Inducible System in Human Hepatoma Cell Lines for Hepatitis C Virus Production

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We cloned the complete complementary DNA of an isolate of the hepatitis C virus, HCV-S1, into a tetracycline-inducible expression vector and stably transfected it into two human hepatoma cell lines, Huh7 and HepG2. Twenty-six Huh7 and two HepG2-positive clones were obtained after preliminary screening. Two Huh7 (SH-7 and -9) and one HepG2 (G-19) clones were chosen for further characterisation. Expression of HCV proteins in these cells accumulated from 6 h to 4 days posttreatment. Full-length viral plus-strand RNA was detected by Northern analyses. Using RT-PCR and ribonuclease protection assay, we also detected the synthesis of minus-strand HCV RNA. Plus- and minus-strand viral RNA was still detected after treatment with actinomycin D. Indirect immunofluorescence staining with anti-E2, NS4B, and NS5A revealed that these proteins were mostly localised to the endoplasmic reticulum (ER). Culture media from tet-induced SH-9 cells was separated on sucrose density gradients and analysed for the presence of HCV RNA. Viral RNA levels peaked at two separate ranges, one with a buoyant density of 1.08 g/ml and another from 1.17 to 1.39 g/ml. Electron microscopy demonstrated the presence of subviral-like particles (approximately 20–25 nm in diameter) in the cytoplasm of SH-9 and G-19 cells, which were positively labelled by anti-HCV core antibodies. Anti-E2 antibodies strongly labelled cytoplasmic vesicular structures and some viral-like particles. Complete viral particles of about 50 nm which reacted with anti-E2 antibodies were observed in the culture media of tet-induced SH-9 cells following negative staining. Supernatant from tet-treated SH-9 cells was found to infect naïve Huh7 and stable Huh7-human CD81 cells. © 2002 Elsevier Science (USA)

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped virus with a single positive-strand RNA genome of about 9.5 kb (6). It belongs to the family of Flaviviridae, as a separate genus from pestiviruses and flaviviruses (25, 43, 57). The HCV polyprotein is cleaved by both host and viral proteases to yield structural proteins (core, E1, and E2) and nonstructural proteins (NS2 to 5B) (46). More than 170 million people worldwide are infected with HCV (1, 5, 13). About 80% of the infected individuals remain chronically infected for life, of which an estimated 15–20% eventually develop liver cirrhosis and hepatocellular carcinoma (8).

The understanding of the pathogenesis and replication of HCV has been hampered by the lack of a satisfactory cell-culture system which allows efficient propagation of the virus. Numerous groups have reported the replication of HCV in various human T (38, 39, 54, 55), B (3, 41, 63), and hepatocytic (17, 22, 51, 59) cell lines using infected sera as inocula. In addition, HCV-producing hepatocytic cell lines derived from patients with chronic hepatitis have also been described (19, 62). Persistent, long-term HCV-infected cell lines have also been achieved by transfection of human hepatoma cell line, Huh7, with a cDNA (15) or in vitro transcribed viral RNA (7, 66). In all these cases, although HCV infection could be maintained for long periods of time, even up to over a year (41), viral RNA was not always detectable in the cells or culture supernatant, suggesting that viral replication is not very efficient and viral progeny is at best, intermittently produced (reviewed in 23, 50).

For reasons that are yet unclear, it is difficult to establish continuous cell lines constitutively expressing full-length HCV cDNAs (40 and S. P. Lim, personal communication). This could be attributable to transcriptional repression, hypermethylation of the transgene, or toxicity of HCV gene products (28, 34, 40). Recently, the continuous production of inducible HCV proteins from a tetrarepressive system in the osteosarcoma cell line U-2 OS was described (40). Clones from this cell line allow the tightly regulated expression of the entire HCV ORF. Nevertheless, as both the 5’ and 3’ noncoding viral sequences are not included in the transgene, viral replica-
tion does not take place in these cells. Attempts to generate a similar system in the human hepatoma cell line, Huh7, were apparently unsuccessful (40).

We recently reported the cloning of nine overlapping cDNA clones covering the entire viral genome of a HCV isolate (HCV-S1) of genotype 1b including the complete 5' and 3' UTRs (30). We then generated a full-length genome from these overlapping clones and placed it under a tetracycline (tet)-inducible promoter in the construct pSTAR (69). This vector offers the advantage of having incorporated all the features of the rTANl system in one single vector and eliminates the necessity to establish stable double transfectants normally required for tet-inducible/repressor systems (69). Following transfection of the human hepatoma cell lines, Huh7 and HepG2, we obtained stable clones that contained this full-length HCV genome. These lines can be induced to express HCV structural and nonstructural proteins. In addition, these stable cell lines permit replication of HCV and the production of infectious viral particles. Thus they maybe potentially advantageous for the study of the life cycle and molecular biology of the virus and may also be useful for screening antiviral agents as well as the large-scale production of viral particles.

RESULTS

Generation and characterisation of tetracycline-inducible HCV expressing cell lines

We utilized for our cloning the pSTAR vector which offers the advantage of having incorporated all the features of the rTANls system for tet induction in one single vector (69). The HCV genome is cloned under the control of the promoter TetOmP in pSTAR and is activated by rTANls, which are driven by a CMV promoter and enhancer located also in pSTAR (69). Thus transcription of the HCV genome is mediated by rTANls in association with tet. It has been reported that cloned HCV genome sequences often harbour deleterious mutations (11). Since the construction of pSTAR-HCV-S1 involved several rounds of cloning, five constructs bearing the full-length HCV cDNA, HCV-S1 (30, 58), in pSTAR were tested by transient transfection in 293T cells. To determine if the HCV proteins would be properly translated, we examined the expression of core, E2, and NS5A in these clones. All constructs expressed similar levels of these HCV proteins, 2 and 4 days posttransfection, indicating that the HCV genomic cDNAs were not defective (Fig. 1). Huh7 and HepG2 cells were then transfected with one of these pSTAR-HCV-S1 plasmids and selected in G418 for 6 to 8 weeks. Individual clones were picked and screened by Western analyses. Sixty-six Huh7 clones were obtained and 26 analysed. All these clones expressed comparable levels of the various HCV proteins upon tet induction, with little or no basal expression (data not shown). Two clones, SH-7 and -9, were selected for further analysis. In

the case of HepG2 cells, from 36 clones that were picked and analysed, only two clones were positive (G-19 and -22) (data not shown). Clone G-19 was found to express high levels of HCV proteins and was picked for further analysis.

