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Replication of a novel subgenomic HCV genotype 1a replicon expressing a puromycin resistance gene in Huh-7 cells

Chengyu Liang[a], Elizabeth Rieder[a,1], Bumsuk Hahm[b], Sung Key Jang[b], Aniko Paul[a], Eckard Wimmer[a,*]

[a]*Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, NY 11790, USA*
[b]*Department of Life Science, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31, Hyoja-Dong, Pohang, Kyungbuk 790-784, Korea*

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Abstract

Genotype 1a is a most prevalent genotype of hepatitis C virus in North America yet HCV replication has been studied predominantly with genotype 1b subgenomic replicons under neomycin selection in Huh-7 cells. Development of 1a-related dicistronic replicons under neo selection proved difficult and required either "conditioned" Huh-7 cells and/or chimeric genomes harboring pre-engineered adaptive mutations. We report the construction of a novel dicistronic genotype 1a(H77C) replicon expressing the *puromycin* N-*acetyltransferase* (PAC) gene as a selectable marker that, without prior introduction of adaptive mutations, allows establishment of puromycin-resistant Huh-7 colonies after transfection of naïve Huh-7 cells. The large majority of HCV1a/PAC replicons did not reveal any adaptive mutations on short-term passage of Huh-7 cells. Continued passage led to mutations in the non-structural genes although these mutations did not significantly enhance replication of the original replicon. Transfection with total cellular RNA isolated from HCV1a/PAC replicon-containing cells led to a significant increase in colony-forming ability. The data identify PAC as an efficient selectable marker for studies of HCV replication, which may be useful with different genotypes in different host cell systems.

Keywords: Hepatitis C virus; RNA replication; Genotype 1a replicon; Puromycin resistance gene

Introduction

Hepatitis C virus (HCV), a human virus, causes inflammation of the liver, a condition that frequently progresses to persistent infection and very serious chronic liver disease. With an estimated 200 million people infected worldwide, HCV, the sole member of the genus Hepacivirus of Flaviviridae, has emerged as a major public health problem (Wasley and Alter, 2000). Currently, there are no specific anti-HCV vaccines or drugs available although combination therapy with interferon and ribavirin has led to limited success in viral clearance and cure (Medina et al., 2003; Poynard et al., 1998; Reichard et al., 1998).

HCV genomes are distinct by broad sequence diversity. They have been separated into six major genotypes of which various combinations cluster in different parts of the world. Genotypes 1a and 1b are the most prevalent genotypes in North America (Zein et al., 1996). Unfortunately, the success rate in treatment of genotype 1-caused persistent infections with interferon/ribavirin is low as compared to the treatment of persistent infections of other genotypes (Zein, 2000). The HCV genome expresses a polyprotein with an order of coding regions typical for Flaviviridae (Choo et al., 1989; Reed and Rice, 2000; Fig. 1A). The most pronounced difference between HCV and member viruses of the genus Flavivirus (e.g., yellow

* Corresponding author. Fax: +1 631 632 8891.
E-mail address: ewimmer@ms.cc.sunysb.edu (E. Wimmer).
[1] Present address: Foot-and-Mouth Disease Research Unit, USDA, ARS, Plum Island Animal Disease Center, Greenport, NY 11944-0848, USA.

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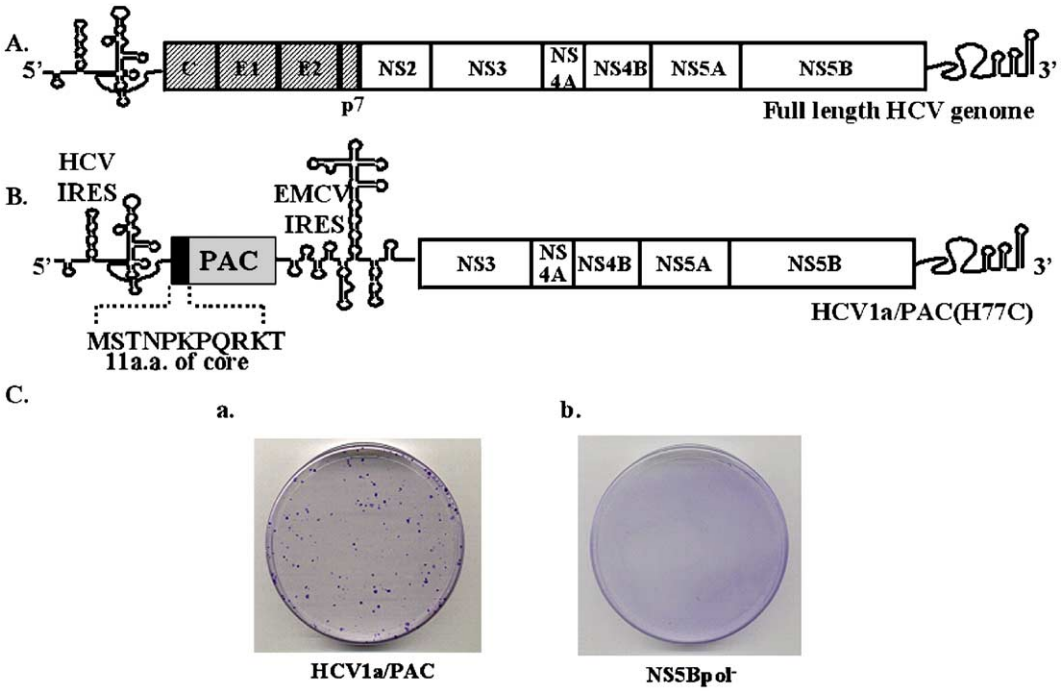

Fig. 1. Schematic representation of the HCV genome and the subgenomic HCV replicon HCV1a/PAC and selection of puromycin-resistant colonies. (A) Structure of the HCV genome. The open reading frame indicated by boxes is flanked by the 5′ and 3′NTR. The structural proteins are located in the NH_2-terminal part of the polyprotein (stripped boxes), and the remainder (NS2 to NS5B) encodes the nonstructural proteins. (B) Structure of the HCV1a-based replicon designated as HCV1a/PAC. It is composed of the HCV 5′NTR (solid line), the first 11 amino acids of the core region (solid box) fused to the PAC gene (shaded box), the EMCV-IRES (solid line), and HCV sequences from NS3 to NS5B and the 3′NTR. PAC, *puromycin* N-*acetyltransferase*. (C) Puromycin-resistant colonies produced after transfection with wild type (wt) and mutant HCV replicon RNAs. Transfections of Huh-7 cells with the wt (Left) and NS5B (pol^-) (Right) mutant replicon RNAs were carried out as described in Materials and methods.

fever virus, dengue virus) is the presence of an internal ribosomal entry site (IRES) in the 5′ non-translated region (NTR) (Tsukiyama-Kohara et al., 1992). There is no evidence of a 5′-terminal capping group in viral RNA, which may explain the adoption of an IRES by the HCV genome. The precise nature of the 5′ end in virion RNA is still uncertain.

All HCV genotypes notoriously resist cultivation in tissue culture. Studies of the molecular biology of viral proliferation have, therefore, relied on subgenomic dicistronic replicons (Lohmann et al., 1999), which resemble in architecture dicistronic polioviruses and dicistronic poliovirus expression vectors (Alexander et al., 1994; Molla et al., 1992). In these dicistronic HCV replicons, the open reading frame of the HCV polyprotein, or a segment thereof, is driven by the IRES of encephalomyocarditis virus (Jang et al., 1988), while the HCV IRES is used to drive the gene of a selectable marker, neomycin phosphotransferase (neo) (Lohmann et al., 1999). The neo gene confers resistance to neomycin sulfate (G418). Interestingly, Huh-7 cells, derived from human hepatoma, have been found to serve as the only efficient cell line in which replication of replicons was scored after transfection of in vitro transcribed RNA. Following neo selection, cell colonies have been isolated, which contained efficiently replicating dicistronic subgenomes. These replicon RNAs revealed distinct adaptive mutations, mapping to different coding regions of the polyprotein (Blight et al., 2000; Lohmann et al., 2001; Guo et al., 2001; Krieger et al., 2001). Interestingly, adaptive mutations rapidly evolved in HCV[Con1] replicons (Con1 referring to the 1b HCV strain from which the replicon was constructed) and were found to be required for efficient replication. In contrast, replicons based on different strains of genotype 1b, e.g., HCV-N (Ikeda et al., 2002) or genotype 2a JFH-1 (Kato et al., 2003), did not display such degree of needed genetic variation for survival in neomycin-treated cells. This is surprising and indicates some degree of genotype-related dependence of neo-selected replicons.

