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Short Communication

Development and characterization of a transient-replication assay for the genotype 2a hepatitis C virus subgenomic replicon

Paul Targett-Adams and John McLauchlan

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

Correspondence
Paul Targett-Adams
p.targett-adams@vir.gla.ac.uk

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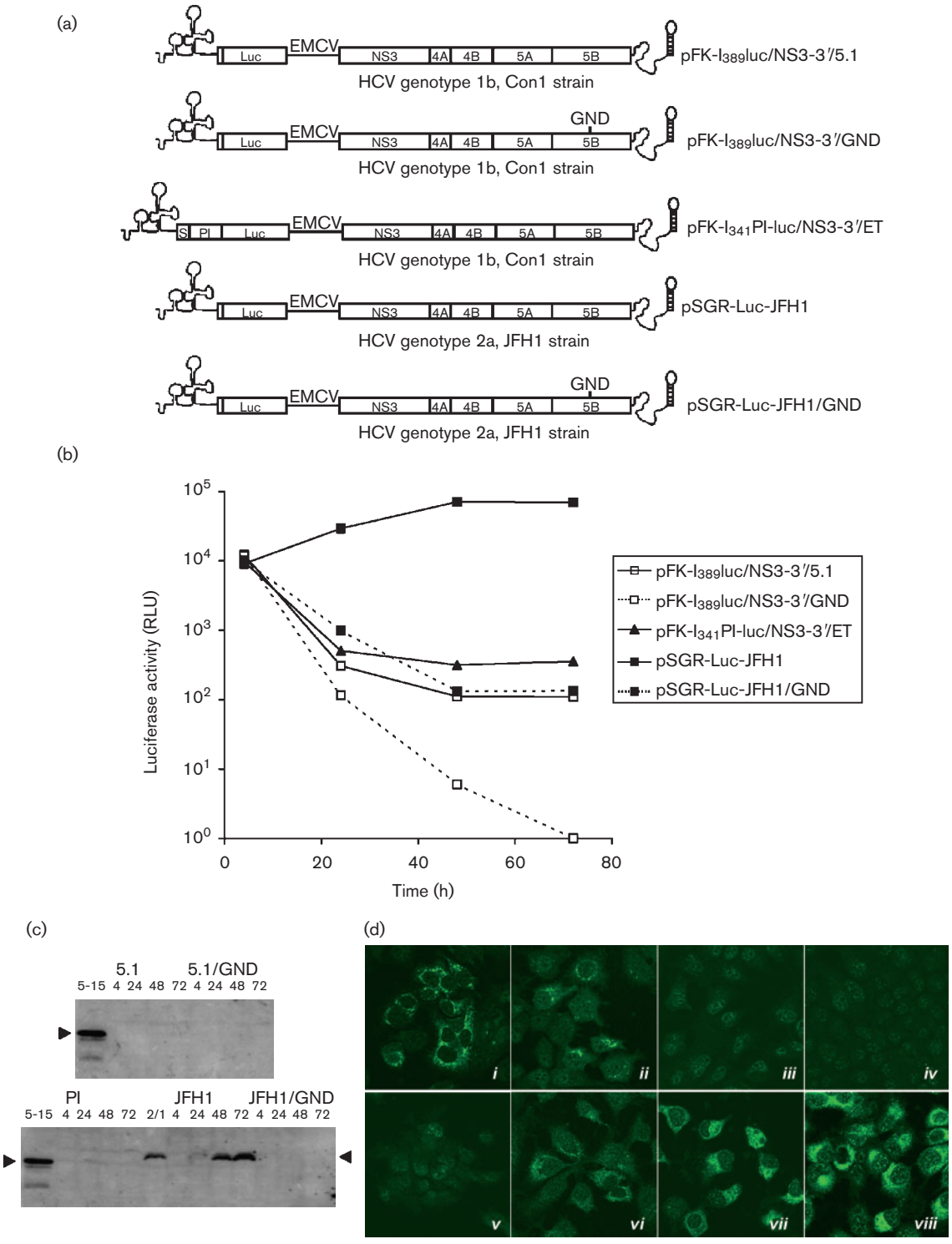
Dicistronic, subgenomic hepatitis C virus (HCV) replicons were constructed containing sequences from JFH1, a genotype 2a strain, that also incorporated the firefly luciferase gene under the control of the HCV internal ribosome entry site element. Luciferase activity in Huh-7 cell extracts containing *in vitro*-transcribed subgenomic JFH1 RNA was monitored over a 72 h period to examine early stages of HCV replication in the absence of any selective pressure. Enzyme activities produced by the replicon were almost 200-fold greater than those generated from corresponding genotype 1b replicons and correlated with an accumulation of NS5A protein and replicon RNA. Transient replication was sensitive to IFN treatment in a dose-dependent manner and, in addition to Huh-7 cells, the U2OS human osteosarcoma cell line supported efficient replication of the JFH1 replicon. Thus, this system based on JFH1 sequences offers improvements over prior genotype 1b replicons for quantitative measurement of viral RNA replication.

