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Expression of hepatitis C virus structural genes controlled by alphaviral recombinant replicons

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Abstract

Since no highly efficient treatment for the hepatitis caused by hepatitis C virus (HCV) is available yet, the development of an anti-HCV vaccine candidate remains a goal of high priority. In our study, two different alphaviral expression systems were applied for expression of HCV structural protein genes in cell culture. For this purpose, the HCV genome fragment encoding the core-E1-E2-p7 polyprotein, and HCV core and E2 genes, separately, were cloned in Semliki Forest virus (pSFV1) and Sindbis virus (pCytTS) expression vectors. Efficient synthesis of structural HCV proteins in a BHK-21 cell line was demonstrated. HCV polyprotein precursor was authentically post-translationally processed. Identification of HCV proteins was confirmed by immunoprecipitation, immunocytochemical analysis, and by Western blotting with corresponding anti-HCV antibodies.

Key words: alphavirus, expression, Hepatitis C virus, Semliki Forest virus, Sindbis virus.

Introduction

Viral hepatitis C, caused by hepatitis C virus (HCV), is a major public health concern worldwide since more than 3 % of the world population is infected with this virus. Worst of all, up to 4 million persons are becoming newly infected each year. Approximately 80% of infected patients develop chronic hepatitis, among which 20 % to 30 % progress to liver cirrhosis and end-stage liver disease. Current therapy relies on the use of pegylated interferon and ribavirin, but outcomes are unsatisfactory, since only 42 % of patients infected with HCV genotype 1 prevalent in Latvia respond positively to treatment. Therefore, despite many obstacles, there is a compelling need to develop an effective anti-HCV vaccine. Unfortunately, the traditional approach to use whole-organism vaccines containing inactivated whole or live attenuated viruses cannot be adapted for HCV, because no cell culture system is available to propagate HCV so far. Our research develops the newer types of anti-HCV vaccine candidates, based on the general concept that one or several genes of the viral pathogen (HCV in this study) are incorporated into the genome of a viral carrier (in our case, alphavirus) for amplification and expression of the putative immunogens.

HCV virion carries RNA genome of 9.5 kb. The genome consists of a highly conserved 5' noncoding region (Baumert et al. 1998) followed by a long open reading frame of 9,030

to 9,099 nucleotides that is translated into a single polyprotein of 3,010 to 3,030 amino acids (Krekulova 2006). The polyprotein is co- and post-translationally processed by cellular and viral proteases to yield ten mature protein products (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV genome encodes three structural proteins: a core protein (core) and two envelope glycoproteins (E1 and E2) (Penin et al. 2004). These proteins are released from the N-terminal region of the polyprotein by signal peptidase cleavage (Dubuisson 2002). In addition, processing at the C-terminal region of the core protein by a signal peptidase leads to generation of a mature core protein (McLauchlan 2002).

Non-mature HCV core protein (aa 1-191) appears to be conserved among several HCV strains isolated in different regions worldwide (for review see Krekulova 2006). HCV core is not only involved in the formation of the HCV virion, but also has a number of regulatory functions: it effects host cell gene expression and modulates apoptosis. The C-terminal domain of HCV core is essential for virus secretion (Suzuki at al. 2007). HCV envelope glycoproteins E1 (aa 192-383) and E2 (aa 384-746) are believed to be type 1 of transmembrane proteins, with N-terminal ectodomains and C-terminal hydrophobic anchors and together are expected to form the viral envelope. HCV E1 protein displays two main glycosylated forms with molecular weights of 33 kDa and 35 kDa (30 kDa and 35 kDa in Baumert et al. 1998). HCV E2 is a 70-kDa glycoprotein, which demonstrates large variation among HCV genotypes. N-terminal 27 residues of E2 (aa 384-410) show an especially high degree of variation, also within genotypes. This portion of the E2 sequence is known as a hypervariable region 1 (HVR1). The HVR1 is likely to be involved in virus infection, since virus-neutralizing antisera to HVR1 have been reported in vitro and in vivo models, although other studies showed that HCV with the deleted HVR1 remains infectious (Keck et al. 2004). Mature E1 and E2 proteins assemble into a non-covalent heterodimer, although the exact nature of this interaction is still poorly understood (Patel et al. 1999).

Therefore, structural HCV proteins seem to be perspective as a source of the immunological antigens for the development of anti-HCV vaccine, especially when combination of two or more proteins with different immunological properties in one genetic construction is possible.

Viral vectors with the property to self-replicate in transfected cells are a favourable approach to be used as candidate vehicles in the development of the genetic vaccines. Alphaviral vectors with their ability of efficient cytoplasmic replication established themselves rapidly as tools for basic research and biotechnology. Semliki Forest virus (SFV) (Liljestrom, Garoff 1991) and Sindbis virus (SINV) (Boorsma et al. 2000) are the most commonly used alphaviral expression vectors. Since all alphaviruses have very similar biology, the major structural elements of these expression vectors are identical. The vectors are generated in such a way that the heterologous gene insert should be placed, replacing the viral structural genes, under the subgenomic 26S promoter. In general, the vectors have a non-structural coding region, which is required for the production of the alphaviral replicase complex (nsP1-4), 26S subgenomic promoter, and a polylinker with several unique restriction sites for foreign gene insertion. An SP6 promoter is introduced upstream of the recombinant alphaviral replicon for RNA transcription *in vitro*. Recombinant replicons are packed into viral envelopes, which are provided with co-transfected defective "helper" RNAs *in trans* (Smerdou et al. 1999). In the first generation

of alphaviral expression vectors, the high-level expression of a foreign gene is coupled with a shutdown of the host protein translation followed by cell death within 12 to 24 h after infection. The cytotoxicity of these alphaviral vectors is caused by p53 independent apoptosis (Glasgow et al. 1998), which is induced by the alphaviral non-structural region. This is a major drawback for many applications, and the first generation of RNA-based vectors is used only for transient gene expression. HCV structural proteins (genotype 1a) have also been expressed by using recombinant SFV replicon in mammalian cells; this system has been shown to allow the visualization of HCV budding events *in situ*, but budding is abortive or slow (Blanchard et al. 2002; Hourioux at al. 2007).

