МОЛЕКУЛЯРНАЯ БИОЛОГИЯ, 2012, том 46, № 2, с. 251–259

МОЛЕКУЛЯРНАЯ БИОЛОГИЯ КЛЕТКИ

UDC 578.224

EXPRESSION AND CHARACTERIZATION OF *Escherichia coli* DERIVED HEPATITIS C VIRUS ARFP/F PROTEIN

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Received March 29, 2011
Accepted for publication July 05, 2011

Genome of the hepatitis C virus (HCV) contains a long open reading frame encoding a polyprotein that is cleaved into 10 proteins. Recently, a novel, so called "ARFP/F", or "core+1", protein, which is expressed through a ribosomal frame shift within the capsid-coding sequence, has been described. Herein, to produce and characterize a recombinant form of this protein, the DNA sequence corresponding to the ARFP/F protein (amino acid 11–161) was amplified using a frame-shifted forward primer exploiting the capsid sequence of the 1b-subtype as a template. The amplicon was cloned into the pET-24a vector and expressed in different *Escherichia coli* strains. The expressed protein (mostly as insoluble inclusion bodies) was purified under denaturing conditions on a nickel-nitrilotriacetic acid (Ni-NTA) affinity column in a single step with a yield of 5 mg/L of culture media. After refolding steps, characterization of expressed ARFP/F was performed by SDS-PAGE and Western blot assay using specific antibodies. Antigenic properties of the protein were verified by ELISA using HCV-infected human sera and by its ability for a strong and specific interaction with sera of mice immunized with the peptide encoding a dominant ARFP/F B-cell epitope. The antigenicity plot revealed 3 major antigenic domains in the first half of the ARFP/F sequence. Immunization of BALB/c mice with the ARFP/F protein elicited high titers of IgG indicating the relevance of produced protein for induction of a humoral response. In conclusion, possibility of ARFP/F expression with a high yield and immunogenic potency of this protein in a mouse model have been demonstrated.

Keywords: **Hepatitis C virus, ARFP/F, Core +1, expression, immunization.**

Hepatitis C virus (HCV) infection is a public health problem with the global prevalence of 2.2%, corresponding to about 130 million people [1]. Chronic HCV infection frequently leads to cirrhosis and hepatocellular carcinoma. Currently, there is no vaccine against this virus and therapeutic approaches obliterate the virus in only 50% of the patients [2, 3]. HCV belongs to the *flaviviridae* family and its single-strand positive RNA encodes a polyprotein that is cleaved by cellular and viral proteases, generating at least 10 mature proteins including three structural (core, E1 and E2) and six non-structural (NS) proteins [4]. The HCV core protein (HCV_{cp}) is located at the N-terminus of the polyprotein and consists of 191 amino acid residues with a molecular mass of 23 kDa (p23) that is further processed to a mature 173–182-residue fragment (p21) [5–6]. Recently, it has been shown that another 16 kDa protein is expressed from the HCV core coding region by a +1 ribosomal frameshift mutation, starting at codon 11 and ending at codon 161 of the core open reading frame (ORF) [7]. Presence of this so called "core+1", or "ARFP" (alternative reading frame protein), or "ARFP/F" protein [7–9] was initially recognized by detection of anti-(ARFP) antibodies in serum of HCV-positive patients via application of synthetic peptides coding portions of this protein [9, 10]. Subsequently, other alternative forms of this protein like ARFP/DF (double-frameshift) and ARFP/S (short form of core+ю1) were also described. The ARFP/DF is composed of the first 42 amino acids of HCV_{cp} linked to the next 101 amino acids encoded in the alternate reading frame (ARF), followed by the C-terminus of the HCV_{cp}, while the ARFP/S is derived from an internal initiation event around the codons 85/87 of HCV_{cp} that may generate products in the +1 reading frame [11, 12].

The exact role of core+1 in the development of chronicity and HCV pathogenesis is still not well characterized; however, some of the functional properties that were attributed to the core [5, 6] were recently assigned to the ARFP. In fact, recent studies indicated that ARFP is an unstable protein that associates with the endoplasmic reticulum [13] and may play a role in

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the regulation of gene expression, cell signaling and apoptosis [14–17], as well as in altering immune responses [18]. These potential properties of the ARFP have attracted recently much attention and intensified the studies of its features. However, in order to characterize the ARFP/F protein and to study its functional properties, as well as to produce poly/monoclonal antibodies for clinical studies, a source of high amounts of this protein in a pure form should be available. Although in previous studies, expression of different recombinant forms of ARFP (the full-length or truncated forms) of different HCV genotypes 1a, 1b, 1c and 2 in *E. coli* was decribed [19–23], optimization of expression and purification conditions was only reported for the ARFP/S form of ARFP (amino acid residues 85–142 and 85–160) with a maximum achieved yield of 1 mg/L [24]. Moreover, accurate evaluation of antigenic properties or potential immunogenicity of these proteins was not addressed in prior studies.

The aim of present study was to develop and optimize a prokaryotic system for high level expression and simple purification of ARFP/F (amino acid residues 11–161) protein. Alignment of different ARFP/F amino acid sequences from NCBI Gene Bank database with the ARFP/F sequence employed in our study [25] was conducted. In addition, antigenic properties of this HCV-1b-derived ARFP/F protein were investigated theoretically using the Hopp & Woods method [26] and experimentally using HCV-infected human sera and sera of mice immunized with a specific epitopic peptide (P_f) of ARFP/F protein. Finally, by immunization of BALB/c mice with this produced recombinant protein, immunogenic properties of ARFP/F were evaluated. According to our knowledge, this study is the first report on the potency of ARFP/F protein for the induction of humoral response in an animal model.

EXPERIMENTAL

Construction of ARFP/F sequence and cloning. A $pIVEX_{2.3}$-HCV_{cp} plasmid containing the HCV core gene [25] was used as a template for constructing the core+1 sequence and production of the capsid protein (HCV_{cp}). Amplification of the DNA corresponding to the ARFP/F protein coding sequence in the +1 frame of the core starting from amino acid residue 11 (i.e. lacking the first 10 core-derived amino acids) to amino acid 161, was performed by PCR using C1F (forward) and C1R (reverse) primers harboring BamHI and XhoI restriction sites respectively (C1F: 5'-CAA***GGATCC***CCAAACGTAACACCAACCG-3', C1R: 5'-CCC***CTCGAG***CGCCGTCTTCCAGAAC-3'; restriction sites are in bolded italics). Subsequently, the PCR product was double digested by BamHI/XhoI restriction enzymes and inserted into the same sites of the expression vector pET-24a ("Novagen", USA) under the control of a T7 promoter and upstream of a 6xHis-tag (fig. 1). The resulting chimeric construct pET-HCV_{FP} after confirmation by restriction and sequencing analysis (Seq lab) was transfered into *E. coli*. All plasmid manipulation and cloning steps were performed according to standard procedures [27].

