

Effect of the 5' non-translated region on self-assembly of hepatitis C virus genotype 1a structural proteins produced in insect cells

Christel Girard,¹ Marc Ravallec,¹ Marcel Mariller,¹ Jean-Pierre Bossy,¹ Annie Cahour,² Miguel López-Ferber,¹ Gérard Devauchelle,¹ Geneviève Inchauspé³ and Martine Duonor-Cérutti¹

Correspondence

Martine Duonor-Cérutti
duonor@ensam.inra.fr

¹Station de Pathologie Comparée, INRA-CNRS, 30380 Saint-Christol-lès-Alès, France

²CERVI Laboratoire de Virologie, UPRES EA 2387 Hospital Pitié-Salpêtrière, 83 boulevard de l'Hôpital, 75651 Paris Cedex 13, France

³Unité Mixte CNRS-bioMérieux, École Normale Supérieure, 69000 Lyon, France

The effect of the 5' non-translated region (5'NTR) on hepatitis C virus (HCV) morphogenesis in insect cells is investigated in this study. Expression in baculovirus-infected cells of a sequence encoding the C and E1 structural proteins under the control of the very late promoter P10 (AcSLP10-C-E1) led to the synthesis of C and C-E1 complexes, essentially found in dense reticular material associated with the ER and sedimenting at a density of 1·24–1·26 g ml⁻¹. Addition of the 5'NTR upstream of the C-E1 sequence (AcSLP10-5'NTR-E1) prevents translation from the initiating codon, probably because of the presence of five AUG codons in this sequence. When cells were co-infected with these two viruses, virus-like particles (VLPs) were found in the cytoplasm. The size and shape of these VLPs were variable. Concomitantly, a shift in the sedimentation profile from 1·24–1·26 to 1·15–1·18 g ml⁻¹ was observed, suggesting an association of C/E1 with the ER membrane. A unique vector was then constructed bearing a mutated 5'NTR (mutation of the five AUGs) and the sequence encoding all of the structural proteins and part of NS2 (5'NTRm-C-E1-E2-p7-NS2Δ). Translation of structural proteins was restored and electron microscopic observation of a cytoplasmic extract showed the presence of icosahedral particles with a density of 1·15–1·18 g ml⁻¹.

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INTRODUCTION

Known for a long time as non-A/non-B hepatitis, hepatitis C has been known for about 20 years (Feinstone *et al.*, 1975). The positive single-stranded RNA virus hepatitis C virus (HCV) is a member of the family *Flaviviridae* (Choo *et al.*, 1989; Kaito *et al.*, 1994). The genome varies little in length between genotypes, being around 9·6 kb, and consists of a unique open reading frame (ORF) encoding a 3011–3033 aa polyprotein, surrounded at its 5' end by an approximately 340 nt non-coding region and at its 3' end by a non-translated region of variable size (5'- and 3'-NTR). The general genome organization is as follows: 5'-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3' (Grakoui *et al.*, 1993). Structural protein sequences are located on the 5' part of the genome and represent a quarter of the entire ORF; the remaining part encodes non-structural proteins.

No mammalian cell lines are known that allow efficient replication of this virus (Bertolini *et al.*, 1993; Shimizu *et al.*, 1993; Shimizu & Yoshikura, 1994; Tagawa *et al.*, 1995; Kato *et al.*, 1995, 1996; Nakajima *et al.*, 1996). Infection of

primary cultures of either hepatocytes or lymphocytes resulted only in limited virus replication with, in some cases, release of viral particles into the culture medium (Ito *et al.*, 1996; Iacovacci *et al.*, 1997).

The absence of a suitable cellular model for HCV propagation has led to the use of various heterologous expression systems. Among these, the baculovirus–insect cell system has proved useful for analysis of the production and maturation of HCV structural proteins (Matsuura *et al.*, 1992, 1994; Lanford *et al.*, 1993; Hsu *et al.*, 1993; Hüsey *et al.*, 1996a, 1997) and non-structural proteins (Hirowatari *et al.*, 1993; Nishihara *et al.*, 1993; Overton *et al.*, 1995; Suzuki *et al.*, 1995; Hwang *et al.*, 1997). In addition, this system is a valuable tool (i) for the production of large amount of antigens for immunological studies (Chiba *et al.*, 1991; Chien *et al.*, 1992; Inoue *et al.*, 1992; Basset *et al.*, 1999) and (ii) for the investigation of viral protein interactions and assembly (Hüsey *et al.*, 1996b; Wang *et al.*, 1997). Using recombinant baculovirus, Baumert *et al.* (1998) showed that pseudovirus particles could be generated.

The 5'NTR is a highly conserved region, 341–349 nt in length, thought to fold into a complex secondary and tertiary structure comprising four major domains, I to IV, a pseudoknot and a helical structure (Brown *et al.*, 1992; Honda *et al.*, 1996; Smith *et al.*, 1995; Le *et al.*, 1995; Wang *et al.*, 1995). The 5'NTR of HCV contains several AUG codons (three to six, depending on the genotype), two of which (at positions 85 and 215) are highly conserved between HCV and pestiviruses. This structure functions as an internal ribosome entry site (IRES), probably as a type II element (Reynolds *et al.*, 1995). It also binds different cellular factors such as polypyrimidine-tract-binding (PTB) protein (Ali & Siddiqui, 1995) and proteins of 25, 87 and 120 kDa (Fukushi *et al.*, 1997; Yen *et al.*, 1995), which are thought to be important (except for p87) for IRES function. The core protein is an RNA-binding protein with RNA-binding domains localized to the N-terminal 75 amino acids (Santolini *et al.*, 1994), and a specific interaction between the core protein and the 5' NTR has been reported (Shimoike *et al.*, 1999).

Recombinant baculoviruses able to transcribe wild-type HCV RNAs, RNAs mutated in the 5'NTR or with this region deleted were used to analyse the role of this domain in the assembly process. We present evidence of the involvement of the 5'NTR in the self-assembly of HCV structural proteins in insect cells.

METHODS

Cells and viruses. The *Spodoptera frugiperda* cell line Sf9 (Vaughn *et al.*, 1977) was maintained at 28 °C in TC100 medium supplemented with 5% fetal calf serum and used for propagation of both wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) clone 1.2 (Croizier *et al.*, 1988) and recombinant baculoviruses. For infection, cells (4×10^6 per 25 cm² flask) were inoculated with a viral suspension at an m.o.i. of 10 p.f.u. per cell. After 1 h adsorption at room temperature, the viral inoculum was removed and fresh culture medium was added. Cells were further incubated at 28 °C.

Construction of plasmids. Two baculovirus transfer vectors, p118, derived from pGm8022 (Chaabihi *et al.*, 1993; a kind gift of M. H. Ogliaastro), and pGmAc118, were used to generate recombinant viruses. pGmAc118 has a *Bgl*II linker inserted at a deletion in the polyhedrin gene sequence spanning nucleotides –10 to +483 (position +1 is the first nucleotide of the polyhedrin ATG start codon). This vector presents a deletion in the polyhedrin promoter sequence in order to decrease the expression level of the foreign gene (Gaymard *et al.*, 1996). Different constructs (Fig. 1) were made using the cDNA of the HCV-H strain, genotype 1a (Inchauspé *et al.*, 1991; Simmonds *et al.*, 1993).