In this work we applied SFV and Sindbis virus-based expression systems for the efficient expression of the structural HCV genes (core, E1 and E2) in cell culture, to assess the applicability of recombinant alphaviruses as anti-HCV vaccine prototypes.

Materials and methods

Constructs carrying HCV genes

Constructs used in this work are presented in Fig. 1. The HCV isolate 274933RU (GeneBank accession no. AF176573) genome (Mokhonov at al. 2002) was used for amplification of target genes with corresponding primers. These HCV cDNA fragments were inserted after SP6 promoter to construct a plasmid pSFV1/HCVpolyP, which contained core, E1, E2, and p7 genes, as well as the pSFV1/HCVcore gene and pSFV1/HCVE2p7 gene separately. Recombinant plasmids were multiplied, linearized at the 3′ end of the HCV cDNA by *Bcu*I (*Spe*I) restriction endonuclease. The linearized DNAs were then purified and used as templates for in vitro transcription.

Primers used for HCV core-E1-E2-p7 genes amplification to create pSFV1/HCVpolyP recombinant plasmid were: 5′-GTG AGA TCT GCA CCA TGA GCA CGA ATC CTA AAC-3′ and 5′-CTC TAG ATC TTT AGG CGT ATG CTC GTG GTG GTA G-3′. After amplification, the PCR fragment was cut out with *Bgl*II restriction endonuclease and ligated into pSFV1 vector, which was cleaved with *Bam*HI restriction endonuclease and dephosphorylated with bacterial alkaline phosphatase (Fermentas).

Primers for HCV core gene amplification to create pSFV1/HCVcore recombinant plasmid were: 5′-GTG AGA TCT GCA CCA TGA GCA CGA ATC CTA AAC-3′ and 5′-GAT CGT TAA CTA AGC GGA AGC TGG AAT GG-3′. After amplification, the PCR fragment was cut out with *Bgl*II and *Hpa*I restriction endonucleases and ligated into pSFV1 vector, which was cleaved with *Sma*I and then with *Bam*HI restriction endonucleases.

In order to construct recombinant pSFV1/HCVE2p7, a DNA fragment carrying E2 and p7 genes was cut out with *Sal*I and *Hpa*I restriction endonucleases from plasmid pGMV-E2p7.9.3 (obtained from Maria Isaguliants, Ivanovsky Institute, Moscow). The DNA fragment was treated with Klenow fragment (Fermentas) to blunt the DNA end after SalI digestion and ligated into pSFV1 vector, which was cleaved with *Sma*I restriction endonuclease.

In order to construct the recombinant plasmid pCyt/HCVpolyP containing core, E1, E2, and p7 protein genes, the pCytTS vector was cleaved with *Xba*I and *Xho*I restriction endonucleases. The HCV genome fragment was amplified with primers 5′-GCA TCT CTA GAC GTA GAC CGT GCA CCA TGA GCA CG-3′ and 5′-GCA TCC TCG AGT TAG GCG TAT GCT CGT GGT GGT AGT G-3′, cut out with *Xba*I and *Xho*I restriction

endonucleases and ligated into pCytTS vector.

The recombinant plasmid pSFV1/EGFP carrying Enhanced green fluorescence protein (EGFP) gene was used as a control for infection of BHK-21 cells. In order to generate this plasmid, the DNA fragment carrying the EGFP gene was cut out from pEGFP-C1 plasmid (Clontech) with *Nhe*I and *Hpa*I restriction endonucleases and treated with T4 DNA polymerase (Fermentas) to blunt the DNA end after *Nhe*I digestion. The DNA fragment was ligated into pSFV1 vector, which was cleaved with *Sma*I restriction endonuclease.

Cell culture

Baby hamster kidney (BHK-21) 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 % CO_2 atmosphere at 37 °C.

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-21cells together with SFV "helper" RNA under the above-mentioned conditions. After 24 to 48 h, SFV particles were collected from the culture medium 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. The achieved titres were from 1×10^7 to 5×10^7 viral particles per ml. Infection of BHK-21 cells was carried out in serum-free medium with the appropriate dilution of the virus stocks, which enabled 100 % infection of the cells.

Metabolic labelling

Metabolic labelling of transfected cells and immunoprecipitation of proteins from cells lysates were performed as described previously (Kozlovska et al. 2004).

RNA transcription and transfection

RNA transcripts were produced *in vitro* from 3 mg of SpeI-linearized recombinant plasmids in reactions containing 7mG(5′)ppp(5′G) (New England Biolabs) using SP6 RNA polymerase (Fermentas). RNA (3 to 5 mg) was transfected into BHK-21 cells by electroporation at 850 V, 25 mF, pulsed twice using Bio-Rad Gene Pulser apparatus without the pulse controller unit. Electroporated cells were diluted in 15 mL of complete BHK medium, transferred into tissue culture plates, and incubated at 37 °C (5 % CO_2).

DNA transfection and selection of puromycin-resistant cell populations

Slightly sub-confluent monolayers (60 to 70 %) of BHK-21 cells in 35-mm tissue culture plates were used for DNA transfection. DNA was transfected by using ExGene (Fermentas) according to the manufacturer's recommendations. After transfection, cells were incubated 18 to 48 h at 37 °C. For termoinduction of target gene expression, transfected cells were incubated at 29 °C. For selection of puromycin-resistant cell populations, cells were passaged after 18 to 24 h, media was changed then at 24 h to standard growth media containing 5 μg mL^{-1} of puromycin (Sigma). Thereafter, cells were maintained in media with puromycin and were passaged or frozen as standard BHK-21 cells.

