*Journal of General Virology* (2002), **83**, 561–566. Printed in Great Britain SHORT COMMUNICATION

# Expression of hepatitis C virus envelope glycoproteins by herpes simplex virus type 1-based amplicon vectors

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**Herpes simplex virus type 1 (HSV-1)-based amplicon vectors expressing hepatitis C virus (HCV) E1 and E2 glycoproteins were investigated. HSV-1 amplicon vectors carrying the E1E2p7- or E2p7-coding sequences of HCV type 1a under the control of the HSV-1 IE4 (α22/α47) promoter were constructed. Studies of infected HepG2, WRL 68 or Vero cells indicated that HSV-1-based amplicon vectors express high levels of HCV glycoproteins that are processed correctly. Immunofluorescence microscopy combined with immunoprecipitation and endoglycosidase treatment of cells infected with the HSV-1-based vectors expressing E1 and E2 showed that the two glycoproteins were retained in the endoplasmic reticulum and had the expected glycosylation patterns. Furthermore, although most of the E1 and E2 proteins formed disulfide-linked aggregates, significant amounts of monomeric forms of the two proteins were detected by SDS–PAGE under non-reducing conditions, suggesting the presence of non-covalently associated E1 and E2. Similar results were produced by a replication-competent recombinant HSV-1 vector expressing HCV E1 and E2. These results indicated that HSV-1-based amplicon vectors represent a useful expression system for the study of HCV glycoproteins.**

Hepatitis C virus (HCV) is the major causative agent of parenterally transmitted non-A, non-B hepatitis and is associated frequently with chronic hepatitis, which often progresses to liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Saito *et al.*, 1990). At present, no prophylactic vaccine against HCV infection is available. HCV is a small, enveloped, single-stranded, positive-sense RNA virus belonging to the family *Flaviviridae* (Francki *et al.*, 1991). The ~9·6 kb viral genome encodes a polypeptide of approximately 3000 aa. This polypeptide is cleaved by host peptidases to release the core protein and the two putative envelope glycoproteins E1 and E2; viral proteases cleave the polypeptide to produce the non-structural proteins (NS2–5) (Reed & Rice, 2000).

HCV E1 and E2 are heavily glycosylated type I transmembrane proteins. A hydrophobic domain at their carboxy terminus acts as a retention signal for the endoplasmic reticulum (ER) and is required for the correct assembly of the two glycoproteins (Cocquerel *et al.*, 1998, 1999, 2000; Dubuisson, 2000; Flint & McKeating, 1999; Patel *et al.*, 2001). A number of independent studies have shown that E1 and E2 interact to form two types of complexes. One type consists of non-covalently associated E1/E2 heterodimers and is believed to result from the productive folding and assembly of the two glycoproteins (Deleersnyder *et al.*, 1997; Michalak *et al.*, 1997). The other type consists of disulfide-linked E1/E2 heterodimers, which fail to acquire their correct conformation and form aggregates that show prolonged association with ER chaperones (Choukhi *et al.*, 1998, 1999; Deleersnyder *et al.*, 1997; Dubuisson *et al.*, 1994). Interestingly, recent studies suggest that both types of E1–E2 complex may actually occur *in vivo* and may play distinct roles in the life cycle and pathogenesis of the virus (Liberman *et al.*, 1999).

To date, despite the many efforts, an efficient tissue culture system for the propagation of HCV is still not available. Thus, studies on HCV protein structure and function rely on the use of heterologous expression systems. Herpes simplex virus type 1 (HSV-1) amplicons represent unique virus expression vectors because their genome comprises multiple copies of plasmid DNA. The amplicon plasmid contains one copy of an HSV-1 origin of replication (usually *ori*-S), a packaging signal sequence (pac), which is contained within the repeated '*α*' sequence of the HSV genome, and the transgene (Freese *et al.*, 1990; Frenkel *et al.*, 1994; Spaete & Frenkel, 1982, 1985). In the presence of HSV-1 helper virus, the plasmid DNA is amplified (presumably by a rolling-circle mechanism) into a head-to-tail