Expression and purification of recombinant ARFP/F protein. Expression of the recombinant ARFP/F protein was analyzed in four bacterial hosts: BL21 (DE3), BL21 (DE3) pLysS, Rosetta (DE3), BL21-CodonPlus (DE3)-RIL ("Novagen"). The *E. coli* cells containing pET/HCV_{FP} were grown in LB medium at 37°C. Protein expression was induced by addition of 1 mM IPTG at mid-log phase ($OD_{600} = 0.6$). To increase the yield, the expression of the recombinant ARFP/F protein was optimized at different temperatures (22°C, 30°C and 37°C), various concentrations of IPTG (0.5, 1 and 1.5 mM), and varying duration of induction time (1, 2, 3, 4 and 5 h). The ARFP/F protein was purified in denaturizing conditions on a nickel-nitrilotriacetic acid (Ni-NTA)-agarose column according to the manufacturer's protocol ("Qiagen", Germany). Briefly, the cell pellet was resuspended in the lysis buffer containing 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8.0). Subsequently, the lysate was centrifuged at $12000 \times g$ for 30 min and the supernatant was applied onto a Ni-NTA column. Following two washing steps by 4 mL of the wash buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 6.5); the His-tagged recombinant protein was eluted 5 times with 0.5 mL of the elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 4.5). Subsequently, the eluted fractions of the purified ARFP/F protein were dialyzed against the refolding buffer: 25 mM Tris-HCl (pH 7.4), 10% (v/v) glycerol, 250 mM NaCl, 10 mM glycine, 25% sucrose and protease inhibitor cocktail ("Roche", Germany) – with decreasing concentrations of urea (6, 4, 2, 1, 0.5, 0.25, 0 M) at 4°C for 20 h. Protein concentration was determined by the BCA protein assay method ("Pierce", USA) and the endotoxin level was measured with the QCL-1000 Chromogenic Limulus amoebocyte lysate test (Bio-Whittaker) [27].

Characterization of the recombinant ARFP/F protein and its antigenicity. Accuracy of recombination and the authenticity of the ARFP/F protein were evaluated by SDS-PAGE and Western blotting assays. Western blotting was performed in separate experiments with a set of different antibodies: anti-His tag monoclonal antibody, sera collected from mice immunized with a B-cell specific ARFP/F related epitopic peptide (anti-$P_f$sera), which was shown to produce highly specific ARFP/F antibodies (*paper in preparation*), and a mouse monoclonal antibodies against the HCV_{cp} ("Alexis Biochemical", UK) as positive and negative controls, respectively. Briefly, after SDS-PAGE the protein bands were transferred to a nitrocellulose membrane ("Sigma", USA) using a Bio-Rad Mini-protein II System. The membrane was blocked in PBS buffer containing 3% BSA for 2 h, and treated with either