A time course was carried out to determine the kinetics of induction of HCV proteins in these lines. HCV proteins were synthesized as early as 6 h after tetracycline treatment and the levels continued to accumulate up to 4 days after induction (Figs. 2A and 2B). In both SH-9 and G-19 cells, we observed that the induced HCV core protein had a molecular weight of about 16–17 kDa, corresponding to the mature processed form. Subsequently, at 96 h tet-treatment, we were also able to detect the full-length unprocessed core of about 21 kDa in G-19 cells (Fig. 2B). Interestingly, in G-19 an increase in truncated NS5A products was observed with time (Fig. 2B). We have demonstrated that HCV core can induce pro-
teolytic cleavage of NS5A through a caspase-mediated mechanism (16). It is highly likely that the truncated NS5A products seen at the later time points are the outcome of such an effect. Nevertheless, the protein levels were maintained for up to 10–12 days post-tet treatment with little evidence of morphological damage or cell death in both SH-7, -9, and G-19 cells, suggesting that the HCV proteins were initially relatively well-tolerated by both the hepatoma cell lines (data not shown). Withdrawal of tet from the culture media after day 8 led to a gradual shut-down in HCV protein synthesis, suggesting the induction process is reversible (data not shown). Prolonged culture of the clones in tet beyond 12–15 days, however, did result in increased cell death.

Although the expression of E2, NS3, NS4B, and NS5A were readily detectable in the two stable Huh7 cells, we were unable to detect core, E1, and NS5B from 6–48 h. However all three proteins became detectable after 4 days of tet-treatment (Fig. 2A). The reason for this disparity is unknown but maybe due to a higher turnover of these proteins in the cells. The expression of these proteins were readily observed in clone G-19 after 24 h of tet-treatment (Fig. 2B).

**HCV transgenes in stable HCV-inducible cell lines**

We next performed Southern analyses to examine the integration of HCV transgene(s) in the stable hepatoma clones G-19, SH-7, and -9 (Fig. 3). Plasmid pSTAR-FL HCV (clone 16) and genomic DNA were digested with EcoRI and Xhol (Fig. 3A) and probed with the pSTAR FL HCV construct. In the case of the plasmid DNA, an additional
digest with EcoR5 was also carried out. This digest produced seven bands, of molecular weights 4.42, 3.00, 2.70, 2.65, 2.30, 2.29, and 0.45 kb. The smallest fragment, consisting of a 0.45-kb band, had migrated out of the gel and hence was not observed (Fig. 3A). Similar restriction patterns were observed for both plasmid and genomic DNA (Fig. 3A). This suggested that a single copy of the transgene had integrated into the cell lines, as an additional band of 5.65 kb would have been observed had two or more copies of the plasmid integrated into the
chromosome in tandem repeats in a head-to-tail fashion (Fig. 3A). To verify the results observed, we redigested the DNAs with BglII and BstEII, which gave rise to three bands, of molecular weights 13.12, 3.8, and 1.8 (Fig. 3B). Plasmid DNA was further digested with EcoR5 as before. Similar to the previous finding, plasmid and genomic DNAs gave similar restriction patterns (Fig. 3B). No additional band of 5.6 kb was obtained, confirming that indeed only a single copy of the plasmid had integrated into the chromosome.

Expression of HCV transcripts in stable HCV-inducible cell lines

To study the expression of HCV transcripts upon tetracycline treatment, we carried out Northern blot analyses on the stable cell lines. We also in vitro transcribed the full-length plus-strand HCV-S1 and included it in our Northern as a positive control (Fig. 4). In all three cell lines, the viral genomic RNA migrated to a similar position as the full-length in vitro transcribed (IVT) product (Figs. 4A and 4B). Full-length viral mRNA was detected in SH-7, -9, and G-19 cells from 6 h of tet-induction (Figs. 4A and 4B). In general, the levels of transcripts were lower in SH-7 and -9 compared to G-19 cells. In the latter, they increased progressively from 6 h to 4 days after tet treatment (Fig. 4B). In SH-7, they peaked at 48 h post-tet addition and declined by day 4, while in SH-9 cells, they peaked around 24 h (Fig. 4A). We proceeded to assay for viral replication by using a specific HCV positive-strand riboprobe that binds minus-strand HCV RNA. Unfortunately we observed only very faint bands at 48 and 96 h post-induction in the Northern assays (data not shown). Hence, we turned to the more sensitive RT-PCR, adopting a strand-specific method devised by Lanford et al. (29), who used a tagged primer to determine the expression of minus-strand viral RNA (Figs. 4C and 4D). Plus-strand RNA was detected as early as 1 h post-tet induction in all three clones. Minus-strand RNA was also observed, although the expression was lower than the plus-strand RNA. In SH-7 and -9 cells, they were found 12 h after tet induction and persisted during the 4 days of tet-treatment (Fig. 4C). Similarly, minus-strand RNA were also present from 12–96 h after tet addition in G-19 cells (Fig. 4D). In contrast, control RNA from Hu7 and HepG2 cells failed to give rise to bands in either the Northern analyses or the RT-PCR (Figs. 4A–4D).

To ascertain the authenticity of these results, we repeated the experiments with actinomycin D (actD) treatment. We first induced G-19 cells with tet for 4 days and continued the incubation with [32P]-labelled UTP, in the presence or absence of actD for another 24 h. Total cellular RNA was harvested and Northern hybridized against plus- or minus-strand full-length IVT HCV-S1 (Fig. 4E). As a control RNA was hybridized against full-length minus-strand IVT HIV-1 (Fig. 4E). RNA from both untreated and actD-treated G-19 cells gave rise to a positive band with IVT minus-strand HCV-S1 (Fig. 4F, lanes 2 and 6). The signal from untreated cells was much stronger than actD-treated cells. Similarly, a weak but discernible signal was obtained when RNA from untreated and actD-treated G-19 cells were hybridized against IVT full-length plus-strand HCV-S1 (Fig. 4F, lanes 4 and 8). In all cases, no signal was detected when hybridization was performed with IVT full-length minus-strand HIV-1 (Fig. 4F, lanes 1, 3, 5, and 7). This shows that the binding of radiolabelled RNA from G-19 cells were HCV specific. These results indicate that authentic viral RNA synthesis was taking place in G-19 cells as actD selectively inhibits transcription from DNA templates but not from RNA templates.

We next repeated the actD experiments using RNA obtained from untreated and actD-treated FL-Δ5B-28 cells, a clone which was isolated from stable transfection of the HepG2 cell line with the construct, pSTAR-HCV-S1-Δ5B. The latter contains a HCV-S1 genome with a truncated NS5B sequence and expresses all HCV structural and nonstructural proteins with the exception of NS5B (Fig. 4G). We observed a relatively strong band after Northern hybridization of RNA from untreated FL-Δ5B-28 cells with IVT minus-strand HCV-S1 (Fig. 4F, lane 10). On the other hand, we failed to detect any signal after Northern hybridization with IVT plus-strand HCV-S1 (Fig. 4F, lane 12). In addition, FL-Δ5B-28 cells did not produce any signal with both plus- and minus-strand IVT HCV-S1 after treatment with actD (Fig. 4F, lanes 14 and 16). These results further show that the hybridization signals observed in actD-treated G-19 cells were HCV specific.