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concatamer, which is then packaged into defective HSV-1 particles that are up to one genome size (~ 150 kb) (Kwong & Frenkel, 1984). Amplicons have been employed successfully as vectors for the transfer of a variety of genes of neurobiological or therapeutical interest, as well as genes encoding proteins from heterologous virus families (Savard *et al.*, 1997; Sena-Esteves *et al.*, 1999, 2000; Costantini *et al.*, 1999). HSV-1-based amplicon vectors combine a number of features that make them attractive virus vectors for the expression of heterologous genes. These include the ability to efficiently infect various cell lines, the ability to carry high copy numbers of the transgene and the simplicity of constructing these vectors. Most importantly, new strategies have been developed recently, allowing the generation of either limited amounts of helper-free amplicon vectors (Saeki *et al.*, 2001) or large amounts of vector stocks presenting a high amplicon to non-pathogenic helper virus ratio (Logvinoff & Epstein, 2001), thus providing safe virus vectors for vaccine development.

The goal of this study was to explore the potential of HSV-1-based amplicon vectors as alternative expression systems for the study of HCV envelope glycoproteins. For this purpose, the regions encoding the E1E2p7 (aa 191–807) or E2p7 (aa 383–807) polypeptides, amplified by PCR from a plasmid vector containing the cDNA sequence of HCV-1a (H) (kindly provided by G. Inchauspe, INSERM, Lyon, France), were cloned into the pA-SK*lac*Z amplicon plasmid under the control of the HSV-1 IE4 (α22/α47) promoter. To ensure efficient processing of the HCV glycoproteins in the context of the HSV-1-based vectors, the signal sequences of E1 in the E1E2p7 construct and E2 in the E2p7 construct have been substituted by the signal sequence of the HSV-1 glycoprotein E (gE) (nt 141183–141309), corresponding to wild-type HSV-1 (F). The sequence of gE was obtained from plasmid pHPI400 by PCR (Miriagou *et al.*, 1995). pA-SK*lac*Z is a pBluescript II plasmid (Stratagene), which contains the amplicon module (*ori*-S and α sequences) from the pA-SF1 plasmid (Lowenstein *et al.*, 1994) and also a LacZ/β-galactosidase expression cassette [based on the human cytomegalovirus (HCMV) immediate-early promoter and the simian virus type 40 poly(A) sequences, kindly provided by P. Lowenstein, University of Manchester, Manchester, UK] (Fig. 1a).

For the generation of amplicon vectors, 50–70% confluent BHK-21 cells (ATCC) seeded in 100 mm tissue culture dishes were transfected with the amplicon plasmids using the Effectene (Qiagen) or Transfast Transfection (Promega) reagents, according to the protocols of the manufacturers. At 24 h after transfections, cells were superinfected with HSV-1 strain LaL (Logvinoff & Epstein, 2000) at an m.o.i. of 0·1–0·5 and incubated in 199 V medium (Gibco BRL) supplemented with 1% foetal bovine serum until 100% cytopathic effect (CPE) was observed. The infected cells were subjected to three freeze–thaw cycles at −80 °C and 37 °C to release amplicon vector progeny (P0). In order to amplify the amplicon vector stocks, one-third of the P0 progeny was used to infect Vero cells (ATCC) seeded in F75 flasks, thus generating P1 progeny. P1 was amplified further by infecting Vero cells grown in F150 flasks to generate a high-titre P2 amplicon vector stock with $10^8$–$10^9$ amplicons/ml and an amplicon to helper virus (A:H) ratio of ~ 1. Notably, the helper virus, HSV-1 LaL, is completely non-pathogenic, even after high-dose intracerebral inoculation of mice (A. Epstein, unpublished data). Furthermore, this virus contains a single packaging signal flanked by two loxP sites. Recent studies have shown that during infection of TE-CRE30, a cell line expressing Cre recombinase, the single packaging signal of HSV-1 LaL is excised efficiently from the viral genome by site-specific recombination, resulting in a defective 'unpackageable' viral genome (Logvinoff & Epstein, 2001). Thus, HSV-1 LaL recombinant virus presents the unique advantage of generating both high-titre non-pathogenic helper-dependent amplicon stocks and potentially helper-free amplicon vectors.

