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Short communication

Chimeric hepatitis A virus particles presenting a foreign epitope (HIV gp41) at their surface

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Abstract

Hepatitis A virus (HAV) protein 2A has been demonstrated to be involved in virus morphogenesis and suggested to be located on the surface of the particle. To determine whether this protein can function as a target structure to harbor and expose foreign epitopes on HAV particles, a full-length HAV cDNA, containing a seven amino acid stretch of human immunodeficiency virus type 1 (HIV-1) envelope protein gp41, was constructed. Following vaccinia virus MVA-T7-mediated expression of the cDNA in COS7 and Huh-T7 cells, chimeric HAV particles, exposing the foreign epitope gp41 on their surface, were produced. These particles were found to be empty capsids (70S), as judged by immunospecific enzyme linked immunosorbent assay (ELISA) on sucrose gradient fractions and immunoelectron microscopy. The immunological detection of VP1-2A harboring the gp41 epitope of HIV suggests that the 2A domain of HAV is suitable to present foreign antigenic epitopes.

Keywords: HAV; Protein 2A; HIV gp41; Chimeric particles

In recent years, considerable interest has been focused in developing epitope presentation systems in which defined peptide sequences, corresponding to antigenic sites from a pathogen, are fused to a carrier protein capable of assembly into a macromolecular structure. Many of these systems employ coat proteins of animal or plant viruses as carrier molecule and produce non-pathogenic virus-like particles bearing the foreign epitope. In addition, the construction of chimeric infectious viruses carrying foreign antigenic domains has been described (Porta et al., 1994; Garcia-Sastre and Palese, 1995; Fernandez-Fernandez et al., 1998; Casal et al., 1999; Pumpens and Grens, 1999). To date, different members of the picornavirus family have been evaluated as vectors for foreign gene delivery (Girard et al., 1993; Altmeyer et al., 1995; Arnold et al., 1996; Zhang and Kaplan, 1998; Obuchi et al., 1999).

The genome of picornaviruses encodes a large polyprotein which undergoes autoproteolytic cleavage to yield the structural proteins of the domain

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P1 (VP1–VP4) as well as the non-structural proteins of domains P2 and P3. Only three structural proteins and some non-structural proteins are well conserved among all picornaviruses. In particular, the role of the non-structural protein 2A is greatly variable (Molla et al., 1993; Michiels et al., 1997; Zoll et al., 1998; Seipelt et al., 1999). Sequence analysis of hepatitis A virus (HAV) protein 2A shows no obvious homology with the corresponding proteins of the other picornaviruses. Protein 2A of HAV is proteolytically inactive and forms part of the capsid protein precursor, P1-2A, that is cleaved by protease 3C to generate VP0 (VP4–VP2), VP3 and PX (VP1–2A) (Borovec and Anderson, 1993; Probst et al., 1999). Recently, it has been demonstrated that 2A is involved in morphogenesis of HAV, being required for the initial assembly step leading to the formation of pentamers; it has been suggested that 2A, as part of VP1–2A, might be located near the 5-fold vertices on the outside of pentamers and empty capsids (Probst et al., 1999). Furthermore, the presence of a small quantity of VP1–2A has been reported in infectious virus particles (Borovec and Anderson, 1993). It has also been observed that antibodies present in sera of HAV infected patients react with an immunodominant antigenic domain containing the C-terminal part of VP1 and the entire 2A protein (Khudyakov et al., 1999). Harmon et al. reported that deletion of ten or 15 amino acid residues from the central portion of HAV protein 2A does not significantly affect polyprotein processing and virion production and that the resulting virus maintains its ability to grow in cell culture and to infect marmosets (Harmon et al., 1995).

Based on these unique properties of HAV 2A, we investigated the possibility of obtaining chimeric particles exposing a foreign antigen on their surface by introducing extraneous sequences within the context of the 2A gene of HAV. For that purpose, the amino acid residues 44–53 of protein 2A were replaced by a foreign antigenic epitope and the particles obtained after expressing the chimeric HAV open reading frame were analyzed. The data suggest that domain 2A on the viral particles can tolerate foreign epitopes and that chimeric HAV particles could be used as the basis for new diagnostic and prophylactic antigens.