Dicistronic, subgenomic replicon systems have been developed to examine RNA synthesis of hepatitis C virus (HCV) genotypes 1a (Blight *et al.*, 2000), 1b (Lohmann *et al.*, 1999) and 2a (Kato *et al.*, 2003). These replicons consist of the HCV internal ribosome entry site (IRES), which directs expression of the G418-selectable marker *neo*^{r} in the first cistron, and the encephalomyocarditis virus (EMCV) IRES to control production of the HCV non-structural proteins NS3–NS5B in the second cistron (Fig. 1a). Following G418 selection of Huh-7 human hepatoma cells containing *in vitro*-transcribed subgenomic replicon RNA, clonal lines capable of supporting virus replication can be isolated to enable assessment of HCV RNA synthesis. Sequence analysis of HCV RNAs from clonal lines has identified adaptive mutations in replicons that modulate replication efficiency. However, limited resources exist to study the early stages of RNA synthesis in the absence of selective pressure, which promotes the emergence of cell culture-adapted mutations. Genotype 1b subgenomic replicons containing the firefly luciferase gene in place of the *neo*^{r} gene have been constructed, but detectable levels of replication require adaptive mutations in the HCV coding sequence that have been shown to prevent productive replication in chimpanzees (Krieger *et al.*, 2001; Bukh *et al.*, 2002). Second-generation versions of the genotype 1b subgenomic replicon containing the poliovirus IRES to direct expression of the luciferase gene sequence in the first cistron replicate RNA to higher levels than the original luciferase gene-containing replicons (Lohmann *et al.*, 2003). However, they also require the presence of cell culture-adapted mutations for improved RNA synthesis (Krieger *et al.*, 2001; Friebe *et al.*, 2001; Lohmann *et al.*, 2003). Subgenomic replicons of JFH1, an HCV genotype 2a strain originally isolated from a patient with fulminant hepatitis, replicate efficiently in Huh-7 cells without the need for adaptive mutations (Kato *et al.*, 2001, 2003). Furthermore, it has been reported recently that cells transfected with JFH1 genomic RNA can secrete viral particles, which can be propagated in Huh-7 cells and establish an infection in the chimpanzee model (Wakita *et al.*, 2005; Zhong *et al.*, 2005). The JFH1 genome can accommodate additional sequences, since insertion of the luciferase gene does not disrupt secretion of infectious progeny (Wakita *et al.*, 2005). Here, we report the development and characterization of a robust transient-replication assay for JFH1 using a subgenomic replicon encoding the firefly luciferase gene.