To assess initially the ability of HSV-1-based amplicon vectors to express the HCV glycoproteins, HepG2 cells (ATCC) were infected with pA-SK *lac*ZE1E2p7 or pA-SK *lac*ZE2p7 amplicon vectors at an m.o.i. of 1 and an A:H ratio of ~ 1 for both stocks. Cells were lysed at different times post-infection (p.i.) in TBS buffer (10 mM Tris–HCl pH 7·5, 150 mM NaCl and 2 mM EDTA) containing 0·5% Igepal CA630 (Sigma), 0·1 mM PMSF and 20 mM iodocetamide. Cell lysates were analysed by SDS–PAGE and immunoblot analysis using anti-E2, anti-E1 (kindly provided by J. Dubuisson, Pasteur Lille, Lille, France) or anti-β-gal (Gibco BRL) monoclonal antibodies (mAbs). As shown in Fig. 1(b), both amplicon vectors supported efficient expression of E2 (66–68 kDa) and E1 (31–35 kDa) proteins. Similar results were obtained with amplicon stocks with A:H ratios of 100, prepared following infection of TE-CRE30 cells (data not shown). Consistent with previous observations, the E2 protein from the E1E2p7-expressing amplicon vector resolved into two bands, which probably arise from inefficient cleavage of the E2p7 site (Dubuisson *et al.*, 1994). Notably, in the case of the pA-SK *lac*ZE2p7 amplicon vector, the expression levels of E2 were repeatedly lower and only the faster migrating band was the major E2 protein observed after 15 h p.i. Since similar amounts of β-galactosidase were produced by the two vectors [Fig. 1b, (i) and (iv)], the possibility of an intrinsic problem of the amplicon vector stock was unlikely, suggesting that the presence of upstream nucleotide sequences and/or the presence of E1 may affect the processing and the levels of E2 expressed in this system.

Secondly, in order to assess the levels of expression obtained from the amplicon vectors, we analysed the expression of E1 and E2 in cells infected in parallel with the pA-SK *lac*ZE1E2p7 amplicon vector or with the replication-competent recombinant HSV-1 rHPI A2/E1E2p7 virus. This virus contains identical E1E2p7-coding sequences expressed from the strong chimeric αγ1 promoter (kindly provided by B. Roizman, University of Chicago, Chicago, USA) and was