When virus particles are to be used for presentation of foreign epitopes, it is essential to demonstrate that the presence of the foreign sequence does not interfere with virus structure and assembly and that the epitope is expressed on the surface of the particles. The potential site for insertion of foreign sequences on the HAV capsid surface was chosen on the basis of the data reported in the literature. The recent suggestion of the possible exposure of protein 2A onto the surface of HAV empty particles (Probst et al., 1999) and the report that some deletions covering the central amino acids of 2A had no significant effect on viral infectivity (Harmon et al., 1995), prompted us to choose the central region of the 2A protein as the insertion site of foreign amino acids for the production of HAV chimeric particles. The parent plasmid used to prepare the constructs was pT7-18f, which contains the P1 region of the attenuated HAV strain HM175 in the background of the cytopathic HAV strain 18f, placed under control of the T7 promoter (Kusov and Gauss-Müller, 1999). The selected foreign epitope was the immunodominant determinant of gp41, an envelope transmembrane glycoprotein of human immunodeficiency virus type 1 (HIV-1), ELDKWAS. This seven amino acids epitope has been shown to be capable of raising protective antibodies in

Fig. 1. (A) Oligonucleotide primers used for mutagenesis. The asterisk indicates the deletion of 30 nucleotides. The nucleotides coding for the foreign epitope are underlined. (B) Schematic representation of the HAV genome and amino acid sequence of the wild type (wt) and the mutated 2A central region. (C) Demonstration of the specificity of the monoclonal 7E7 for assembled HAV structural proteins. The ELISA was performed using antibody 7E7 as capture and detection antibody on crude lysates of pT7-18f and pEXT7-HM/HAS-P1-2A (P1–2A) expressed in COS7 cells. (D) Expression of recombinant cDNAs in COS7 cells. Lysates of cells transfected with the control HAV cDNA (pT7-18f), the mutated constructs (pHAV-gp41 and pHAV-2Adel) or mock transfected and infected with vMVA-T7 were analyzed by ELISA. (a) The monoclonal antibody 7E7 was used as capture antibody to detect the expression level of the constructs. (b) The monoclonal antibody 2F5 was used as capture antibody to reveal the presence of the foreign sequence. In both cases, antibody 7E7 conjugated to horse radish peroxidase was used for detection.

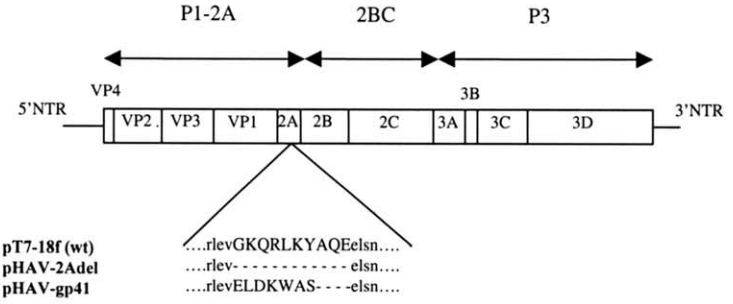
Primers for creating the deletion mutant (pHAV-2Adel), nucleotide 3126 to 3214 (HM175):

Sense: 5'- AAGCCATATAAAGAACTGAGATTAGAAGTT*GAATTGTCAAATGAAGT ACTTCCACCCCCT - 3'
Antisense: 5'- AGGGGGTGGAAGTACTTCATTTGACAATTC*AACTTCTAATCTCAG TTCTTTATATGGCTT- 3'

Primers for creating the insertion mutant (pHAV-gp41), nucleotide 3164 to 3205 (HM175):

Sense: 5'- AAAGAACTGAGATTAGAAGTTGAATTAGATAAATGGGCAAGTGAATT GTCAAATGAAGTACTT - 3'
Antisense: 5' - AAGTACTTCATTTGACAATTCACTTGCCCATTTATCTAATTCAACT TCTAATCTCAGTTCTTT - 3'

A



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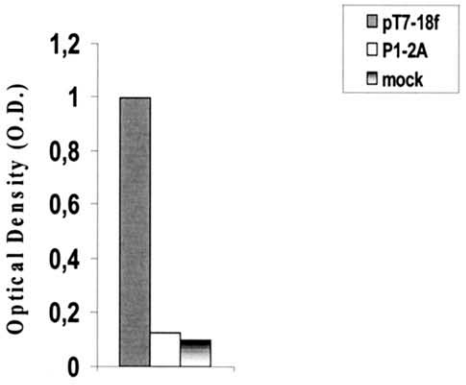