(i) **p118-C-E1.** The sequence encoding the first 88 aa of the capsid protein was amplified using the primers 5'-CCGGGAGCTCTCGTAGACTCTGCACC-3' (forward) and 5'-CTCATTGCCATAGAGGGGCC-3' (backward) and the plasmid pMINK containing the complete cDNA of the HCV-H strain (Inchauspé *et al.*, 1991) as DNA matrix. The fragment was cloned into a modified pUC plasmid (a *Bgl*II site was inserted in place of the *Sac*I site) giving pUC-CA. A 902-bp *Asp*718–*Sall* fragment encoding the C-terminal part of the capsid and the first 191 aa of E1 was excised from pMINK and inserted into the *Asp*718–*Sall* sites of pUC-CA. The last

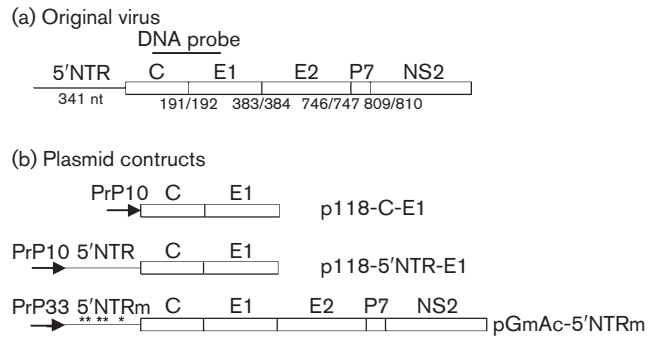


Fig. 1. Plasmid constructs. (a) HCV-H strain (genotype 1a) genomic organization. Positions of cleavage sites of the structural polyprotein by cellular signalases are indicated under the diagram. (b) Schematic representation of HCV sequences inserted into the baculovirus transfer vectors: p118-C-E1 (encompassing nt +1 to 1146), p118-5'NTR-E1 (nt –341 to +1016) and pGmAc-5'NTR_m-NS2Δ (nt –341 to +2895). PrP10 and Pr33 are P10 and polyhedrin gene promoters, respectively. Mutated AUG codons in the 5'NTR are indicated by asterisks; details are given in Table 1.

codon of E1 and a stop codon were finally inserted via a *Sall* linker before subcloning into the p118 transfer vector, resulting in a plasmid called p118-C-E1. The *Sall* linker was reconstituted after hybridization of the oligonucleotides 5'-GTCGACGCGTAAAGATCTGAGCT-3' and 5'-CAGATCTTACGCGTCGACAGCT-3'.

(ii) **p118-5'NTR-E1.** A 1369-bp *Bam*HI fragment containing the 5'NTR and the sequence encoding the capsid protein and 149 aa of the glycoprotein E1 was inserted into the unique *Bgl*II site of p118-stop plasmid (p118 with a stop codon downstream of the *Bgl*II site), giving a plasmid called p118-5'NTR-E1.

(iii) **pGmAc-5'NTR_m-NS2Δ.** A DNA fragment of 256 bp containing a modified 5'NTR with mutations of the five silent AUG codons (5'NTR_m) was reconstituted using a set of 14 overlapping oligonucleotides. Mutations were chosen in order to create convenient sites for subsequent cloning steps, but not to preserve the secondary structure of the 5'NTR (Table 1). A 2987-bp *Nhe*I–*Bgl*II fragment containing 93 bp of the wild-type 5'NTR and the sequence encoding C, E1, E2 p7 and 158 aa of NS2 was introduced downstream of the mutated 5'NTR in the transfer vector pGmAc118. A *Bgl*II linker inserted in the NS2 gene introduced an in-frame stop codon, resulting in plasmid pGmAc-5'NTR_m-NS2Δ.

Table 1. Mutation of the 5'NTR

Positions of AUG codons are given as the position of the adenosine residue. Mutated bases are given in bold.

AUG position	Original sequence	Mutated sequence
–328	ugAUGgg	ugAUCgg
–310	ccAUGaa	ccAUCaa
–257	ccAUGgc	ccUAGgc
–246	guAUGgag	guAUCgag
–127	caAUGcc	cgAGGcc

Cotransfection and purification of recombinant baculoviruses.

Sf9 cells were cotransfected with viral DNA and recombinant transfer vector DNA by the lipofection method (Felgner & Ringold, 1989) (DOTAP; Roche). Recombinations into the P10 locus were obtained after cotransfection with transfer vector and AcSLP10 DNA (Chaabihi *et al.*, 1993). Screening and purification of recombinant viruses were carried out as described by Summers & Smith (1987). The two recombinant viruses generated with p118-C-E1 and p118-5'NTR-E1 were named AcSLP10-C-E1 and AcSLP10-5'NTR-E1. The recombinant virus obtained after recombination between pGmAc-5'NTRm-NS2Δ and DNA purified from wild-type virus was named AcPH-5'NTRm-NS2Δ.

Protein analysis. Infected cells were collected and washed with cold PBS and resuspended in reducing sample buffer (Laemmli, 1970). After boiling (100 °C for 5 min), protein samples were resolved by 12.5% SDS-PAGE under denaturing conditions (Laemmli, 1970). The proteins were then transferred onto a nitrocellulose filter (BAS 85, 0.45 μm; Schleicher & Schuell) with a semi-dry electroblotter apparatus (Ancos). The nitrocellulose membrane was stained with Ponceau red (Ponceau-S; Sigma) and subsequently blocked with a solution of Tris-buffered saline (TBS; 0.05 M Tris/HCl, pH 7.4, 0.2 M NaCl) containing 0.05% Tween 20 and 5% dry skimmed milk (TBS-sat). HCV capsid protein was detected using the mouse monoclonal antibody 27D5C5 (1:500 in TBS-sat) as the primary antibody and a rabbit anti-mouse IgG-horseradish peroxidase conjugate as the secondary antibody (1:1000 in TBS-sat; Sigma). Glycoprotein E1 was detected by using a rabbit polyclonal anti-E1 hyperimmune serum (1:200 in TBS-sat) as the primary antibody and a goat anti-rabbit IgG-horseradish peroxidase conjugate or a mouse anti-rabbit IgG-alkaline phosphatase conjugate as the secondary antibody (1:10 000 in TBS-sat; Sigma). Glycoprotein E2 was detected by using a rabbit polyclonal anti-E2 hyperimmune sera (1b8356 or WU105, 1:500 in TBS-sat) as the primary antibody and a goat anti-rabbit IgG-horseradish peroxidase conjugate or a mouse anti-rabbit IgG-alkaline phosphatase conjugate as the secondary antibody. Blots were also probed with anti-HCV-positive human serum (1:500 in TBS-sat) containing antibodies against the three structural proteins and revealed using an anti-human Ig-alkaline phosphatase conjugate. Immunoreactive bands were visualized by using either 1-amino-3-ethyl carbazol or NBT/BCIP as chromogenic agents.

Evaluation of glycosylation. Tunicamycin at a final concentration of 10 μg ml⁻¹ was added to the cell culture medium at 7 h post-infection (p.i.). Infected cells were collected at 52 h p.i. and protein samples were analysed by Western blotting as described above. As a control, cells were infected under the same conditions but without tunicamycin.

Isolation of total RNA and Northern blot hybridization.

Isolation of total RNA was performed using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions followed by chloroform extraction and isopropanol precipitation. Total RNA was resuspended in RNase-free water and quantified by spectrophotometry. After addition of RNA sample buffer (Promega), 10 μg of each sample was electrophoresed on a 1% non-denaturing agarose gel and transferred to a nylon membrane (Roche). Blots were hybridized with a 417-bp probe (encompassing 205 bp at the 3'-end encoding the capsid protein and 212 bp at the 5'-end encoding glycoprotein E1) radio-labelled with ³²P using a random-primed DNA-labelling kit (Roche).