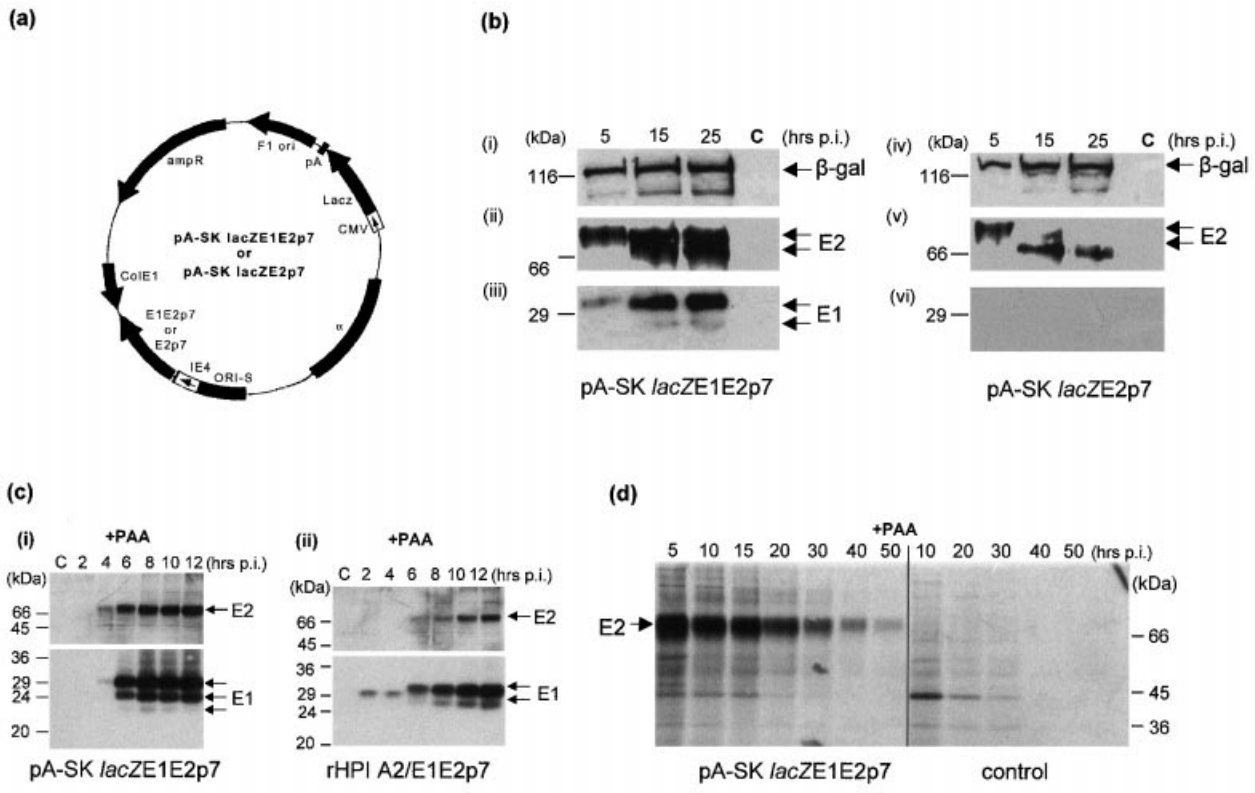


Fig. 1. (a) Schematic representation of the amplicon plasmids pA-SK *lac*ZE1E2p7 and pA-SK *lac*ZE2p7 used for the generation of amplicon vectors. α and *ori*-S represent the packaging sequence and the origin of replication of HSV-1 DNA, respectively. The *lacZ* gene is under the control of the immediate-early HCMV promoter and the HCV envelope proteins are under the control of the HSV-1 IE4 (α22/α47) promoter. (b) Expression of HCV E1 and E2 in HepG2 cells infected with the pA-SK *Lac*ZE1E2p7 or the pA-SK *Lac*ZE2p7 amplicon vectors (m.o.i. of 1, A:H ratio of ~ 1). Cells were lysed at the indicated times p.i., analysed by SDS–PAGE (12% acrylamide gel) and immunoblotted using the anti-E2 (ii and v), anti-E1 (iii and vi) and anti-β-gal (i and iv) mAbs. C, cells infected only with the helper virus. (c) Expression of HCV E1 and E2 in Vero cells infected with (i) pA-SK *Lac*ZE1E2p7 amplicon vector (m.o.i. of 0·3, A:H ratio of ~ 1) or (ii) the rHPI A2/E1E2p7 recombinant virus (m.o.i. of 3) in the presence of PAA. Cells were harvested at the indicated times p.i. and cell lysates were analysed by SDS–PAGE (12% acrylamide gel) and immunoblotted using the anti-E1 and anti-E2 mAbs. Arrows indicate E2 and the various forms of E1. C, cells infected with HSV-1 LaL in the case of the amplicon or wild-type HSV-1 for the recombinant virus. It should be noted that, similar to previous data, the anti-E1 mAb revealed a set of bands ranging from 19 to 35 kDa corresponding to the different glycosylated forms of E1 (Dubuisson *et al.*, 2000; Patel *et al.*, 2001). (d) Vero cells were infected with the pA-SK *Lac*ZE1E2p7 amplicon vector (m.o.i. of 2·5) in the presence of PAA. Cells were labelled for 2 h before harvesting at the indicated times p.i. The cell lysates were immunoprecipitated with the anti-E2 mAb. After immunoprecipitation, the proteins were analysed by SDS–PAGE (12% acrylamide gel), transferred to a nitrocellulose membrane and subjected to autoradiography. Control, cells infected with HSV-1 LaL.

generated by homologous recombination between wild-type HSV-1(F) viral DNA and a plasmid shuttle vector (U. Georgopoulou, A. Caravokiri and P. Mavromara; unpublished data) containing the HCV sequences flanked by HSV-1 thymidine kinase homologous sequences, as described previously (Post & Roizman, 1981). The chimeric αγ1 promoter was designed to combine the potency of an HSV-1 α promoter and the ability of long-term expression of a γ1 promoter. Infections were performed in the presence of phosphonoacetic acid (PAA) (300 μg/ml). Under these conditions, viral DNA replication is inhibited and expression from the γ1 HSV-1 promoter is reduced significantly (Roizman, 1996). Therefore, the activity of the αγ1 promoter would be due primarily to its α component. This allows an indirect comparison between the two expression systems. As shown in Fig. 1[c, (i) and (ii)], Vero cells infected with the pA-SK *lac*ZE1E2p7 amplicon vector at an m.o.i. of 0·3 supported higher levels of E1 and E2 expression than cells infected with the HSV-1 rHPI A2/E1E2p7 recombinant virus at an m.o.i. of 3. This result suggests that the amplicon vectors efficiently produce high levels of the HCV glycoproteins.