Ribonuclease protection assay for HCV minus-strand RNA in stable HCV-inducible cell lines

Next we carried out ribonuclease protection assay (RPA) for minus-strand viral RNA using a positive-strand riboprobe corresponding to the first 221 nucleotides of the NS3 5’ coding sequence. In the G-19 clone, a protected band similar in size to the probe was obtained from cells treated with tet for days 2, 5, and 10 (Fig. 4H). A positive control carried out with full-length negative-strand IVT HCV-S1 yielded similar results (Fig. 4H, lane 1). Notably, the intensity of the bands increased with the length of time the cells were induced with tet (Fig. 4H, lanes 5–7). No band was found in untreated G-19 cells nor in control tet-treated GSR-1 cells (a stable clone derived from the HepG2 cell line, bearing the pSTAR vector) (Fig. 4H, lanes 2 and 3).

Subcellular localisation of HCV proteins

We then proceeded to examine the subcellular localisation of HCV proteins in the HCV stable cell lines by indirect immunofluorescence microscopy. We chose the
FIG. 4. Northern analyses, RT-PCR, and ribonuclease protection assays for HCV RNA in total cellular RNA from stable HCV-hepatoma cell lines treated with tet for the indicated times. (A and B) Northern analyses. Detection of full-length HCV plus RNA in SH-7 and SH-9 (A) and G-19 (B) clones was observed as early as 6 h after tet treatment and comigrated with full-length in vitro transcribed (IVT) HCV RNA. Control RNA from Huh7 (A) and HepG2 (B) cells failed to give rise to any viral transcript. Levels of HCV RNA were normalised against endogenous GAPDH levels (A and B). (C and D) RT-PCR. Plus- and minus-strand HCV RNA were detected by Southern hybridisation after RT-PCR of total cellular RNA harvested from SH-7 and SH-9 (C) and G-19 (D) clones as described. Plus-strand transcripts were detected at 1 h (SH-7, -9, and G-19) post-tet induction while minus-strand transcripts were detected at 12 h post-tet induction (SH-7, -9, and G-19). (E) IVT. Full length HCV-S1 (2.5 μl) plus- (lane 2) and minus- (lane 3) strand IVT products were run on a denaturing agarose-formaldehyde gel. Lane 4 contains full-length HIV-1 minus-strand IVT product and lane 1 contains RNA ladder. (F) ActD treatment. Control untreated G-19 (i and ii) or FLΔ5B-28 cells (v and vi) and actD-treated G19 (iii and iv) and FLΔ5B-28 cells (vii and viii) were extracted for total cellular RNA as described. Hybridisation was performed with IVT full-length HCV-S1 minus- (lanes 2, 6, 10 and 14) and plus- (lanes 4, 8, 12, 16) strand products as described. As a control, they were also hybridised against IVT full-length HIV-1 minus-strand product (lanes 1, 3, 5, 7, 9, 11, 13, 15). Membranes were exposed to autoradiographic films for 1 (i), 2 (iii and vi), or 5 (ii, iv, vi–viii) days. (G) Western analyses for FLΔ5B-28 cells showing expression of various HCV structural and nonstructural proteins cultured for 4 days in the absence (−) or presence (+) of tet. Expression of NS5B was not detected in these cells. (H) RPA for the detection of HCV negative strand in G-19 cells. G-19 cells were induced with tet over a period of 10 days. RPA was performed using a probe corresponding to the first 221 nt of NS3 coding sequence as described. In vitro transcribed full-length negative-strand HCV RNA (IVT) was used as a positive control (G–I, lane 1). Control untreated G-19 (lane 4) and GSR-1 (lane 3) failed to give rise to the protected band observed in G-19 tet-treated cells (lanes 5–7). A lower exposure of lane 7 is provided (lane 8). Arrows indicate position of full-length viral transcript (A and B), RT-PCR product (C and D), full-length IVT products (E), and the RPA protected band (H), respectively.
clone SH-9 for these studies instead of G-19 cells, as the latter tended to grow in nonadherent clumps which were not suitable for these studies. With anti-E2, -NS4B, and -5A antibodies, strong membranous reticular staining concentrated at the perinuclear regions was observed, which was characteristic of localisation of these proteins in the endoplasmic reticulum (Fig. 5, right panel). In the uninduced cells, only a few cells exhibited a similar pattern of staining, at much lower levels (Fig. 5, left panel). Antibodies to core, E1, NS3, and -5B failed to produce good immunostaining (data not shown).

Capture of viral particles from culture media of tet-treated SH-9 and G-19

We next assessed the stable lines for their ability to release viral particles into the cell media. To do this, we induced SH-9 and G-19 cells with tet for 5 days, after which the culture media was incubated overnight.
with anti-E2 beads. As controls, we used media from untreated cells. All samples were subsequently treated with DNaseI and RNaseA after binding to the beads. Half of the samples were then extracted for RNA and treated again with DNaseI prior to RT-PCR analyses. A single strong PCR product of correct size was obtained in culture media of tet-treated SH-9 and G-19 cells, but not in uninduced cells (Fig. 6A and B, lanes 1, untreated and 3, tet-treated). This product was still present following pretreatment with DNaseI and RNaseA (Figs. 6A and 6B, lanes 2, untreated and 4, tet-treated). These results indicate that the PCR products were not due to contaminating viral RNA or plasmid DNA present in the culture media, but rather were derived from encapsidated viral particles that bound to the anti-E2 antibody beads.

Detection of viral RNA in sucrose density gradients at two major fractions

To further verify the secretion of viral particles by tet-induced stable HCV cells, we subjected the culture media of tet-treated SH-9 to sucrose density gradient centrifugation and analysed the fractions collected for viral RNA (Fig. 7). In the fractions we collected from media of untreated SH-9 cells, we observed only one band at the density of 1.28 g/ml (Fig. 7A). After tet-induction for 5 days, we obtained positive PCR bands to

FIG. 5. Indirect immunofluorescence staining of HCV proteins E2 (A), NS4B (B), and NS5A (C) in SH-9 cells after treatment with tet for 5 days. Cells were prepared for immunostaining as described under Materials and Methods. Control untreated cells gave little background staining (left) while induced cells showed strong perinuclear staining (right).
viral RNA at two separate ranges, one with a single peak at buoyant density of about 1.08 g/ml (fraction 4) and another that spanned from about 1.17 to 1.39 g/ml (fractions 7–12; Fig. 7B). As a control, we mixed the media from tet-induced cells with chloroform and performed the centrifugation as before. Not only did the two positive ranges disappear, the positive bands also shifted to the higher range of 1.35–1.43 g/ml, suggesting that chloroform had disrupted the envelope of viral particles present in the fractions of lower densities. We also repeated the experiment using culture media from tet-treated G-19 cells and obtained similar results (data not shown). To check the sensitivity of our RT-PCR assay, we repeated the RT-PCR using decreasing amounts of in vitro transcribed full-length HCV RNA from 100 ng to 0.01 fg. We observed RT-PCR products only when 100 ng to 1 pg of IVT RNA was used as template (Fig. 7D). From this experiment, we conclude that our assay had a detection limit of about 1 pg or the equivalent of about $10^5$ copies of viral RNA (Fig. 7D).