Isopycnic centrifugation on sucrose gradients. At 48 or 72 h p.i., cells were resuspended in cold low-salt buffer (10 mM HEPES/NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP40) with protease inhibitors (*O*-Complete; Roche) and were disrupted with a potter (Bioblock Scientific). After low-speed centrifugation (10 min at 4 °C and 10 000 g), the supernatant was layered onto a linear 20–60% (w/w) sucrose gradient (in TEN buffer; 50 mM

Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA) and subjected to isopycnic centrifugation for 24 h at 4 °C and 150 000 g. Fractions (500 μl) were collected from the bottom and analysed by Western blotting. In some experiments, 1% NP40 was added to the crude protein extracts before layering onto the sucrose gradient.

Particles present in the cell culture supernatant were isolated as follows: after low-speed centrifugation (10 min at 4 °C and 10 000 g) the supernatant was pelleted (1–2 h at 4 °C and 100 000 g). This pellet was then resuspended in TEN (containing protease inhibitors) and subjected to isopycnic sucrose gradient centrifugation. Under these conditions, the supernatant was concentrated 20- to 100-fold. Fractions (500 μl) were collected and analysed as described below.

Electron microscopy. Antigen-positive fractions isolated by isopycnic centrifugation were examined directly with a Zeiss EM 10C/CR electron microscope after negative-staining with uranyl acetate. For morphological studies, cells were harvested at 48 or 72 h p.i. by centrifugation at 1000 g for 5 min, washed with PBS and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After post-fixation with 2% osmium tetroxide in the same buffer, cells were dehydrated in a graded series of ethanol solutions followed by propylene oxide and embedded in Epon 812 resin (Fluka). Ultrathin sections were stained with uranyl acetate and lead citrate before observation.

For immunogold labelling, cells were fixed with 4% paraformaldehyde/0.25% glutaraldehyde in PBS. Two different sample preparation methods were then used. (i) Following dehydration in ethanol solutions, cells were embedded in Unicryl resin (TEBU) and polymerized under a UV source at 4 °C for 2 days; ultrathin sections were cut with a diamond knife in an LKB Ultratome III apparatus. (ii) Preparation of cryosections was performed as described by Reggio & Boller (1989). Briefly, after washing in PBS/50 mM NH₄Cl, cells were embedded in gelatin, kept on ice for 2 h and allowed to infuse for at least 30 min in 2.3 M sucrose (in 0.1 M phosphate buffer, pH 7.2). After rapid freezing of the sample in liquid nitrogen, ultrathin sections were cut using an RMC Ultracryotom MT-7 apparatus. Ultrathin sections prepared as described in (i) or (ii) were collected on nickel grids and pre-incubated for 10 min in NH₄Cl (50 mM in PBS) before saturation for 30 min in PBS containing 1% casein. Preparations were incubated for 2 h with anti-HCV patient serum (1:50 in 0.1% casein/PBS), washed three times with PBS and probed with gold-conjugated anti-human Ig (1:50 in 0.1% casein/PBS). After several washes with PBS and distilled water, sections were stained with uranyl acetate and observed. Cryosections were covered with a film of 70% methylcellulose in uranyl acetate.

Alternatively, infected cells were immunolabelled after permeabilization with Triton X-100 (1–3% in PBS). Cells were incubated with the anti-capsid polyclonal antibody WU105 (1:100 in 0.1% casein/PBS) and gold-conjugated secondary anti-rabbit IgG (10 nm; Biocell) diluted 1:50 in 0.1% casein/PBS before subsequent treatment for morphological analysis, as described before.

Cell lysis control. Lysis of infected cells was assessed using the Promega LDH detection kit (Cytotox 96 non-radioactive cytotoxicity assay).

RESULTS

The 5'NTR placed *in cis* towards the structural polyprotein sequence inhibits translation of C–E1

SDS-PAGE and immunoblotting analysis of proteins expressed in cells infected with AcSLP10-C-E1 showed the