Finally, to investigate the long-term kinetics of expression by the amplicon vectors, we performed a pulse-labelling experiment to detect newly synthesized E2 at several h p.i. In order to avoid CPE of the infected cells due to the replication of helper virus, the experiment was performed in the presence of PAA. Vero cell monolayers cultured in 25 $cm^2$ flasks were infected with the pA-SK *Lac*ZE1E2p7 amplicon vector at an

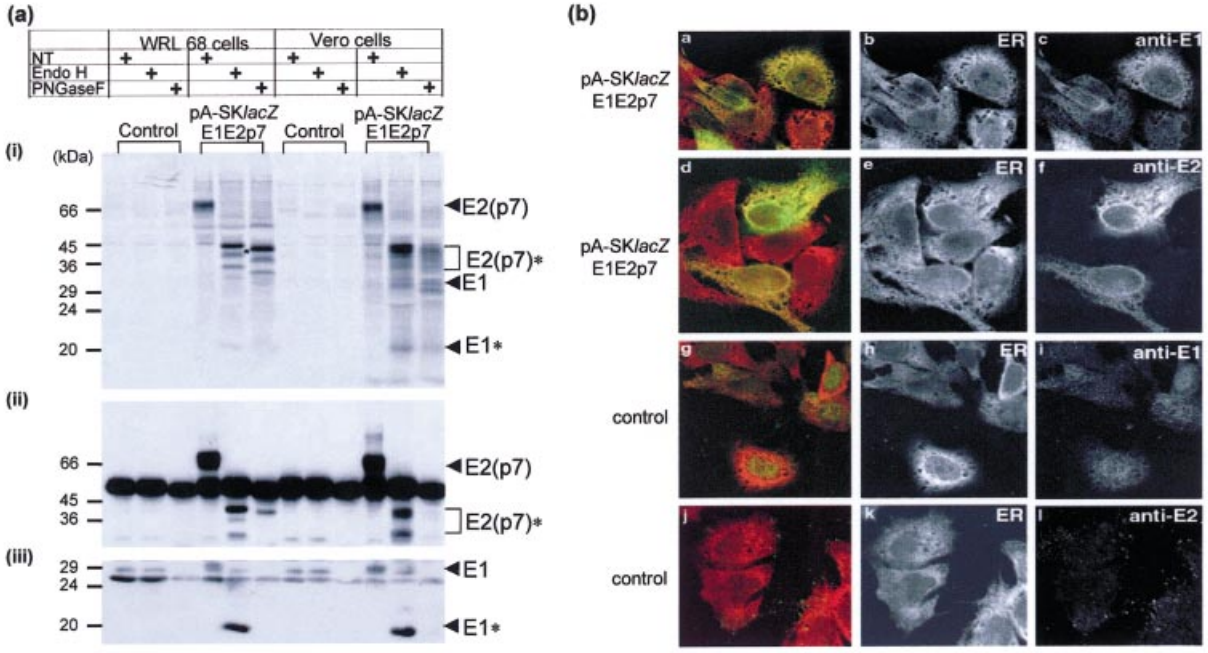


Fig. 2. Endoglycosidase treatment of HCV E1 and E2 expressed by the HSV-1-based vectors. (a) WRL 68 or Vero cell monolayers infected with the pA-SK *lacZ*E1E2p7 amplicon vector (m.o.i. of 2·5) or with the helper virus HSV-1 LaL (control lanes) were labelled from 2 to 18 h p.i. Lysates were immunoprecipitated with the anti-E2 mAb. Immunoprecipitates were divided in three equal aliquots for digestion with either EndoH or PNGaseF enzymes or were mock-digested (NT). The proteins were analysed by SDS–PAGE (12% acrylamide gel), transferred to a nitrocellulose membrane and subjected to autoradiography (i). The same membrane was also analysed by immunoblot analysis using the anti-E2 (ii) or anti-E1 (iii) mAbs. For reasons unknown, the anti-E1 and anti-E2 mAbs showed decreased reactivity with the PNGaseF-treated samples. The bands present in (ii), around 55 kDa, and in (iii), around 26 kDa, correspond to immunoglobulin heavy and light chains reacting with the secondary antibody used during immunoblot analysis. (b) Immunofluorescence analysis of the pA-SK*Lac*ZgEE1E2p7 amplicon vector-infected cells expressing E1 and E2. HepG2 cells infected with the pA-SK *lacZ*E1E2p7 amplicon vector or the LaL helper virus (control) labelled with either the anti-E1 mAb (panels c and i) or the anti-E2 mAb (panels f and l), followed by goat anti-mouse antibody conjugated to Alexa fluor 488 (green) are shown. ER staining using the anti-ER polyclonal antibody followed by goat anti-rabbit antibody conjugated to Alexa fluor 568 (red) (panels b, e, h and k) are also shown. The colour images represent the superimposition of the ER and E1 or E2. The colour yellow in (a) and (d) represents the co-localization of the ER protein marker and the E1 and E2 proteins in the amplicon-infected cells, respectively.

m.o.i. of 2·5. Cells were labelled with 100 μCi/ml $S^{35}$-trans label (ICN) for 2 h before harvesting at the different times p.i. The cell lysates were then immunoprecipitated with the anti-E2 mAb. After immunoprecipitation, the proteins were analysed by SDS–PAGE, transferred to a nitrocellulose membrane and subjected to autoradiography. We found that E2 was produced even after 50 h p.i. (Fig. 1d), providing evidence for the long-term expression of the HCV glycoproteins in the HSV-1 amplicon-infected cells.