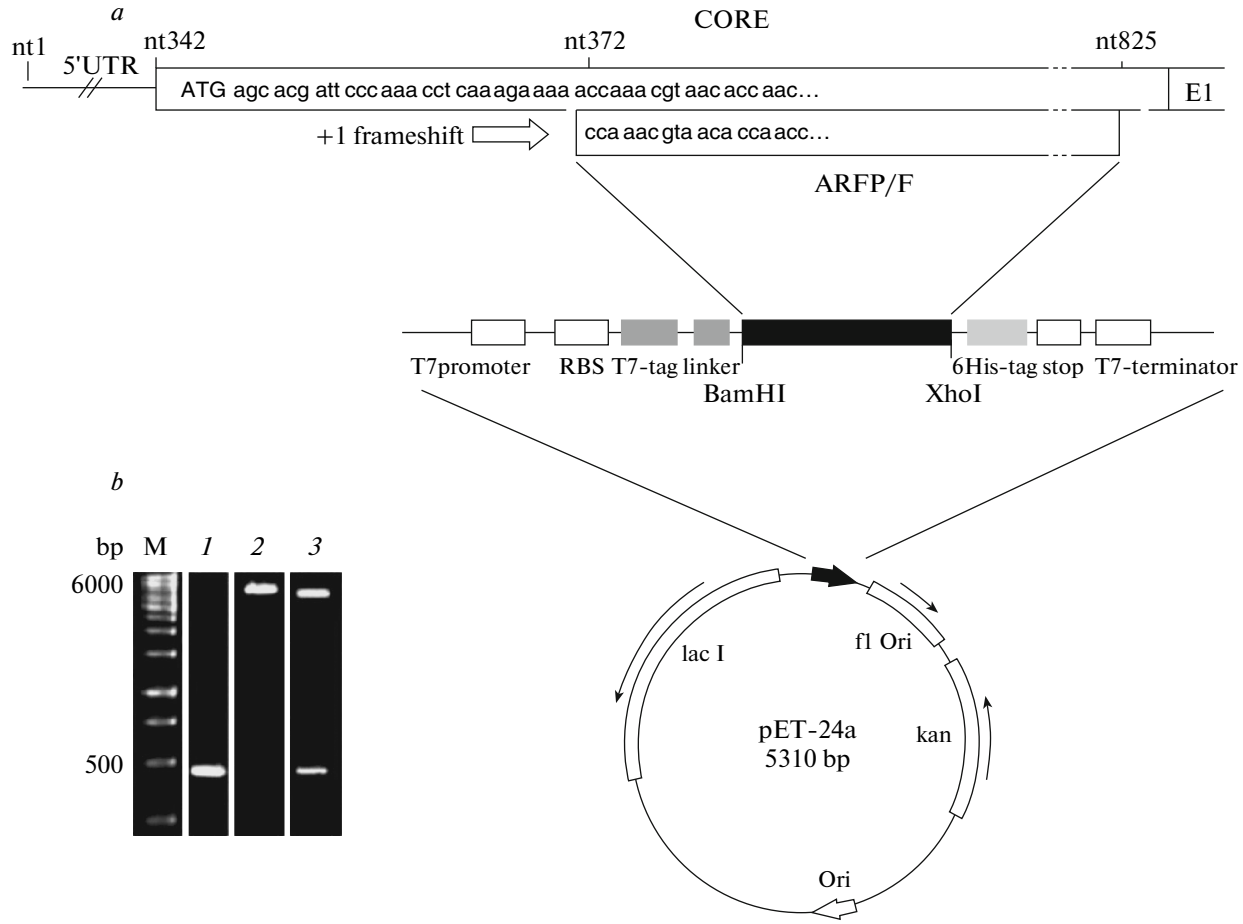

Fig. 1. Cloning strategies for constructing the pET-HCV_{FP} expression vector. *a* – Schematic representation of the expression elements in the pET/HCV_{FP} plasmid. The HCV ARFP nucleotide sequence corresponding to amino acid residues 11–161 (nucleotides 372–825 of the core sequence with a +1 frame shift) was amplified by PCR using a frameshifted-forward primer and ligated into the BamHI/ XhoI sites of the pET24a plasmid. This cloning strategy permitted to fuse a T7-tag to the N-terminal and a 6xHis-tag to the C-terminal of the core+1 fragment. *b* – Agarose gel electrophoresis of the PCR-amplified fragment and restriction analysis of the pET/HCV_{FP} construct. Lane M – DNA marker ("Fermentas", LV), *1* – PCR product, *2* and *3* – undigested and digested forms of the pET-HCV_{FP} by BamHI/XhoI enzymes, respectively.

anti-HCV core (1/10000) or anti-His-tag (1/2000) monoclonal antibodies or mice sera (1/100) diluted in PBST. After washing step, the membrane was incubated for 2 h with 1/10000 dilution of HRP-conjugated goat anti-mouse antibody ("Sigma") in PBST. Following more washing steps, the bands were visualized using the DAB (3,3'-diaminodbenzidine) as a substrate ("Roche", Germany). In parallel and separate assays, the non-immunized mice sera were used as a control.

The antigenicity and immune-reactivity of the recombinant ARFP/F protein to sera of mice immunized with a peptide encoding the dominant core+1 B-cell epitope (anti-P_f sera) and 11 blood sera samples from the HCV positive patients (Tehran Blood Transfusion Centre) were also analyzed by ELISA according to standard protocols [27]. In these sets of analyses, the ELISA plates were coated with either purified recombinant ARFP/F protein (3 μg/mL) or P_f peptide or HCV_{cp} (3 μg/mL) as positive or negative controls, respectively.

Immunization of mice against HCV ARFP/F protein. Seven inbreed female BALB/c mice aged 6–8 weeks were housed under standard pathogen-free conditions and commercial food and water were provided. Mice were immunized subcutaneously (s.c.) with 25 μg of the purified ARFP/F antigen in a complete Freund's adjuvant. Three weeks later, the same dose of antigen in an incomplete Freund's adjuvant was injected s.c. as a booster. Blood samples were obtained from the retroorbital sinus of mice at weeks 0, 3 and 6 and stored at –20°C prior to analysis.

Evaluation of humoral response against ARFP/F protein in immunized mice. The level of anti-ARFP/F antibodies in mice sera was measured by ELISA. Briefly, the purified ARFP/F protein (3 μg/mL) was used as a capture molecule to coat 96-well plates ("Nunc", Denmark). After washing with PBS and blocking with BSA

(1% w/v), wells were probed with 100 μL of serially-diluted sera (from 1 : 650 to 1 : 166400) obtained from mice immunized with the recombinant ARFP/F protein, and incubated for 1 h at 37°C. After three washing steps with PBST, the plates were incubated at 37°C for 1 h with a goat anti-mouse IgGγ chain specific HRP-labeled conjugate ("Sigma", USA) diluted to 1/10000. Finally, conjugate binding was visualized with TMB (3,3',5,5'-Tetramethylbenzidine; "Sigma") as a substrate. After the color developed, the reaction was stopped by addition of 50 μL of 1N HCl, and absorbance (at 450 nm) was measured. Sera prepared from 3 naive BALB/c mice were used as a negative control, and each sample was tested in triplicate. The positive cut-off value was taken as twice the mean values of the negative control sera.

Sequence analysis of ARFPs. The sequence alignment of the putative $ARFP_{1\text{-}161}$ from 2 different HCV genotypes (1a and 1b) and ARFP sequence employed in this study (1b genotype) was performed to analyze the degree of amino acid conservation among these sequences using the Bioedit program. An antigenicity plot of the studied F protein was generated using the algorithm proposed by Hopp & Wood [26].

RESULTS

Expression, purification and characterization of recombinant ARFP/F

The PET/HCV_{FP} plasmid containing 459 bp ARFP/F coding sequence downstream of the T7 promoter (fig. 1) was transformed into various *E. coli* strains, and the positive transformants were analyzed for the expression of the ARFP/F protein by SDS-PAGE. Several expression parameters including IPTG concentrations, temperatures and induction times were evaluated to optimize the expression conditions. The highest expression levels were achieved at the 1 mM IPTG at 37°C. Finally, by screening various incubation times, maximal expression levels of the ARFP/F protein were observed after the 3-h induction periods (fig. 2*b*). Accordingly, analysis of bacterial lysates resulted in detection of protein bands at the calculated (theoretically expected) molecular weight (18.1 kDa) for both BL21 (DE3) and Rosetta (DE3) *E. coli* strains. However, the expected bands were not observed in case of BL21 (DE3) pLysS and BL21-CodonPlus (DE3)-RIL strains (fig. 2*a*).

