Subgenomic replicons (SGRs) of hepatitis C virus (HCV), based on sequences from genotype 1a and 1b strains, have been invaluable for examining viral RNA replication (Blight et al., 2000; Lohmann et al., 1999). Recent reports have demonstrated that SGRs possessing sequences from JFH1, an HCV genotype 2a strain, replicate far more efficiently than those from genotype 1b in both colony-forming assays and quantitative transient replication assays (TRAs) using reporters such as luciferase (Kato et al., 2003; Miyamoto et al., 2006; Targett-Adams & McLauchlan, 2005; Windisch et al., 2005). Importantly, efficient replication with JFH1 SGRs is not dependent on either highly permissive Huh-7 cells or tissue-culture-adapted mutations that are needed to enhance genotype 1b replicon competence (Krieger et al., 2001; Lohmann et al., 2001). By contrast to genotype 1b SGRs, the JFH1-based replicon exhibits large increases in luciferase activity over the initial 24 h following transfection of Huh-7 cells with SGR RNA (Targett-Adams & McLauchlan, 2005). Hence, this replicon offers unique possibilities to examine the early stages of viral RNA replication.

Previous studies have reported stable cell lines that support steady-state replication of SGRs containing genotype 1b sequences that incorporate GFP-tagged NS5A (Liu et al., 2006; McCormick et al., 2006b; Moradpour et al., 2004). However, replication in TRAs with such replicons was barely detectable (Appel et al., 2005b). Here, we describe development of JFH1-based SGRs that incorporate GFP and a photoactivatable variant of GFP (PAGFP) (Patterson & Lippincott-Schwartz, 2002, 2004) into the C-terminal region of the NS5A protein.

To construct tagged JFH1 SGRs, the GFP and PAGFP open reading frames were inserted into the 3' coding sequence of JFH1-derived NS5A at a site previously shown to be capable of accommodating GFP within genotype 1b-based replicons (Liu et al., 2006; McCormick et al., 2006b; Moradpour et al., 2004). DNA fragments encoding NS5A–GFP/PAGFP fusion proteins were engineered into pSGR-Luc-JFH1 (Targett-Adams & McLauchlan, 2005), generating pSGR-Luc-GFP-JFH1 and pSGR-Luc-PAGFP-JFH1, respectively (Fig. 1a). Replication-incompetent GND derivatives of the GFP- and PAGFP-tagged JFH1 replicons were also constructed as controls. To investigate whether the GFP- and PAGFP-tagged JFH1 SGRs could initiate and sustain transient replication, RNAs generated from wild-type (wt) and GND constructs were introduced into Huh-7 cells as described previously (Targett-Adams & McLauchlan, 2005) and luciferase activity was measured at regular time intervals for 72 h (Fig. 1b). Replication of both GFP- and PAGFP-tagged wt SGRs was delayed compared with the unmodified JFH1 replicon (pSGR-Luc-JFH1) (Fig. 1b). By 24 h post-electroporation, luciferase activities exhibited by the GFP- and PAGFP-tagged SGRs were respectively 35 and 29 % of that for the unmodified JFH1 replicon; however, by 72 h, these respective values had risen to 91 and 77 % (Fig. 1b). Both tagged variants of the wt JFH1 SGR generated luciferase activities, which were more than three orders of magnitude greater at 72 h post-electroporation compared to the unmodified JFH1 replicon (pSGR-Luc-JFH1) (Fig. 1b).
with the tagged GND replicons (Fig. 1b). Hence, we concluded that insertion of GFP into NSSA did not have any significant inhibitory effect on replication compared with the untagged replicon. To determine whether the NSSA fusion proteins were stable during transient replication, cell extracts were prepared at various times post-electroporation and analysed for the presence of NSSA–GFP and NSSA–PAGFP proteins by Western blot analysis (Fig. 1c). The two fusion proteins were identified in extracts from cells electroporated with SGR-Luc-GFP-JFH1 and SGR-Luc-PAGFP-JFH1 RNAs, respectively, at 48 and 72 h post-electroporation (Fig. 1c) and there was no evidence of proteolytic breakdown. The levels of NSSA fusion proteins observed at later time points were comparable to those of untagged NSSA expressed by SGR-Luc-JFH1 RNA (Fig. 1c). By contrast, no NSSA species were recognized in cells electroporated with any GND mutants (data not shown). Thus, incorporation of GFP and PAGFP into the C-terminal region of NSSA gave replicons that remained capable of robust transient replication.