Cell lysis and anti-HCV antibodies

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

For analysis of total cell lysates on PAGE, Laemmli buffer was used for lysis of cells.

The polyclonal rabbit anti-core and anti-E2 antibodies were obtained from Maria Isaguliants (Ivanovsky Institute, Moscow) and used as described earlier (Sominskaya et al. 2005).

Western blot analysis of HCV proteins

The protein samples prepared from pSFV1/HCVpolyP- or pSFV1/HCVcore-infected and pCyt/HCVpolyP- or pCyt/HCVcore-transfected BHK-21 cells were separated by SDS-PAGE (15 %). The Prestained Protein Molecular Weight Markers (Fermentas) were used as molecular mass markers. After electrophoresis, proteins were electrotransferred to Hybond-P membranes (Amersham Pharmacia Biotech) with a blotting apparatus. After transfer, membranes were incubated with blocking buffer (5 % non-fat dry milk in PBS). Anti-HCV core polyclonal rabbit antibodies or anti-HCV E2 polyclonal rabbit antibodies diluted 1 : 1000 in blocking buffer containing 2.5 % of non-fat dry milk and 0.05 % Tween 20 were used as primary antibodies. Membranes were subsequently treated with horseradish peroxidase-conjugated anti-rabbit Ig (Amersham Pharmacia Biotech) at 1 : 3000 dilution for 60 min at room temperature. Protein binding was detected with the ECL Plus system (Amersham Pharmacia Biotech).

Indirect immunofluorescence microscopy

BHK-21 cells cultured on glass slides (Nagle Nunc International) were infected with pSFV1/HCVpolyP or pSFV1/HCVcore recombinant viruses and fixed after designated cultivating periods. The fixed cells were first incubated with antibody dilution buffer (1 % bovine serum albumin, 2.5 mM EDTA, phosphate-buffered saline, pH 7.4) for 10 min at room temperature. Then, cells were incubated for 60 min at room temperature with anti-HCV core or anti-E2 polyclonal antibodies as primary antibodies at 1 : 1000 dilution. After incubation, cells were washed three times with PBS. Anti-rabbit TRITC-labelled antibodies were used as secondary antibodies at 1 : 100 dilution. Cells were mounted then with PermaFluor aqueous mounting medium (Immunon, Pittsburgh, USA) and analyzed under a Leica DM 6000 fluorescence microscope (Leica Camera AG, Solms, Germany). DAPI was used for visualization of nucleus.

Purification of HCV-like particles

At 48 h postinfection or postelectroporation, cells were lysed in 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 % Nonidet P40 buffer with 10 μg mL^{-1} of PMSF. Lysate was subjected to low-speed centrifugation (15 min at 4 °C and 15000 g) to remove cell debris. Supernatant was layered over a 20 % (w/v) sucrose cushion in TNE buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA) and centrifuged at 30000 rpm, rotor TLS-55 (TL-100 centrifuge, Beckman) for 18 h at 4 °C. Pellet containing HCV-like particles was resuspended in TNE buffer and analyzed by Western blotting and electron microscopy.

Results

Generation of recombinant plasmids carrying HCV genes

In order to test the ability of the SFV expression system for translation of HCV genes, we generated constructs expressing poly P (core-E1-E2-p7), HCV core and E2p7 genes (Fig. 1). DNA copies of poly P (core-E1-E2-p7), HCV core, and E2p7 genes were amplified and cloned in the pSFV1 expression vector under the control of the SFV subgenomic 26S promoter. Resulting plasmids pSFV1/HCVpolyP, pSFV1/HCV core, and pSFV1/HCV E2p7 were used as templates for *in vitro* transcription of the recombinant SFV region by SP6 RNA polymerase. Subgenomic RNAs carrying in our case different HCV sequences (depending on the construct) served as templates for translation of HCV genes.

Recombinant SFV particles were produced for each construct by simultaneous electroporation of cells with the subgenomic RNA of the appropriate constructs and helper SFV RNA. BHK-21 cells, which were used in these experiments, are not natural host cells for HCV. However, they are optimal for infection with and production of recombinant SFV particles, allowing the highest yields of recombinant proteins.

HCV polyP fragment encoding structural proteins and protein p7 was cloned also in the pCytTS vector. The pCytTS is an expression vector based on Sindbis virus. The recombinant plasmid specified as pCyt/HCV polyP was used for transfection of BHK-21 cells to express HCV proteins using another alphavirus besides SFV (Fig. 1). HCV sequences in all DNA constructs were verified by direct nucleotide sequencing.

Expression of HCV genes in BHK-21 cells

BHK-21 cells were transfected with recombinant RNA or, alternatively, infected with recombinant SFV viral particles. At 24 h postinfection, cells were metabolically labelled with ^{35}S-methionine for 1 h and lysed with NP-40 containing lysis buffer. The lysates were analyzed for the presence of HCV proteins. The analysis of cells lysate in SDS-PAGE is demonstrated in Fig. 2. HCV proteins were observed in cell lysate without concentration of lysates. The pattern of proteins showed a band about 70 kDa corresponding to E2 protein, 33 and 35 kDa bands corresponding to major glycosylated forms of the E1 and bands corresponding to the HCV core (Fig. 2, lane 3). The heaviest form of the HCV core was not detected in all experiments, probably because the level of protein production was not equivalent in different cases and minor forms of the HCV core protein remained undetectable. The molecular mass of proteins expressed from a construct that encoded the HCV core gene alone was identical to the molecular mass of the HCV core protein expressed from the construct, which encoded HCV structural proteins (Fig. 2, Fig. 3A). Expression of the HCV core gene was more efficient in the case of infection than in the case of electroporation (data not shown), perhaps due to strong reduction of the growth rate of electroporated cells and damage of many cells.