To construct subgenomic JFH1 replicons containing the luciferase gene, a fragment composed of the firefly luciferase gene was amplified from pGL3-Basic (Promega) using forward and reverse primers (5′-AGATCTATGGAAGACGCCAAAAACATA-3′ and 5′-GTTTAAACTTACACGGCGATCTTTCCGCCCTT-3′, respectively) to produce a DNA fragment with *Bgl*II and *Pme*I sites at the 5′ and 3′ termini, respectively. A second PCR amplification, using pSGR-JFH1 (Kato *et al.*, 2003) as a template together with forward (5′-GTCTGCGGAACCGGTGAGTACACC-3′) and reverse (5′-AGATCTTGGGCGACGGTTGGTGTTTCTTTTGGT-3′) primers, was employed to generate a product

Quantitative RT-PCR results are available as supplementary material in JGV Online.



containing the *Age*I site from the 5′ non-translated region (5′NTR) of JFH1 at the 5′ terminus and the first 57 nt of the core open reading frame (ORF) followed by a *Bgl*II site at the 3′ terminus. The PCR products were digested with *Bgl*II/*Pme*I and *Age*I/*Bgl*II, respectively, and ligated to *Age*I/*Pme*I-digested pSGR-JFH1 or pSGR-JFH1/GND (a replication-deficient subgenomic replicon encoding a GDD to GND mutation in NS5B; Kato *et al.*, 2003) to give pSGR-Luc-JFH1 and pSGR-Luc-JFH1/GND, respectively (Fig. 1a). Linearized plasmids were used to programme *in vitro* transcription of RNA as described previously (Krieger *et al.*, 2001; Kato *et al.*, 2003). RNA (5 μg) was used to electroporate 4×10^6 Huh-7 cells, which were then divided among four 35 mm dishes. Cell extracts were prepared at

Fig. 1. Development of a transient-replication assay for the HCV JFH1 subgenomic replicon. (a) Features of dicistronic HCV subgenomic replicons for wt HCV genotype 1b (pFK-I_{389}luc/NS3-3′/5.1), genotype 2a (pSGR-Luc-JFH1) and their counterparts encoding the GDD to GND mutation in the NS5B sequence (pFK-I_{389}luc/NS3-3′/GND and pSGR-Luc-JFH1/GND, respectively) are shown. The 5′ HCV NTR and 36 nt of the core ORF were fused to the firefly luciferase gene (Luc) to form the first cistron for the pFK plasmids, whereas 57 nt of the core ORF were included in the pSGR constructs. The second-generation genotype 1b subgenomic replicon, pFK-I_{341}PI-luc/NS3-3′/ET, has been described by Lohmann *et al.* (2003). The second cistron of all replicons contained the EMCV IRES located upstream from the HCV NS3–5B coding region, followed by the HCV 3′NTR derived from strains Con-1 (genotype 1b) and JFH1 (genotype 2a). (b) RNA was electroporated into Huh-7 cells and luciferase activities contained within cell extracts prepared at 4, 24, 48 and 72 h post-electroporation were determined. RLU, Relative light units. (c) Extracts from Huh-7 cells electroporated with pFK-I_{389}luc/NS3-3′/5.1 (5.1), pFK-I_{389}luc/NS3-3′/GND (5.1/GND), pFK-I_{341}PI-luc/NS3-3′/ET (PI), pSGR-Luc-JFH1 (JFH1) and pSGR-Luc-JFH1/GND (JFH1/GND) RNAs were prepared at the times indicated. Samples were examined by Western blot analysis with anti-NS5A antisera (Huang *et al.*, 2004). Lanes designated 5-15 and 2/1 correspond to extracts derived from cell lines containing subgenomic HCV replicons from genotypes 1b and 2a, respectively. Arrowheads indicate NS5A protein. (d) Cells were electroporated with pFK-I_{341}PI-luc/NS3-3′/ET (i–iv) and pSGR-Luc-JFH1 RNAs (v–viii) and fixed with methanol at 4 (i and v), 24 (ii and vi), 48 (iii and vii) and 72 (iv and viii) h after electroporation. The presence of intracellular NS5A was tested with anti-NS5A antisera.

various times after electroporation and assayed for luciferase activity using the Luciferase Assay System (Promega) and an M3 luminometer (Biotrace). Experiments were repeated at least three times and representative data are given for each experiment.