Analysis of recombinant ARFP/F solubility under native conditions using imidazole and in the absence of urea, (i.e: lysis of the bacterial pellet in the native buffer and evaluation of the supernatant after centrifugation and separation of bacterial debris) indicated lower amounts of the ARFP/F protein in the cell lysate supernatant (containing soluble proteins) (fig. 2*c*) as compared to denaturing conditions (i.e: lysis of the bacterial pellet in a denaturing buffer containing urea and evaluation of the supernatant after centrifugation and separation of bacterial debris). This observation indicated the presence of a higher amount of the expressed protein in the insoluble fraction rather than soluble one (fig. 2*c*). In addition, these results implied that purification of the ARFP/F protein under native conditions (which is based on purifying soluble fraction) should not be efficient because most of the expressed protein was in the form of inclusion bodies rather than in the soluble cytoplasmic fraction. Therefore, by preparing the lysate in 8 M urea buffer, the recombinant His-tagged ARFP/F protein was purified by Ni-NTA chromatography under denaturing conditions (fig. 2*d*), refolded during a dialysis procedure and analyzed by 12% SDS-PAGE under non-reducing conditions (without β-mercaptoethanol in the sample buffer). SDS-PAGE analysis of ARFP/F purification steps (binding, washing and elution) on Ni-NTA column is shown in fig. 2*e*. As shown, one sharp band with MW of 18.1 kDa corresponding to ARFP/F as well as two other faint bands with approximate MWs of 35 kDa and 70 kDa could be detected in SDS-PAGE (non-reducing). These heavier proteins are most likely the oligomerized forms of the ARFP/F (see discussion section). Finally, the production yield of the purified and refolded ARFP/F protein was shown to be 5 mg/L of the induced culture media for the *E. coli* BL21 (DE3) strain.

Western blot analysis by either commercial anti-His monoclonal antibodies (mAbs) or anti-P_f sera indicated specific bands with the expected size while no interaction was observed when commercial anti-core mAb was applied, indicating to the specificity of the produced ARFP/F protein (fig. 2*f*).

To evaluate antigenic features of the recombinant ARFP/F, refolded protein was analyzed by anti-P_f sera using the ELISA assay. As shown in fig. 3*a*, recombinant ARFP/F protein was detected by the anti-P_f sera. In addition, while all 11 HCV positive human sera used in this study were anti-HCV core positive, two of them also produced significant signals against either the P_f peptide or ARFP/F protein (fig. 3*b*). Therefore, 2 HCV positive sera were ARFP/F positive and further implied the discrimination power of the expressed ARFP/F protein from HCV_{cp}. In fact, core positive human sera that did not bind to P_f peptide (i.e. ARFP/F negative sera) did not react with the recombinant ARFP/F either. Thus, cross-reactivity between HCV_{cp} and recombinant ARFP/F was not observed. Furthermore, this finding proposed a similar antigenicity for the expressed protein and P_f peptide, which was an ARFP/F-derived B-cell determinant.

Immunogenicity of the recombinant ARFP/F in BALB/c mice

As shown in fig. 4, all animals immunized with the ARFP/F protein displayed different levels of specific IgG antibody response, while sera of negative control