Expression of HCV genes was analysed in BHK-21 cells also after transfection by pCyt/HCVpolyP plasmid. Cells were lysed at 72 h after thermoinduction in lysis buffer containing NP-40 or in Laemmli buffer. Efficiency of expression of the HCV genes and immunological specificity of the corresponding proteins were demonstrated by Western blotting. Two species of HCV core protein with molecular mass 21 kDa and 23 kDa were identified in the total cell lysate. Bands, which were observed under major p23 and p21 forms of HCV core protein, corresponded to HCV core form 16 kDa (Fig. 3B).

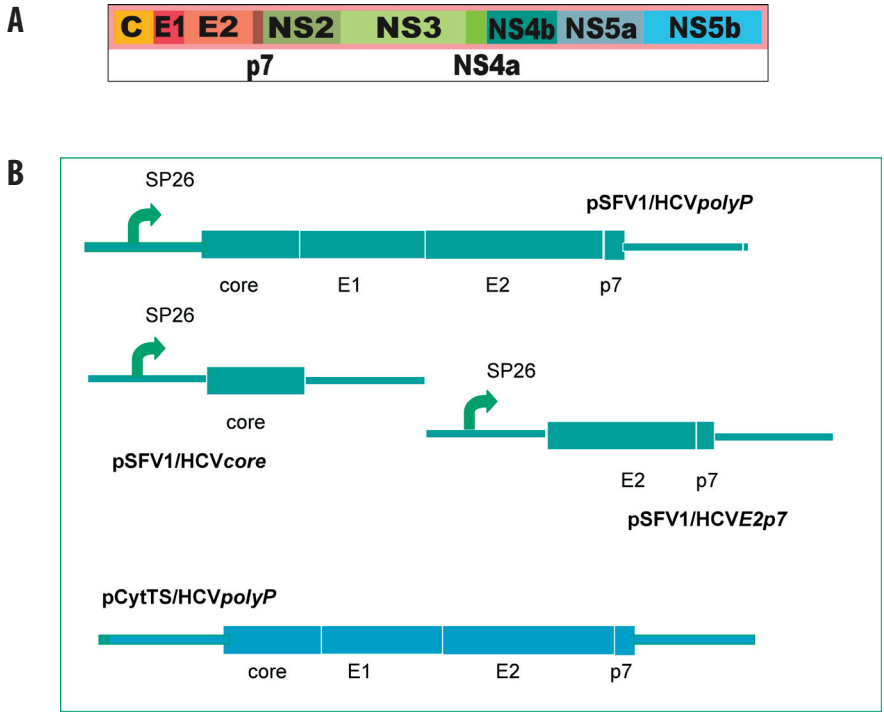

Fig. 1. Schematic diagram of recombinant constructs for analysis of HCV protein translation. A, HCV proteins: core, E1, E2 – structural proteins, NS2, NS3, NS4A, NS4B, NS5A, NS5B -nonstructural proteins, p7 – small protein with unclear functions. B, HCV proteins genes used in this study for recombinant plasmids construction.

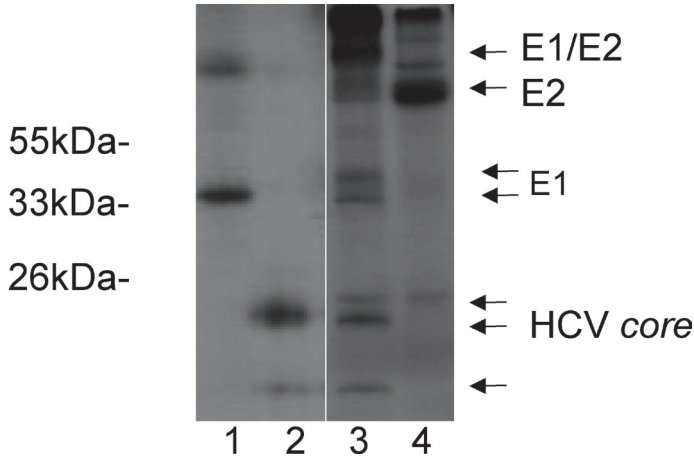

Fig. 2. HCV structural proteins genes expression in BHK-21 cells after infection by recombinant viruses. Lane1. BHK-21 cells lysate (NP-40 contained buffer) after infection by SFV1/EGFP as control; lane 2. BHK-21 cells lysate (NP-40 contained buffer) after infection by SFV1/HCVcore; lane 3. BHK-21 cells lysate (NP-40 contained buffer) after infection by SFV1/HCVpolyP; lane 4. BHK-21 cells lysate (NP-40 contained buffer) after infection by rSFV1/HCV E2p7. The Prestained Protein Molecular Weight Markers (Fermentas) were used as molecular mass markers.