Previous studies have shown that the HCV E1 and E2 glycoproteins, when expressed in mammalian cells, are retained in the ER and remain sensitive to endo-*N*-acetylglucosaminidase H (EndoH). Notably, all available data indicate that, under these conditions, the majority of E1 and E2 proteins have the tendency for aberrant disulfide bond formation, while the efficiency of non-covalently associated E1/E2 heterodimers is low (Deleersnyder *et al.*, 1997; Michalak *et al.*, 1997; Patel *et al.*, 1999). In order to study the behaviour of E1 and E2 in the context of the HSV-1-based vectors, we performed three series of experiments. Initially, the sensitivity of E1 and E2 to the EndoH and *N*-glycosidase F (PNGaseF) endoglycosidases was studied in cells infected with the pA-SK *lacZ*E1E2p7 vector. Sensitivity of a glycoprotein to EndoH treatment indicates that the protein is resident in the ER or the *cis*-Golgi and does not migrate further in the secretory pathway. Infected WRL 68 cells (kindly provided by A. Budkowska) or Vero cells were labelled from 2 to 18 h p.i. with 30 μCi/ml $S^{35}$-trans label and cell lysates were immunoprecipitated, as described previously by Dubuisson *et al.* (1994), using the anti-E2 mAb. Immunoprecipitates were divided in three aliquots and were digested subsequently with either EndoH or PNGaseF (New England Biolabs), according to the manufacturer's protocol, or left untreated. Samples were analysed by SDS–PAGE and autoradiography [Fig. 2a, (i)]. The presence of E1 and E2 in the immunoprecipitated materials was confirmed by immunoblot analysis using the anti-E1 and anti-E2 mAbs [Fig. 2a, (ii) and (iii)]. As shown in Fig. 2(a), the patterns observed with EndoH or PNGaseF were essentially the same for both glycoproteins,

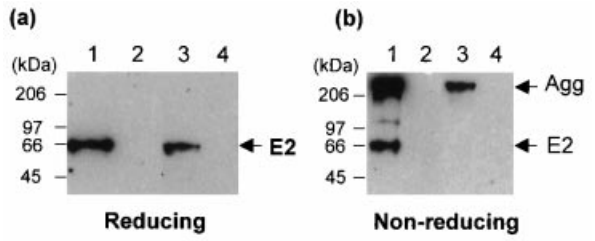


Fig. 3. Analysis of the E2 protein expressed by the amplicon vector under reducing and non-reducing conditions. Lanes 1 and 2, BHK-21 cells infected with the pA-SK *lacZ*E1E2p7 amplicon vector (m.o.i. of 0·2); 3 and 4, BHK-21 cells transiently transfected with plasmid pHPI 682 containing identical gEE1E2p7-coding sequences. Infected cells were lysed after 24 h and transfected cells after 48 h. Cell lysates were analysed by SDS–PAGE under (*a*) reducing or (*b*) non-reducing conditions and immunoblotted with the anti-E2 mAb.