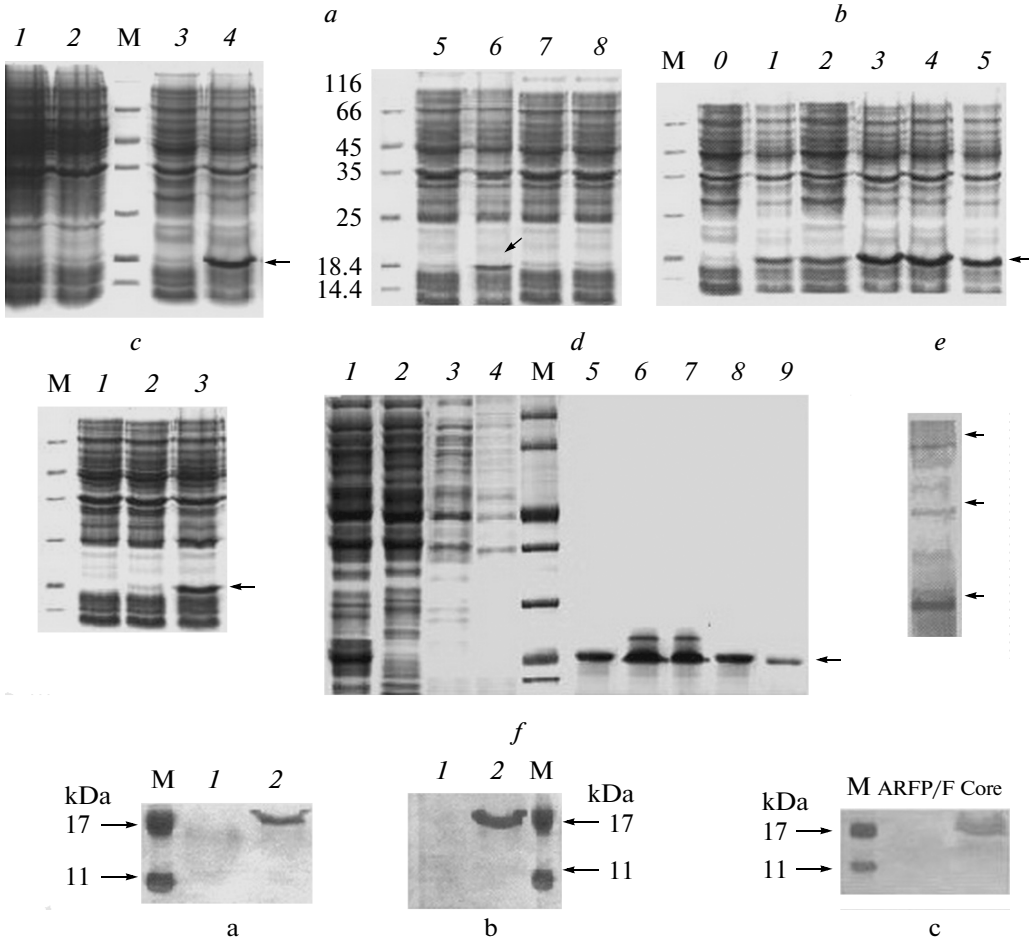

Fig. 2. Analysis of expression and purification of the ARFP/F protein by SDS-PAGE and Western blot. *a* – SDS-PAGE analysis of bacterial lysates of *E. coli* strains harboring the pET-HCV_{FP} recombinant plasmid after induction with IPTG: lanes *1* and *2* – bacterial lysate of BL21 (DE3) pLysS before and 3 h after induction, respectively; *3*, *5* and *7* – total bacterial lysates of BL21 (DE3), Rosetta (DE3) and BL21-CodonPlus (DE3)-RIL strains before induction; *4*, *6* and *8* – total bacterial lysates of the same bacterial cells after induction, respectively. *b* – Time course inductions of the ARFP/F protein (lanes *1* to *6* correspond to the induction time course of 0 to 5 h in the BL21 (DE3) host, respectively). *c* – Protein solubility analysis: lane *1* – total bacterial lysate before induction; *2* – the bacterial pellet after induction was lyzed in the "native" buffer (containing immidazole) and the supernatant after centrifugation were loaded; *3* – exactly as lane *2* but the denaturing buffer (containing urea) was used. *d* – Protein purification under denaturizing conditions: *1* – bacterial lysate supernatants, *2* – Ni-NTA unbounded material, *3* and *4* – wash flows and lanes *5–9* – elution aliquots from the Ni-NTA column. *e* – The refolded ARFP/F protein with one sharp band at 18.1 kDa and two other faint bands at approximately 35 kDa and 70 kDa (indicated by arrows) in a SDS-PAGE analysis under non-reducing conditions. *f* – Western blot analysis of the recombinant ARFP/F protein. BL21 (DE3) bacterial lysate before (lane *1*) and after (lane *2*) induction was treated either with the anti-His monoclonal antibodies (a) or with the anti-P_f peptide mouse anti-sera (b). Purified recombinant ARFP/F and core proteins were treated with anti-core mAb (c) to discriminate between the two proteins.

groups did not show any reactivity to the ARFP/F protein. Moreover, an average end-point antibody titer of 1/83200 was detected in two mice sera.

Sequences analysis

Sequence analysis of ARFP/F proteins showed a high degree of conservation, especially in the early N-terminal (amino acid residues 1–58) and late C-terminal (amino acid residues 148–160) fragments (fig. 5*a*). The antigenicity plot of the ARFP/F sequence employed in the study indicated 3 major antigenic domains in the first half of the protein fragment (residues 1–28, 48–75 and 85–98) (fig. 5*b*).

DISCUSSION

The ARFP/F protein is encoded by ORF that overlaps the core ORF in the first 10 codons and is differentiated by a +1 frameshift mutation at codon 11 [7]. The ARFP/DF (double-frameshift) and ARFP/S (short form of core+1), are also described as two other forms of the ARFP protein [11, 12]. There is lots of evidence of ARFP expression during the life cycle of the

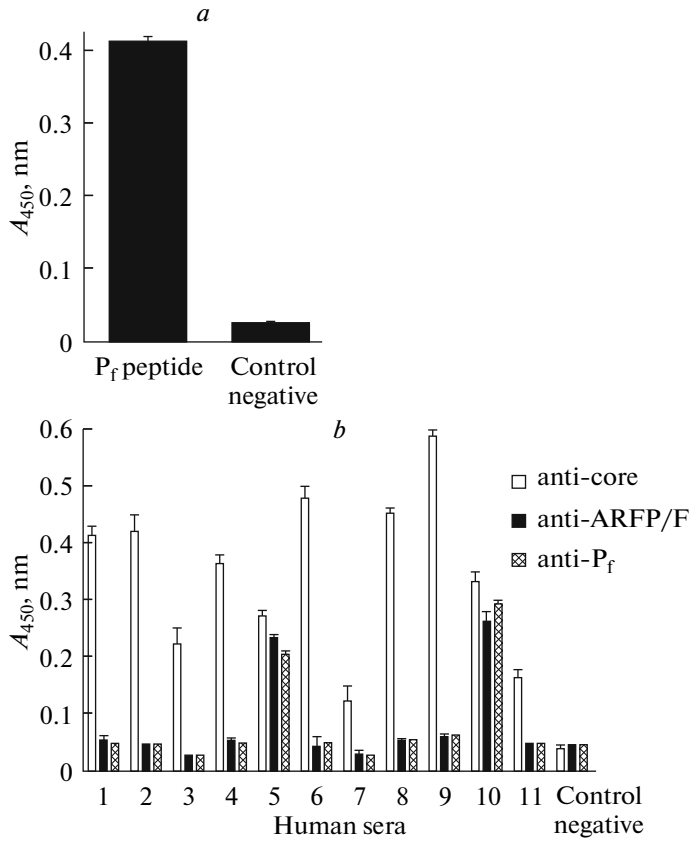

Fig. 3. Analysis of the antigenicity of the ARFP/F protein by ELISA. *a* – ARFP/F-specific epitopic peptide (P_f): immunized and naive mouse sera were used as positive and negative ones, respectively. *b* – Eleven samples of HCV-positive human serum and one of HCV-negative normal human serum were analyzed on presence of anti-core, anti-ARFP/F and anti- P_f antibodies. The positive cut-off value was taken as twice the mean value of the negative control sera. Error bars in the diagram indicate SD.

HCV and stimulation of specific immune responses. In some HCV positive patients expression of the core+1 proteins has been confirmed by detecting both the specific T-cell and the B-cell mediated immune responces against the ARFP antigen [9, 10, 18, 22, 28]. Furthermore, ARFP proteins were found to interfere with apoptosis and cell cycle regulation [14–16], suggesting a role of these proteins in HCV pathogenesis.

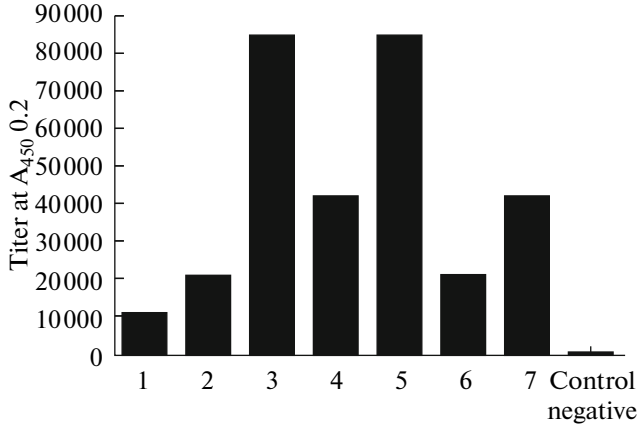

Fig. 4. Antibody response of BALB/c mice immunized with the recombinant ARFP/F protein. Seven BALB/c mice were immunized two times with 25 μg of ARFP/F protein at 3-week intervals. Mice were bled 3 weeks after the last immunization and anti-ARFP/F antibody response was evaluated by ELISA. The bars represent antibody titers in mice sera.