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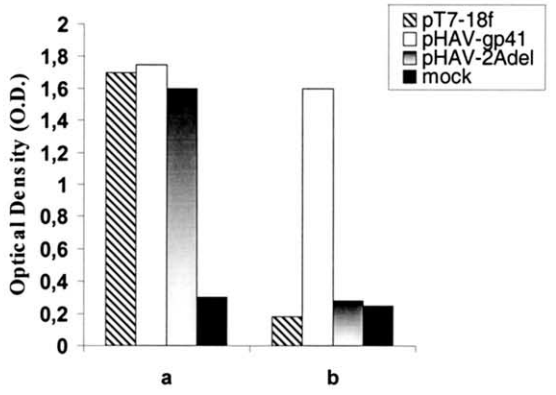

D

Fig. 1

humans and experimental animals (Muster et al., 1994). The HIV sequence was positioned into the full-length HAV pT7-18f in place of the nucleotides coding for the amino acid residues 44–53 of 2A (based on the suggested N-terminus of 2A, Probst et al., 1997), maintaining the translational reading frame of the polyprotein. The resulting construct was named pHAV-gp41. A mutant cDNA carrying the deletion of amino acids 44–53 of 2A (pHAV-2Adel) was also constructed. Mutagenesis was carried out by the polymerase chain reaction (PCR) using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers reported in Fig. 1A. The cycling parameters used were: one cycle at 95 °C for 30 s, followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 18 min (2 min/kb of plasmid length) for 18 cycles. The constructs were sequenced by the dideoxy-termination method to verify their identity and the correctness of the reading frame. The amino acid sequence of the mutated region of 2A is shown in Fig. 1B.

The ability of the recombinant cDNA to retain its competence for expression, regardless of the presence of a foreign sequence, was examined by transient expression of pHAV-gp41 in COS7 and Huh-T7 cells. Eighty percent confluent cell monolayers grown in wells of 35 mm diameter were transfected with 1 μg of purified cDNA and 9 μl of LipofectAmine according to the instructions of the manufacturer (Life Technologies, Inc.). One to 3 h after transfection, infection with the replication-deficient vaccinia virus MVA-T7 providing T7 RNA polymerase to transcribe the HAV RNA (Wyatt et al., 1995), was performed. After 30 min incubation, the medium was replaced by 2 ml of Dulbecco's modified Eagle's medium supplemented by 10% fetal calf serum, and incubation was continued for 18 h. Then the cells were scraped off the plate in 300 μl of phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20. Cells trans-infected with pT7-18f and pHAV-2Adel were used as control. To examine the expression level of the constructs, the crude extracts of trans-infected cells were clarified by centrifugation at $13\,000 \times g$ and the supernatants were analyzed by a particle-specific enzyme linked immunosorbent assay (ELISA), with the neutralizing monoclonal antibody 7E7 as capture and 7E7 conjugated to peroxidase (Mediagnost, GMBH) as detection antibody. This antibody is directed against an immunodominant antigenic site on the viral capsid (Ping and Lemon, 1992) and is specific in recognizing processed and assembled structural proteins but not their unprocessed precursors. The latter feature was demonstrated in the absence of reactivity with the uncleaved precursor polypeptide P1-2A, obtained by expression of the construct pEXT7-HM/HAS-P1-2A (Probst et al., 1999) in the presence of MVA-T7 (Fig. 1C). Fig. 1D (part a) reports the mean optical densities obtained in multiple replicates for all constructs and shows that both pHAV-gp41 and the deletion mutant (pHAV-2Adel) can be expressed in this system with an efficiency similar to the parental construct pT7-18f. These data agree with those reported by Harmon et al. that deletions in the central portion of 2A cause only minor effects on the viral growth and they suggest that replacement of HAV sequences with seven amino acids of gp41 does not interfere with viral protein synthesis, processing and particle formation. To determine whether the gp41 epitope was expressed in this system, an enzyme immunoassay was performed on the crude extracts with the human monoclonal antibody CHO2F5 (2F5), a neutralizing antibody that recognizes the amino acid sequence ELDKWA in gp41 of HIV-1 (Muster et al., 1994), as capture antibody and 7E7 conjugated to horse radish peroxidase, as detecting antibody (Fig. 1D, part b). The optical density values obtained for pHAV-gp41 in comparison to the absence of antigenic reactivity of pHAV-2Adel and pT7-18f demonstrate the specificity of the ELISA for the gp41 epitope and suggest that the foreign epitope is exposed on the capsid surface.