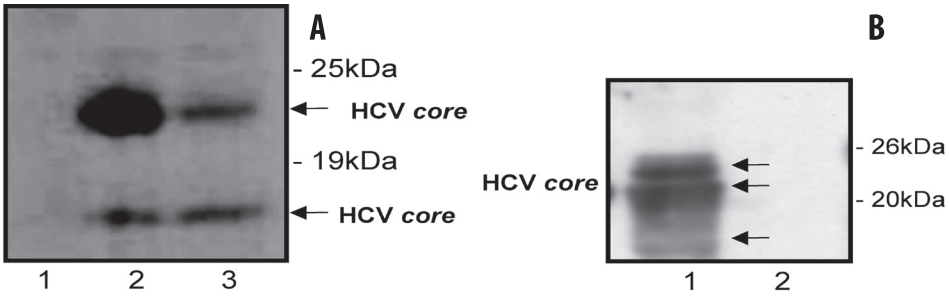

Fig. 3. HCV core protein gene expression in BHK-21 cells: Western-blot with polyclonal anti-HCV core antibody from rabbit. A, HCV core protein gene expression in BHK-21 cells after infection by recombinant viruses rSFV1/HCVpolyP and rSFV1/HCVcore. Lane 1: BHK-21 cell lysate (NP-40 contained buffer) after infection by SFV1/EGFP as control. Lane 2: BHK-21 cell lysate (NP-40 contained buffer) after infection by SFV1/HCVcore. Lane 3: BHK-21 cell lysate (NP-40 contained buffer) after infection by rSFV1/HCVpolyP. B, HCV core gene expression in BHK-21 cells after plasmid pCyt/HCVpolyP transfection by commertial kit (Fermentas). Western-blot with polyclonal anti-HCV core antibody from rabbit. Lane 1: BHK-21 cell total lysate (Laemmli buffer) after transfection by pCyt/HCVpolyP. Lane 2: negative control – noninfected BHK-21 cell lysate (Laemmli buffer). The Prestained Protein Molecular Weight Markers (Fermentas) were used as molecular mass markers.

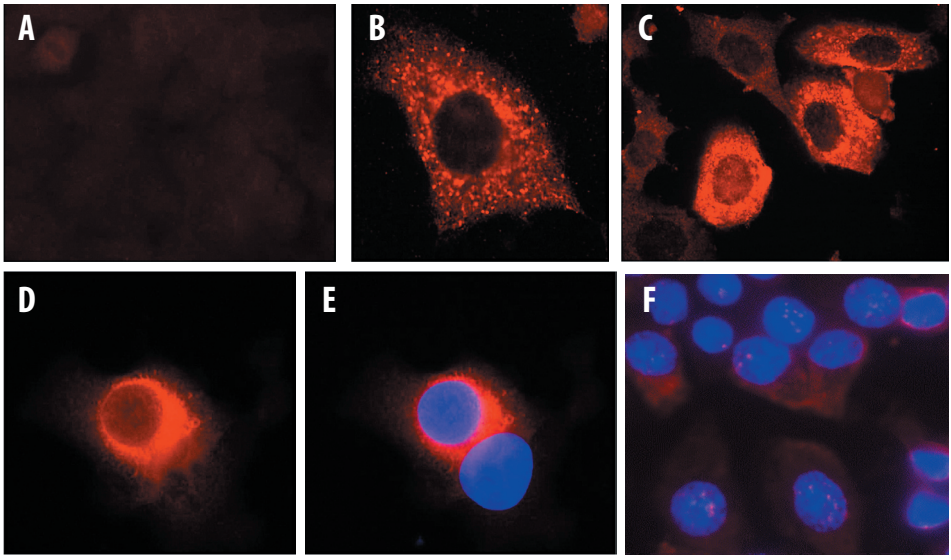

Fig. 5. Immunocytochemical detection of HCV core protein gene expression. BHK-21 cells were infected were with recombinant virus SFV1/HCVcore. Infected cells were stained with rabbit anti-HCV core antibodies (1:1000) and secondary anti-rabbit TRITC-antibodies (1:100). A, noninfected BHK-21 cells stained with rabbit anti-HCV core antibodies (1:1000) and secondary anti-rabbit TRITC-antibodies (1:100). Core protein distribution at 4 h (B), 8 h (C), 16 h (D) post infection. Nucleus visualization with DAPI 16 h (E) and 24 h (F) post infection.

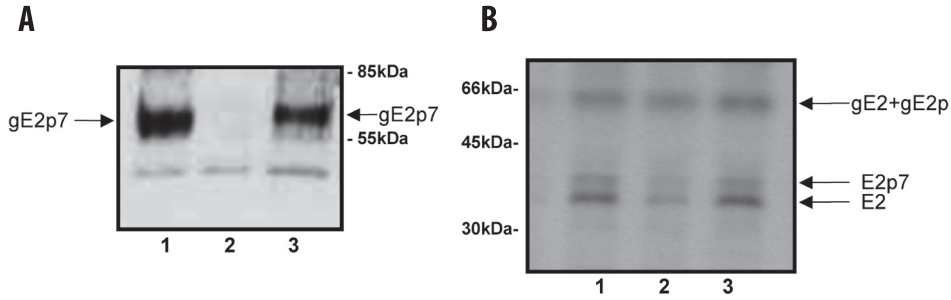

Fig. 4. HCV E2 protein gene expression in BHK-21 cells after infection by recombinant viruses SFV1/HCVpolyP and SFV1/HCVE2p7. A, Western-blot with polyclonal anti-HCV E2 antibody from rabbit. Lane1. BHK-21 cell total lysate (Laemmli buffer) after infection by SFV1/HCVpolyP. Lane 2: negative control – noninfected BHK-21 cell lysate (Laemmli buffer). Lane 3: BHK-21 cell total lysate (Laemmli buffer) after infection by SFV1/HCV E2p7. B, Immunoprecipitation of E2 and E2p7 proteins from cells lysates after infection by recombinant viruses SFV1/HCVpolyP and SFV1/HCVE2p7. Lane 1, 2, 3: immunoprecipitation with different polyclonal rabbit anti-E2 antibodies. The Prestained Protein Molecular Weight Markers (Fermentas) were used as molecular mass markers.