Recently, the host range for HCV dicistronic replicons has been expanded to human HeLa cells (derived from a human cervical carcinoma) and mouse hepatoma cells (Zhu et al., 2003). However, cell colonies under neomycin selection could not be established when transcript RNAs were transfected into these cells (Zhu et al., 2003). It was necessary to use total RNA of replicon-carrying cells for transfections. This interesting restriction will be discussed later.

So far, all HCV replicons are based on selection to neomycin resistance, and by far the largest number of studies has been carried out with subgenomic RNA of the 1b genotype. For unknown reasons, constructs with the genotype 1a sequence, nearly identical in architecture

to those of 1b, did not yield stably transformed cells that would support replication under neomycin selection. Nonetheless, specific cell lines harboring 1a-specific replicons under neomycin selection have been developed recently (Blight et al., 2003; Grobler et al., 2003). However, the cells used to establish persistent replication of 1a replicons were not "naïve" Huh-7 cells but Huh-7 cells that had been "cured" previously from 1b HCV replicons with interferon (Blight et al., 2002; Murray et al., 2003). The phenotype of the more permissive cells, referred to by Blight et al. (2002) as Huh-7.5 cells, is not well understood, but the previous presence of replicating HCV replicons must have left persistent imprints to render the cells more accommodating for uptake, translation, or RNA synthesis of the HCV replicons.

Gu et al. (2003) and Yi and Lemon (2004) have also succeeded recently in the construction of 1a replicons supporting the establishment of naïve Huh-7 cells under neo selection. However, these 1a replicons are chimeras in which the 5′-terminal 225 nt of the NS3 reading frame of 1a was replaced with those of genotype 1b to facilitate better translation of the 1a-specific ORF (Guo et al., 2001). Moreover, replication of these chimeric 1a replicons in naïve Huh-7 cells was achieved only if a mutation (S2204I), known previously from studies with 1b replicons to greatly enhance replication, was engineered into the 1a NS5A coding sequence (Gu et al., 2003; Yi and Lemon, 2004).

In pursuit of an alternative model system for HCV replicons, we describe here the development of a dicistronic replicon expressing the *puromycin* N-*acetyltransferase* (PAC) gene, a selectable marker conferring puromycin resistance to transfected cells. As the model HCV RNA, we chose genotype 1a [H77C] HCV the genome of which had been cloned previously by Yanagi et al. (1997). Puromycin-resistant colonies were easily recovered after transfection of naïve Huh-7 cells under appropriate conditions and replication of HCV1a/PAC replicon RNA was observed with ~500 copies per cell. Establishment of cells harboring the HCV1a/PAC replicon under puromycin selection did not require prior mutation of the HCV genome. A high percentage of clonal Huh-7 cell lines were able to support HCV replication under puromycin selection independent of adaptive mutations in the replicons. On long-term passage of Huh-7 cells harboring 1a/PAC replicon RNA under continuous selection revealed, depending upon the clone selected for analyses, some mutations of which the S2204I mutation in NS5A dominated. However, in vitro reconstructed mutant replicon RNA harboring the S2204I mutation had only a modest effect on the initiation of RNA replication whereas transfection with total cellular RNA isolated from genotype 1a replicon containing naïve cells led to a significant increase in the number of resistant colonies.

The data show that a dicistronic replicon expressing the PAC selectable marker offers an attractive new avenue for the study of HCV genotypes other than genotype 1b.

Results

An HCV1a/PAC replicon conferring puromycin resistance to naïve Huh-7 cells

The difficulties of transferring the strategy of dicistronic 1b replicons expressing the neo gene in Huh-7 cells (Blight et al., 2000; Ikeda et al., 2002; Kato et al., 2003; Lohmann et al., 1999, 2001) to the equivalent 1a genotype (Blight et al., 2000; Guo et al., 2001; Ikeda et al., 2002) are unexplained. It could be due to the intrinsic properties of proteins expressed by HCV 1a in combination with neomycin selection in Huh-7 cells. Whatever the reason, the restriction has been overcome recently by, first, using either "cured" Huh-7 cells (Blight et al., 2002; Murray et al., 2003) that were permissive to replication of 1a replicons under neomycin selection (Blight et al., 2003; Grobler et al., 2003) or, second, by using 1a/1b chimera harboring an engineered mutation in the coding region of NS5A (Gu et al., 2003; Yi and Lemon, 2004). These modified chimeric replicons allowed replication in naïve Huh-7 cells.

We have explored the possibility whether a different model of selection in Huh-7 cells may favor the establishment of cell lines harboring efficiently replicating 1a-related dicistronic replicons. We speculated that the neo selection might block in some manner the establishment of stable cell lines when naïve Huh-7 cells or when the unchanged 1a genotype is used. Therefore, we constructed a dicistronic replicon similar to that originally described by Lohmann et al. (1999) except that the neo resistance gene was replaced by a gene encoding *puromycin* N-*acetyltransferase* (PAC). Expression of the small PAC gene (597 bp) in transfected cells confers resistance to puromycin, a potent inhibitor of translation. PAC has been used successfully in alphavirus replicons for the selection of specific alphavirus phenotypes (Frolov et al., 1999). We speculated that a transdominant marker different from neomycin might allow the 1a-specific replicon to establish cell colonies under puromycin selection.

The HCV1a/PAC replicon used in our studies is based on the infectious HCV 1a-cDNA clone H77C, originally constructed by Yanagi et al. (1997) (Fig. 1A). The genetic map of the HCV1a/PAC replicon, [HCV]5′NTR-Δcore-PAC-[EMCV]IRES-[HCV]NS3-NS4-NS5-3′NTR (Fig. 1B), entails the HCV 5′ NTR including the IRES that regulates the synthesis of PAC, and the EMCV IRES regulating the expression of the truncated 1a HCV polyprotein. The gene for PAC has been fused at the 5′ end to a small segment of the core protein reading frame (33 nt) since a nucleotide sequence downstream of the initiating AUG is required for HCV IRES-mediated translation (Lu and Wimmer, 1996; Reynolds et al., 1995). The NS3 to NS5 coding regions specify proteins absolutely essential for replicon replication (Bartenschlager and Lohmann, 2001; Reed and Rice, 2000). NS3 is a serine proteinase and a helicase, NS4A a co-factor for the NS3 proteinase by forming a heterodimer, and NS5B

the RNA-dependent RNA polymerase. NS4B can induce the formation of cytoplasmic vesicles that are thought to function as platform for RNA replication (Egger et al., 2002; Hugle et al., 2001). The function(s) of NS5A, a highly phosphorylated protein, is not yet known. Recently, it has been reported that hyper-phosphorylation of NS5A can down-regulate HCV RNA replication in cell culture (Neddermann et al., 2004).