To determine whether the recombinant particles correctly expressed and processed the viral proteins containing the insertion of foreign amino acids, the cell lysates were evaluated by immunoblot analyses with rabbit antisera against VP1 and against 2A of HAV and the monoclonal antibody anti-gp41 (2F5), after separation of the crude cellular extracts on a discontinuous 12% SDS-polyacrylamide minigel. Expression of the con-

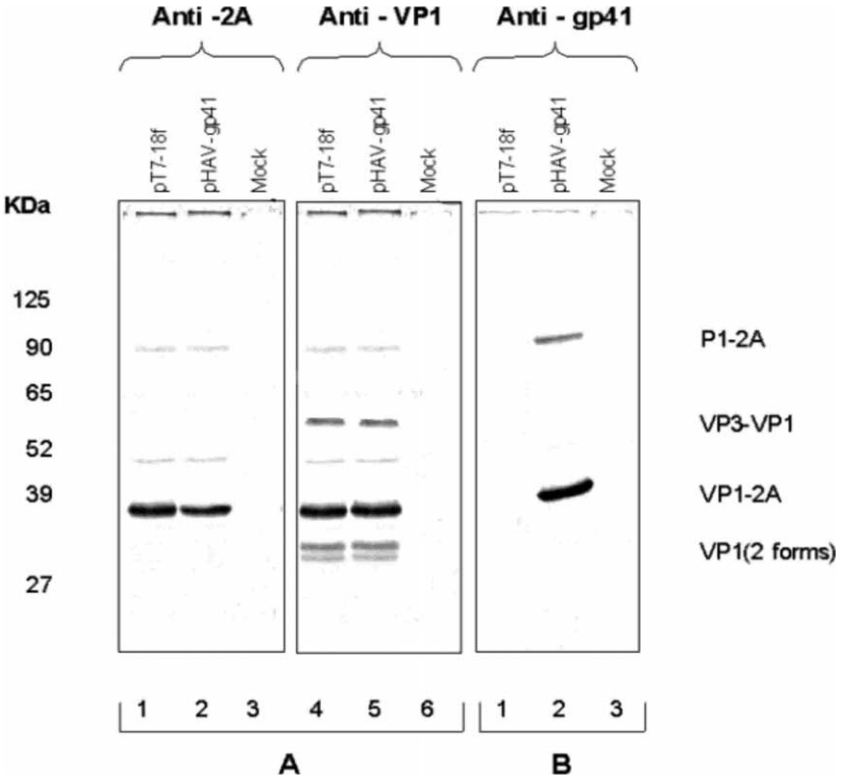

Fig. 2. Immunoblot analysis of HAV proteins expressed in COS7 cells transfected with cDNAs or mock transfected and infected with vMVA-T7. After 18 h, cells were harvested and proteins were fractionated on 12% SDS-polyacrylamide gel, transferred on nitrocellulose and probed with (A) rabbit antisera against 2A (lanes 1–3) and against VP1 (lanes 4–6) or (B) human monoclonal antibody 2F5. Sizes of protein molecular standards run on the same gels are indicated on the left.

structs resulted in products of the expected molecular masses which were detected by anti-2A and anti-VP1 antibodies (Fig. 2A). The chimeric construct pHAV-gp41 was found to produce and process viral capsid proteins in a manner similar to the parental plasmid pT7-18f (Fig. 2A, lane 1 and 2, 4 and 5). When the two lysates were probed with the anti-epitope antibody, two proteins with apparent molecular weights corresponding to P1–2A and VP1–2A were detected in the pHAV-gp41 extract (Fig. 2B, lane 2). This immunoreactivity was absent in the pT7-18f extract and in the mock-infected cell extract (Fig. 2B, lanes 1 and 3, respectively). These results demonstrate that P1-2A and VP1-2A of pHAV-gp41 contain both HAV and gp41 immunoreactivity and that the central region of 2A can be replaced by foreign sequences without abrogating P1-2A processing.

The results obtained with the antibodies 7E7 and 2F5 strongly suggested the formation of chimeric viral particles in COS7 cells trans-infected with pHAV-gp41. To verify the formation of chimeric particles, rate zonal centrifugation on a sucrose gradient was performed with extracts generated by the expression of pHAV-gp41. pT7-18f was used as negative control. After lysis of 1×10^7 cells in 1 ml of NPT (100 mM NaCl, 0.5% (v/v) Nonidet P-40, 10 mM Tris, pH 7.3) the extracts were clarified by centrifugation at $1300 \times g$. Ultracentrifugation was performed in a Beckman SW41 rotor at 35 000 rpm, 4 °C for 3 h, through a linear 5–30% (w/w) sucrose gradient in 100 mM NaCl, 10 mM Tris, pH 7.3.The fractions (0.5 ml each) were collected from the top of the tube and analyzed by ELISA. The sucrose concentration of each fraction was determined with a refract-