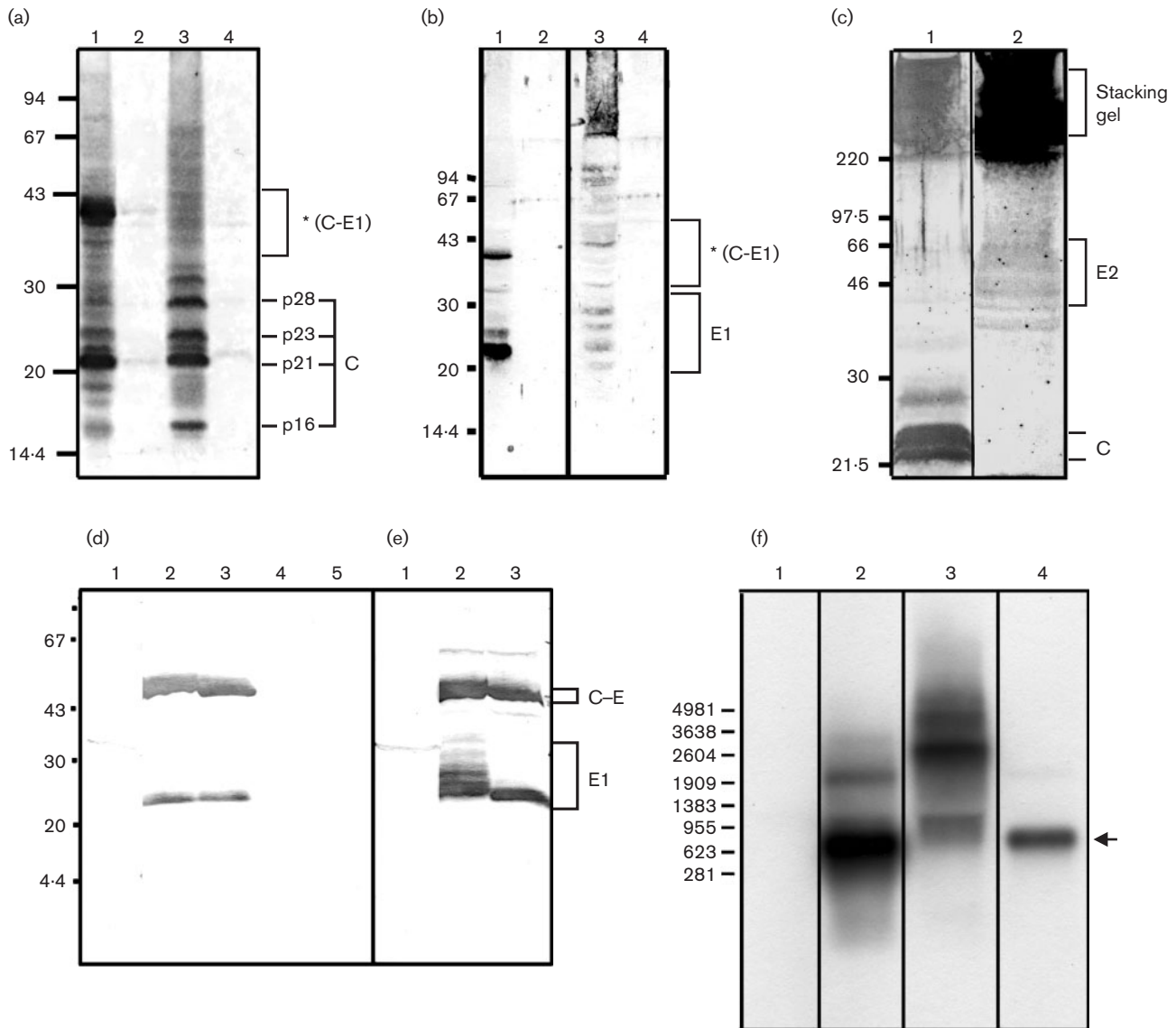


Fig. 2. Analysis of expression of HCV structural proteins in Sf9 cells. (a–b) Sf9 cells were infected with AcSLP10-C-E1 (lanes 1), AcSLP10-5′NTR-E1 (lanes 2), AcPH-5′NTR_m-NS2Δ (lane 3) or wild-type AcMNPV (lane 4). Cells were harvested at 48 h p.i. and cell lysates were analysed by SDS-PAGE and immunoblotting with an anti-HCV-positive human serum (a) or a polyclonal anti-E1 serum (b). Asterisks indicate precursor proteins. (c) Sf9 cells infected with AcPH-5′NTR_m-NS2Δ were collected at 72 h p.i. Cell lysates were analysed by SDS-PAGE and immunoblotting with polyclonal anti-capsid serum #57 (lane 1) and a polyclonal anti-E2 serum 1b8356 (lane 2). (d–e) Effect of tunicamycin on the synthesis of recombinant proteins. Sf9 cells infected with wild-type virus (lanes 1) or with AcSLP10-C-E1 (lanes 2–5) were treated (lanes 3 and 5) or not (lanes 2 and 4) with tunicamycin. At 7 h p.i., the supernatant was replaced with fresh culture medium containing 10 μg tunicamycin ml⁻¹ or with medium alone as a control. At 52 h p.i., cells were harvested and proteins from crude cell extracts (lanes 1–3) or from cell culture supernatants (lanes 4 and 5) were analysed by Western blotting. Membranes were incubated with anti-capsid monoclonal antibody (27D5C5) (d) or anti-E1 polyclonal serum (e). HCV-specific proteins are indicated on the right and positions of molecular mass markers (in kDa) on the left. (f) Analysis of RNA synthesis in cells infected with recombinant viruses. Total RNA isolated from cells infected with wild-type AcMNPV (lane 1), AcSLP10-C-E1 (2), AcPH-5′NTR_m-NS2Δ (3) and AcSLP10-5′NTR-E1 (4) was analysed by Northern blot. Hybridization was carried out with a ³²P-labelled probe overlapping capsid and E1 sequences. The arrowhead indicates the RNA transcript produced in cells infected with AcSLP10-5′NTR-E1. Positions of molecular size markers (in nt) are shown on the left.

presence of a major 21 kDa product and two minor bands of 23 kDa, probably corresponding to the capsid protein uncleaved at its C terminus (Fig. 2a, lane 1). Three additional products of lower (16 and 18 kDa) and higher (22 kDa) molecular mass were revealed using the anti-HCV-positive human serum. A 42–55 kDa band was labelled with the anti-E1 antibody (Fig. 2b, lane 1), suggesting that it results from inefficient processing of the precursor polyprotein C–E1. The glycosylation status of these molecules was analysed by infecting cells in the presence of tunicamycin, a fungal antibiotic that blocks N-glycosylation. Under these conditions, the high molecular mass forms of E1 and C–E1 disappeared (Fig. 2d, e), confirming the glycosylated status of these products. These different structural proteins were never found in the cell culture medium.

Transmission electron microscopy (TEM) observation of AcSLP10-C-E1-infected cells revealed a dense reticular material with perinuclear sparing, characteristic of proteins associated with the ER (Fig. 3b). Labelling of cells with the anti-HCV-positive human serum showed colocalization of immunoreactive materials with these dense intracytoplasmic structures (Fig. 3c), suggesting that the expressed proteins accumulate in the ER.

No production of the capsid or of the E1 glycoprotein was detected at 48 h p.i. in cells infected with AcSLP10-5'NTR-E1 compared with AcSLP10-C-E1 (Fig. 2a, b; compare lanes 1 and 2). Using a specific DNA probe overlapping the sequence encoding the C-terminal part of the capsid and the N-terminal part of E1, a specific transcript with the expected size (about 750 nt) was detected in cells infected with 5'NTR-C-E1 but in smaller amounts compared with AcSLP10-C-E1. The failure to detect the products with this vector may be the consequence of this lower transcription. More likely, as reported by Croizier *et al.* (2000), who expressed structural proteins of *Junonia coenia* densovirus under the control of the P10 promoter in the baculovirus system, the polypeptide starting at the first AUG immediately downstream from the P10 promoter was always the most abundantly expressed in infected cells. So, previous initiation(s) at upstream AUGs present in the 5'NTR would easily explain the absence or very low synthesis of the genuine products C and E1.

TEM observations of cells infected with AcSLP10-5'NTR-E1 showed all of the characteristic features found in cells infected with a recombinant baculovirus, nuclear hypertrophy, the presence of large amounts of virus in the nucleus and the absence of polyhedrin or P10 structures

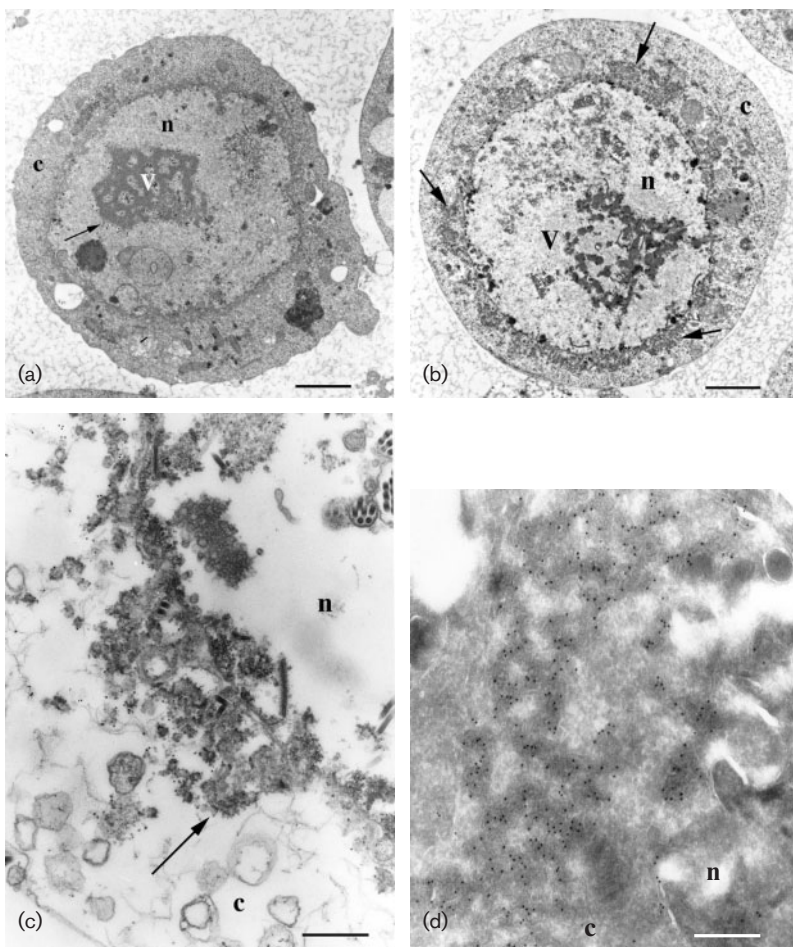


Fig. 3. Electron microscopy analysis of cells infected with AcSLP10-5'NTR-E1 (a) or with AcSLP10-C-E1 (b–d). n, Nucleus; c, cytoplasm; V, virogenic stroma. (a) Baculovirus particles, indicated by an arrow, are observed in the nucleus. Bar, 2.6 μ m. (b) Accumulation of dense perinuclear material. Bar, 2.6 μ m. (c) Observation at higher magnification of dense perinuclear material. Bar, 350 nm. (d) Cryosection electron microscopy. Immunogold labelling with an anti-HCV-positive human serum shows colocalization of HCV structural proteins with the dense perinuclear material. Bar, 235 nm.