Efficient expression of HCV E2 protein gene from pCyt/HCVpolyP, pSFV1/HCVpolyP, and pSFV1/HCVE2p7 plasmids was also demonstrated. Specificity of the E2 protein was confirmed by Western blotting, imunoprecipitation, and imunocytochemical analysis with corresponding anti-E2 antibodies. Fig. 4 shows an analysis of BHK-21 cells lysates after infection by recombinant SFV1/HCVE2p7 and SFV1/HCVpolyP viruses. In our study, both protein patterns after infection with recombinant viruses were similar and E2 protein existed in a glycosylated form of about 70 kDa (Fig. 4A). Different E2 proteins forms represented by glycosylated E2 (about 70kDa), ngE2p7 (41 kDa), and ngE2 (36 kDa) were concentrated from cells lysates after infection with recombinant SFV1/HCV E2p7 virus using imunoprecipitation with corresponding anti-E2 antibody (Fig. 4B). Localization of the HCV E2 protein in the cytoplasm of infected cells was oserved using immunocytochemical analysis (data not shown).

Localization of HCV core protein in the cells

Localization of the HCV core protein was monitored by immunocytochemical analysis using binding of polyclonal rabbit antibody to HCV antigens. The percentage of cells stained positive increased during the first 24 h post infection, in most cases reaching a maximum in 24 h. For all constructs, the number of cells stained positive reached a value of up to 80 %. HCV core protein was localized mainly in the cytoplasm, but its localization was different at different times post infection. At 4 h and 8 h after infection of cells with recombinant viruses, HCV core protein was found in cytoplasm only. At 16 h after infection, HCV core protein concentrated in the perinuclear region. Only 24 h after infection, about 15 % of the HCV core protein was found in the nucleus (Fig. 5). However, correlation of each HCV core protein form with its localization was not established. At 4 h postinfection, a p21 form was detectable only in cell lysate. At 8 h postinfection, the p21 form and the same amount of a p23 form were detected in cell lysate. The smallest form of the HCV core protein of p16 kDa was detected at 24 h postinfection only (Fig. 6).

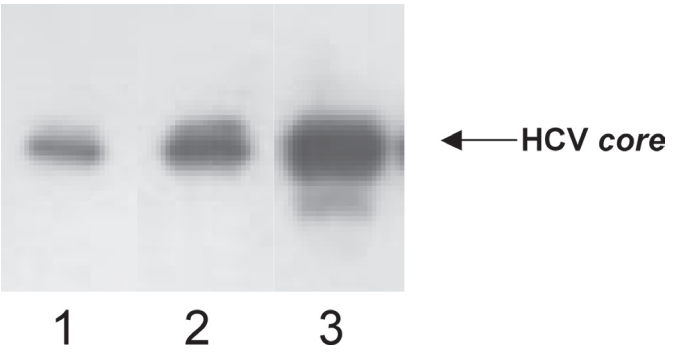

Fig. 6. HCV core protein gene expression in BHK-21 cells after infection by recombinant virus SFV1/HCV core at different times postinfection: different forms of HCV core proteins detection by Western-blot with polyclonal anti-HCV core antibody from rabbit. Lane 1: HCV core protein p21 form in BHK-21 cell total lysate (Laemmli buffer) at 4 h post infection. Lane 2: HCV core protein p21 form and some amount of p23 form in BHK-21 cells total lysate (Laemmli buffer) at 8 h post infection. Lane 3: HCV core protein p21 form, p23 form and p16 in BHK-21 cells total lysate (Laemmli buffer) at 24 h post infection. The Prestained Protein Molecular Weight Markers (Fermentas) were used as molecular mass markers.

Assembly of HCV structural proteins

To assess the ability of HCV structural proteins to be associated into virus-like particles, clarified cell lysates after infection with recombinant viruses pSFV1/HCVpolyP or pSFV1/HCVcore (or electroporation by the corresponding RNAs) were subjected to centrifugation through a sucrose cushion. The pellets were dissolved in TNE buffer and analyzed by Western blotting for two HCV structural proteins: core and E2. Both proteins were found in pellets (data not shown). Viral HCV particles were visualized by electron microscopy. First, HCV core particles of 35 to 37 nm in diameter were detected after infection with SFV1/HCVpolyP recombinant virus or SFV1/HCVcore (Fig. 7A, B). HCV-like particles with a diameter of 60 nm formed by HCV structural proteins were observed in the cells infected with SFV1/HCVpolyP recombinant virus (or electroporated with the corresponding RNAs), but the number of such particles in cell lysates was low (Fig. 7C).

Discussion

The absence of a suitable cellular model for HCV propagation has led to the use of various heterologous expression systems. Among others, the baculovirus insect cell system has proved useful for production and maturation of HCV structural (Baumert et al. 1998) and non-structural proteins (Overton et al. 1995). In addition, this system was a valuable tool for the (i) production of large amounts of antigens for immunological studies (Chiba et al. 1991) and (ii) investigation of viral protein interactions and assembly. Using recombinant baculovirus, Baumert et al. (1998) showed formation of pseudoviral particles. Pseudotyped and recombinant viruses carrying HCV envelope proteins were developed on the basis of Vesicular Stomatitis virus (VSV) vectors. Recombinant VSV expressed incorporated HCV genes, the products of which became fully processed and assembled into HCV-like particles (Ezelle et al. 2002). Moreover, recombinant VSV carrying unmodified HCV E1 and E2 proteins was able to infect Huh7 cells (Tani et al. 2007).