Although, expression of ARFP/F has been already addressed in mammalian cells [29, 30] and *E. coli* [19–23], however, our study is the first attempt towards optimization of prokaryotic expression of ARFP/F using different *E. coli* strains, induction systems or vectors (results not shown), various temperatures, IPTG concentration and incubation times in order to provide a high amount of the protein for further investigation. Our results indicated that the vector and host are the most important factors for effective expression of ARFP/F protein in a bacterial system. It is noteworthy that our primary attempts to express this novel protein by the pIVEX2.3 vector ("Roche") in the BL21-AI host were not successful (*data not shown*). However, changing the plasmid to pET system that encodes the protein in fusion with a large N-terminal tag (T7-tag) resulted to the expression of ARFP/F protein. This observation is consistent with previous reports, demonstrating that expression of recombinant proteins could be increased

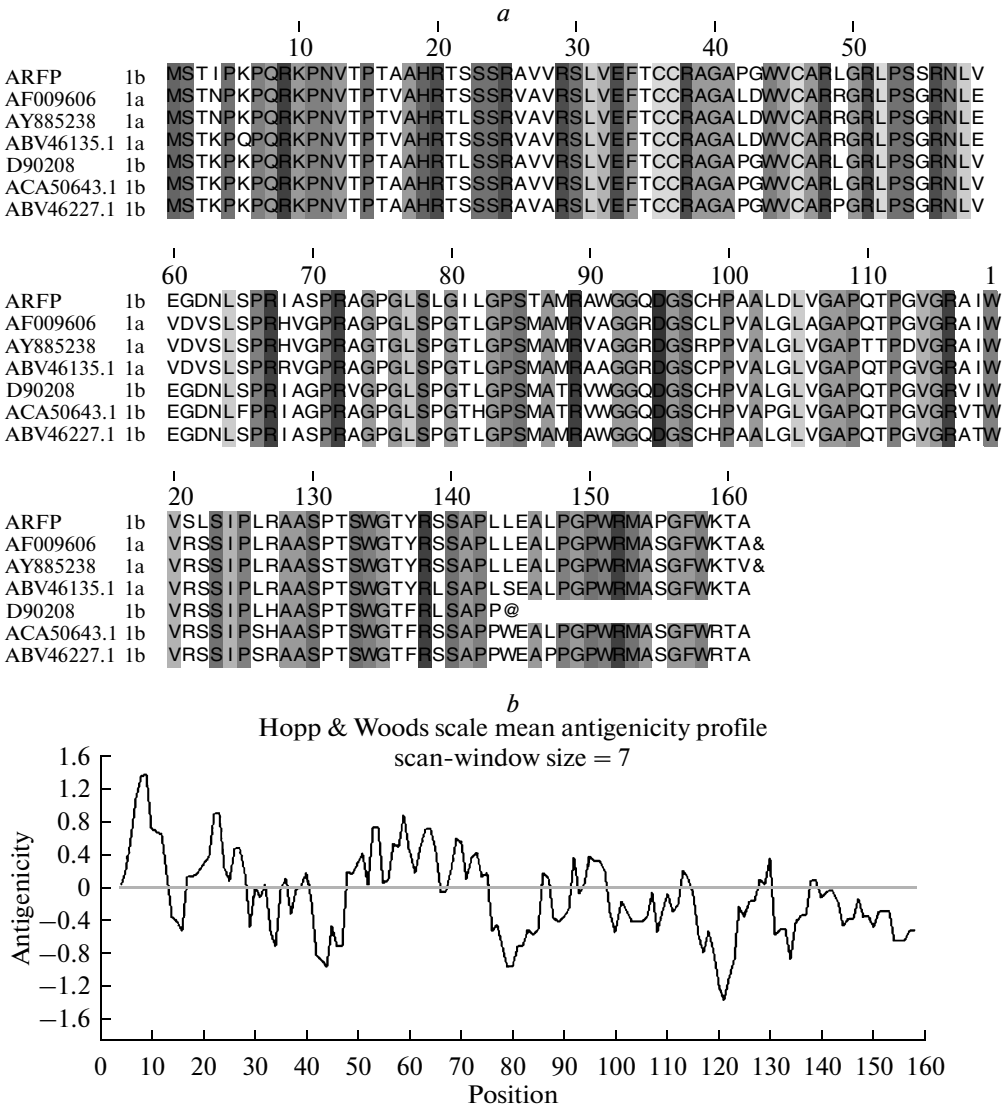

Fig. 5. Sequence alignment of six different ARFP/F amino acid reference sequences of the 1a and 1b genotypes with the ARFP/F sequence employed in this study (first row). *a* – The ARFP/F sequences were obtained after translation of nucleotide sequences retrieved from the NCBI GeneBank database (accession numbers are shown) in the +1 ORF (with 10 amino acids of the core protein in the N-terminal of the ARFP/F protein). *b* – The ARFP/F (amino acid 1–161) antigenicity plot according to the Hopp & Woods method [26]. Positive values indicate hydrophilic or antigenic areas.

by addition of fusion polypeptides to the N-terminus [31–34].

Here, the ARFP/F protein has been effectively expressed in the BL21 (DE3) *E. coli* strain in accordance with previous studies [19–22, 24, 28]; furthermore, the ARFP/F expression has been achieved for the first time in the Rosetta (DE3) strain, but not in the BL21 (DE3)-pLysS or BL21-CodonPlus (DE3)-RIL strains (fig. 2*a*). Although there is no clear explanation for these results, optimization of the vector/host combination might be one of the most important issues that should be considered in recombinant protein expression.

In previous studies the reported expression of ARFP/S (residues 85–142 and 85–160) in *E. coli* cells was at most up to 1 mg/L [24]. The optimization of expression parameters allowed us to increase the productivity of ARFP/F (amino acid residues 11–161) up to 5 mg of protein per liter of bacterial culture. This difference in yields might be due to differences in expression conditions, variations in the length of expressed ARFPs and gene sequences.