Huh-7 cells, which allow replication of HCV 1b/neo replicon RNA, were also chosen for transfections with the 1a-specific replicons. Run-off transcripts of the HCV1a/PAC replicon plasmid (see Materials and methods) were introduced into naïve Huh-7 cells, and the transfected cells were grown under the selection of puromycin. As reported previously, 1a replicons expressing neomycin resistance failed to yield colonies under neomycin selection in similar experiments (Blight et al., 2000; Guo et al., 2001). However, transfection of HCV1a/PAC replicon RNA followed by selection resulted reproducibly in puromycin-resistant colonies, ranging from 20 to 200 colonies per microgram of RNA (Fig. 1C). A mutant construct carrying a NS5B RNA polymerase-defective lethal mutation failed to produce colonies, as expected (Fig. 1C). Transfection efficiency was affected by the condition of cell growth: 80–85% subconfluent Huh-7 cells passaged at regular intervals were optimal for RNA transfection. The relative efficiency of transfection was consistent across different samples. No obvious changes in cell morphology were observed in isolated colonies except a slight retardation of growth in comparison with naïve Huh-7 cells. All selected cell lines displayed persistent resistance to puromycin in the medium at 2–4 μg/ml. These results suggest that PAC selection can be used to isolate colonies harboring replicating 1a-dicistronic replicons.

Multiple independent clonal Huh-7 cell lines supporting HCV1a/PAC subgenomic RNA replication post transfection, as well as a cell line derived from pooled puromycin-resistant colonies post-selection, were examined and showed similar replication profiles (data now shown). Data presented in the following for RNA and protein analyses have been derived from the fastest growing clone, clone A.

Evidence of viral RNA replication and viral protein expression in Huh-7 cells transfected with HCV1a/PAC replicon transcripts

The presence of replicon RNA in puromycin-resistant Huh-7 cells (clone A) after the first few consecutive passages was analyzed by RT-PCR with HCV-specific primers (see Materials and methods). An RT-PCR fragment of the expected size was generated from RNA of the transformed cells (Fig. 2). There was no amplification of DNA fragments that may have resulted from carry-over of plasmid DNA or from integration of plasmid DNA because direct PCR assay of total cellular DNA with viral-specific or PAC-specific primers, bypassing the step of reverse transcription, did not yield any positive PCR products (data not shown).

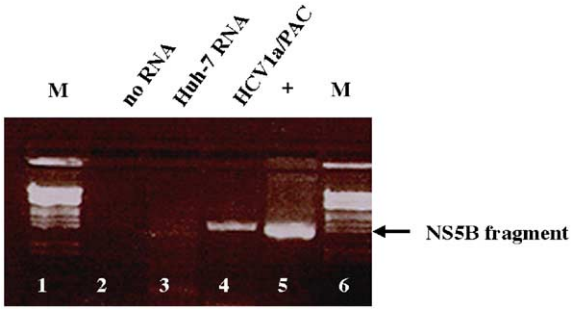

Fig. 2. RT-PCR analysis of the total cellular RNA from a selected Huh-7 clone. NS5B-specific primers were used for the standard RT-PCR analysis, as described in Materials and methods. As control, RT-PCR reaction with naïve Huh-7 cellular RNA, with (Lane 3) and without RNA template (Lane 2), was included. Lanes 1 and 6: DNA molecular weight marker III (M); Lane 4: RT-PCR of the total cellular RNA from a selected Huh-7 clone; Lane 5: RT-PCR of the respective transcript RNA (+). The resulting 1540-bp fragment DNA was detected on an agarose gel and identified as NS5B sequence by DNA sequencing.

Replication of 1a-specific replicon RNA was then demonstrated by metabolic labeling with [^{3}H]-uridine in the presence of 5 μg/ml actinomycin D (ActD), a potent inhibitor of cellular DNA-dependent RNA synthesis at this concentration. This method has been previously applied for 1b replicons (Lohmann et al., 1999). Clone A cells (10^6) harboring the HCV1a/PAC replicon and the same number of naïve Huh-7 cells were preincubated with actinomycin D for 1 h after which [^{3}H]-uridine was added (see Materials and methods). Fig. 3A shows that the level of labeled RNA in clone A cells increased more than 10-fold during the first 30 h of incubation, then dropped sharply. No labeling of cellular RNA was observed under the same conditions, a result strongly suggesting that the labeled RNA corresponded to replicon RNA. Indeed, autoradiography of total RNA revealed a band with the estimated molecular weight (~7.8 kb) expected for a 1a replicon RNA (data not shown). The loss of [^{3}H]-uridine-labeled RNA after 30 h is most likely due to degradation under the condition of Act D-induced host cell death.

In further support that replicon RNA of correct size was present in persistently selected clone A cells, total cellular RNA was isolated from 10^6 cells and examined by Northern blot analysis. As shown in Fig. 3B, a band approximating 7.8 kb was observed, corresponding to the molecular weight of intact subgenomic RNA. This is in agreement with the result of RNA labeled metabolically with [^{3}H]-uridine in the presence of actinomycin D. Control samples involving naïve Huh-7 cells did not reveal any RNA corresponding to replicon RNA.

Using two specific monoclonal antibodies for Western blot analyses, it was subsequently shown that the cells transfected with clone A contained viral polypeptides NS3 and NS5B, as would be expected if the replicons proliferate and express their encoded proteins (Fig. 4A). A rough

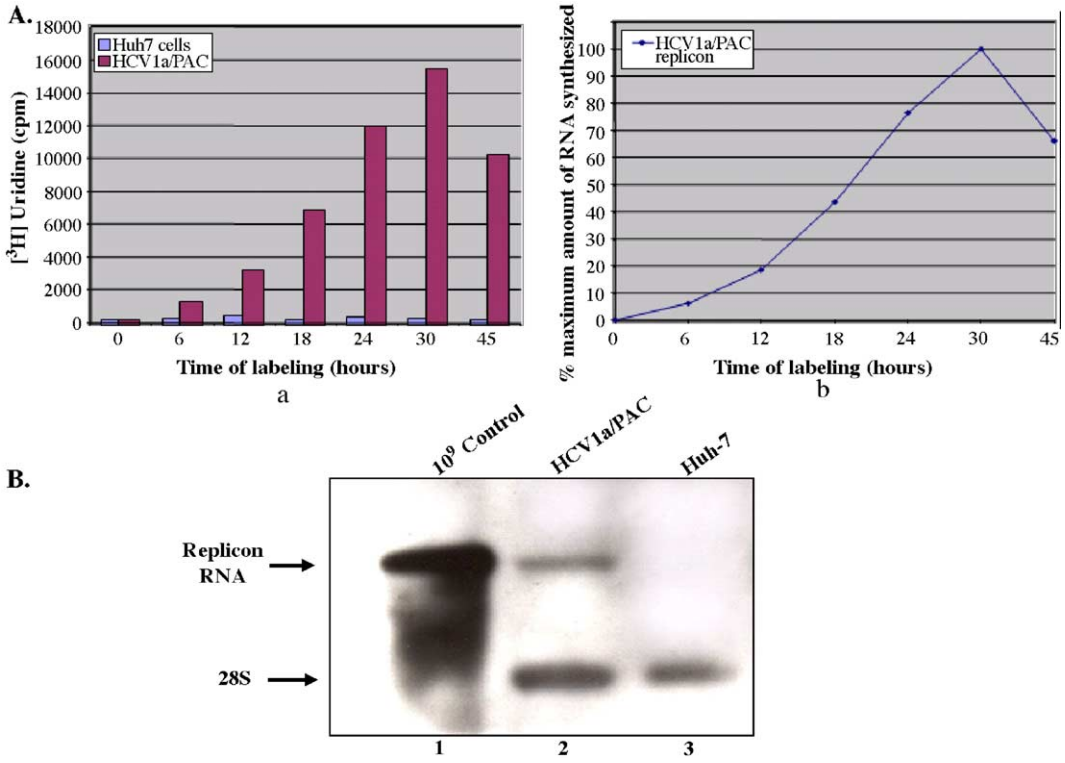

Fig. 3. Replication of HCV1a/PAC subgenomic replicon in Huh-7 cells. (A) In vivo [^{3}H]-labeling of HCV-specific RNA. (a) A series of 6-cm cultured naïve Huh-7 cells and HCV1a/PAC-containing Huh-7 cells were preincubated with 5 μg/ml actinomycin D for 1 h before the addition of [H^3]-uridine (50 μCi/ml). The plates were incubated for various times as indicated on the figure. Total cellular RNAs were extracted and the amount of [H^3]-labeled RNA was measured by scintillation counting (Materials and methods). (b) CPM of RNA in replicon-containing Huh7 cells was corrected by subtraction of the CPM in naive Huh-7 and converted to the percentage of the maximal number of CPM. (B) Northern blot analysis of total RNA extracted from HCV1a/PAC replicon containing Clone A cells. Total RNA (5 μg; lane 2) was resolved in a denaturing formaldehyde agarose gel and hybridized with an HCV-specific DIG-labeled IRES probe. Lane 1 is control of in vitro synthesized replicon RNA (equivalent to 10^9 copies of HCV genome). Lane 3 is total RNA isolated from naive Huh-7 cells.