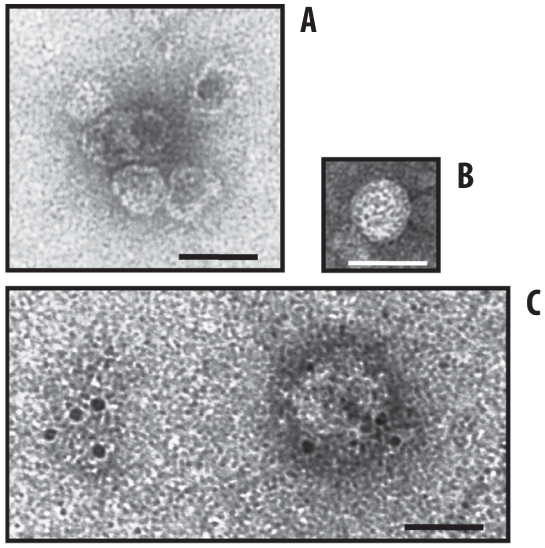

Fig. 7. Electron microscopy of purified HCV-like particles. A, HCV core particles in the NP-40 containing lysate of SFV1/HCVcore-transfected cells. B, HCV core particle. C, mmunogold labeling of HCV-like particles with anti-E2 polyclonal antibodies. The bar represents 50 nm.

Recently, a mammalian cell culture for a JFH1 strain of the 2a genotype (HCVcc) isolated from a fulminant HCV patient was developed for HCV replication *in vitro* (Zhong et al. 2005). However, a robust cell culture system for HCV of the 1a and 1b genotypes, the most prevalent genotypes in the world, has not yet been successfully developed, except for the cell culture producing H77 or H77-S strains of HCV, genotype 1a (Yi et al. 2006) and the replicons derived from Con1, a strain of HCV, genotype 1b (Lohmann et al. 1999).

The recombinant SFV replicon was used for production of HCV, genotype 1a, structural proteins in mammalian cells (Blanchard et al. 2002; Hourioux et al. 2007). Interestingly, the expression level of the HCV E1 and E2 proteins seems to be higher in the mammalian SFV expression system than that in the baculovirus expression system in insect cells. However, budding of the HCV-like particles in the SFV system seems to be abortive. Nevertheless, more information about translation and processing of HCV proteins, virus replication and maturation, molecular interactions between HCV virions and hosts are required to clarify details of HCV pathogenesis and persistence. In principle, each step of the HCV life cycle is a target for antiviral intervention.

We are developing a non-replicating recombinant SFV with a genome encoding unmodified HCV core, E2, and polyproteins, and not SFV envelope proteins, in cultivated cell lines. Expression of HCV, genotype 1b, structural genes provides novel information about translation and posttranslational processing of HCV structural proteins in cell cultures, as well as about their self-assembly. All structural HCV proteins appear in lysates after infection of BHK-21 cells with recombinant viruses. The pattern of HCV proteins after infection with SFV1/HCVpolyP virus presented several bands: a band of about 70 kDa corresponding to E2 protein, 33 and 35 kDa bands corresponding to major glycosylated forms of the E1, and bands of 23 kDa, 21 kDa, and 26 kDa corresponding to different forms of the HCV core (Fig. 2, lane 3). The heaviest form and smallest form of the HCV core are

not detectable; only the major form of the HCV core with the molecular mass of 21 kDa is observed. HCV core protein products are identical after infection with recombinant SFV1/HCVcore and SFV1/HCVpolyP viruses. However, expression of HCV core protein by SFV1/HCVcore virus is more efficient (Fig. 3A). The observations on production and posttranslational processing of HCV core proteins obtained in our study are in a good correlation with the literature. HCV core proteins, reviewed by Krekulova et al. (2006), were established as 21 kDa (191 aa), 19 kDa (173 aa), and 16 kDa. According to more recent observations, the molecular mass of two forms generated by posttranslational splicing at sites near aa residue 191 and near aa residue 173 are 23 kDa and 21 kDa (Vauloup-Fellous et al. 2006). The smallest form of HCV core protein is generated by translation from the alternative reading frame (Xu et al. 2001). The 21 kDa and 23 kDa proteins are bound to membranes of ER, while the 16 kDa form is localized preferentially in the perinuclear space (Lo et al. 1995). However, information about localization of the HCV core proteins in the cells is controversial. Krekulova et al. (2006) notes that a small portion of 19 kDa (21 kDa in other studies) protein was translocated into nucleus. Matured HCV core protein was found in nucleus by Yasui et al. (1998). However, Rouille et al. (2006) did not find HCV core protein in the nucleus. In our study, localization of the HCV core protein monitored by an immunocytochemical method leads to conclusion that the HCV core protein appears mainly in cytoplasm, but it is present also in nucleus (Fig. 5). During early stages of cell infection with recombinant viruses, HCV core protein is detectable in cytoplasm only. In 16 h after infection, HCV core protein moves to the perinuclear region and is detectable in nucleus only after 24 h after infection (Fig. 5).

Based on the observation of the variable location of the HCV core protein forms, one can estimate multiple roles of HCV core protein in the HCV replication cycle and in the course of HCV infection. Earlier publications assumed that the p21 form of the HCV core protein provides a regulatory function in nucleus and affects expression of the host cell genome (Ray at al. 1996). Later papers showed that the ratio of specific HCV core protein variants, as well as their location within the host cell, is important for cell transformation (Chang et al. 1998).

The HCV envelope glycoprotein E2 binds to CD81 receptor on human cells. Therefore, it is a prime candidate for inclusion in HCV vaccines. Expression of the HCV E2 protein gene in BHK-21 cells is shown in Fig. 4. The E2 protein exists in a glycosylated form of about 70 kDa in BHK-21 cell lysates, after infection with recombinant SFV1/HCVpolyP or SFV1/HCVE2p7 viruses (Fig. 4A).

It is known that HCV core, envelope glycoproteins E1 and E2, and p7 are cleaved from the polyprotein by host signal peptidases. The precursor product E2-p7 was described by Lin et al. (1994). This protein is relatively stable and it is cleaved slowly to produce the E2 protein. It seems that cleavage at sites of E2p7/NS2 and E2/p7 is not necessarily co-translational, in contrast to the C/E1 and E1/E2 cleavages. Forms of the HCV E2 proteins observed in this study in cells infected with recombinant SFV1/HCV E2p7 after imunoprecipitation with corresponding anti-E2 antibody consist of glycosylated E2 (about 70kDa), ngE2p7 (41 kDa), and ngE2 (36 kDa) (Fig. 4B). Therefore, correct processing of HCV E2 protein is possible also in the SFV based expression system. Moreover, a surrogate SFV model system for expression of HCV structural genes exhibits high efficiency in BHK-21 cells.