RNAs transcribed from pSGR-Luc-JFH1 and pSGR-Luc-JFH1/GND were electroporated into Huh-7 cells and luciferase activity within cell extracts was assayed 4, 24, 48 and 72 h later. Genotype 1b subgenomic replicons containing the firefly luciferase gene were used for comparative analysis (Fig. 1a; Krieger *et al.*, 2001; Friebe *et al.*, 2001; Lohmann *et al.*, 2003). Luciferase activities for the wild-type (wt) and GND mutants of both genotype 1b and 2a replicons were comparable at 4 h and taken to represent translation of input RNA. The pattern of luciferase activity displayed by the genotype 1b replicons beyond 4 h was similar to previously published data (Fig. 1b; Krieger *et al.*, 2001; Lohmann *et al.*, 2003). For the wt genotype 1b replicons, luciferase activity gradually decreased after the initial time point, but stabilized by 72 h. By comparison, activity displayed by the genotype 1b subgenomic replicon encoding the GND mutation in the NS5B sequence decreased sharply between 4 and 24 h and was undetectable by 72 h (Fig. 1b). In contrast to the pattern shown by the wt genotype 1b replicons, luciferase levels generated by the JFH1 replicon rose steadily from 4 h and, by 72 h, were approximately 10-fold higher than initial values (Fig. 1b). From 48 h onwards, we consistently observed that luciferase activity from the JFH1 replicon was about 200- to 400-fold higher than that exhibited by either the wt genotype 1b replicon or the improved version containing the poliovirus IRES (Fig. 1b). Indeed, the GND mutant of the JFH1 subgenomic replicon frequently gave enzyme levels that were comparable to those for the wt genotype 1b replicons. However, by day 7 after electroporation, luciferase activity was reduced to background levels (data not shown). Therefore, we did not consider that the comparable levels in activity between the GND mutant of JFH1 and the wt genotype 1b replicons arose from replication of the JFH1 mutant. It is possible that either greater stability of JFH1 RNA in cells or differences in the translational efficiencies of the JFH1 and Con-1 5′NTR elements could contribute to the luciferase activity obtained with the GND mutant for JFH1. Either of these possibilities could combine with other, as yet unknown, characteristics of the wt JFH1 replicon to account for its greater replication efficiency over the genotype 1b replicon. In a previous report, different passages of Huh-7 cells from the same stock gave fluctuations in replication efficiency in short-term assays for the genotype 1b replicons (Lohmann *et al.*, 2003). With the JFH1 replicon, the absolute levels of luciferase activity were consistent over several cell passages. Therefore, passage number did not appear to influence the efficiency of RNA synthesis from the JFH1 replicon in the same manner as for genotype 1b replicons. We also found that enzyme levels from pSGR-Luc-JFH1 were not enhanced using 2/1C, a Huh-7 cell line that had been cured of the JFH1 subgenomic replicon by IFN-α treatment (data not shown).

To determine whether there was a correlation between luciferase activity and viral protein synthesis, the production of NS5A was examined. Western blot analysis revealed that NS5A could be identified in extracts from cells electroporated with pSGR-Luc-JFH1 RNA at 24 h, and the abundance of the protein had increased by 48 and 72 h (Fig. 1c). By contrast, NS5A was either difficult to detect or undetectable with both of the wt genotype 1b subgenomic replicons and the GND mutants. From indirect immunofluorescence studies, intracellular NS5A protein could be detected up to 24 h post-electroporation for the wt genotype 1b and JFH1 subgenomic replicons, but not at later times for the genotype 1b replicons (Fig. 1d and data not shown). By contrast, high levels of NS5A were present in 80–90 % of cells by 72 h for the JFH1 replicon (Fig. 1d, viii). The levels of luciferase activity generated by the JFH1 replicon were also reflected in an accumulation of intracellular replicon RNA, as judged by quantitative RT-PCR

(see Supplementary Fig. S1, available in JGV Online). Taken together, our data indicate that the high levels of luciferase activity generated by the JFH1 subgenomic replicon in this transient-replication assay represented efficient RNA replication in Huh-7 cells in the absence of any selective pressure.

