Saudi Journal of Biological Sciences (2011) **18**, 213–218

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Subgenomic HCV RNA replication and its localization in the nucleus of the infected cells

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Received 3 August 2010; revised 30 January 2011; accepted 30 January 2011
Available online 3 February 2011

KEYWORD
Hepatitis C Virus

Abstract Cell culture systems have been established, where a hepatitis C virus (HCV) subgenomic replicon was efficiently replicated and maintained for a long period. It is known that HCV contains proteins which interact host cell proteins.

To see whether a HCV RNA replicon can interact in the same way with host cell proteins, HCV RNA replicon was transfected in Huh7 cells. In most infected cells, HCV replicon is present in the cytoplasm; however, in a minority of HCV-infected cells, both the cytoplasm and the nucleus or the nucleus on its own is positive for NS3. The presence of NS3 in the nuclei of Huh7 cells indicates that the protein may play a role other than in virus replication, such as in persistence of HCV infection.

1. Introduction

Hepatitis C virus (HCV) was identified as the causative agent for most post-transfusion and sporadic non-A, non-B hepatitis cases in 1989 (Ramadori and Meier, 2001). According to recent estimates, about 170 million individuals worldwide are infected and 3–4 million people are newly infected each year (Cohen, 1999). One important characteristic of HCV is its strong propensity to persist in the infected individual, which leads to severe liver damage, ranging from chronic hepatitis to liver cirrhosis and hepatocellular carcinoma (Khu et al., 2001). It is a serious infection, affecting 1–2% of the population in most developed countries. Around 90% of HCV infections become chronic, up to 20% of these develop into liver cirrhosis, and 1–5% of the cases lead to hepatocellular carcinoma (Lanford et al., 2001). Although treatment of this infection using interferon-α and ribavirin has been effective in a few cases (about 40%), more than 60% of the patients, however, show no response to the treatment. The development of new antiviral agents against HCV infections has been hampered by the lack of a cell culture system for propagating the virus and practical animal model systems.

1.1. Cell Culture Systems

There have been a number of attempts at propagating HCV in cell culture using different cell lines but this has been difficult to achieve. Studies have involved infection of primary cell lines

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Peer review under responsibility of King Saud University.
doi:10.1016/j.sjbs.2011.01.008

Production and hosting by Elsevier

with HCV, such as the inoculation of primary foetal human hepatocytes (Iacovacci et al.,1993, 1997), primary human hepatocytes (Rumin et al., 1999) and primary hepatocytes from chimpanzees (Lanford et al., 1994), as well as cultivation of infected cells from patients harbouring the virus, such as peripheral blood mononuclear cells (PBMCs) and primary hepatocytes prepared from liver biopsies (Ito et al., 1996). The experimental infection of PBMCs has also been reported (Cribier et al., 1995). In addition to infection of primary cell lines, HCV inoculation of human hepatoma cell lines, such as HepG2, Huh7 and PH5CH cells, as well as, B- and T-cell lines, such as, Molt-4, MT-2 and Daudi cells, have been studied in detail (Bartenschlager and Lohmann, 2001). Of the cell lines tested the PH5CH cell line appears to be the most susceptible to HCV infection (Kato et al., 1996).

The problem with these systems is that they are poorly reproducible and low levels of HCV are present within the cultures, requiring sensitive detection techniques to identify the virus. Techniques such as RT-PCR, sequencing of the HCV genome fragments in cells, and the detection of viral antigens have been used to demonstrate HCV replication within cells (Bartenschlager and Lohmann, 2001).

2. Materials and methods

2.1. Tissue culture materials and reagents

All tissue culture plasticware were purchased from VWR. All tissue culture reagents were purchased from Invitrogen: RPMI 1640 and media supplements (FCS, glutamine, geneticin, penicillin and streptomycin). Cell lines were maintained in DMEM growth media supplemented with 2 mM L-glutamine, 10% Fetal Calf Serum (FCS), 100 International Units/ml penicillin and 100 μg/ml streptomycin. Inactivation of complement in FCS was achieved by incubation at 56 °C for 60 min.

Replicons were kind gift from Dr. Charles Rice.

2.2. Mammalian cell culture techniques

2.2.1. Maintenance and handling

The cell lines described in the materials were propagated as follows:

The media incubated with the cells in 75 cm^2 (T75) tissue culture flasks was discarded and cells were washed with 5 ml of PBS. The PBS was discarded and replaced with 2 ml of trypsin (5×) (Invitrogen) diluted 1/5 in PBS. Thirty millilitres of the correct tissue culture medium (depending on the cell line) was added to the trypsinised cells and 10 ml was distributed into new 75 cm^2 tissue culture flasks. Cells were propagated in this manner every 4–5 days.