(Fig. 3a). Neither reticulate cytoplasmic retention nor aggregate formation were noticed, and no intracellular proteins were immunolabelled with the anti-HCV-positive human serum (data not shown), confirming the Western blot results.

Trans-acting effect of the 5'NTR on capsid protein multimerization

The interaction between the 5'NTR and capsid protein was analysed by coexpressing the two baculoviruses AcSLP10-5'NTR-E1 and AcSLP10-C-E1, thus combining a 5'NTR-containing, non-translatable transcript with a 5'NTR-deleted, translation-competent transcript. Isopycnic centrifugation analysis of crude cellular extracts showed a new distribution of the capsid protein and C-E1 polypeptide in the gradient (compare Fig. 4a and b). While C and C-E1 proteins sedimented at a density of 1.21–1.26 g ml⁻¹ when expressed in cells infected solely with AcSLP10-C-E1, the two proteins sedimented at a density of 1.15–1.18 g ml⁻¹

in double-infected cells also expressing the RNA transcript 5'NTR-C-E1. This shift in the sedimentation profile could be explained by the formation of lipid-associated multimers. This hypothesis was confirmed by treatment of the crude extract with a mild non-ionic detergent (1% NP-40) prior to analysis of the cellular extract on the sucrose gradient. In this case, immunoreactive material was detected at higher density (1.24–1.26 g ml⁻¹; Fig. 4c).

TEM observation of cells coinfecting with AcSLP10-5'NTR-E1 and AcSLP10-C-E1 showed a reticular distribution of dense material with perinuclear sparing similar to that described above (Fig. 5a). Observation at high magnification of the cytoplasmic region of cells late in infection revealed the presence of virus-like particles (VLPs) varying in size and shape (Fig. 5b). These particles were surrounded with knobs that might consist of either structural protein complexes or associated ribosome-like structures. Pre-embedding labelling with the anti-capsid polyclonal antibody of detergent-permeabilized infected cells showed colocalization of the capsid protein with these particles (Fig. 5c). Cells infected with wild-type baculovirus or other recombinant baculoviruses expressing one or both of the HCV glycoproteins without the capsid protein never displayed such a pattern (data not shown).

Mutations of AUG codons located in the 5'NTR restore translation of C and E1 proteins

Taking into account the 5'NTR *trans*-acting effect on capsid multimerization described above, we investigated the potential role of this sequence on structural protein assembly when placed *in cis*. For this purpose, we constructed a recombinant baculovirus AcPH-5'NTRm-NS2Δ encompassing the whole structural polyprotein sequence of HCV preceded by a mutated 5' NTR, where the five AUG codons were modified as outlined in Table 1. Sf9 cells were infected with this virus and cell lysates were prepared at 72 h p.i. and analysed by Western blotting using either the anti-capsid mouse monoclonal antibody 27D5C5, the anti-E1 polyclonal hyperimmune serum, the anti-E2 polyclonal serum 1b8356 or the human anti-HCV-positive serum. As shown in Fig. 2, translation of the structural proteins was restored. Two major immunoreactive products with molecular masses of 21 and 23 kDa were revealed with an anti-capsid antibody (Fig. 2c, lane 1), corresponding to the two different forms of capsid protein. To a lesser extent, two minor species of 16 and 28 kDa were also detected. The anti-E1 polyclonal antibody (Fig. 2b, lane 3) reacted with several bands between 21 and 35 kDa, probably corresponding to different glycosylation levels of E1 glycoprotein. Additional bands distributed between 35 and 110 kDa may represent uncleaved and partially glycosylated C-E1, E1-E2 or C-E1-E2 polypeptides. E2 glycoforms were revealed using an anti-E2 polyclonal antibody at molecular masses ranging from 40 to 66 kDa (Fig. 2c, lane 2). The two glycoproteins E1 and E2 and, to a lesser extent, the core protein were also detected in the stacking gel as insoluble aggregates.

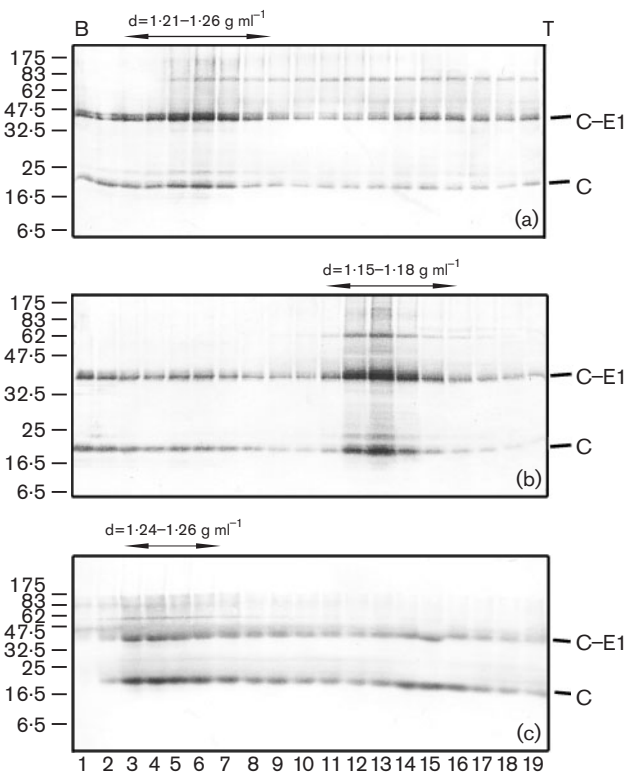


Fig. 4. Isopycnic centrifugation analysis on sucrose gradients of cytoplasmic extracts prepared from cells infected with AcSLP10-C-E1 alone (a) or coinfecting with AcSLP10-C-E1 and AcSLP10-5'NTR-E1, before (b) and after (c) treatment with 1% NP40. Fractions were collected from the bottom and analysed by Western blotting using an anti-HCV-positive human serum. Fractions and densities (d) are indicated. Positions of molecular mass markers (in kDa) are indicated on the left. T, Top; B, bottom.