distribution of these polypeptides in HCV1a/PAC replicon-transfected cells by indirect immunofluorescence then revealed a cytoplasmic location for NS3 (Fig. 4B, panels b and c) and for NS5B (Fig. 4B, panels e and f). There was no viral protein detectable in naïve Huh-7 cells (Fig. 4B, panels a and d). These expression patterns of the viral enzymes NS3 and NS5B resemble those observed in 1b neo/replicons (Lohmann et al., 1999; Pietschmann et al., 2001).

These combined data allow us to conclude that a 1a subgenomic PAC/replicon, based on the infectious HCV 1a[H77C] cDNA clone (Yanagi et al., 1997), can replicate in Huh-7 cells and establish stable cell lines under puromycin selection.

Establishment of puromycin-resistant Huh-7 cell colonies is not dependent upon adaptive mutations in the HCV1a/PAC replicon

Adaptive mutations are a hallmark of the genetics of dicistronic 1b[Con1] replicons proliferating in Huh-7 cells. They are naturally selected among the quasi species of the replicating RNAs because they confer vastly better proliferation properties to the replicon within transfected Huh-7 cells and, thus, better resistance of the host cells to the selecting drug. The significance of adaptive mutations has been recognized originally by Blight et al. (2000) and have now been found in nearly all dicistronic HCV replicon systems (Blight et al., 2000, 2002, 2003; Grobler et al., 2003; Guo et al., 2001; Krieger et al., 2001; Lohmann et al., 2001, 2003; Yi and Lemon, 2004). Adaptive mutations can map to any coding region of the NS polyprotein, but there is a distinct hierarchy in the effect they exert, singly or in combination, on replication (Lohmann et al., 2003 and references therein).

Not all replicons constructed from naturally occurring genotypes seem to need adaptive mutations for efficient replication. For example, the genotype 1b HCV-N replicon did not seem to depend on adaptive mutations for efficient proliferation in the selection of stable neo-resistant Huh-7 cells lines. However, the HCV-N replicon was peculiar in that it revealed a four amino acid insertion in the reading fame of NS5A (Ikeda et al., 2002), which appeared responsible for the phenotype. On the other hand, a wild-type genotype 2a replicon, constructed to express the neomycin resistant marker, revealed high colony forming efficiency without carrying adaptive mutations (Kato et al., 2003).

To address whether autonomous replication of HCV1a/PAC replicon required adaptive mutations for initial replication in clonal cell lines, nearly full-length replicon RNAs of clone A cells were amplified by RT-PCR using

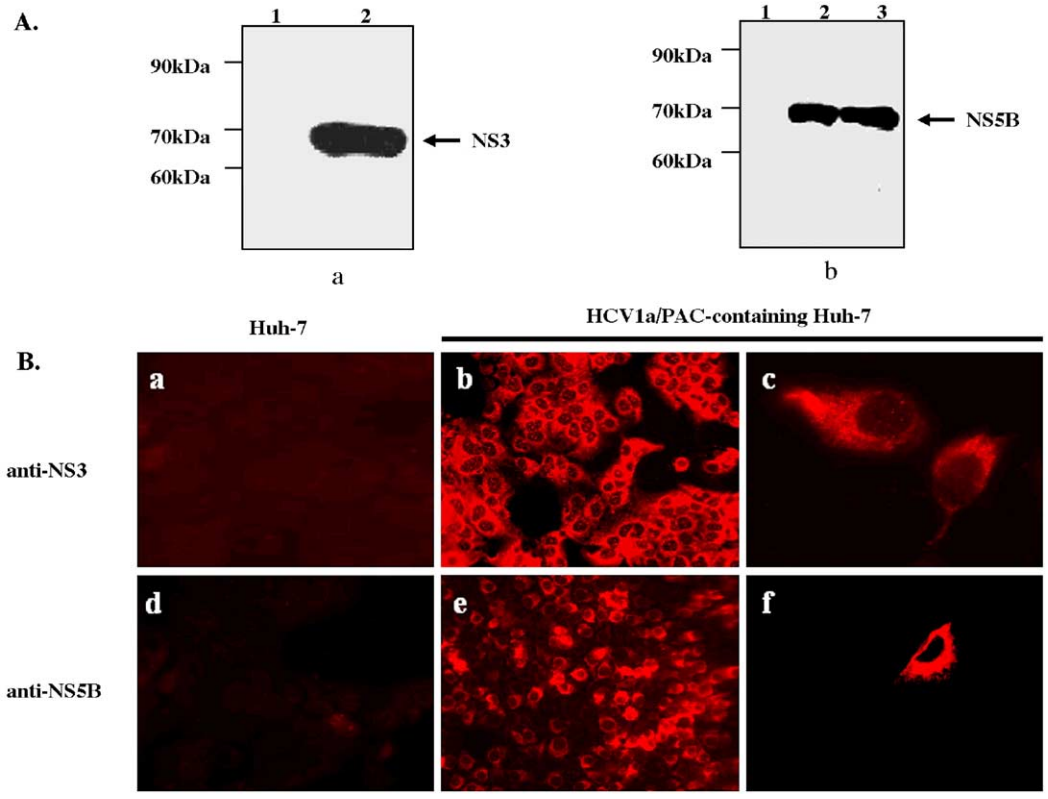

Fig. 4. Viral protein expression in 1a replicon-containing cells. (A) Western blot analysis of cytoplasmic extracts from naïve and replicon-containing Huh-7 cells. Western blot analysis was carried out as described in Materials and methods with monoclonal antibodies to NS3 or NS5B. (a) Antibody to NS3 used for probing. Lane 1: naïve Huh-7 cells; Lane 2: replicon containing cells. (b) Antibody to NS5B used for probing. Lane 1: naïve Huh-7 cells; Lane 2: replicon containing cells; Lane 3: purified NS5B. (B) Immunofluorescence assay of naïve Huh-7 cells and cells containing HCV1a/PAC replicons. Immunofluorescence analysis was carried out as described in Materials and methods using either NS3 (a–c) or NS5B (d–f) specific monoclonal antibodies. Naïve Huh-7 cells (a and d); replicon-containing cells (b, c, e, and f).

primers near the 5′ end (nt 59–82) and 3′ terminal (nt 9599–9580). The amplicon was then directly sequenced. Notably, except for a hitherto unnoticed change of five bases in the NS3 reading frame of the original genotype of the HCV1a/PAC replicon (see below), no mutations were observed in this viral RNA population in Clone A after 2-month passage (~20 passages). These sequence analyses were performed on RT-PCR-amplified products derived from bulk RNA of collected cells. Thus, mutations in individual RNA species would have escaped detection unless a dominant mutation, for example, S2204I in NS5A, was required so that the HCV1a/PAC replicon could form Huh-7 colonies after transfection. However, such a dominant mutation was not found. Indeed, an analysis of replicon RNAs present in puromycin-resistant cells derived from pooled colonies did not reveal any dominant nucleotide changes. These data strongly suggest, but do not prove, that the selection of puromycin-resistant colonies did not depend on a dominant mutant genotype within the population of replicating RNAs.