Having established a short-term replication assay using the JFH1 replicon, we sought to determine whether the amount of RNA used to electroporate cells influenced the levels of replication (Fig. 2a). Huh-7 cells were electroporated with 1, 2·5, 5 or 10 μg pSGR-Luc-JFH1 RNA and the luciferase activity contained within cell extracts was monitored over 72 h (Fig. 2a). By 4 h, luciferase activities were enhanced as the amount of RNA introduced into cells was increased. However, the enzyme levels reached at the end of the experiment were similar, irrespective of the amount of input RNA. Thus, in agreement with Lohmann *et al.* (2003), overall replication efficiency was highest for 1 μg input RNA and decreased as larger amounts of RNA were introduced into cells. These findings, along with those described above, suggest that Huh-7 cells support efficient replication from the JFH1 replicon, but that there is a finite limit to the levels of replication that can be achieved.

Since HCV replicons are sensitive to IFN treatment (Blight *et al.*, 2000; Frese *et al.*, 2001; Guo *et al.*, 2001; Yi *et al.*, 2002), we next tested whether JFH1 replication was affected by various concentrations of IFN-α. Replication of the wt replicon was inhibited in a dose-dependent manner in response to IFN-α treatment and, at a concentration of 100 IU ml^{-1}, luciferase activity was reduced to 0·2 % of the untreated control by 72 h (Fig. 2b). Interestingly, enzyme levels generated by the JFH1 replicon carrying the GND mutation were similarly lowered by IFN-α treatment (Fig. 2c). This result suggested that IFN-α inhibits translation of input, non-replicating RNA, which is in agreement with previous studies of its action on HCV replicons (Wang *et al.*, 2003). Thus, the assay system we have established offers the potential to distinguish rapidly between compounds that act against either translation or replication of viral RNA.

From previous reports, the JFH1 replicon has been shown to replicate in hepatic-derived Huh-7, HepG2 and IMY-N9 cells and non-hepatic HeLa cells (Kato *et al.*, 2003, 2005; Date *et al.*, 2004). In these studies, replication was assessed on the ability of the subgenomic JFH1 replicon to form G418-resistant colonies following drug selection of cells for 3–4 weeks. To determine whether our assay was suitable for screening the ability of different cell types to support JFH1 replication, we used pSGR-Luc-JFH1 to assess transient replication in various cell lines. Cells tested were HeLa (human cervical adenocarcinoma), U2OS (human osteosarcoma), HUVEC (human umbilical vein endothelial), HFF (human foreskin fibroblasts), BHK (baby hamster kidney) and McA-RH7777 (rat hepatoma) (Fig. 3a and data not shown). In the case of HeLa cells, any replication as determined by luciferase activity was approximately 1000-fold less efficient than in Huh-7 cells (Fig. 3a). The JFH1