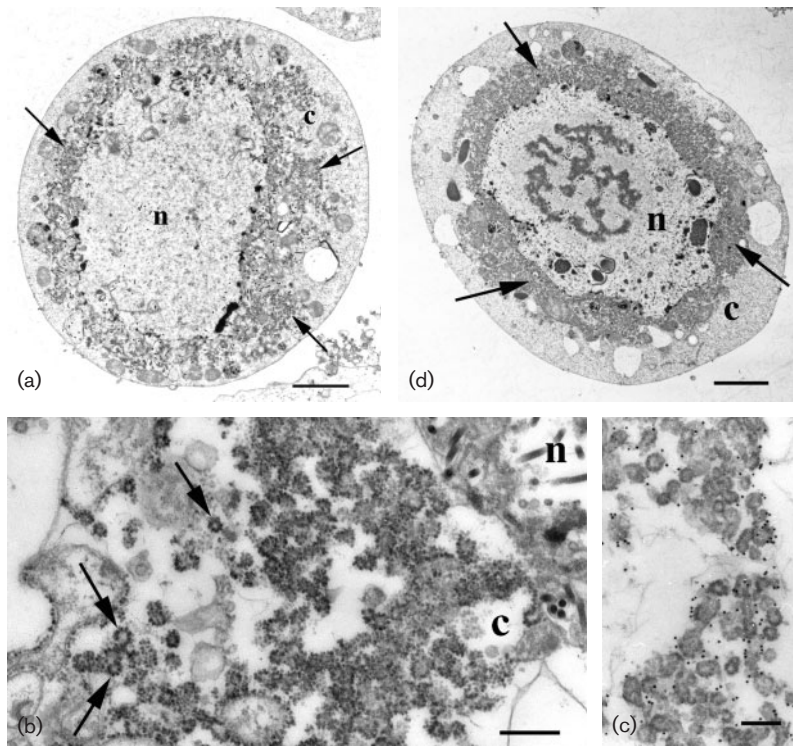


Fig. 5. Electron microscopic analysis of cells co-infected with AcSLP10-C-E1 and AcSLP10-5'NTR-E1 (a–c) or AcPH-5'NTRm-NS2Δ (d). n, Nucleus; c, cytoplasm. In (a) and (d), arrows point to the dense perinuclear material observed in the cytoplasm. Bars, 1.9 μm. A higher magnification image (b) shows VLP formation; arrows indicate VLPs presenting a 'cog-wheel' structure. Bar, 200 nm. In (c), pre-embedding labelling of cells using an anti-capsid polyclonal antibody indicates colocalization of HCV capsid protein with the dense perinuclear material. Bar, 170 nm.

TEM observation of cells infected with the recombinant AcPH-5'NTRm-NS2Δ showed a perinuclear ring of very dense material probably resulting from the accumulation of HCV structural proteins associated with the ER (Fig. 5d). Immunolabelling of cryosections of infected cells with the anti-HCV-positive human serum confirmed the colocalization of HCV proteins in this cytoplasmic area. Faint labelling was also observed in the nuclei (not shown).

Analysis of cytoplasmic extracts prepared from cells infected with AcPH-5'NTRm-NS2Δ showed sedimentation of immunoreactive proteins in fractions with a density of 1.14–1.18 g ml⁻¹ when probed with the anti-HCV-positive human serum (Fig. 6a). Under these conditions, the major product detected was the capsid protein, essentially as the p21 processed form. Treatment of cell extracts with NP40 (1% final in the cell lysis buffer) resulted in a shift of the particle density from 1.14–1.18 g ml⁻¹ to 1.23–1.26 g ml⁻¹ (Fig. 6b). TEM observation of these anti-HCV-positive fractions after negative staining showed icosahedral particles of about 30 nm diameter (Fig. 7a). The difficulties encountered in observing complete particles may be related to their high intrinsic instability or to their high sensitivity to the purification process.

Finally, cell culture supernatant collected from cells either coinfecting with AcSLP10-5'NTR-E1 and AcSLP10-C-E1 or infected with AcPH-5'NTRm-NS2Δ alone were concentrated by centrifugation at 150 000 g and analysed by isopycnic centrifugation on sucrose gradients. No anti-HCV-positive

product was detected when cells were coinfecting with AcSLP10-5'NTR-E1 and AcSLP10-C-E1 viruses (not shown), whereas capsid protein was detected when cells were infected with the AcPH-5'NTRm-NS2Δ recombinant (Fig. 6c). The major HCV-positive fraction (number 12) had a density of 1.18 g ml⁻¹ and reacted with anti-capsid and, to a lesser extent, with anti-E1 and anti-E2 antibodies (Fig. 6e). Signals obtained with those two anti-envelope antibodies were very weak and might reflect a low level of incorporation of the E1 and E2 glycoproteins into these particles. Treatment of pelleted particles with 1% NP40 before sucrose gradient centrifugation resulted in the same density shift from 1.18 to 1.25 g ml⁻¹ as observed previously for particles from cytoplasmic extracts (Fig. 6d).

To verify that particles were not released in the cell culture medium as a consequence of premature cell lysis, the time-course of secretion of the HCV capsid and of a cytosolic control protein, lactate dehydrogenase (LDH), was followed using Western blot analysis and an LDH detection kit (Fig. 7b). Our results indicated that core protein could be detected in the culture medium as early as 30 h p.i. At this time, cell lysis could not account for release of cellular components. Moreover, detailed observation of ultrathin sections of cells infected with AcPH-5'NTRm-NS2Δ and labelled with the anti-HCV-positive human serum revealed budding of some enveloped, immunogold-decorated particles (Fig. 7b). This phenomenon might have occurred via an exocytosis process, as described for other members of the family *Flaviviridae* (Rice, 1996).