A SFV replicon carrying HCV NS3/4A protein genes was used to enhance

immunogenicity of NS3/4A by mRNA amplification (Frelin et al. 2004). However, in Brinster´s (Brinster et al. 2002) study replicating SFV DNA vaccines and rSFVs expressing HCV core or E2 antigens were compared with classical CMV-driven plasmids (pCMV) in single or bimodal vaccine protocols. Comparison of different injection modes did not show any increased efficacy of the SFV-core and SFV-E2 plasmids or rSFVs compared with the CMV-driven vectors (Vidalin et al. 2000). Despite the contradictory data, combination of "genetic vaccines" (DNA, RNA, or viral particles, carrying target proteins gene) and corresponding proteins for prime-boost immunization in order to enhance immunoresponce remains very promising. These results indicate that the recombinant SFV carrying HCV structural proteins genes developed in this study can be used to study HCV proteins with respect to not only the biological functions in the virus life cycle, but also for development of HCV vaccines.

SFV-based expression systems have a serious disadvantage: overproduction of viral proteins stimulates a strong cytotoxic effect on host cells. The pCytTS expression vector, which is based on Sindbis virus, was created specially for routine production of difficult or toxic proteins (Boorsma et al. 2000). In our study, however, Sindbis-driven expression of HCV genes is not more efficient than the initial SFV-driven expression system, which allows to obtain sufficient amount of target proteins in the shortest time.

Immunocytochemical data, imunoprecipitation from cell lysates, and Western blot analysis with appropriate anti-core and anti-E2 polyclonal antibodies show that recombinant alphaviruses can provide high levels of authentically posttranslationally processed HCV core and E2 proteins. As known from literature (Baumert et al. 1998, Blanchard et al., 2002), cooperative production of structural HCV proteins results in self-assembly of the structural proteins into HCV-like particles. Production of HCV-like particles using a recombinant baculovirus in insect cells has already been reported. These particles, 40 to 60 nm in diameter, are polymorphic in appearance. In some electron micrographs (Blanchard et al. 2002; Vauloup-Fellous et al.,2006), HCV core-like particles of 30-40 nm in diameter are surrounded by ER-derived envelope, yielding a particle with a total diameter of 50-60 nm. Some authors explain the morphological heterogeneity of HCV-like particles by incorporation of partially uncleaved structural polypeptides into the envelope of such particles (Khromykh et al. 1998). The functional importance of RNA-protein interactions for assembly of HCV-like particles in heterologous system was reported by Girard et al. (2004). We detected homogeneous HCV core-like particles of 35-37 nm in diameter by electron microscopy (Fig. 7A, B), despite the fact that some of them may be damaged during preparation and concentration. Moreover, HCV-like particles with the diameter of 60 nm are observed randomly (Fig. 7C). This fact confirms once again the ability of correct processing of the HCV proteins in alphaviral expression systems.

As previously reported, formation of HCV-like particles is hampered by inefficient retention of HCV envelope proteins in the aggregated form on the ER (Deleersnyder et al. 1997). Moreover, formation of native HCV glycoprotein complexes is a limiting step in particle morphogenesis, this process varying efficiency from one HCV genotype to another (Girarg et al. 2004). The difficulties encountered in observing complete particles may be related to their high intrinsic instability or to their high sensitivity to the purification process. As known, it is currently not possible to obtain a sufficient amount of HCV particles for biological and physiochemical studies due to low viral load in the sera of hepatitis C patients and the low yield of HCV particles in cell cultures. Thus, HCV-like

particles obtained in the SFV expression system may serve, after optimization, as a useful tool for development of tissue culture model of HCV infection, for a better understanding of the mechanism of HCV assembly, and for the study of HCV morphogenesis. Moreover, the HCV proteins in HCV-like particles presumably are presented in a native, virion-like conformation and may therefore be superior in eliciting protective humoral and cellular immune responses. Since HCV-like particles synthesized in cells cultures are derived from partial viral genomes, without nonstructural genes required for viral replication, they are noninfectious and therefore represent excellent candidates for an HCV vaccine.

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Hepatīta C vīrusa strukturālo gēnu ekspresija alfavirālo rekombinanto replikonu kontrolē

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Kopsavilkums

Tā kā pašlaik vēl nav pieejami efektīvi hepatīta C vīrusa (HCV) izraisītā hepatīta ārstēšanas paņēmieni, anti-HCV vakcīnas kandidāta izstrādāšana ir mērķis ar augstu prioritāti. Mūsu pētījumos izmantotas divas dažādas alfavirālās ekspresijas sistēmas HCV strukturālo proteīnu gēnu ekspresijai šūnu kultūrā. Šī mērķa sasniegšanai HCV genoma fragmentu, kas kodē serdes-E1-E2-p7 poliproteīnu, kā arī HCV serdes un E2 gēnus atsevišķi klonēja Semliki meža vīrusa (pSFV1) un Sindbis vīrusa (pCytTS) ekspresijas vektoros. Parādīta efektīga HCV strukturālo proteīnu sintēze BHK-21 līnijas šūnās. HCV poliproteīna priekštecis bija autentiski procesēts pēctranslācijas laikā. HCV proteīnus identificēja ar imunoprecipitāciju, imunocitoķīmisko analīzi, kā arī ar *Western blotting* analīzi ar atbilstošajām anti-HCV antivielām.