2.2.2. Linearization of plasmid DNA

Ten micrograms of each replicon encoding plasmid (Pol, Bart, A1174T (Rep 3), and S1179I (Rep 4)) was linearized by digestion with *Sca*1 restriction enzyme for 2 h at 37 °C. Each plasmid DNA was checked to ensure that complete linearization had been achieved by agarose gel electrophoresis.

2.3. RNA transcription reaction

Transcription reactions were carried out by using MEGAscript Kit (Ambion).

2.4. RNA transfection

Transfection was carried out in two ways:

2.4.1. Transfection using lipofectin

Twenty-five microlitres of each RNA sample was mixed with 275 μl of PBS and 30 μl of lipofectin was mixed with 270 μl of PBS. Then, the RNA mixture was added to lipofectin mixture and incubated on ice for 10 min. The culture medium was removed from 80% confluent Huh7 cells and they were washed with 2 ml of PBS. One hundred and fifty microlitres of the RNA–lipofectin mixture was further diluted in 3 ml of PBS, was then added to each flask and incubated for 30 min. The RNA–lipofectin mixture was removed and the cells were washed with 1 ml of PBS. Ten millilitres of culture medium was added to the cells and incubated at 37 °C for 24 h. The medium was removed and replaced with new culture medium containing 0.5 mg/ml of G418. Huh7 cells which had not been transfected with HCV RNA was also grown in RPMI/G418 medium, as a negative control.

2.4.2. Transfection using electroporation

A 75 cm^2 tissue culture flask of 80% confluent Huh7 cells was washed with 5 ml of PBS and replaced by another 2 ml of PBS. Two millilitres of trypsin/EDTA (5×) diluted 1/5 in PBS solution was added to the flask, and incubated for 2 min at 37 °C. Ten millilitres of RPMI medium was added to the flask to stop trypsinization and the cells were then transferred to a 20 ml tube. Five to ten microlitres of RNA (10 μg) was added to the cell suspension. The electroporation apparatus, Gene Pulser® (BIO-RAD), was set up to give a field strength of 1.5 kV/cm at 25 μF capacitance. The cuvette was placed in the electroporator and the cell suspension was pulsed twice. The cells were placed on ice for 5 min to allow them to recover. The electroporated cells were transferred to a new 75 cm^2 flask which contained 9.5 ml of RPMI medium. The flask was incubated at 37 °C for 24 h. The following day, the medium was replaced with 10 ml of RPMI medium and 0.1 ml of G418 (0.5 mg/ml). A negative control of untransfected Huh7 cells was also incubated in RPMI/G418 medium.

2.5. In situ protein staining

Glass cover slips were sterilised (dipped in alcohol and flamed) and placed in the bottom of the wells of Sterilin 6 well plates. HCV RNA replicon cells were seeded in half of the wells while the rest of the wells were seeded with Huh7 cells. The cells left to grow up to 80% confluence.

2.6. Staining of cells to detect HCV NS3

Three millilitres of methanol:acetone mix was added to the wells which contained growing cells onto cover slips within a six well plate for a minimum of 1 h at −20 °C. The mix was then removed from the wells and the cells were re-hydrated by washing with PBS. To block non-specific binding of antibodies the slides were incubated with 1:25 dilution of swine normal serum (Dako) for 30 min. The swine serum was discarded and the excess was wiped away. Each of the cover slips were removed from the corresponding well and placed, cell coated face down, onto 50 μl of rabbit polyclonal anti-NS3

antibody (NS3–7) (Errington et al., 1999) diluted 1 in 50 in sterile PBS at room temperature on a 2 cm by 2 cm square of parafilm for 30 min. Unbound antibody was washed off by washing three times in PBS and the coverslips were then placed face down onto 50 µl of FITC conjugated swine anti-rabbit antibody (Dako), diluted 1 in 40 in sterile PBS for 30 min. The slide was washed again three times in sterile PBS, once in water, and placed face down on mounting medium (Vectashield) on a clean microscope slide. The cells were viewed at 400× magnification with an inverted microscope.

2.7. DNA gel electrophoresis techniques

2.7.1. Agarose gel electrophoresis of DNA

Agarose gels were formed using a Horizon gel electrophoresis apparatus (Invitrogen). 0.7–2.0% agarose (w/v) in 1× TAE or 1× TBE buffer was melted in a microwave oven 5 µg/ml ethidium bromide was added and it was allowed to cool to 65 °C and then poured into the assembled apparatus taking care not to introduce air bubbles.