**Quantification of viral particles in culture media of tet-treated SH9 cells**

We next set out to quantify the amount of viral particles secreted into the culture media of tet-treated cells, by employing the Chiron bDNA signal amplification assay system. Culture media was harvested from tet-treated and untreated cells, and first treated with DNasel and RNaseA to eliminate any contamination from lysed cells. The culture media was then extracted for RNA and treated with DNasel again before being assayed. From four separate experiments (Table 1, samples A–D), we obtained an average value of $1 \times 10^5$ to $3 \times 10^5$ copies/ml of viral RNA in the culture media of SH-9 and G-19, respectively, after 5 days tet-induction (Table 1). The control culture media consisting of uninduced SH9 and G-19 cells failed to give detectable viral particles (Table 1).

**Presence of viral-like particles in stable SH9 cells and its culture media**

To investigate if viral particles were indeed made intracellularly, we first carried out transmission electron microscopy of tet-treated SH9 cells. We observed a relatively abundant number of electron dense spherical particles of approximately 20–25 nm in diameter located in the cytoplasm, often along the endoplasmic reticulum (Fig. 8A). These particles were consistently absent in control Huh7 and untreated SH9 cells (data not shown). The localisations in the cytoplasm and appearance of these particles resemble those of HCV nucleocapsids reported by other workers (37, 52, 53, 60). We proceeded to perform immunostaining experiments using anti-HCV core antibodies on the stable HCV-cell lines. In both SH-9 and G-19 cells we observed specific immunolabeling in the cytoplasm and cell surface (Figs. 8B–8D). Furthermore, spherical structures present on the cell surface (Figs. 8B and 8D) and in the cytoplasm (Figs. 8C and 8E) were labeled. These structures were mainly 20–30 nm in diameter, although staining of larger structures (30–50 nm) at the surface were also detected (Figs. 8B and 8D).

We next performed immunolabeling with anti-HCV E2 antibodies. In general we found specific strong labeling of ER and cytoplasmic vesicular membranous structures in tet-treated SH9 (Fig. 8F) and G-19 (Figs. 8G and 8H) cells. This observation is in agreement with that of Baumert et al. (2). Untreated cells failed to give any labeling (Fig. 8I). It is believed that HCV virions acquire their envelope proteins from host intracellular membranes and buds out of the cell via fusion of cytoplasm vesicles with the plasma membrane (51) and our findings with these stable cell lines concur with this hypothesis. Nevertheless, we failed to detect any HCV particles with enclosed envelopes corresponding to the full viral particles in SH-9 cells and only a low number in G-19 cells (Figs. 8G and 8H). One possible explanation for the absence/low numbers of complete virions may be that the process of envelope acquisition is slow or transient and affected by specific cellular host protein(s). We then checked for viral particles in the culture media from tet-SH9 cells after fractionation on sucrose density gra-
dient centrifugation. Complete viral-like particles of approximately 50 nm were observed by transmission electron microscopy after negative staining (Figs. 9B–9D). They were exclusively found in fractions of buoyant densities from 1.08 to 1.15 g/ml, which were also positive for viral RNA by RT-PCR (Fig. 9A). The nature of these particles was confirmed by their positive staining with anti-E2 antibodies (Figs. 9E and 9F).

Determination of 5' and 3' ends of HCV transcripts recovered from sucrose density gradient centrifugation

As described, the 5' end of the HCV-S1 transcript contained an additional 6 nt at its 5' UTR and an additional 8 nt at its 3' UTR. Extraneous nonviral sequences at either 5' and/or 3' ends of viral genomes have been reported to be removed during viral replication in cells and shown to be absent in progeny virus (24, 67, 68). It has been suggested that this in vivo repair can occur either through host or viral-encoded exonuclease activities (24, 67, 68). To investigate whether the extra nucleotides added to HCV-S1 RNA were retained or lost in progeny virions, we sequenced the ends of the RNA recovered from fractions 1.08–1.11 g/ml of the sucrose density gradient runs of culture media from tet-treated SH-9 and G-19. Purified viral RNA was first reverse ligated, followed by RT-PCR amplification, and then cloned as described under Materials and Methods. A band corresponding to about 195 nt was observed in the samples after two rounds of PCR amplification (Fig. 10).
After TA cloning, individual clones were selected for sequence analyses. Four clones were analysed from each sample. In all cases, the extraneous sequences at the 5′ and 3′ ends of viral RNA were found to be trimmed from the viral genome.

Infection of naïve Huh7 cells using culture media from tet-treated stable Huh7 clones

To affirm if the viral particles released infectious, we collected culture media from SH-9 cells following tet treatment and layered them onto Huh7 cells, or stable Huh7 cells expressing the putative receptor for HCV, CD81 (hCD81). As controls we used culture media from Huh7 cells expressing the pSTAR vector alone. Viral plus-strand RNA was detected by RT-PCR in cells layered with media from tet-treated SH9, but not control cells (Fig. 11A). In addition, using the strand-specific method devised by Lanford et al. (29), we also found negative-strand viral RNA, indicating that viral replication was taking place. We further observed that stable Huh7-hCD81 cells expressed more plus- and minus-strand viral RNAs compared to parental HuH7 cells (Fig. 11A).

To further verify that infection of the Huh7-hCD81 cells was mediated by HCV virions, we checked if infection could be abolished by anti-E2 antibodies or recombinant E2 proteins. First, culture media from tet-treated SH-9 cells was preincubated with protein AG-bound anti-E2 or human c-myc antibodies before being layered onto Huh7-hCD81 cells. The experiment was then carried out as before. Media precleared with anti-E2 but not c-myc antibodies failed to produce viral infection, as no RT-PCR bands were obtained for either plus-strand viral RNA (Fig. 11B, lanes 3 and 4). Next, we preincubated Huh7-hCD81 cells with recombinant truncated E2 proteins and repeated the experiment. As was expected, recombinant E2 completely blocked viral infection as we failed to obtain any bands after RT-PCR (Fig. 11B, lane 5).