If adaptive mutations were required to establish replication of HCV1a/PAC replicons in Huh-7 cells at a level sufficient for conferring continuous drug resistance, they should have evolved in most of the clones during early selection. This was, however, not the case for the majority of the HCV1a/PAC replicons in Huh-7 cells as demonstrated in analyses of the pooled Huh-7 cell population. To further address this issue, the NS3-5B coding region was amplified by RT-PCR from total cellular RNA isolated from 12 additional independent puromycin-resistant clones selected after 10 passages (termed clones No. 1 to 12). Individual RNAs of clones 1 to 12 were then sequenced (Table 1). Significantly, no mutations in the RNA and, hence, no amino acid changes were identified in ten of the twelve (~83%) replicons. Clone No. 12 contained a mixture of replicons encoding both serine (S) and isoleucine (I) at position 2204, an observation indicating that some replicons in this cell line had acquired the adaptive mutation S2204I. Replicon RNA of clone No. 6 showed the mutation S2204I and one silent A to G change at position 6791 within NS5A. No other mutations were identified.

These data show that the establishment of puromycin-resistant Huh-7 cells upon transfection with an HCV1a/PAC replicon is not dependent upon adaptive mutations in the HCV coding region. However, a S2204I mutation does evolve in a minority of clones. The significance of this mutation on the transduction efficiency will be analyzed further below.

Table 1
12 independent Huh-7 cell clones tested in this study

Cell clone	Nucleotide change[a]	Amino acid change[b]
1	–	–
2	–	–
3	–	–
4	–	–
5	–	–
6	G6952T	S2204I
	A6791G	Silent
7	–	–
8	–	–
9	–	–
10	–	–
11	–	–
12	6952G/T[c]	2204S/I[d]

[a] Numbers refer to the nucleotide position of HCV type 1a isolate H77C (NCBI accession no. AF011751).
[b] Numbering of amino acids starts from the beginning of the HCV polyprotein.
[c] Both G and T at position 6952 in nucleotide sequence were present in replicon RNA population of clone 12.
[d] Both serine (S) and isoleucine (I) at amino acid position 2204 were present in replicon RNA population of clone 12 (Table 2).

We then analyzed multiple genome species in clone A cells after serial passage for 18 months under puromycin selection to determine whether dominant mutations emerged under conditions of long-term cultivation. Total RNA from clone A cells was amplified by long distance RT-PCR, producing two fragments spanning almost the complete genome of replicon RNA (see Materials and methods). These segments were then subcloned into the pGEM-T vector for clonal sequencing. The major mutations found in 30 individual clones spanning the left half (nt 59–4654) and 37 individual clones spanning the right half of the replicon (nucleotides 4229–7747) are summarized in Table 2. Whereas mutations in NS4A and NS4B were not detected, mutations were scored in NS3, NS5A, and NS5B. Twenty-four out of 37 clones (65%) sequenced contained mutation G6952T, resulting in an amino acid substitution S2204I in NS5A, a genetic change that has been identified in many 1b/neo replicons passaged in Huh-7 cells (see Bartenschlager and Lohmann, 2001; Blight et al., 2000; Lohmann et al., 2003; and references therein). As mentioned before, the S2204I change in NS5A was a mutation that was engineered into the chimeric 1a/neo replicon and found necessary to facilitate initiation of colony formation in Huh-7 cells (Yi and Lemon, 2004). The S2197P mutation was found by Krieger et al. (2001) to greatly facilitate the replication of the HCV[Con 1] replicons of the 1b genotype but here it was found in only 4/37 clones of continuously passaged cells harboring the HCV1a/PAC replicon. Except for the S2204I and S2197P mutations, other mutations listed in Table 2 are rare or have not been reported before to occur in efficiently replicating 1b/neo or 1a/neo replicons.

As can be seen in Table 2, all clones that we have sequenced revealed five nucleotides in NS3 that are in variance to that of the published genotype 1a[H77C] sequence (Yanagi et al., 1997; NCBI accession no. AF011751). Two of these are silent mutations whereas three result in amino acid substitutions (A1336T, C1394Y, and R1484G). These mutations, the origin of which is obscure, are not adaptive mutations that evolved during the cellular passages as they were, surprisingly, present also in our original HCV1a/PAC replicon. Currently, we have no explanation at what stage of the construction of the HCV1a/PAC replicon these deviations from the original 1a(H77C) sequence evolved. Modeling of the NS3 polypeptide indicated that the three amino acids, which differ from the published sequence of NS3, are not located at the surface of NS3 (data not shown). It is unlikely, therefore, that these substitutions alter significantly the function of NS3. Nevertheless, by whatever mechanism, they could be a factor in the HCV1a/PAC replicon's ability to establish stably transformed colonies in naïve Huh-7 cells under puromycin selection. To test this hypothesis, we analyzed the effect of back mutations of the amino acid substitutions in NS3. Each of the residues in the NS3 region was individually reverted to the residues reported in the published sequence of 1a(H77C). None of the back mutations exerted beneficial effect on RNA replication as measured by the colony forming efficiency (data not shown). We then analyzed whether a combination of all three back mutations influenced the colony forming phenotype. Again, no effect was seen (data not shown). The sequence variation relative to 1a(H77C), which we have found in NS3 of our HCV1a/PAC replicon, therefore, is unrelated to the efficient replication of this replicon in naïve Huh-7 cells.

We have also tested the possibility that these mutations in NS3 could affect the colony forming phenotype of a 1a genomic replicon whose selection was controlled by neomycin phosphotransferase. An HCV1a/neo replicon harboring these mutations in NS3 could not transduce the

Table 2
Conserved mutations in HCV1a/PAC replicon (in Clone A cell)

Protein	Nucleotide change[a]	Amino acid change[b]
NS3	G3434C	Silent(30/30)[c]
	G4347A	A1336T (30/30)[c]
	G4522A	C1394Y (30/30)[c]
	G4721A	Silent (30/30)[c]
	A4791G	R1484G (30/30)[c]
	A3754G	D1138G (3/30)
	G5049A	A1570I (4/30)
NS5A	A6454G	H2038R (8/37)
	T6930C, T6932G	S2197P (4/37)
	G6952T	S2204I (24/37)
NS5B	A8835G	I2832V (3/37)
	T8958C	S2873P (3/37)
	G9185T	Silent (5/37)

[a] Numbers refer to the nucleotide position of HCV type 1a isolate H77C (NCBI accession no. AF011751).
[b] Numbering of amino acids starts from the beginning of the HCV polyprotein.
[c] Mutations already present in the original pHCV1a/PAC plasmid.

selection of G418-resistant colonies in naïve Huh-7 cells (data not shown).

Effect of the adaptive mutation S2204I in NS5A on HCV1a/PAC replication

As has been pointed out before, a S2204I mutation in NS5A seems to confer advantage to stable replication in Huh-7 cells of many of the replicons analyzed before. The fact that 24 of 37 HCV1a/PAC replicons in the 1a RNA population of the Clone A harbor the same mutation and the observation that 2 out of 12 clones have evolved this mutation in early stages of selection suggest a replication advantage of replicons carrying the S2204I mutation in Huh-7 cells. To test this hypothesis, replicon HCV1a/PAC (S2204I) was constructed, carrying an isoleucine at position 2204. On transfection, this mutant replicon HCV1a/PAC(S2204I), however, yielded only a modest increase (2- to 5-fold increase on average) in the number of puromycin-resistant colonies as compared to transfection of the wild type 1a replicon (Fig. 5; compare bar 1 with bar 2).

Quantitative analyses revealed that the level of replicon RNA present in HCV1a/PAC(S2204I)-transfected Huh-7 cells (passage 60) was only slightly higher than that in HCV1a/PAC-transfected Huh-7 cells (passage > 200) (Table 3). Nevertheless, the S2204I mutation exerted a beneficial but small effect on the replicon's ability to establish puromycin-resistant colonies although the increase was low as compared to the effect of the same mutation in other neo/replicon systems (Blight et al., 2000, 2003; Yi and Lemon, 2004).