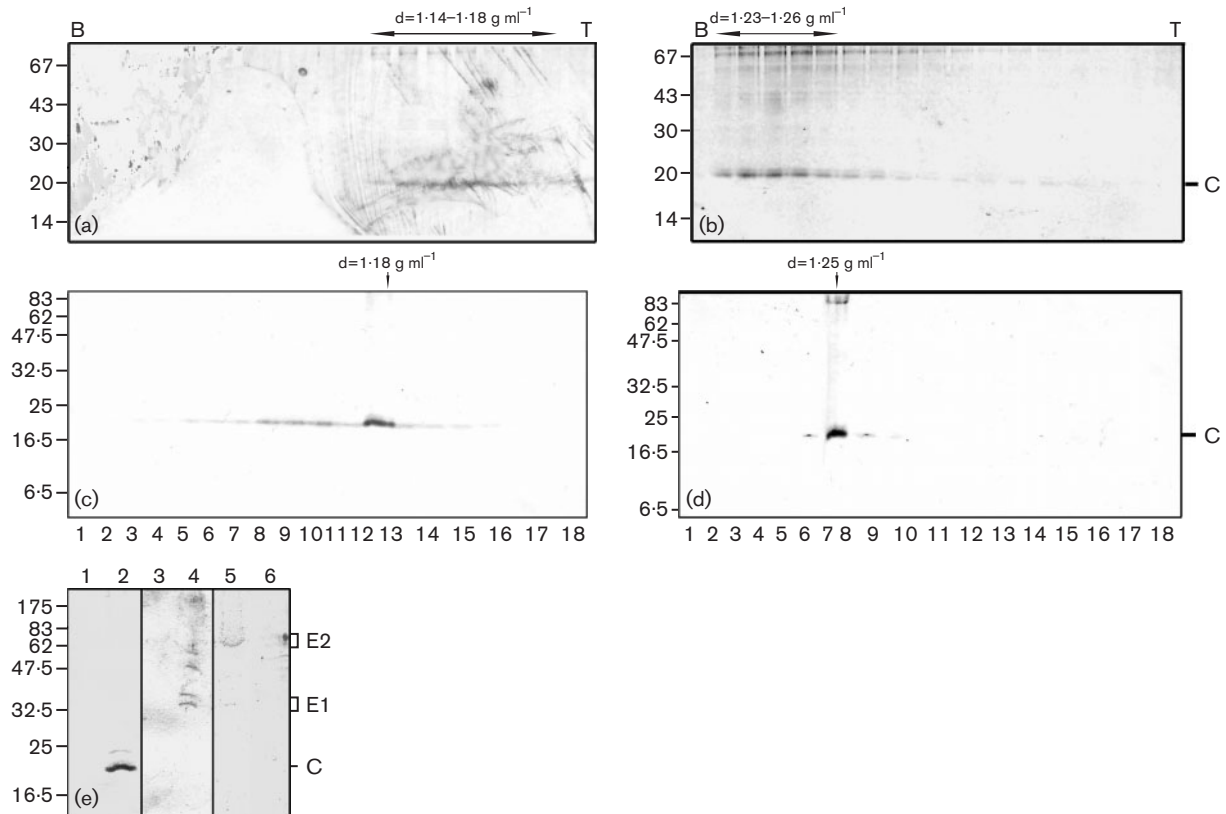


Fig. 6. Analysis of HCV VLPs present in cytoplasmic extracts or cell culture medium collected from cells infected with AcPH-5'NTR_m-NS2Δ. (a–b) Isopycnic centrifugation analysis of cytoplasmic extracts prepared from cells infected with AcPH-5'NTR_m-NS2Δ, before (a) and after (b) treatment with 1% NP40. Sucrose gradient fractions were analysed by SDS-PAGE and immunoblotting using an anti-HCV-positive human serum. Fraction numbers and densities (d) are indicated. (c–e) Characterization of VLPs present in the supernatant. (c–d) Isopycnic centrifugation analysis of concentrated cell culture medium collected from cells infected with AcPH-5'NTR_m-NS2Δ, before (c) or after (d) treatment with 1% NP40. Sucrose fractions were collected and analysed by immunoblotting using an anti-HCV-positive human serum. Fraction numbers and densities (d) are indicated. (e) Immunoblotting of fraction 12 of the gradient shown in (c) using a monoclonal anti-capsid antibody 27D5C5 (lane 2), a polyclonal anti-E1 antibody (lane 4) and a polyclonal anti-E2 antibody (lane 5). Equivalent fractions isolated from concentrated cell culture supernatant collected from cells infected with wild-type baculovirus were analysed by immunoblotting using the same antibodies (lanes 1, 3 and 6, respectively). HCV-specific proteins are indicated on the right and positions of molecular mass markers (in kDa) on the left. T, Top; B, bottom.

DISCUSSION

Particle assembly of RNA viruses occurs after the recognition of specific structures on the viral genomic RNA by the capsid protein. In the family *Flaviviridae*, RNA–protein interactions involved in the particle assembly process have been recently identified for the Kunjin flavivirus (Khromykh *et al.*, 1998). In the case of HCV, an RNA-binding domain potentially implicated in particle morphogenesis initiation was localized in the highly basic N-terminal region of the core protein (Santolini *et al.*, 1994). Interaction between the HCV capsid and the 5'NTR has also been reported (Shimoike *et al.*, 1999). This highly conserved 5' non-coding sequence has been shown to form a complex secondary structure with functional importance (Rijnbrand & Lemon, 2000). In addition, the 5'NTR is able to direct the translation

of adjacent transcripts via an IRES (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993) in *in vitro* and *in vivo* mammalian systems. In contrast, the IRES fails to initiate translation of proteins in infected insect cells (Wang *et al.*, 1997).

We investigated the effect of the 5'NTR on translation and assembly of HCV genotype 1a structural proteins in baculovirus-infected cells. When the 5'NTR of HCV was maintained *in cis*, i.e. upstream of the C–E1 coding region, no production of structural proteins was observed in insect cells, despite efficient transcription of this region. Deletion of the 5'NTR led to the expression of capsid and E1 proteins with the expected sizes, 21 kDa for the capsid protein and 30–35 kDa for the fully processed E1 glycoprotein, as reported previously (Baumert *et al.*, 1998).