In prior studies, existence of relatively high molecular mass particles in protein samples due to partial oligomerization of the HCV Core+1/S proteins (although puried in a monomeric form by size exclusion chromatography) were reported [24]. This oligomerization phenomenon was attributed to residues Ile33 and Val35 of the core protein. Accordingly, SDS-

PAGE (under non-reducing conditions) analysis of purified and refolded ARFP/F protein obtained in our study detected the presence of extra protein bands with molecular mass of ~35 and ~70 kDa (fig. 2*e*). These bands were most probably related to the oligomerized forms of ARFP/F. However a more detailed analysis is needed to bring the evidence of such oligomerization.

Availability of the recombinant ARFP/F protein in high amounts and with adequate antigenic properties is required for its application as a diagnostic and prognostic tool. In this context, results of a recent study have demonstrated that the presence or absence of specific antibodies to the ARFP/F protein may serve as an indicator for predicting the efficiency of antiviral treatment in HCV patients [20]. Although, purified ARFP/F obtained here could be successfully used as a primary diagnostic tool for the detection of mouse and human anti-ARFP antibodies (fig. 3*a*, *b*) and demonstrated no cross-reactivity with anti-core specific antibodies from human HCV positive sera, still more detailed studies are required to address the real diagnostic value of this protein.

In accordance with previous studies [9, 10, 18, 22, 28], anti-ARFP/F antibodies in HCV-infected individuals were detected in 2 of 11 (18%) serum samples from Iranian HCV-positive patients. These two serum samples reacted with the produced recombinant genotype 1b ARFP/F protein in an in-house ELISA test. The specificity of anti-ARFP/F antibodies has been also confirmed using a synthetic ARFP epitopic peptide (P_f). In previous investigations, different frequencies (from 13% [9] to 89% [27]) of anti-ARFP/F antibodies in HCV-infected patients were reported. The prevalence of anti-ARFP/F antibodies (18%) obtained in our study is compatible with data reported by Pawlotsky et al. [35], namely: specific anti-ARFP/F antibodies were detected in 17.6% (3 of 17) of HCV-infected patients using a genotype 1b $ARFP_{43-141}$ synthetic peptide. Of note, this frequency variability for the presence of anti-ARFP/F antibodies in HCV-infected patients might be due to the different sample sizes (number of investigated sera) and the different ARFP antigens (different source, length and sequences) employed for detection of the antibodies.

According to Hopp & Woods antigenicity plot [26], 3 antigenic domains are found in the ARFP/F protein sequence (fig. 5*b*); the first one is located in the N-terminal part (residues 1–28) and others are in the middle part of the protein (residues 48–75 and 85–98). Since the first domain seems to be a well conserved region among the different analyzed ARFP sequences (fig. 5*a*), it could be a suitable candidate epitope for further immunological investigations such as monoclonal antibody production.

Several studies already reported the presence of anti-ARFP antibodies and T-cell response in some HCV-infected patients [9, 10, 18, 22, 28]. Zhang et al. [36] detected the anti-ARFP antibodies in mice immunized with a DNA vaccine containing the core-E1–E2 fragments of the HCV genome. Results of these prior studies indicated that ARFP was expressed from the core sequence and could be an immunogenic protein. However, detailed information about the immunogenic properties of this protein was not revealed. We studied the induction of humoral immune response by immunization of BALB/c mice with the recombinant ARFP/F protein. Accordingly, all of the injected mice promoted high titers of specific anti-ARFP/F antibody response (fig 4). To our knowledge, this study is the first attempt to show an immunogenic potency of the ARFP/F protein in an animal model. Although, these experiments provide some basic information on the immunogenicity of the ARFP/F protein, more investigations, especially on the evaluation of the CTL response in mice, are required. This approach is important for understanding the type and mechanisms of immune response against the ARFP proteins and the immunogenic nature of this protein, as well as its use as a candidate vaccine against HCV.

Taken together, in present study an efficient and simple system for high-yield expression (5 mg/L of culture) and purification of the ARFP/F protein was developed, and the proper antigenicity and immunogenicity of this protein was demonstrated. This expression system is currently in use for a large-scale production of the ARFP protein for its structural and functional analysis as well as mAb preparation.

ACKNOWLEDGEMENTS

This work was financially supported by grant no. 393 from the Research Council of the Pasteur Institute of Iran. F.B. was partially supported by the Hepatitis and AIDS Department of the Pasteur Institute of Iran to pursue her studies in the PhD. program.

REFERENCES

1. Alter M. 2007. Epidemiology of hepatitis C virus infection. *World J. Gastroenterol.* **13**, 2436–2441.
2. Houghton M., Abrignani S. 2005. Prospects for a vaccine against the hepatitis C virus. *Nature*. **436**, 961–966.
3. Gremion C., Cerny A. 2005. Hepatitis C virus and the immune system. *Rev. Med. Virol.* **15**, 235–268.
4. Schulze zur Wiesch J., Schmitz H., Borowski E., Borowski P. 2003. The proteins of the Hepatitis C virus: their features and interactions with intracellular protein phosphorylation. *Arch. Virol.* **148**, 1247–1267.
5. Ray R.B., Ray R. 2001. Hepatitis C virus core protein: intriguing properties and functional relevance. *FEMS Microbiol. Lett.* **202**, 149–156.
6. McLauchlan J. 2000. Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J. Viral. Hepatitis*. **7**, 2–14.
7. Xu Z., Choi J., Yen T., Lu W., Strohecker A., Govindarajan S., Chien D., Selby M., Ou J. 2001. Synthesis