Table 3
Quantification of replicon RNA in Huh-7 cells

Origin of replicon RNA	No. of copies per cell[a]	ECF[b]
WT[c]	392	~100
S2204I[c]	537	~300
Clone A total RNA[d]	746	>5000

[a] Number of HCV replicon RNA molecules per cell as determined by LightCycler quantification method. Values are the mean of three independent experiments.
[b] Efficiency of colony formation per microgram of HCV replicon RNA (mean values derived from six independent transformations of each replicon RNA).
[c] Transfection with in vitro synthesized RNA transcripts.
[d] Transfection with total cellular RNA of Clone A cells.

Whereas the number of colonies formed per microgram of RNA was between 100 and 300 in transfections with HCV1a/PAC and HCV1a/PAC(S2204I) transcript RNA, respectively, transfections with HCV replicon RNA isolated from HCV1a/PAC clone A cells produced >5000 colonies per microgram replicon RNA (Fig. 5A, bar 3). In view of the quite similar levels of replicon RNAs in different cell lines (Table 3), which corresponded to approximately 400 to 800 copies of replicon RNA per cell, this 50-fold increase in colony forming efficiency is surprising. The possible reason for this phenomenon will be discussed later.

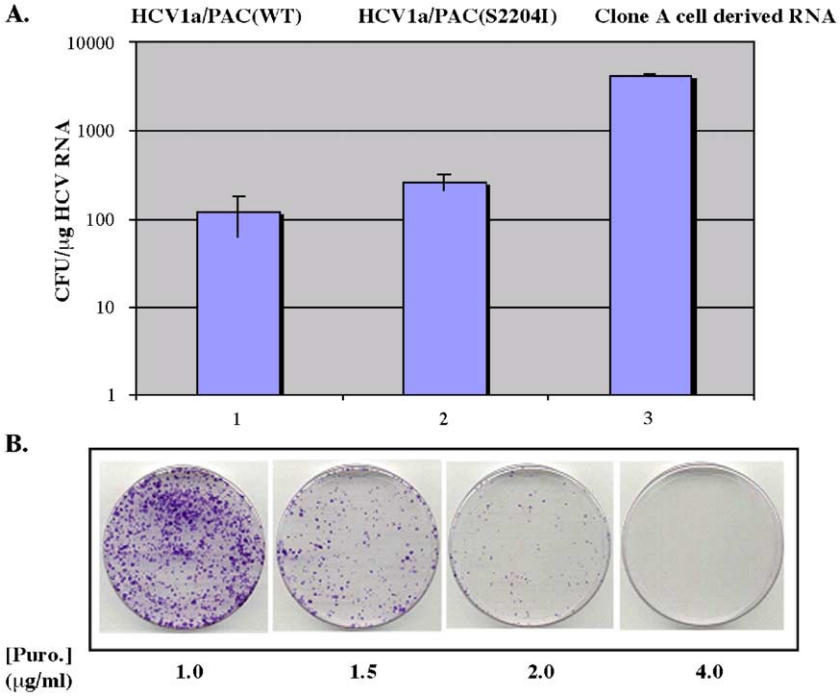

Fig. 5. (A) Colony forming units (CFU) per microgram of HCV replicon RNA obtained from different sources. Transfections were carried out as described in Materials and methods. Bar 1: HCV1a/PAC RNA transcript; Bar 2: mutant (S2204I) replicon RNA transcript; Bar 3: total cellular RNA isolated from replicon containing clone A cell line. (B) Transfer of puromycin resistance by transfection of total cellular RNA containing type 1a replicon. 10 μg total cellular RNA isolated from replicon-containing clone A cells was transfected into naïve Huh-7 cells and subjected to puromycin selection under different concentration of the drug, as described in Materials and methods.

The experiments shown in Fig. 5A have been carried out at a puromycin concentration of 2 μg/ml. It should be noted, however, that the transfer of puromycin resistance to Huh-7 cells occurs in a drug-dependent manner (Fig. 5B).

Discussion

For reasons that cannot be explained presently, studies of a dicistronic HCV replicon of genotype 1a, expressing a neomycin selectable marker as described by Lohmann et al. (2001) for HCV genotype 1b, were unsuccessful because no stable Huh-7 cell lines could be established in the presence of neomycin (see, for example, Blight et al., 2000). This changed when Blight et al. (2003) and Grobler et al. (2003) used Huh-7 cells that had been "cured" from previously carrying replicating HCV replicons of genotype 1b by treatment with interferon (Blight et al., 2002). Transfection of these cured cells with 1a[H77C] dicistronic replicon transcript RNAs allowed the establishment of stable cell lines under neomycin selection, whereas transfection of naïve Huh-7 cells with the same RNA did not (Blight et al., 2003). The permissive phenotype of these cured Huh-7 cells to HCV replicon replication is most interesting. It may result from permanent genetic imprints on Huh-7 cells by previous HCV 1b replicons that inactivated or weakened a protective mechanism against a new transfection and neomycin selection with replicons (Blight et al., 2002; Lohmann et al., 2003). Alternatively, the "cured" cells may present a class of highly permissive cells selected by their previous encounter with HCV replicons (Blight et al., 2002; Lohmann et al., 2003).

In contrast, transfection followed by drug selection with the HCV1a[H77C]/PAC replicon described here did not require "cured" Huh-7 cells. At a concentration of 2 μg/ml in the tissue culture media, where all naïve Huh-7 cells are killed, we reproducibly scored 20–200 colonies. A colony of an expanded replicon-containing cell line was stable under drug selection for 2 years. We expected that we would also select for replicon genotypes carrying adaptive mutations conferring a highly advantageous replication phenotype to the HCV dicistronic RNA. This was not the case. Except for the five nucleotide changes in the NS3 reading frame that were present in the original replicon clone (see below), no dominant mutations were found in replicon RNAs isolated from cells after <20 passages. Analysis of replicon RNAs of several independently isolated transduced cells at <20 passages revealed that only two of thirteen replicons harbored a sole amino acid change (S2204I) in NS5A. However, when replicon RNA was analyzed after >200 passages by subcloning and sequencing, mutations in NS3, NS5A, and NS5B were found of which only the S2204I in NS5A was frequent (24 out of 37 subclones).

The selection for the S2204I mutation in our system as a preferred mutation in a majority of replicons occurred only over continued passages. Transduction of naïve Huh-7 cells with an HCV1a/PAC(S2204I) replicon into which this mutations had been introduced prior to transfection experiments did not yield a large increase in the ability of colony formation. This strongly suggests that the S2204I mutation is not essential in our system to establish stable Huh-7 colonies. This distinguishes the HCV1a[H77C]/PAC replicon from the 1a/neo replicon described by Yi and Lemon (2004) and indicates an advantage of using puromycin selection over neomycin selection for the HCV 1a genotype. Although in a minority of transduced cells the S2204I mutation evolved early in selection, our data clearly show that the S2204I mutation is not imperative for efficient replication of HCV1a[H77C]/PAC replicons in naïve Huh-7 cells under puromycin selection.

Transfection with total RNA from the established HCV1a/PAC clone A yielded a significant increase in colony formation when compared to either HCV1a/PAC or HCV1a/PAC(S2204I) transcript RNAs. This observation is surprising as the prominent S2204I mutation in NS5A alone had only a very modest beneficial effect on transfection with HCV1a/PAC RNA and the other mutations were found only in a small minority of subclones (Table 2). Zhu et al. (2003) have made a similar observation when they studied the replication of HCV1b/neo replicons in HeLa and mouse hepatoma cells. Indeed, transfection of HeLa cells with replicon transcript RNAs, even if they carried the most beneficial adaptive mutations, yielded only a few colonies after selection whereas similar transfection of mouse hepatoma cells yielded none (Zhu et al., 2003). Kato et al. (2003) also observed an increase in the colony-forming ability of RNA isolated from replicon-carrying cells. Zhu et al. (2003) considered two possibilities to explain this interesting phenomenon. Hitherto unknown mutations may aide in the establishment of clones under the conditions of selections. These mutations may then be selected against as the established cell lines are propagated and, thus, they may escape detection. Alternatively, the replicon RNA contained in total cellular RNA may be physically different from transcript RNAs, and this difference may be crucial in the first steps of the initiation of replication leading to drug resistance.