ometer. Sedimentation standards were obtained from a parallel gradient on which HAV particles from infected cells were separated. Rate zonal centrifugation showed that the major antigenic product detected by 7E7 sedimented with approximately 70S, corresponding to empty capsids. Only low amounts of antigenicity were demonstrated in fractions corresponding to faster-sedimenting particles (complete virions), perhaps due to their low production in this system (Fig. 3A). The fractions were also analyzed for their immunoreaction with the epitope-specific antibody, using antibody 2F5 as capture and antibody 7E7 as detection antibody (Fig. 3B). Strong 2F5 reactivity against the recombinant VP1-2A-gp41 antigen produced by expression of pHAV-gp41 was found in the pentamer and empty capsid fractions and, in very low amount, in the virion fractions from cells transfected with pHAV-gp41 but not in those transfected with pT7-18f (Fig. 3B) or pHAV-2Adel (data not shown).

To confirm those results by direct visualizing the surface exposure of the foreign epitope, solid phase immunoelectron microscopy was performed. Formvar-carbon coated nickel grids were placed for 30 min on 50 μl droplets of PBS, pH 7.4

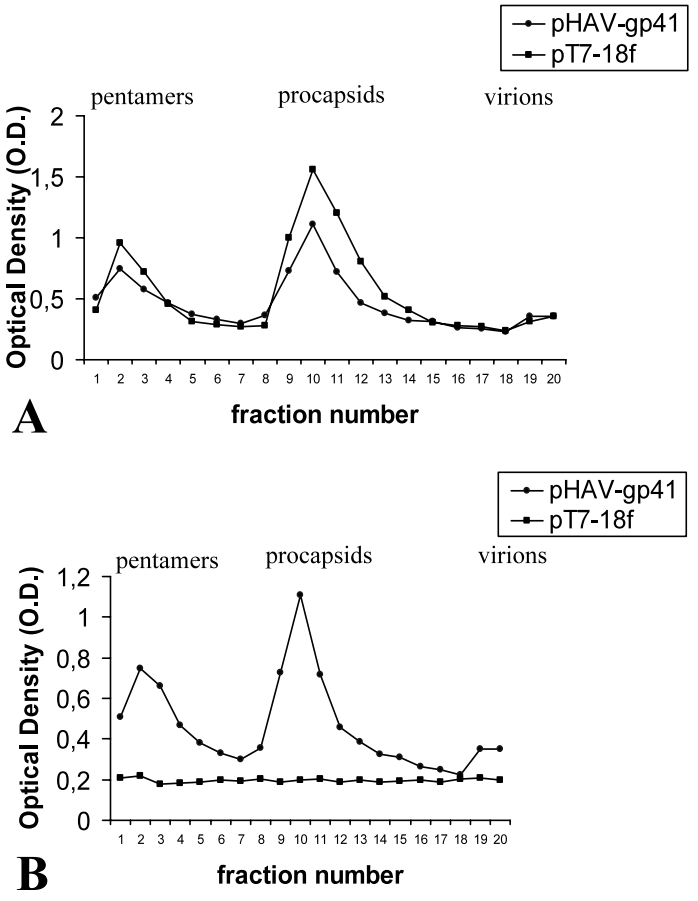

Fig. 3. (A) HAV particle formation after expression of pHAV-gp41and pT7-18f. Lysates of COS7 cells expressing pHAV-gp41 or pT7-18f were separated on 5–30% sucrose gradient (w/w) and the fractions were analyzed by ELISA, using the monoclonal antibody 7E7 as capture and detection antibody. (B) Chimeric HAV particle formation after expression of pHAV-gp41. The same fractions were analyzed by ELISA for the presence of the foreign epitope gp41 on HAV capsids, using the human monoclonal antibody 2F5 as capture and monoclonal antibody 7E7 as detection antibody.

containing protein A (5 μg/ml, Sigma). After rinsing for 1 min on two droplets of PBS, the grids were incubated for 1 h on droplets containing monoclonal 7E7, polyclonal HAV anti-2A (Probst et al., 1997) and 2F5 anti-gp41 human antibodies, respectively. After rinsing two times with PBS as above, the antibody-coated grids were placed on lysates of mock- or cDNA trans-infected Huh-T7 cells. After 2 h at room temperature, the preparations were negatively stained by incubating the grids for 1 min with 1% phosphotungstic acid (PTA). Finally, the grids were air-dried on a filter paper, and examined with a Philips EM 400 at 60 kV. As expected, empty particles were detected by the anti-HAV and anti-2A antibodies in all the supernatants of HAV-expressing lysates (pHAV-gp41, pHAV-2Adel and pT7-18f) (Fig. 4, a–b, d–e, g–h), while recombinant particles, expressing the gp41 epitope at their surface, could be demonstrated only on the grids incubated with the lysates of cells trans-infected with pHAVgp41 and coated with 2F5 antibody (Fig. 4, f). Absence of particles was observed on the grids incubated with the mock cell lysates, independently of the coating antibody. Taken together, these data demonstrate the specificity of the reactions and the presence of the foreign epitope on the surface of the chimeric empty particles.