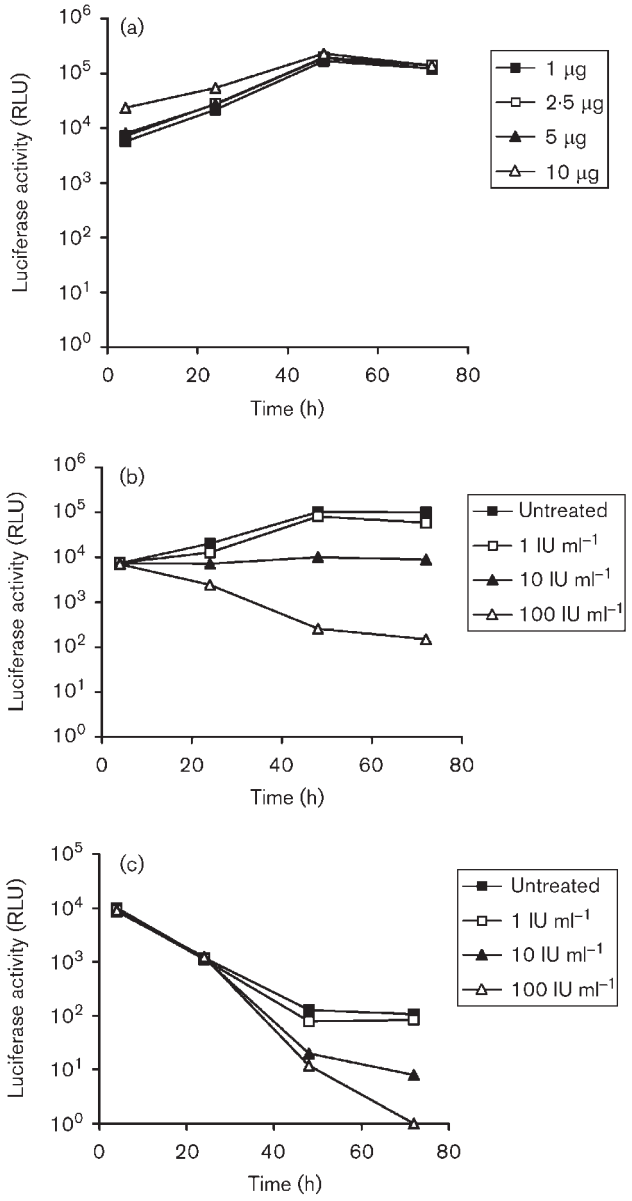


Fig. 2. Replication of the JFH1 subgenomic replicon is not greatly influenced by levels of input RNA, but is sensitive to IFN. (a) Increasing amounts of RNA transcribed from pSGR-Luc-JFH1 were electroporated into Huh-7 cells. The total amount of transfected RNA was adjusted to 10 μg by adding Huh-7 RNA. Luciferase activity in cell extracts was determined at 4, 24, 48 and 72 h post-electroporation. (b, c) Huh-7 cells were electroporated with 5 μg of either pSGR-Luc-JFH1 (b) or pSGR-Luc-JFH1/GND RNA (c) and luciferase activity was determined at 4 h post-electroporation. At this point the remaining cells were either untreated or treated with increasing concentrations of IFN-α and luciferase activities were determined again at 24, 48 and 72 h post-electroporation. The medium was removed at 24 h intervals and replaced with fresh IFN-containing medium. RLU, Relative light units.