To ascertain whether fluorescence from NSSA–GFP could be visualized directly, cells were electroporated with RNAs transcribed from pSGR-Luc-GFP-JFH1 and its GND counterpart and examined at 4, 24, 48 and 72 h post-electroporation (Fig. 2a). In contrast to Western blot analysis (Fig. 1c), fluorescence was detected as early as 4 h post-electroporation in cells harbouring the GFP-tagged replicons for wt and the GND derivative (Fig. 2a). This very early fluorescence presumably resulted from translation of input RNA, since it was not evident in cells containing the GND derivative by 24 h post-electroporation (data not shown). With longer incubation times for the wt replicon, not only did the proportion of fluorescent cells increase, but individual cells displayed greater fluorescence intensity and a larger number of punctuate structures was observed (Fig. 2a and data not shown). At all time points, the GFP signal was associated with cytoplasmic punctuate structures, which have been suggested to represent membrane-bound vesicles where HCV RNA replication may occur (Moradpour et al., 2004). From antibody staining for...
NS5A in paraformaldehyde-fixed cells, the punctuate appearance of GFP fluorescence colocalized with NS5A (Fig. 2b). This result demonstrated that GFP fluorescence was an accurate marker of NS5A distribution and indicated possible sites of HCV RNA replication in replicon-containing cells. These findings describe, for the first time, the development of a GFP-tagged HCV replicon not only competent for transient replication but also capable of producing sufficient NS5A–GFP protein that could be visualized at earlier times than conventional immunodetection methods (Fig. 1c).

The above results prompted us to determine the first period during which replication could be detected. To this end, cells were electroporated with SGR RNAs together with their GND counterparts and luciferase activities were assayed at 2 h intervals over a 24 h period (Fig. 2c). Enzyme activities produced by all replicons initially rose for up to 6 h post-electroporation, presumably representing translation of input RNA (Fig. 2c). For the wt untagged SGR, luciferase values then decreased until 12 h post-electroporation, but rose sharply thereafter over the remaining 12 h. By contrast, the activity generated by the GND mutant continued to fall beyond 12 h after electroporation (Fig. 2c). Thus, replication of the untagged SGR could be detected between 12 and 14 h post-electroporation. Results for the wt and GND versions of the GFP-tagged replicon gave the same pattern except that rises in luciferase activity were delayed to 18 h post-electroporation for wt RNA (Fig. 2c); this lag in replication is consistent with the data shown in Fig. 1(b).

Having established that replication had initiated from the GFP-tagged SGR by 14–18 h post-electroporation, this time-frame was used to examine the biophysical properties of NS5A–GFP. We opted to study movement of NS5A–GFP in live cells by fluorescence recovery after photobleaching (FRAP). Using this approach, we had demonstrated that GFP–NS4B expressed alone is mobile on the ER membrane but relatively immobile on foci that it generates in cells (Gretton et al., 2005). To test whether NS5A–GFP expressed by the replicon was mobile, recovery of GFP fluorescence was examined after photobleaching of selected intracellular regions (Fig. 3a, b). Circular areas of 38 μm² were exposed to six bleaching iterations (100 % laser power, 488 nm laser...
Fig. 3. Biophysical analysis of NS5A tagged with fluorescent proteins. (a, b) FRAP analysis of GFP-tagged NS5A. Cells were either electroporated with RNA produced from pSGR-Luc-GFP-JFH1 or transfected with plasmids pGFP-DNase X and pCMV-NS5A-GFP. pCMV-NS5A-GFP was constructed by amplifying the coding region for NS5A–GFP from pSGR-Luc-GFP-JFH1 and inserting the resultant DNA fragment into pCMV10 (Stow et al., 1993). Cells were incubated at 37 °C for 14–16 h prior to photobleaching. Images were recorded at approximately 2 s intervals before and after bleaching (circled in (a) and indicated by an arrow in (b)) and the percentage fluorescence intensity in photobleached regions was determined. Mean values for the percentage fluorescence intensity and standard errors were calculated from data obtained from several cells (n = 10). Bars, 10 μm. (c) A selected region of a cell electroporated with SGR-Luc-PAGFP-JFH1 RNA was activated with three pulses of a 405 nm laser (100% laser power) at 16 h post-electroporation. Images were recorded both pre- and immediately post-activation and for the times indicated. At the end of the time-course, the entire cell was photoactivated. Bar, 10 μm.
line) to reduce fluorescence intensity to about 20% (± 3%). Before and after bleaching, images were taken with 2% laser power. For comparative purposes, we included a plasmid expressing GFP–DNase X, a mobile ER membrane protein (Fig. 3a, b; Gretton et al., 2005). NS5A–GFP expressed from SGR-Luc-GFP-JFH1 RNA at 16–18 h after electroporation gave low levels of fluorescence recovery to only 30% after photobleaching (Fig. 3a, b); the extent of recovery did not improve in cells harbouring the replicon for up to 72 h (data not shown). These data indicated that NS5A–GFP was relatively immobile within cells containing HCV RNA replication complexes. By contrast, NS5A–GFP expressed from a plasmid, pCMV-NS5A-GFP, recovered to 65% and thus exhibited a higher degree of mobility (Fig. 3a, b). This mobility for NS5A–GFP was somewhat less than that for GFP–DNase X, which recovered to 82.5%, but was far greater than that observed for the GFP fusion protein expressed from the replicon. From these observations, we concluded that NS5A is mobile in the absence of other HCV non-structural proteins but is anchored in cells containing HCV replicon RNA. We propose that the lack of NS5A mobility in replicon-bearing cells arises from interactions with other HCV proteins/genomic RNA (Gao et al., 2004; Huang et al., 2005; Lim et al., 2006; Shiruta et al., 2002), and/or host-cell factors (Hamamoto et al., 2003; Lan et al., 2002; Macdonald et al., 2004; Nanda et al., 2006; Park et al., 2003; Wang et al., 2005).