The HCV1a/PAC replicon used in our studies has one engineered *Xba*I site at the 3′ terminus, which generates 1a replicon RNA with an additional 4 nucleotides at the 3′ end of the genome. It has been shown (Yi and Lemon, 2003) that these exogenous nucleotides might reduce the replication ability of HCV RNA but were subsequently removed during in vivo replication. This reduction, however, was only 2- to 3-fold as compared to replicons with a wild-type 3′ end (Yi and Lemon, 2003), a result that we have confirmed in our own studies (Liang, Lee, Paul, and Wimmer, unpublished results), whereas the increase of colony formation with total cellular RNA was 50-fold.

A third possibility to explain the remarkable transduction efficiency of total RNA isolated from Huh-7 harboring HCV1a[H77C]/PAC replicons is that the total RNA con-

tained cellular mRNAs whose translation will alter the intracellular milieu favorable for the establishment of stable cell lines under drug selection (Abe et al., 2004).

We were surprised to find five nucleotide changes in the coding region of NS3 in variance of the published sequence of the genotype 1a[H77C] RNA (Yanagi et al., 1997). However, none of these changes confer beneficial effects on transducing resistance to puromycin. Modeling of the NS3 structure from published coordinates (see Blight et al., 2003, for references) indicated that the amino acid replacements in 1a NS3 of our replicon are all buried within the NS3 polypeptide (data not shown). Thus, a direct influence of the amino acid substitutions on the properties of NS3 may not be expected.

Studies with hepatitis C virus replicons have enormously stimulated basic research on HCV. HCV occurs in numerous genotypes of which only genotype 1b has been subject of intense investigations of its replication phenotypes due to the availability of replicating replicons conferring highly efficient resistance to neomycin selection in transfected Huh-7 cells. Constructing replicons of genotypes other than 1b, using the exact same strategy as Lohmann et al. (1999), may require genetic alterations of the replicons prior to transfection (Yi and Lemon, 2004) or specially "cured" cells (Blight et al., 2003; Grobler et al., 2003). An alternative selection system such as described here will expand the possibilities to study the multitude of HCV genotypes, perhaps in different host cell systems. This is crucial for the development of anti HCV drugs (Evans and Rice, 2003).

Materials and methods

Cell cultures

Monolayers of human hepatoma cell line (Huh-7) were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 units penicillin, 100 μg streptomycin, and 10% fetal bovine serum. In cells harboring HCV replicons, 2 μg puromycin (Sigma) per ml was added to the growth medium. Cells were passaged two or three times per week after treatment with 0.05% trypsin plus 0.02% EDTA and seeded at a dilution of 1:3.

Plasmid construction

Plasmid pHCV1a/PAC (Fig. 1B) contains the sequence of a dicistronic HCV replicon in which the 5′NTR, NS3-NS5B sequence and the 3′ NTR are derived from an infectious molecular clone of the genotype 1a virus, HCV-H77C (Yanagi et al., 1997). All amino acids and nucleotides are referred to according to the location of their position within the original full-length H77C HCV genome. Standard recombinant DNA technology was used to generate this construct. All PCR fragments and final constructs were sequenced using the ABI Prism DNA sequencing kit. In oligonucleotides, restriction sites or mutations are underlined.

Specifically, the IRES (nucleotides 1–374) of HCV was amplified by PCR from pCV H77C (a gift of Robert H. Purcell) and inserted into a pSK(−) vector (Stratagene, La Jolla, CA) restricted with *Kpn*I and *Bam*HI to generate pSK-HCV IRES. The PAC gene was amplified by PCR using the pPUR vector (Clontech) as template with oligonucleotide 5′-CGC**GGTACC**GGATCCATGACCGAGTACAAGCC-3′ (plus-strand sequence) and oligonucleotide 5′-TGC**TCTAGA**ATTCTAGGCACCGGGCTTGCGGG-3′ (minus-strand sequence). The amplified PAC fragment was cut with *Bam*HI and *Xba*I then ligated into the same site of pSK-HCV IRES to generate pSK-HCV IRES-PAC. Next, DNA fragments representing the EMCV IRES and HCV NS3 protein-coding sequence were fused by overlapping PCRs. The resulting DNA was digested with *Eco*RI and *Nsi*I and then ligated with the *Age*I–*Eco*RI fragment of pSK-HCV IRES-PAC and the *Nsi*I–*Age*I fragment of pCV H77C to engineer the dicistronic HCV 1a replicon, which is composed of HCV IRES-PAC-EMCV IRES-HCV NS3-4-5-HCV 3′NTR (Fig. 1B). The HCV 1a replicon is designated HCV1a/PAC. This recombinant replicon plasmid was later sequenced to confirm its authentic type 1a H77C sequence except for five nucleotide changes in NS3. Two of them [G3434C and G4721A] were silent mutations, whereas mutations G4347A, G4522A, and A4791G led to amino acid substitutions of A1336T, C1394Y, and R1484G, respectively. Presently, we do not know the origin of these mutations in our HCV1a/PAC clone.

The cell culture-adaptive mutation S2204I was introduced into the plasmid replicon HCV1a/PAC by PCR mutagenesis using the QuickChange PCR mutagenesis kit (Stratagene). A subclone of pHCV(*Sna*BI–*Bsp*EI) of the HCV replicon in plasmid pGEM-T was constructed and used as template for mutagenesis. The following primers were used for site-directed mutagenesis 5′-CCAGCTCCTCGGCTA**T**CC-AGCTGTCCGCTCC-3′(plus-strand sequence) and 5′-GGAGCGGACAGCTGG**A**TAGCCGAGGAGCTGG-3′(minus-strand sequence). The mutant fragment was restricted with *Sna*BI and *Bsp*EI and then transferred back into the original pHCV1a/PAC replicon.

NS5B polymerase defective derivatives were generated by changing the Gly-Asp-Asp (GDD) motif to Ala-Ala-Gly(AAG) (Kolykhalov et al., 2000) using the QuickChange PCR mutagenesis kit (Stratagene) and primer pair 5′-CCATGCTCGTGTGTG**C**CG**C**CG**G**CTTA GTCGT-TATCTG-3′ (plus-strand sequence) and 5′-CAGATAACG-ACTAAG**C**CG**G**CG**G**CA CACACGAGCATGG-3′(minus-strand sequence).

The plasmid of the subgenomic replicon (pFK-I389neo/NS3-3′/5.1, abbreviated as pNK5.1) was generously provided by R. Bartenschlager (Krieger et al., 2001). It is a dicistronic construct containing the HCV 5′NTR, 16 amino acids of the core coding region, the neo gene, and the

EMCV IRES for the translation of HCV sequences NS3-NS5B, followed by the 3′NTR.