DISCUSSION

We report here the generation of inducible stable clonal lines from the hepatoma cells, Huh7 and HepG2, that contain a full-length HCV genome, HCV-S1, bearing both the complete 5′ and 3′ UTRs (30, 58). It also includes the highly conserved 98-nt sequence found on the extreme 3′ end of the viral genome which is believed to be essential for replication in vivo (25, 65). These clones synthesized and accumulated HCV proteins following tet-treatment in a time-dependent manner (Fig. 2). Furthermore viral replication in these stable cells was demonstrated by treatment of the cells with actinomycin D (Fig. 4F) and the detection of the presence of negative-strand RNA using RPA (Fig. 4H) and strand-specific RT-PCR method (29, Figs. 4C and 4D).

Preliminary electron microscopic work revealed the presence of electron-dense subviral-like particles (approximately 20–25 nm in diameter) in the ER and cytoplasm of tet-treated SH-9 cells that were absent in the parental Huh7 or uninduced SH-9 cells (Fig. 8A). These particles resemble HCV nucleocapsids reported by other workers (37, 52, 53, 60). Their localisation at the ER within the cytoplasm as well as their immunolabeling by anti-HCV core antibodies (Figs. 8B and 8D) support this (37, 52, 53, 60). Although the diameters of the particles (about 20–25 nm) in SH-9 cells were smaller than those reported by other workers (30–35 nm) (37, 52, 53, 60), they concur with the 20–30 nm viral-like particles (VLPs) produced in the Pichia pastoris yeast transfected with an expression construct for the first N-terminal 399 aa of the HCV polyprotein (9). One plausible reason for this observed disparity in the sizes of VLPs is that the observed larger VLPs were surrounded by envelope-like structures derived from either the viral envelope proteins and/or the cell-membrane components (9). Indeed, following detergent treatment of the VLPs in P. pastoris, the authors obtained a homogenous population of particles of about 20 nm in diameter (9).

Interestingly, anti-core antibodies showed strong immunolabeling on the cell surfaces of tet-treated HCV cells (Figs. 8C and 8E), including spherical membranous structures of 30–60 nm in diameter. A recent report has indicated that nonenveloped HCV nucleocapsids are found in the serum of HCV-infected patients and chimpanzees (34). They were rather heterogenous in size, mainly from 38 to 43 nm in diameter, with some larger 54-to 62-nm particles (34). It is highly possible that these spherical structures observed in our HCV-cell lines correspond to HCV nucleocapsids being released from their cell surfaces. Nevertheless, few complete viral particles were detected in these HCV cell lines, despite strong staining by anti-E2 antibodies in the ER and Golgi apparatus (Figs. 8C, 8D, and 8E). The process of envelope acquisition may be slow or transient and affected by specific cellular host protein(s), which makes it difficult to visualize complete virions. In addition, it may also be due to the technical difficulties as optimal preservations of cellular and viral structures are critical parameters for viral visualization (2).

More importantly, these stable cells released infectious viral particles into their culture media after tet-induction. Viral transcripts were specifically detected in culture media of tet-treated cells after incubation with anti-E2 beads (Fig. 6) and were similarly detected by Chiron bDNA assay kit (Table 1). Complete viral-like particles of approximately 50 nm were further observed in the culture media of tet-treated SH-9 cells after sucrose density gradient centrifugation (Fig. 9). Specifically, these particles were found only in fractions with buoyant densities of between 1.08 and 1.15 g/ml, which were positive for viral transcripts, and in addition, were specifically immunostained with anti-E2 antibodies (Figs. 9E and 9F). These findings are in line with the reported low...
buoyant density of HCV particles obtained from HCV-positive patient sera (14, 21, 36). The sizes of these complete viral particles concurred with the findings of other workers where the size of HCV particles were estimated to be between 30 and 60 nm (12) and 40 and 60 nm (3, 20, 37, 56, 60). Hence, taken together with the results from the sucrose density gradient centrifugations and anti-E2 capture experiments, the EM/IEM studies...

FIG. 9. Electron microscopy of HCV-like particles in culture media of tet-induced SH-9 cells. (A) Ethidium bromide stained gels of RT-PCR-amplified DNA fragments from fractions 1–6 obtained after sucrose density gradient centrifugation of culture media from tet-treated SH-9 cells. The buoyant density of the fractions are shown at the top. (B–D) Fractions 4–6 were collected and stained with phosphotungstic acid as described under Materials and Methods. Viral-like particles of about 50 nm in diameter were observed in these three fractions with buoyant densities of about 1.08–1.15 g/ml (B to D). The bar indicates 100 nm. (E and F) Immunogold labeling by anti-E2 antibodies and protein A-gold (6 nm) of viral-like particles (arrows) similar to those observed in (B–D). The bar indicates 100 nm.

FIG. 8. Electron microscopy of HCV-like subviral particles in tet-treated SH-9 cells. At 5 days post-tet induction, cells were cryofixed and processed for electron microscopy as described. (A) Subviral-like particles of about 20–25 nm in diameter (arrows) were observed adjacent to endoplasmic reticulum (ER). Inset shows a higher magnification of the viral-like particles (arrowheads). Bar indicates 100 nm. (B–F) Immunogold staining of subviral-like particles (arrows) by anticores antibodies and protein-gold (10 nm), on cell surface of SH-9 (B) and G-19 (D) or in the cytoplasm of SH-9 (C) and G-19 (E) cells, affiliated with membraneous structures (arrowheads). Bar indicates 200 nm. (F–H) Immunogold labeling by anti-E2 antibodies and protein A-gold (10 nm) of rough endoplasmic reticulum (ER) in SH-9 (F) and G-19 (G) cells and Golgi apparatus (GA) in G-19 cells (H). Arrows indicate viral-like particles. Control untreated cells failed to give any labeling (I). Bar indicates 200 nm.
provide strong evidence that the stable HCV hepatoma cell lines are capable of releasing intact HCV particles into the culture media after tet-treatment.

Huh7 cells have been reported to be capable of supporting HCV replication (50). Human CD81 is a tetraspanning transmembrane receptor which was previously shown to interact with the HCV E2 envelope protein (45). Hence we tested the culture media from tet-induced SH7-9 and G-19 cells for viral particles by determining their ability to infect HuH7 and stable HuH7-hCD81 cells. Culture media from these tet-treated stable cell lines indeed led to infection of these cells as evidenced by the presence of plus- and minus-strand viral RNA 5 days postinfection. We also showed that this infection is mediated via enveloped virions as it was specifically inhibited by preincubating the media with anti-E2 antibodies but not with anti-c-myc antibodies. Moreover recombinant truncated E2 proteins were also capable of preventing viral infection of naïve unexposed cells.