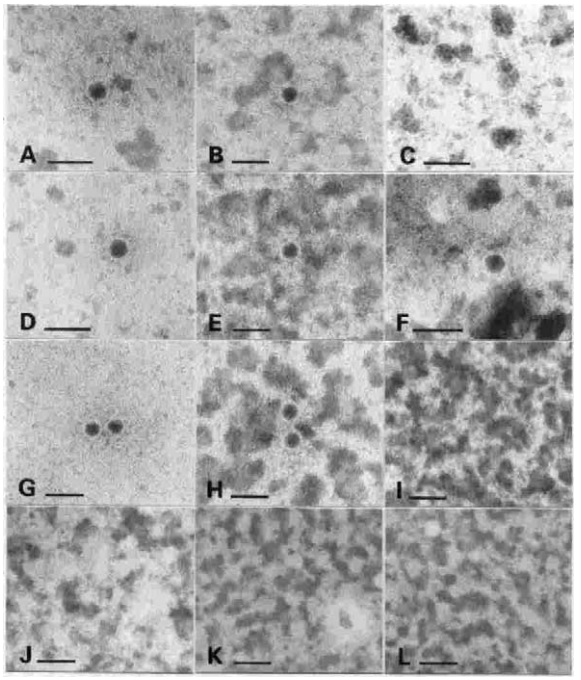

Fig. 4. Solid phase immunoelectron microscopy (SPIEM) after transient expression of pHAV-2Adel (a, b and c), pHAV-gp41 (d–f) and pHAV18f (g–i) in Huh-T7 cells. One day after cDNA transfection and infection with MVA-T7, the cells were lysed and the supernatants analyzed by SPIEM using monoclonal 7E7 (a, d, g and j), polyclonal HAV anti-2A (b, e, h and k) or monoclonal HIV anti-gp41 (c, f, i and l) antibodies, respectively. Mock: mock-infected Huh-T7 cells (j–l).

Picornaviruses have been a successful target of antiviral prevention. In particular, the high efficacy and safety of poliovirus and HAV vaccines, together with their ability of eliciting high-titre specific humoral and cellular immune responses, have been demonstrated in various studies (Katrak et al., 1991; Cederna et al., 1999; Hollinger and Emerson, 2001). The possibility of using chimeric picornaviruses as vectors of foreign epitopes has evoked considerable interest in the last years and, in fact, different members of the family have been tested for this ability. Recently, the insertion of foreign sequences at the N-terminus of the HAV polyprotein and the accumulation of larger-than-VP0 proteins, containing the extraneous peptides, as empty capsid in the infected cells has been reported. However, the exposure of those epitopes on the outer surface of the capsids has not been demonstrated (Zhang and Kaplan, 1998). In this paper we report the feasibility of incorporating a foreign amino acid sequence into a protein component on the surface of HAV particles, without preventing capsid assembly or abolishing its antigenicity. We demonstrate that the chimeric HAV particles have the antigenic property of the inserted epitope, in addition to their HAV immunoreactivity. The results reported here show that protein 2A is functioning as assembly signal in the presence of foreign amino acids substituting amino acids of its central portion. Protein 2A carrying the foreign sequence produced empty particles displaying the epitope on their surface, which supports the recent observation that protein 2A is present (as VP1–2A) on the surface of empty particles and of a limited amount of infectious particles (Borovec and Anderson, 1993; Probst et al., 1999). Although at present we have expressed only a short sequence derived from HIV-1 and we

do not know the size limitation of the peptide which can be inserted in 2A, this system could be useful for the expression of a variety of heterologous peptide sequences. These recombinant particles might be a good starting point for the design and production of HAV chimeric vaccines and/or antigens for diagnostic uses that might contain antigenic epitopes of viral or bacterial origin. Further studies are in progress to determine the antigenicity of the particles in mice and to test various infectious chimeric HAV constructs.

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