In vitro RNA transcription, RNA purification, electroporation, and selection of puromycin-resistant Huh-7 cells

Wild type or mutant plasmid DNAs containing H77C sequence were linearized with *Xba*I and transcribed into RNA with T7 RNA polymerase. The template DNA was removed by twice serial digestion with RNase-free DNase I for 30 min. The RNA was purified with an RNeasy mini kit (Qiagen) and was dissolved in RNase-free water. For electroporation, subconfluent Huh-7 cells were detached from the plate by trypsin treatment, washed 3 times with ice-cold phosphate-buffered saline (PBS), and resuspended at 10^7 cells/ml in Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM potassium phosphate, pH 7.6, 2 mM Hepes, pH 7.6, 2 mM EGTA, pH 7.6, 5 mM $MgCl_2$) (Lohmann et al., 2001). 1–5 μg of purified replicon RNA was mixed with 400 μl of the cell suspensions in a cuvette with gap width of 0.4 cm (GenePulser II System; Bio-Rad, Hercules, CA). The cell–RNA mixture was subjected to electroporation with a Bio-Rad Gene Pulser II at 270 V, 960 μF, and maximum resistance. The cells were immediately transferred into 10 ml complete DMEM (supplemented with 1.25% DMSO) and were seeded into 10-cm diameter culture dishes. After 24- to 48-h incubation, the cells were first washed with PBS and the medium was then replaced by complete DMEM supplemented with 1–2 μg of puromycin (Sigma) per ml. The medium was replaced every 2–3 days with fresh G418. In 3–4 weeks after transfection, viable colonies were visible. They were stained with crystal violet, or individual foci were cloned, expanded, and stored frozen in 10% dimethylsulfoxide and 90% FBS in liquid nitrogen.

RNA extraction and standard one-step RT-PCR

Total cellular RNA was isolated from cell monolayers using the TRIzol regent (Gibo-BRL) according to the suggested protocol by the manufacturer, and further purified with an RNeasy kit (Qiagen). Viral sequences were amplified by the Titan one-tube RT-PCR system kit (Roche Biochemicals) with antisense primer 5′-CGTATTCTGGTT-GTGGGGGG-3′ and sense primer 5′-GCGGGAGGTCTC-CGTACCTGCAG-3′ using the protocol suggested by the manufacturer. The resulting 1540-bp DNA product representing nucleotides 7136–8676 within NS5B was analyzed on a 1% agarose gel stained with ethidium bromide.

In vivo metabolic [^{3}H] labeling assay in the presence of actinomycin D

Cells that were passaged twice a week in the presence of 2 μg/ml puromycin were seeded in multiple cell culture plates and pretreated for 1 h with 5 μg/ml actinomycin D (Sigma). Following incubation, the cells were incubated in a culture medium containing 200 μCi of [^{3}H]-uridine (46 Ci/mmol) (ICN Biomedical) and 1 μg/ml of actinomycin D. At each time point, cells were harvested and total cellular RNAs were isolated using the TRIzol reagent (Gibco-BRL). [^{3}H] uridine incorporation was determined by liquid scintillation counting.

Western blot analysis

Total cells from 35-mm diameter plates were harvested and lysed by a 1-min sonification in denaturing protein sample buffer (50 mM Tris–HCl [pH 8.8], 100 mM dithiothreitol, 0.1% bromophenol blue, 2% SDS, and 10% glycerol). Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide). The proteins were transferred to a nitrocellulose (Roche) membrane by using a SemiDry Trans Blot system (Bio-Rad), and immunostained according to standard protocols. The HCV proteins were specifically labeled with NS3- or NS5B-specific monoclonal antibodies (MAb). The NS5B-specific antibody was a kind gift of D. Moradpour.

Immunofluorescence analysis

Indirect immunofluorescence analysis of HCV NS3 or NS5B proteins in the parental Huh-7 and replicon-containing cells was performed as described by Pietschmann et al. (2001). In brief, cells grown as monolayers on microscope coverslips were fixed in an ice-cold mixture of methanol and acetone (1:1) for 10 min at −20 °C. The fixed cells were washed with PBS and incubated for 1 h in immunofluorescence buffer (PBS, 3% BSA, 0.1% Triton X-100) at 4 °C. Specific primary antibody to NS3 or NS5B protein was added in immunofluorescence buffer, and the cells were incubated for 1 h at room temperature. Bound primary antibody was detected with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. The coverslips were washed with PBS, mounted to a slide using fluorescent mounting medium, and examined under a fluorescence microscope.

Northern blot analysis

Total cellular RNA from replicon containing cells was extracted with TRIzol reagent (Gibco-BRL) and was further purified by using the RNeasy kit (Qiagen). Northern analysis was performed by using the NorthernMax kit (Ambion) according to the manufacturer's protocol. Briefly, 5–10 μg total cellular RNAs were separated by denaturing agarose gel containing 2.2 M formaldehyde, vacuum transferred to a BrightStar-Plus membrane (Ambion) with PosiBlot Pressure Blotter (Stratagene), and immobilized by UV cross-linking (Stratagene). Hybridization was carried out with a digoxigenin-labeled, negative-sense RNA ribo-

probe complementary to nt 59–343 of the IRES region of HCV for 16 h at 65 °C. The membranes were washed twice in 2× SSC–0.1% SDS for 10 min at room temperature and three times in 0.1× SSC–0.1% SDS for a total of 45 min at 65 °C. For detection of the Dig-labeled probe, the membranes were incubated with anti-digoxigenin-alkaline phosphatase conjugate, reacted with CDP-star (Roche Molecular Biochemicals), and exposed to X-ray film.

Quantitative analysis of HCV RNA

The absolute number of HCV RNA copies per cell was determined by quantitative RT-PCR based on the LightCycler sequence detection system (Roche Molecular Biochemicals). Total cellular RNA isolated from a known number of cells was subjected to a one-step real-time RT-PCR reaction, following the protocol of a commercial kit. Reverse transcription and amplification were performed in 20 μl of LightCycler RNA SYBR Green I mix containing 2.5 mM $MgCl_2$ by using 250 ng total RNA obtained with TRIzol reagent (Gibco-BRL) and primer 5′-CACTCCCCTGTGAGGAACTACTGTCT-3′ (plus-strand sequence) and 5′-TGGTGCACGGTCTACGAGACCT-CCC-3′ (minus-strand sequence). This resulted in the amplification of a 305-base pair of the 5′-NTR region corresponding to nt 38–343. LightCycler RT-PCR was performed in 1 cycle of 30 s at 55 °C for reverse transcription, followed by 45 cycles of 5 s at 95 °C, 10 s at 55 °C, and 12 s at 72 °C with fluorescence detection at 88 °C after each cycle. Melting-curve analysis was performed within the range of 65–95 °C to identify the specificity of amplification.

RT-PCR amplification and sequencing of cDNA from replicating HCV RNAs

Total cellular RNA was extracted from replicon-containing Huh-7 cell lines by the TRIzol reagent procedure. First-strand cDNA synthesis was carried out by using SuperScript first-strand synthesis system for RT-PCR kit (Invitrogen) according to the manufacturer's suggested protocol. Briefly, 1 μg total RNA and HCV-specific primer 5′-CATGTT-CCTGCAGGTCCTAGGACCG-3′(minus-strand sequence) or primer 5′-ACATGATCTGCAGAGAGGCC-3′(minus-strand sequence) complementary to a sequence in NS5A (nt 6506–6482) and the 3′NTR (nt 9599–9580) segments of the genome, respectively, were used for cDNA synthesis in a total reaction volume of 20 μl for 1 h at 42 °C. This was followed by denaturation at 70 °C for 15 min and RNaseH digestion to remove the RNA template. 1/10th of the reaction mixture was used for PCR amplification with the ExpandLong Template PCR System (Roche Biochemicals) with 35 PCR cycles involving denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C (for 2 min in the first 10 cycles and was increased by 10 s for each additional cycle). The final extension reaction was at 68 °C for 10 min. PCR primers 5′-TGTCTTCACGCAGAA-AGCG TCTAG-3′(plus strand sequence) and 5′-CATGTT-CCTGCAGGTCCTAGGACCG-3′(minus-strand sequence), or primers 5′-CAATGGATGAACCGGCTAATAGC-3′(plus strand sequence) and 5′-ACATGATCTGCAGAGAGGCC-3′(minus-strand sequence) were used in the PCR amplification to amplify two overlapping segments covering almost the complete genome of HCV1a/PAC replicon. The amplified RT-PCR products were purified and directly sequenced with an ABI 9600 automatic DNA sequencer. Alternatively, these amplified cDNA products were subcloned into a pGEM-T vector (Promega). Up to 30–40 clones of each fragment were sequenced to confirm the existence of mutations.

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