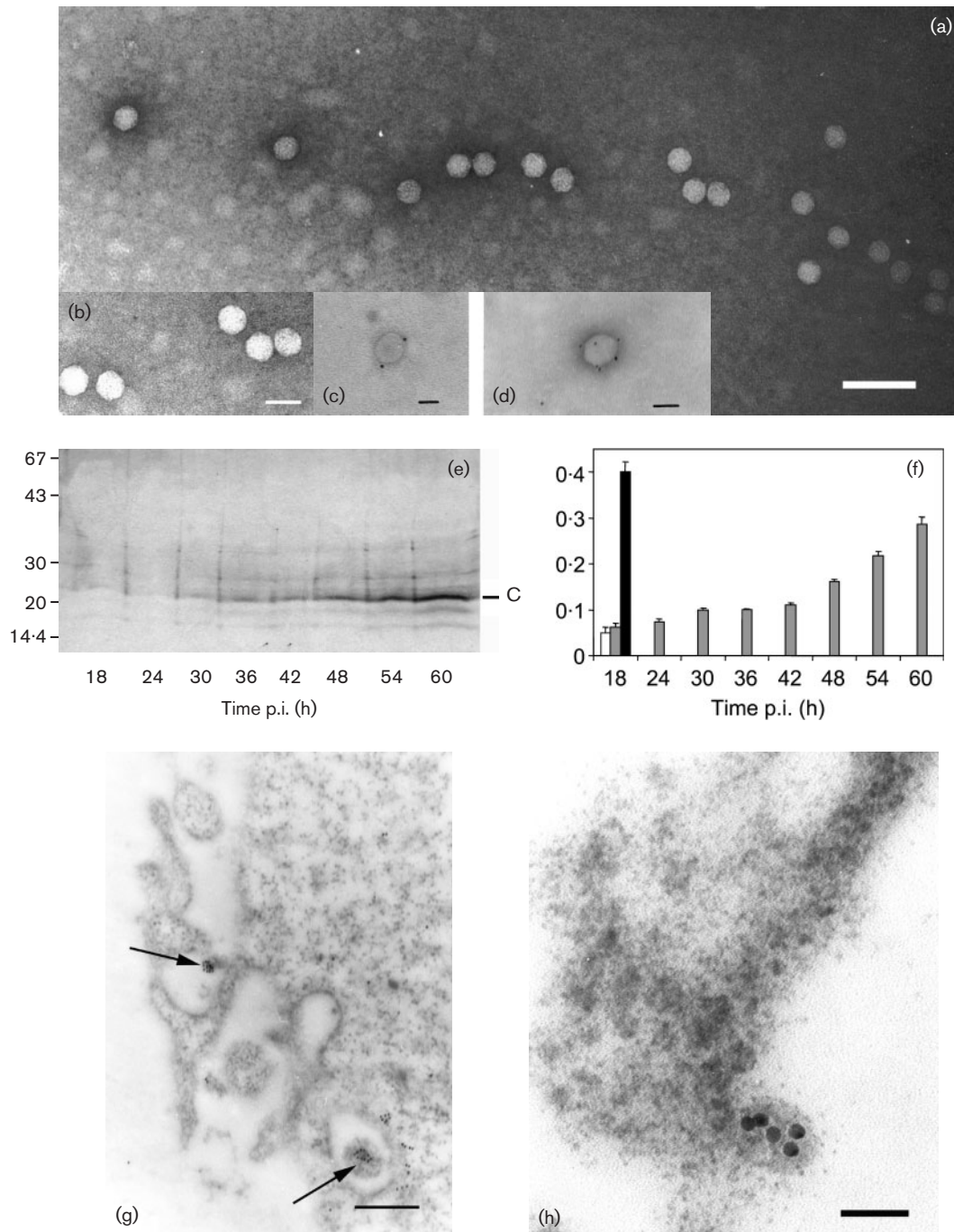


Fig. 7. (a–d) Electron microscopic observation of negatively stained HCV-positive material purified by isopycnic centrifugation on a sucrose gradient of the cytoplasmic fraction extracted from cells infected with AcPH-5'NTR_m-NS2Δ. (a) A rich area with icosahedron-shaped particles about 30 nm in diameter. Bar, 83 nm. (b) Observation of VLPs at higher magnification. Bar, 33 nm. (c–d) Negative staining of VLP purified from the cell culture supernatant immunolabelled with anti-capsid antibody (c) or anti-E1 antibody (d) (bars, 50 nm). (e–h) Analysis of secretion of HCV VLPs in the culture medium of cells infected with AcPH-5'NTR_m-NS2Δ. (e) Supernatant was collected at indicated times p.i., concentrated by centrifugation and analysed by Western blotting using an anti-HCV-positive human serum. The position of the HCV capsid protein is indicated on the right (C) and positions of molecular mass markers (in kDa) on the left. (f) Cell lysis was assessed by determination of the LDH activity (arbitrary units) in the same concentrated cell culture supernatant using an LDH detection kit (see Methods). Uninfected cells are indicated by the open bar and 100% cell lysis by the filled bar. (g–h) Electron microscopic observation of HCV VLP release in the cell culture medium. (g) Immunolabelling of thin sections of cells infected with AcPH-5'NTR_m-NS2Δ with an anti-HCV-positive human serum; immunolabelled particles are indicated by arrows. Bar, 320 nm. (h) Higher magnification of a budding VLP. Bar, 50 nm.

Moreover, mutation of the five AUG codons present in the 5'NTR restored translation of HCV structural proteins. Taken together, these data suggest that, in insect cells, initiation of translation occurs via a ribosome-scanning process, at the first AUG codon encountered, and not by an internal ribosome entry process. In mammalian expression systems, at least three proteins, proteins of 25 and 120 kDa and the PTB protein, have been shown to bind to the HCV 5'NTR. Inhibition of their binding led to a significant reduction in translation efficiency (Ali & Siddiqui, 1995; Fukushi *et al.*, 1997; Yen *et al.*, 1995). One could then hypothesize that specific cellular factors are required for proper initiation of translation by an internal ribosome entry process and that such factors are lacking in infected insect cells.

The high density ($1.21\text{--}1.26\text{ g ml}^{-1}$) of the capsid protein and C-E1 polypeptide in sucrose gradients when expressed in the absence of the 5'NTR-C-E1 transcript could probably be the result of the association of these proteins with the rough ER (Santolini *et al.*, 1994) and/or of multimer formation. In fact, it has been shown that the core protein is capable of homotypic interaction and multimerization both *in vivo* and *in vitro* (Matsumoto *et al.*, 1996). Further analysis revealed that coproduction of structural proteins with the 5'NTR-C-E1 transcript *in trans* or mutated 5'NTR *in cis* led to a shift in density of the anti-HCV-positive material compared with protein density in the absence of the 5'NTR. Instead of sedimenting at a density corresponding to a multimer or a rough ER-associated protein ($1.21\text{--}1.26\text{ g ml}^{-1}$), core protein was found at a density of $1.15\text{--}1.18\text{ g ml}^{-1}$, similar to HCV pseudoparticle density previously reported in a baculovirus expression system (Baumert *et al.*, 1998). TEM observation showed formation of VLPs labelled with the anti-core-positive antibody in the perinuclear area of the cytoplasm of cells coproducing the C and E1 proteins and the 5'NTR-C-E1 transcript *in trans*. Incorporation of partially uncleaved structural polypeptide into the envelope of those particles may be responsible for their morphological heterogeneity. Khromykh *et al.* (1998) reported the *trans*-encapsidation of a Kunjin replicon RNA by Kunjin structural proteins, describing for the first time interaction between RNA and structural proteins of a flavivirus. Our results showed that similar RNA-protein interactions could have functional importance for HCV VLP assembly in our heterologous system. In a previous study, Wang *et al.* (1997) reported that HCV genotype 1b RNA representing the 5'NTR-C (short RNA transcript) or C-E1-E2-p7 (missing the 5'NTR *in cis*) region failed to induce the formation of core particles. Such a discrepancy might be explained by differences (i) in the genotype of the HCV cDNA, (ii) in the constructs used for the expression of structural proteins or (iii) in the constructs used to produce the 5'NTR transcript. A minimal size of the RNA (5'NTR-C-E1) or sequence elements encompassing more than one packaging signal potentially present on the HCV

genome may be necessary for efficient interaction with structural proteins. Indeed, this was observed for a murine leukaemia retrovirus (MoLV) which has a primary encapsidation signal in the 5'NTR and an extended encapsidation signal in the *gag* ORF; this latter is responsible for increased viral RNA packaging efficiency and for viral titre enhancement (Bender *et al.*, 1987). Moreover, we cannot exclude that the length of the encapsidated genomic RNA molecule might have a direct effect on assembly. When wild-type intact proteins were produced in cells infected with a virus bearing a mutated 5'NTR (AcPH-5'NTRm-NS2Δ), the same capsid protein sedimentation profile was observed in lysates prepared from cells coinfecting with AcSLP10-C-E1 and AcSLP10-5'NTR-E1. The weak representation of the E1 and E2 glycoproteins in the core-positive fractions may reflect their low incorporation in the VLP. This could be due to (i) inefficient processing and/or (ii) retention of these proteins in an aggregated form in the ER, as reported previously (Deleersnyder *et al.*, 1997). In addition, the formation of native HCV glycoprotein complexes could represent a limiting step in particle morphogenesis, this process being more or less efficient from one genotype to another. Treatment of the pseudoparticles with NP40 resulted in disruption of membrane-nucleocapsid interactions and the alteration of their sedimentation profile: the anti-HCV-positive fractions were found at a density of $1.24\text{--}1.26\text{ g ml}^{-1}$. This value is in good agreement with the density (1.25 g ml^{-1}) reported for human-serum-derived HCV nucleocapsid treated with the same detergent (Miyamoto *et al.*, 1992). TEM observation of these fractions reveals the presence of few, probably unstable icosahedron-shaped particles about 30 nm in diameter. Finally, weak secretion of VLPs into the cell culture medium was observed for cells infected with AcPH-5'NTRm-NS2Δ. This release occurred early, before cell lysis became generalized (as estimated by LDH activity of the cell culture supernatant). In addition, genuine budding has been assessed by EM (Fig. 7g, h). Such extracellular release of HCV VLPs in insect cells has not so far been reported, to our knowledge. As no VLPs could be observed in the supernatant of cells coproducing the C-E1 structural region and the 5'NTR-C-E1 RNA transcript, we hypothesize that correct self-assembly can only be achieved when the 5'NTR is located *in cis* or that the absence of some HCV structural elements results in a lower stability of HCV VLP.

In this study, we present evidence that the 5'NTR of HCV genotype 1a is implicated in the assembly process of HCV structural proteins in insect cells, although we have no evidence for a direct interaction between the 5'NTR and these structural proteins. Nevertheless, the use of this heterologous baculovirus system for a better understanding of the mechanism of HCV assembly may constitute an interesting alternative to mammalian cell systems, especially in regard to the lack of an efficient cellular system for the replication of the virus.

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