We believe this is the first report of a inducible system which allows for the continuous production of infectious HCV viral particles. An earlier system was described in which stable osteosarcoma cell lines also expressed HCV proteins (40). However, these cells did not enable the assembly of complete viral particles. Besides the absence of both the viral 5’ and 3’ UTRs in the viral transgene, the NS5B protein was also not detected in the stable clones (40). Stable hepatoma cell lines that allow high level replication of subgenomic HCV RNAs have been established (4, 33, 46). In these systems, all the structural proteins were absent in the HCV subgenomic replicons and thus would not permit viral replication or assembly. More recently, transfections of full-length HCV replicons in HuH7 have also been reported (18, 57), although HCV viral particle formation was not observed (57). The authors postulated that HuH7 cells lack host factors to support viral assembly (57). We have instead observed viral particle assembly in stable clones derived from both HuH7 and HepG2 cells. The reason for this difference is unknown, but unlike the authors who observed different subcellular localization of core and E2 proteins (the former was found mainly on the surface of lipid vesicles, and the latter was found mainly in the ER), we detected the presence of both core and E2 in cytoplasm and ER of our stable clones. It is possible that the retention of HCV structural proteins in different cellular compartments prevented viral assembly in their system.
Interestingly, HuH7 cells bearing both HCV sub-genomic and genomic replicons were reported to release low levels of low-density structures containing nuclease-resistant HCV RNA (57). Such membranous vesicles differ from the viral particles we obtained from the supernatant of our stable cells in several ways. First, the viral particles from SH-9 and G-19 cells could be specifically captured by anti-E2 antibodies (Fig. 6) and immunostained with anti-E2 antibodies (Fig. 9). Furthermore, using the supernatant from tet-induced SH-9, we clearly demonstrated reinfection of naïve Huh7 cells (Fig. 11).

Although numerous groups have demonstrated the persistent, long-term infected cell lines, these often produce little HCV proteins, transcripts, and viral particles (see Introduction). Detection of viral transcripts in many cases is often inconsistent. Nevertheless, in our system, the rate of reinfection remains low, as we could only detect viral transcripts by RT-PCR. This may in part be explained by the relatively low numbers of viable viral particles in the culture media (between 1 and 6 × 10^5 copies/ml five days postinduction), and as well the inherent poorer infectivity of Huh7 cells. By generating a stable Huh7 cell line expressing human CD81, we were able to increase the infection efficiency, as demonstrated by the increased levels of plus and minus transcripts in these stable Huh7-CD81 cells compared to parental Huh7 (Fig. 10). Infection could possibly also be enhanced by collecting culture media from cells treated with tet for a longer period of time, and/or concentrating the media, or other mechanisms to enhance viral replication in vivo. On this note, mutations that occur within the various HCV nonstructural proteins, such as the NS5A (4, 37), have been found to confer either increased replicative ability or cellular adaptation. The incorporation of such advantageous mutations into the backbone of our HCV-S1 clone may serve to enhance viral particle production in our system. More work needs to be carried out to explore these various possibilities.

The stable clones reported here could offer a unique system for studying the molecular biology and life cycle of the HCV virus, in a cell type that mimicks the natural infection of HCV in vivo. In so doing, they may serve as a model to allow us to better understand HCV pathogenesis as well as disease onset and progression. In addition, they may also be useful for the screening of antiviral agents as well as the large-scale production of viral particles. Such tet-inducible cell lines are already successfully used in drug-screening activities against hepatitis B virus (HBV) (26, 27). These cell lines offer the advantage of being used in a high-throughout, automated assay system for large-scale evaluation of libraries of candidate anti-HCV compounds. The stable HCV-expressing clones described here could potentially serve as a platform for similar endeavours.

### MATERIALS AND METHODS

#### Cells and cell culture

The human embryonic kidney cell line, 293T, which bears the SV40 large T antigen, and the human hepatoma cell lines, Huh-7 and HepG2, were all purchased from American Type Culture Collection. The cells were cultured in MEM containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum and maintained at 37°C in 5% CO₂.

#### Construction of tetracycline-inducible HCV expression plasmids

The full-length HCV cDNA, HCV-S1, of genotype 1b (30), was cloned into the plasmid vector, pSTAR (69), in two steps. First, a 3-kb EcoRI-blunted XhoI fragment consisting of nt 6699 to 9609 of HCV-S1 was obtained from pKSII(NSP9) and cloned into the EcoRII-blunted BamHI site of pSTAR to generate pSTAR-NSP. Following this, a 6.7-kb EcoRI-EcoRI fragment consisting of nt −341 to 6358 was obtained from pXJ41(S1) (58) and cloned into the EcoRI site of pSTAR-NSP. Five clones bearing inserts of the correct orientation were selected. The construct pKSII(NSP9) contained the fragment from NS2 to 3’ UTR (nt 2428–9609) of HCV-S1 cloned into the SmaI site of pSTAR-FL. This results in an additional 8 nt at the 3’ end of the HCV genome. To generate the construct pSTAR-FLΔ5B, the sequence from nt 202–1579 of NS5B was deleted from construct pSTAR FL HCV by digestion with Bst98I and EcoRI site in front of the 5’ UTR of HCV-S1. An EcoRI site was introduced at the 3’ end of the CMV promotor in pSTAR so that transcription initiation results in an additional six nucleotides at the 5’ end of the HCV genome. To generate the construct pSTAR-FLΔ5B, the sequence from nt 202–1579 of NS5B was deleted from construct pSTAR FL HCV by digestion with Bst98I and Earl, followed by blunt-end ligation. This produces a truncated NS5B protein consisting of only 134 aa. For transfection experiments, both pSTAR HCV constructs were first linearised with EcoRV. This results in an additional 8 nt at the 3’ end of the HCV genome.

#### Sequence analysis

DNA sequencing of all constructs was carried out in the core facility at the Institute of Molecular and Cell Biology. Two hundred nanograms of the double-stranded templates and 10 ng of the primer were used for the dideoxy method with the Taq DyeDeoxy terminator cycle sequencing kit and the automated DNA sequencer 373 from PE Applied Biosystems (Foster City, CA).
Cell transfections

Transfection experiments were performed using Effectene transfection reagent from Qiagen (Valencia, CA). Briefly, 2–5 × 10⁵ cells were plated 14–18 h before and transfected with 1 μg of plasmid DNA according to the manufacturer’s protocol. Cells were incubated for 48–120 h at 37°C with 5% CO₂, after which cells were harvested for RNA isolation, Western analysis, or treated with 1000 μg/ml G418 for selection of stable clones. Media containing G418 were changed every 4–5 days and individual colonies were picked and expanded for analysis. For induction of stable cells, 2 μg/ml tetracycline was added for the periods indicated.

