the length of the 5' end of mRNA and the level of the HBs protein production. Therefore, this suggests that specific IRES elements are not involved in the translation of HBV mRNAs. However, taking into consideration the presence of many sites of 9–16 nt in pgRNA, which are complementary to the 18S rRNA, we cannot completely exclude the possible assistance of internal ribosome assembly for the expression of HBs proteins. Despite the lack of specific experiments unravelling the intimate mechanisms of HBV RNA translation, we propose that the ribosome leaky scanning through pgRNA and pgRNA-like templates results in initiation of HBs proteins synthesis. The translation of HBs proteins decreases from the highest level for the pSFV1/L construct to the pSFV1/Pol and finally to the pSFV1/pgRNA, pSFV1/pgRNA3' Δ , pSFV1/pcRNA constructs. The latter three constructs supported similar low synthesis of HBs proteins, all having long 5' end preceding the start codon of the L gene. The reason for low HBs expression in pgRNA and pgRNA-like templates may also be because of the extensive translation of HBc or preC proteins. The corresponding coding regions occupy the 5' end of the mRNAs and may therefore reduce the capacity for downstream protein translation. One may speculate

that only a small proportion of ribosomes is able to reach the start codons of the remote L, M and S genes in such conditions. HBs proteins synthesized from pgRNA were found in the NP-40 fraction, as when expressed from the L gene transcript. This suggests that the HBs are made by endoplasmic reticulum-associated polysomes in both cases.

We do not know whether this additional synthesis of HBs proteins from pgRNA could play a definite role in HBV biology. Transcription of specific mRNAs in the nucleus is the initial regulation stage of HBV gene expression. This process depends on the activation of promoter/enhancer elements, which are sensitive to the presence of hepatocyte-specific factors (Antonucci & Rutter, 1989; Kosovsky *et al.*, 1996; Tang & McLachlan, 2001). In the case when L and S gene promoters are silent, due to accidental mutations or deficiency of the cellular factors, the pgRNA may appear as a unique source of HBs protein translation. Moreover, the capability of HBV to infect cells of other organs, such as kidney, pancreas (Dejean *et al.*, 1984) or some blood cells (Blum & Vyas, 1983; Lobbiani *et al.*, 1990), where the expression is hampered by the absence of hepatocyte-specific transcription factors, may be provided by additional mechanisms for the HBs protein synthesis including such from pgRNA. Therefore, the pgRNA alone may be able to initiate viral production by ensuring synthesis of Pol and all HBV structural proteins.

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