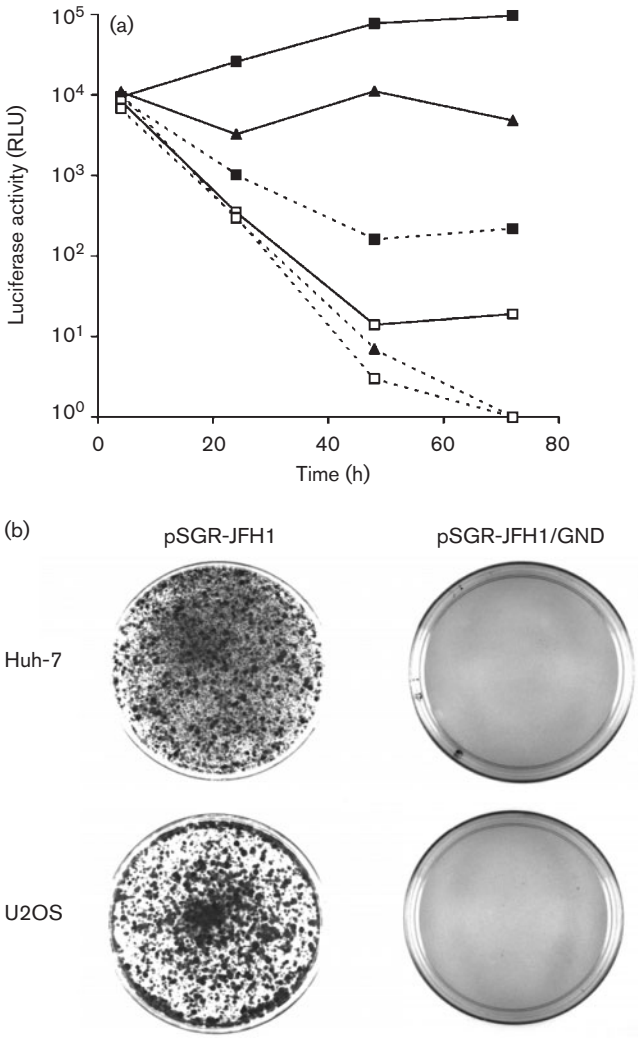


Fig. 3. Transient and stable replication of JFH1 in non-hepatic cell lines. (a) Extracts from Huh-7 (■), HeLa (□) and U2OS (▲) cells, electroporated with either pSGR-Luc-JFH1 (solid lines) or pSGR-Luc-JFH1/GND (dashed lines) RNA, were assayed for luciferase activity at 4, 24, 48 and 72 h post-electroporation. (b) Huh-7 and U2OS cells were electroporated with 3 μg RNA prepared from pSGR-JFH1 or pSGR-JFH1/GND. G418 (1 mg ml^{-1}) was added after 24 h and cells were incubated in the continuous presence of the antibiotic for 3–4 weeks. Drug-resistant colonies were stained with Coomassie brilliant blue.

replicon could establish efficient replication in this stock of cells, as determined by selection of G418-resistant colonies (data not shown), although its colony-forming capacity was about 10-fold less in HeLa cells compared with Huh-7 cells (Kato *et al.*, 2005). The greater reduction in the transient assay may reflect differences in kinetics for RNA synthesis over short compared with long time periods and the selection of cells within the population that can support replication over an extended period. Among the other cell lines tested, evidence for efficient replication was not detected in either the non-human or non-hepatic cells with the exception of U2OS cells (Fig. 3a and data not shown). The difference in luciferase activities between wt and GND replicons in this cell line was approximately 1000-fold by 48 h, which was roughly equivalent to the differential observed in Huh-7 cells (Fig. 3a). Although the absolute enzyme levels for the wt JFH1 replicon cells were about 5–10 % of those produced in Huh-7 cells, our data indicated that U2OS cells represent another cell line in which HCV replication could be examined. To underline the ability of U2OS cells to support replication, G418-resistant colonies could be readily selected 3 weeks after electroporation of cells with pSGR-JFH1 RNA (Fig. 3b). Previous studies have shown that U2OS cells support both constitutive and inducible expression of HCV proteins (Moradpour *et al.*, 1998; Egger *et al.*, 2002). Moreover, the membranous web, which is induced by NS4B expression and represents the site of HCV RNA synthesis, was identified initially in U2OS cells (Egger *et al.*, 2002; Gosert *et al.*, 2003). Such similarities to the morphological changes that occur in Huh-7 cells may indicate that U2OS cells are an alternative model to characterize the processes that contribute to HCV RNA replication.

To conclude, we have developed a simple assay to examine transient replication of the HCV JFH1 subgenomic replicon in Huh-7 cells that is more robust than existing genotype 1b systems. Our assay could provide a convenient means for rapid screening of cell lines that may support replication and candidate antiviral compounds for inhibiting viral RNA synthesis. Moreover, the results indicate that the JFH1 replicon may offer potential for examining the viral and cellular requirements during the early stages of HCV RNA replication.

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