Western blot analyses

Protein samples were resolved on a 10 or 12% sodium dodecyl sulphate (SDS)–polyacrylamide gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat skim milk in PBS, and reacted with primary antibodies followed by anti-mouse or anti-human antibody conjugated with horseradish peroxidase (Sigma). Membrane-bound antibodies were detected with the ECL enhanced chemiluminescence kit (Pierce). The E2 antibodies, H52, and H53 were kind gifts from J. Dubuisson (Institut de Biologie de Lille & Institut Pasteur de Lille, Lille Cedex, France). H52 recognises a linear epitope with aa 585–661 of E2, while H53 recognises conformation-dependent epitopes within E2 (10). Both antibodies inhibit the binding of E2 to CD81-positive cells (10). HCV-S1 bears 84% amino acid homology with HCV-1 in the region recognised by H52. The NS3 monoclonal antibodies were purchased from Devaron, Inc. (NJ) and the NS4B and NS5A monoclonal antibodies were purchased from Biodesign International (ME). To detect core and NS5B, sera from HCV-infected patients were used at dilutions of 1:40–1:100. To screen for sera specific against core and NS5B, we carried out Western analyses with the patient sera on 293T cells transiently transfected with expression constructs for core or NS5B. A total of 15 patient sera samples were tested, three of which were found to react to NS5B, and at least six were found to react to core (data not shown).

RNA extraction

Cells were washed twice in phosphate-buffered saline (PBS) and added with 1.2 ml of the Trizol reagent from Gibco-BRL and resuspended with a pipette. The mixture was allowed to stand for 5 min at room temperature (RT), added with 0.35 ml of chloroform, and inverted for 20 s. It was incubated at RT for another 5 min and spun at 12,000 rpm for 20 min. The upper phase was transferred to a new Eppendorf tube, added with 0.8 ml of isopropanol, and mixed by inversion again. The tube was left at RT for 5 min after which it was spun again at 12,000 rpm for 20 min at 4°C. The RNA pellet was air-dried and redissolved in 50 μl DEPC-treated water.

Northern analyses

Total cellular RNA was extracted as described above using Trizol reagent. Thirty micrograms of RNA was run on 1% formaldehyde-denaturing agarose gels according to standard protocols. Hybridisation was carried out with 25 ng of ³²P-random-primed DNA fragment corresponding to nt 8967–9374 of HCV-S1 in hybridisation buffer containing 5× SSC, 1× Denhardt’s solution, and 50% formamide. The filters were incubated at 42°C for 14–16 h, after which they were washed twice at 50°C in 3× SSC–0.1% SDS followed by 0.3× SSC–0.1% SDS. The filters were then air-dried and exposed to autoradiography films at −70°C for 1–2 days.

RT-PCR

Primers for RT-PCR to detect plus- and minus-strand RNA and GAPDH are listed in Table 2. A 5-μl volume of the RNA isolated from cells was reverse transcribed at 42°C for 30 min using the specific antisense primer and 200 U MMLV RT (New England Biolabs). The cDNA samples were heated at 100°C for 1 h, followed by treatment with 2.5 μg RNase A for 30 min at 37°C. PCR was carried out with Taq polymerase (Roche). The first PCR reaction was performed with 2.5 μl of template in a total volume of 50 μl followed by second round of PCR with 1 μl of the first PCR reaction. For the detection of plus-strand RNA, PCR was performed with P1 and P4, followed by P2 and P3 or P5. For the detection of minus-strand RNA, a strand-specific RT-PCR method devised by Lanford et al. was followed (29). RT was carried out using the tagged primer, P1-tag (29). The first round of PCR was carried out with tag and P4 and the second round with tag and P3 or P5. PCR conditions are as follows: 95°C for 3 min, followed by 30 cycles of 95°C for 20 s, 60°C (for plus-strand) or 49°C (for minus-strand) for 20 s, 72°C for 30 s, and a final extension 72°C for 8 min. In some instances, 40 cycles of the first-round PCR was carried to determine the presence of viral transcripts. In the case of GAPDH, the PCR conditions were 30 cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 30 s. Amplified products were visualised by ethidium bromide staining in a 2.5% agarose gel.

Ribonuclease protection assay

One hundred micrograms of total cellular RNA was treated with DNase1 (1 U/μg RNA) for 2 h at 37°C. RPA was performed using the RNase protection assay from Roche (Mannheim, Germany), according to the manufacturer’s recommendations. Briefly, RNA was coprecipitated with ³²P-labelled probe corresponding to the first 221 nt of NS3 coding sequence (5 × 10⁵ cpm) and the
recovered pellet was resuspended in 30 μl of hybridisation solution (40 mM pipes, 400 mM NaCl, 1 mM EDTA, 80% (v/v) formamide, pH 6.4) at 45°C for 12 h. The reaction mixture was then treated with RNaseA and RNase T1, followed by digestion with proteinase K. It was then extracted with phenol/chloroform/isoamyl alcohol and precipitated in 70% (v/v) ethanol. The pellet was resuspended in 30 μl of hybridisation solution (40 mM Pipes, 400 mM NaCl, 1 mM EDTA, and 80% (v/v) formamide, pH 6.4), heated at 95°C for 5 min, and subjected to electrophoresis in a 6%/7 M urea gel at 30 W for 2.5 h in Tris–borate buffer. The gel was air-dried and exposed to autoradiography films at −70°C for 1–2 days. To detect the presence of the transgene in the stable cell lines, 5 × 10⁷ cells were harvested, washed twice in PBS, and digested overnight at 50°C, with 600 μCi of [32P]UTP using the RiboProbe (USB-Amersham). Hybridisation was carried out with 50 pmol of 32P-end-labelled oligonucleotide corresponding to the 5′ NCR of HCV or GAPDH (Table 2) in hybridisation buffer containing 6× SSC, 1× Denhardt’s solution, and 0.05% Na pyrophosphate. The filters were incubated at 50°C for 14–16 h, after which they were washed twice at 68°C in 3× SSC–0.1% SDS followed by 0.1× SSC–0.1% SDS. The filters were then air-dried and exposed to autoradiography films at −70°C for 1–2 days. To detect the specificity of the products obtained by PCR amplification, 25 μl of the PCR products were hybridised against the entire HCV-S1 cDNA. Hybridisation was carried out as above with 25 ng of each plasmid in the absence or presence of 50 μCi of [α-32P]rCTP using the RiboProbe corresponding to the entire HCV-S1 cDNA.