of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* **20**, 3840–3848.
8. Varaklioti A., Vassilaki N., Georgopoulou U., Mavromara P. 2002. Alternate translation occurs within the core coding region of the hepatitis C viral genome. *J. Biol. Chem.* **277**, 17713–17721.
9. Walewski J., Keller T., Stump D., Branch A. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA.* **7**, 710–721.
10. Walewski J., Keller T., Branch A. 1998. HCV patients have antibodies against a novel protein encoded in a second reading frame. *Hepatology.* **28**, 278A.
11. Boulant S., Becchi M., Penin F., Lavergne J. 2003. Unusual multiple recoding events leading to alternative forms of hepatitis C virus core protein from genotype 1b. *J. Biol. Chem.* **278**, 45785–45792.
12. Vassilaki N., Mavromara P. 2003. Two alternative mechanisms are responsible for the synthesis of the HCV ARFP/F/core+1 protein. *J. Biol. Chem.* **278**, 40503–40513.
13. Vassilaki N., Boleti H., Mavromara P. 2007. Expression studies of the core+1 protein of the hepatitis C virus 1a in mammalian cells. *FEBS. J.* **274**, 4057–4074.
14. Shao S., Wu W., Bian Z., Yu J., Zhao P., Zhao L., Zhu S., Qi Z. 2009. Hepatitis C virus F protein inhibits cell apoptosis by activation of intracellular NF B pathway. *Hepatol. Res.* **39**, 282–289.
15. Wu W., Shao S., Zhao L., Luan J., Cao J., Gao J., Zhu S., Qi Z. 2007. Hepatitis C virus F protein Up-regulates c-myc and down-regulates p53 in human hepatoma HepG 2 cells. *Intervirol.* **50**, 341–346.
16. Ma H., Lin T., Li H., Iguchi A. S., Ariga H., Chuang Y., Ou J., Lo S. 2008. Hepatitis C virus ARFP/F protein interacts with cellular MM-1 protein and enhances the gene trans-activation activity of c-Myc. *J. Biomed. Sci.* **15**, 417–425.
17. Huang Y., Cheng J., Zhang S., Wang L., Guo J., Liu Y., Yang Y., Zhang L., Bai G., Gao X. 2005. Screening of hepatocyte proteins binding to F protein of hepatitis C virus by yeast two-hybrid system. *World J. Gastroenterol.* **11**, 565956–565965.
18. Fiorucci M., Boulant S., Fournillier A., Abraham J., Lavergne J., Paranhos G., Inchauspe G., Bain C. 2007. Expression of the alternative reading frame protein of Hepatitis C virus induces cytokines involved in hepatic injuries. *J. Gen.Virol.* **88**, 1149–1162
19. Komurian Pradel F., Rajoharison A., Berland J., Khouri V., Perret M., van Roosmalen M., Pol S., Negro F., Paranhos Baccalà G. 2004. Antigenic relevance of F protein in chronic hepatitis C virus infection. *Hepatology.* **40**, 900–909.
20. Gao D., Zhang X., Hou G., Jin G., Deng Q., Kong X., Zhang D., Ling Y., Yu D., Gong Q. 2008. Assessment of specific antibodies to F protein in serum samples from chinese hepatitis C patients treated with interferon plus ribavarin. *J. Clin. Microbiol.* **46**, 3746–3751.
21. Shesheer Kumar M., Venkateswara Rao K., Mohammed Habeebullah C., Dashavantha Reddy V. 2008. Expression of alternate reading frame protein (F1) of hepatitis C virus in *Escherichia coli* and detection of antibodies for F1 in Indian patients. *Infect. Genet. Evol.* **8**, 374–377.
22. Troesch M., Jalbert E., Canobio S., Boulassel M., Routy J., Bernard N., Bruneau J., Lapointe N., Boucher M., Soudeyns H. 2005. Characterization of humoral and cell-mediated immune responses directed against hepatitis C virus F protein in subjects co-infected with hepatitis C virus and HIV-1. *AIDS.* **19**, 775–784.
23. Bain C., Parroche P., Lavergne J., Duverger B., Vieux C., Dubois V., Komurian-Pradel F., Trepo C., Gebuhrer L., Paranhos Baccala G. 2004. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. *J. Virol.* **78**, 10460–10469.
24. Boumlic A., Nominé Y., Charbonnier S., Dalagiorgou G., Vassilaki N., Kieffer B., Travé G., Mavromara P., Orfanoudakis G. 2010. Prevalence of intrinsic disorder in the hepatitis C virus ARFP/Core+1/S protein. *FEBS J.* **277**, 774–789.
25. Roohvand F., Aghasadeghi M., Sadat S., Budkowska A., Khabiri A. 2007. HCV core protein immunization with Montanide/CpG elicits strong Th1/Th2 and long-lived CTL responses. *BBRC.* **354**, 641–649.
26. Hopp T., Woods K. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* **78**, 3824–3828.
27. Sambrook J., Fritsch E.E., Maniatis T. 1989. *Molecular cloning:A Laboratory Manual*, 2nd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Lab. Press.
28. Cohen M., Bachmatov L., Ben-Ari Z., Rotman Y., Tur-Kaspa R., Zemel R. 2007. Development of specific antibodies to an ARF protein in treated patients with chronic HCV infection. *Digest. Dis. Sci.* **52**, 2427–2432.
29. Roussel J., Pillez A., Montpellier C., Duverlie G., Cahour A., Dubuisson J., Wychowski C. 2003. Characterization of the expression of the hepatitis C virus F protein. *J. Gen. Virol* .**84**, 1751–1759.
30. Xu Z., Choi J., Lu W., Ou J. 2003. Hepatitis C virus F protein is a short-lived protein associated with the endoplasmic reticulum. *J. Virol.* **77**, 1578–1583.
31. Sati S., Singh S., Kumar N., Sharma A. 2002. Extra terminal residues have a profound effect on the folding and solubility of a *Plasmodium falciparum* sexual stage specific protein over expressed in *Escherichia coli*. *Eur. J. Biochem.* **269**, 5259–5263.
32. Hammarström M., Hellgren N., van den Berg S., Berglund H., Härd T. 2002. Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*. *Protein Sci.* **11**, 313–321.
33. Stevens R. 2000. Design of high-throughput methods of protein production for structural biology. *Structure.* **8**, 177–185.
34. Goncharuk M.V., Schulga A.A., Ermolyuk Ya.S., Tkach E.N., Goncharuk S.A., Pustovalova Yu.E., Mineeva K.S., Bocharova E.V., Maslennikova I.V., Arseniev A.S., Kirpichnikov M.P. 2011. Bacterial synthesis, purification, and solubilization of transmembrane segments of ErbB Family Receptors. *Mol. Biol. (Mosk.).* **45**, 823–832.
35. Pawlotsky J.M., Morice Y., Miladi A., Lavergne J.P. 2005. Germanidis. *Abstr. 12th Int.Conf.* "*Hepatitis C Virus and Related Viruses*", Montreal, Canada, 2005.
36. Zhang X.C.L., Fournillier A. 2004. Vaccine induced immune responses specific of the alternate reading frame protein (ARFP) of HCV. *Abstr.11th Int.Conf.* "*Hepatitis C Virus and Related Viruses*," Heidelberg, Germany, 2004.