To confirm the lack of NS5A mobility in replicon-bearing cells, the characteristics of PAGFP-tagged NS5A expressed from SGR-Luc-PAGFP-JFH1 RNA were examined. Fluorescence was photoactivated in selected intracellular regions and the localization of NS5A–PAGFP was monitored over time (Fig. 3c). Negligible fluorescence was detected prior to exposure to the 405 nm laser, but fluorescence was clearly evident immediately after photoactivation (Fig. 3c). The distribution of fluorescent NS5A–PAGFP was examined for 15 min, but no transfer of fluorescence to non-activated regions of the cell was observed. Indeed, continued monitoring of photoactivated NS5A–PAGFP did not reveal any altered localization for up to 1.5 h (data not shown). These findings confirmed that NS5A does not exhibit any appreciable mobility in cells containing replicating HCV RNA.

Our studies have demonstrated that inserting GFP into the NS5A region of the JFH1 SGR enables detection of HCV protein expression relatively soon after introduction of RNA into cells. By 4 h, NS5A–GFP was located in foci, which are consistent with sites of vesicle formation at the ER membrane (termed the membranous web), that have been identified in cells producing replicating HCV RNA (Gosert et al., 2003). An identical distribution arises with a replication-null mutant, indicating that formation of foci requires translation of input RNA but not active HCV RNA synthesis. Moreover, the punctate distribution for NS5A–GFP preceded the onset of replication, which was 18 h as judged by luciferase activity. RNA synthesis probably initiates at earlier times, but cannot be determined accurately in our system due to high levels of reporter activity from input RNA. Expression of NS4B, a critical component of the replication complex, is sufficient for production of foci that are morphologically indistinguishable from those found in replicon-bearing cells (Egger et al., 2002; Gosert et al., 2003; Gretton et al., 2005). Using GFP-tagged NS4B, we have detected foci as early as 2 h after transfection without any evidence for significant association with the ER membrane. Therefore, foci are not artefacts of overexpression but are generated rapidly after initial translation of the HCV non-structural proteins and possibly act as sites that are primed for initiating viral RNA synthesis.

NS5A–GFP expressed from an SGR encoding the NS3–5B polyprotein was relatively immobile from photobleaching studies. By comparison, fluorescence recovery was detected for NS5A–GFP in the absence of the other HCV proteins. The loss of mobility of NS5A–GFP in the context of other HCV non-structural proteins is presumably a result of additional interactions formed by NS5A, including those that may arise in replication complexes. Interestingly, NS5A is extracted less readily from cells expressing the entire HCV polyprotein than from cells expressing the protein alone (Brass et al., 2002). Thus, the characteristics of NS5A in biochemical assays and photobleaching studies are very similar. Our studies also indicate that NS5A does not transfer rapidly between foci. Among the non-structural proteins, NS5A alone can complement defective replicons (Appel et al., 2005a). One option suggested by these authors was that NS5A could transfer between replication complexes as a consequence of loose association with membranes. Our data do not support such a mechanism, although we do not exclude limited movement of NS5A between replication complexes at different cellular locations. It is perhaps more likely that exchange of NS5A arises between replication complexes within individual foci, which was an alternative model proposed by Appel et al. (2005a). Further evaluation of the mobility of HCV-encoded proteins by photobleaching methods is ongoing and should provide greater insight into the protein–protein and protein–lipid interactions that occur during HCV replication.

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References