Southern analyses

To determine the specificity of the products obtained by PCR amplification, 25 μl of the PCR products were Southern blotted onto Hybond N+ membrane (USB-Amersham). Hybridisation was carried out with 50 pmol of 32P-end-labelled oligonucleotide corresponding to the 5′ NCR of HCV or GAPDH (Table 2) in hybridisation buffer containing 6× SSC, 1× Denhardt’s solution, and 0.05% Na pyrophosphate. The filters were incubated at 50°C for 14–16 h, after which they were washed twice at 68°C in 3× SSC–0.1% SDS followed by 0.1× SSC–0.1% SDS. The filters were then air-dried and exposed to autoradiography films at −70°C for 1–2 days. To detect the presence of the transgene in the stable cell lines, 5 × 10⁷ cells were harvested, washed twice in PBS, and digested overnight at 50°C, with 600 μCi of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS) containing 0.1 mg/ml proteinase K. The samples were extracted twice with phenol/chloroform/isoamyl alcohol and the genomic DNA precipitated with 0.3 M Na acetate and 2× volume of ethanol. Thirty micrograms of DNA was digested with the respective restriction enzymes, run on a 0.8% agarose gel, and blotted onto Hybond N+ membranes. Hybridisation was carried out as above with 25 ng of 32P-random-labelled probe corresponding to the entire HCV-S1 cDNA.

In vitro transcription

The plasmids pcDNA(S1), containing full-length HCV-S1 in pcDNA3.1(+) and pKSII NS3(8), containing full-length NS3 in pKSII (+/−) (68), were linearised with XbaI and BsrGI, respectively. In vitro transcription was performed with 1 μg of each plasmid in the absence or presence of 50 μCi of [α-32P]rCTP using the RiboProbe in vitro transcription system from Promega (WI) accord-
ing to the manufacturer’s recommendations. The DNA template was removed using RQ1 RNase-Free DNase (Promega) (1 U/μg DNA). The sizes of the IVT products were checked by visualisation on a denaturing agarose gel following ethidium bromide staining. The amount of radioactivity incorporated was determined with the scintillation counter. In vitro transcription of full-length plus- and minus-strand HCV-S1 was performed with the pcDNA(S1) (58) and pcDNA(S1-) (S. P. Lim, unpublished results), respectively, and that of full-length minus-strand HIV-1 was performed with pHXB2 (48).

Capture of viral particles from culture media

To determine if viral particles are secreted into the culture media of tet-induced cells, culture media was harvested from 1 × 10⁸ SH-9 or G-19 cells incubated for 5 days with or without tet-induction and clarified at 2000 rpm for 10 min. One-fifth of the media was incubated overnight at 4°C with protein A/G beads precoated with approximately 10 μg of anti-E2 (H52) antibodies. The beads were then spun at 14,000 rpm for 5 min and washed six times with PBS. Beads were then extracted for RNA by Trizol reagent or first incubated with 10 U of DNaseI (Promega) (1 U/μg DNA). The sizes of the IVT products were checked by visualisation on a denaturing agarose gel following ethidium bromide staining. The amount of radioactivity incorporated was determined with the scintillation counter. In vitro transcription of full-length plus- and minus-strand HCV-S1 was performed with the pcDNA(S1) (58) and pcDNA(S1-) (S. P. Lim, unpublished results), respectively, and that of full-length minus-strand HIV-1 was performed with pHXB2 (48).

Sucrose density gradients

Culture media harvested from 1 × 10⁸ SH-9 or G-19 cells incubated for 5 days with or without tet-induction was clarified at 2000 rpm for 10 min and spun at 100,000 g for 14–16 h. The pellet was resuspended in TEN (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, pH 8) and layered onto a 20–60% (wt/vol) sucrose (in TEN) gradient and centrifuged at 150,000 g for 72 h at 5°C. Five hundred microliters fractions were collected from the top, extracted for RNA, followed by DnaseI treatment, and analysed by RT-PCR as described. As a control, culture media from SH9 cells treated with tet was mixed vigorously with chloroform for 10 min and centrifuged after the spin at 100,000 g. It was then spun on the sucrose density gradient and treated as before. The density of each fraction was determined by refractometry.

Determination of 5’ and 3’ ends of HCV RNA

Viral RNA from fractions obtained after sucrose density gradient centrifugation were purified as described above. Viral RNA was ligated with T4 RNA ligase (NEB, MA) at 37°C for 1 h, followed by RT using primer S1extREV (Table 2), and two rounds of PCR amplification, using two sets of primers S1extFOR and S1extREV and S1intFOR and S1intREV (Table 2). The PCR products were purified and TA-cloned into pCRII (Invitrogen). The plasmids were sequenced using M13FOR and REV primers.

Indirect immunofluorescence

SH-9 cells grown on coverslips were untreated or treated with tet for 5 days, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. They were stained with anti-E2 (H52, diluted 1:40), anti-NS4B (Biodesig Int, diluted 1:40), and anti-NSSA (Biodesig Int, diluted 1:40) in 2% BSA in PBS, followed by fluorescence isothiocyanate-conjugated anti-mouse antibody (Sigma) each for 30 min at room temperature. Cells were washed with PBS between steps. Coverslips were mounted and immunofluorescence microscopy analyses performed with a MRC1024 (Bio-Rad) confocal laser scanning system.

Electron microscopy

Cells were harvested and fixed in 2.5% glutaraldehyde followed by processing for electron microscopy as described in Refs. 42, 44, and 63. Immunoelectron microscopy was carried out on cells as described (32, 66). For analysis of viral particles in fractions from sucrose density gradient centrifugation, fractions were spun at 150,000 g for 4 h, after which the pellet obtained was resuspended in 100 μl PBS and applied to formvar-carbon-coated grids and negatively stained with phosphotungstic acid (20). For immunostaining, one drop of the sample was adsorbed on a formvar-carbon-coated copper grid for 10 min at room temperature. It was incubated on a drop of primary antibody (at dilutions of 1:10 for anti-core (a gift from Michinori Kohara, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and 1:20 for anti-E2 (H52) in PBS-BSA) for 30 min, followed by rinses on four drops of PBS. It was then incubated on a drop of anti-mouse antibodies for 30 min, rinsed as before, and incubated with protein A-gold for 15 min. A final wash was carried out twice with PBS and thrice with water before negative staining with uranyl acetate.

Infection experiments

The stable line SH-9 was cultured in tetracycline for 5 days, after which the culture media was removed, spun at 2500 rpm for 5 min, aliquoted, and frozen at −80°C. Four hundred microliters of culture media was layered onto 2.5 × 10⁵ Huh7 cells or stable hCD81-Huh7 cells in 60-mm petri dishes. The full-length CD81 cDNA was RT-PCR amplified from human spleen cDNA library and cloned into plasmid pcDNA3.1. Huh7 cells were stably transfected with this construct and individual clones isolated by selection in G418. Highly expressing CD81-Huh7 clones were isolated by FACscan sorting (Tan et al.,
unpublished data). Cells were added with 4 ml complete media and incubated for 6–8 h at 37°C, after which they were washed six times with PBS, added with 4 ml fresh media, and reincubated for 5 days at 37°C. At the end of the period, the supernatant was removed, the cells were washed two times with PBS, and total cellular RNA was extracted with the Trizol reagent from Gibco-BRL.

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