indicating that all *N*-linked oligosaccharides were of the immature form, indicative of the retention of the protein in the ER. We obtained similar results with the HSV-1 recombinant rHPI A2/E1E2p7 virus (data not shown). The ER localization of E1 and E2 expressed by the amplicon vectors and the recombinant virus was also verified by indirect immunofluorescence. HepG2 cells were infected with the pA-SK*lac*ZE1E2p7 amplicon vector at an m.o.i. of 0·5. At 6 h p.i., cells were fixed with 4% paraformaldehyde, permeabilized with 0·1% Triton X-100 and labelled with the anti-E1 (Fig. 2b, panels c and i) or anti-E2 (Fig. 2b, panels f and l) mAbs, followed by goat anti-mouse antibody conjugated to Alexa fluor 488 (green) (Molecular Probes). For ER-staining, the anti-ER polyclonal antibody (Fig. 2b, panels b, e, h and k), followed by goat anti-rabbit antibody conjugated to Alexa fluor 568 (red) (Molecular Probes) was used. E1 and E2 gave a fine, reticular, ER-like pattern of localization that was confirmed further by extensive co-localization with an ER marker (anti-ER rabbit polyclonal antibody, kindly provided by E. Coudrier, Institute Curie Paris, Paris, France) (Fig. 2b, panels a and d). We conclude, therefore, that E1 and E2 expressed by the HSV-1-based vectors are processed correctly by the host peptidases and show the expected patterns of intracellular localization and post-translational glycosylation.

Finally, in order to distinguish between non-covalently associated E1/E2 molecules and those resulting in aggregation due to disulfide-bond formation, we analysed infected cell lysates by SDS–PAGE under reducing and non-reducing conditions: SDS loading buffer contained 2 mM Tris–HCl pH 6·8, 8% SDS, 40% glycerol, 0·4% bromophenol blue and 4 mM $\beta$-mercaptoethanol for reducing conditions or was without $\beta$-mercaptoethanol for non-reducing conditions (Flint *et al.*, 2000). After SDS–PAGE, immunoblot analysis was carried out using the anti-E2 mAb. BHK-21 cells were infected with the pA-SK *lac*ZE1E2p7 amplicon vector or transiently transfected with a plasmid vector containing identical gEE1E2p7-coding sequences to serve as control. As shown in Fig. 3, under non-reducing conditions, the majority of E2 remained at the top of the gel, as expected for disulfide-bridged aggregates. However, a fraction of the total amount of E2 expressed by the amplicon vector appeared to migrate as a monomeric form (68 kDa) (Fig. 3b). No monomeric form of E2 was detected under similar conditions in transiently transfected cells, probably due to lower levels of expression. Similar results were obtained for E1 (data not shown).

In this report, we showed that HSV-1-based vectors represent a potentially useful alternative expression system for the study of the HCV E1 and E2 glycoproteins. HSV-1-based amplicon vectors support efficient expression of HCV E1 and E2 glycoproteins in a variety of cell lines. Furthermore, the size, glycosylation pattern and cellular localization of both glycoproteins are in agreement with data obtained from previous studies, suggesting that HSV-1-based amplicon vectors represent a promising alternative virus vector for the expression of HCV E1 and E2 in mammalian cells. Consequently, since E1 and E2 are candidate antigens for a vaccine against HCV infection, the development of non-pathogenic amplicon vectors may be valuable for the development of a novel vaccine.

We would like to thank J. Dubuisson for providing the anti-E1 and anti-E2 mouse mAbs and G. Inchauspe for the pMink plasmid. We would also like to thank H. Boleti for critically reading the manuscript and invaluable assistance with indirect immunofluorescence and laser confocal microscopy. We also thank A. Michaelidou and E. Aslanoglou for excellent technical assistance. The work was supported by the European commission (Quality of Life, QLK2-CT-1999-00055) and the French–Hellenic Common Research Grant (Plato).

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Received 24 August 2001; Accepted 8 November 2001