2.8. Northern blotting

Total RNA was extracted from transfected cells and 5 µg of total RNA was loaded onto the gel. NS3 probe was purified by using MicroSpin G-50 columns (Amersham Pharmacia). Blots were visualized and quantitated as described (Yang et al., 2002).

3. Results

3.1. Transcription of HCV RNA replicon plasmids

To make cell lines in which contain replicating HCV replicon RNA, it was first necessary to produce RNA transcripts from the RNA replicon plasmids. Ten micrograms of each purified plasmid DNA was digested with *Sca*1 and 0.2 µg samples of the digested plasmids were run on an agarose gel to ensure that they were completely linearized.

Replicon RNAs were then produced by transcription of the linearized plasmids with *Sca*1. Large scale preparations of HCV replicon RNAs were carried out by transcription of the linearized plasmids using the MEGAscript Kit. Approximately 0.2 µg of each RNA transcript were then examined by agarose gel electrophoresis to ensure that transcripts of the correct size, 7990 nucleotides, had been produced and that there was no apparent degradation of the RNA.

3.2. Transfection of Huh7 cells with replicon RNAs

RNA transfection of Huh7 cells was carried out by using two different methods: lipofectin and electroporation. Due to the presence of G418, only transfected cells will survive in its presence. Forty-eight hours after both transfection procedures, dead cells were observed in the tissue culture flasks. Within 2 weeks, all untransfected cells were dead with no cells remaining attached to the flask. Small discrete colonies formed in the presence of G418. These colonies grew and propagated in the presence of G418 indicating that the neomycin resistance gene present in the RNA replicons was allowing these cells to survive.

3.3. The presence of replicon RNA in replicon transfected cells

To show the presence of replicon RNAs in the transfected cells, Northern blot analysis was carried out. RNA was extracted from untransfected Huh7 cells and RNA from Huh7 cells transfected with A1174T (Rep 3) and S1179I (Rep 4). These RNAs were resolved by formaldehyde gel electrophoresis and Northern blot analysis was carried out using a ^{32}P-labelled DNA probe corresponding to the NS3 coding region (Fig. 1). No hybridization signal was detected with RNA from untransfected Huh7 cells by contrast the cells transfected with Rep 3 and Rep 4 replicon RNAs both showed hybridization signals. The signal intensity of hybridization of Rep 4 RNA with the NS3 probe was greater than that of Rep 3.

3.4. Expression of NS3 in RNA replicon transfected cells

Replicon cell lines were grown on glass cover slips in six well plates. When they were 70% confluent, they were fixed and stained with an anti-NS3 specific antibody (NS3–7). Untransfected Huh7 cells were also used as a negative control. The cells were examined using immunofluorescence microscopy. The Rep 4 cells showed staining indicating the presence of NS3 (Fig. 3A) while the untransfected Huh7 cells showed no staining for NS3 (Fig. 2B).

It was found that NS3 was mainly localized in the cytoplasm (Fig. 3A) however, in some cases, it was found in both the cytoplasm and the nucleus (Fig. 3B) (Errington et al., 1999).

4. Discussion

To make cell lines which contain RNA replicons, plasmids which contain the sequence of RNA replicons were transcribed to produce replicon RNA. The resulting RNA transcripts were analyzed by gel electrophoresis to ensure that the correct size bands had been synthesized and that there was no degradation of the RNA.

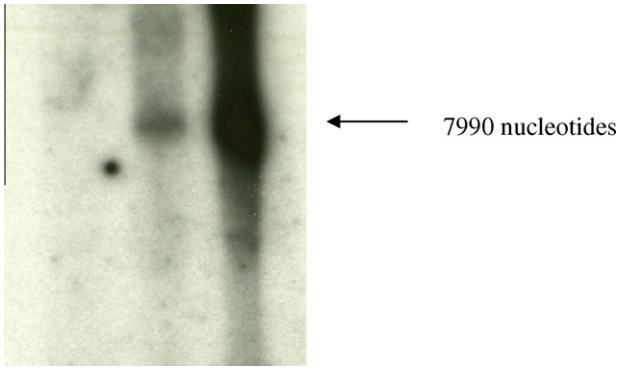

Figure 1 Northern blot hybridization of RNA from cells transfected with replicon RNAs. RNAs resolved by formaldehyde gel electrophoresis were transferred to a nylon membrane and Northern blot analysis was carried out using a ^{32}P-labelled DNA probe corresponding to the NS3 region. X-ray film was developed after 7 days exposure. (1) Untransfected Huh7 cells; (2) Rep 3; (3) Rep 4. The arrow indicates the position of the HCV replicon RNA (7990 nucleotides).

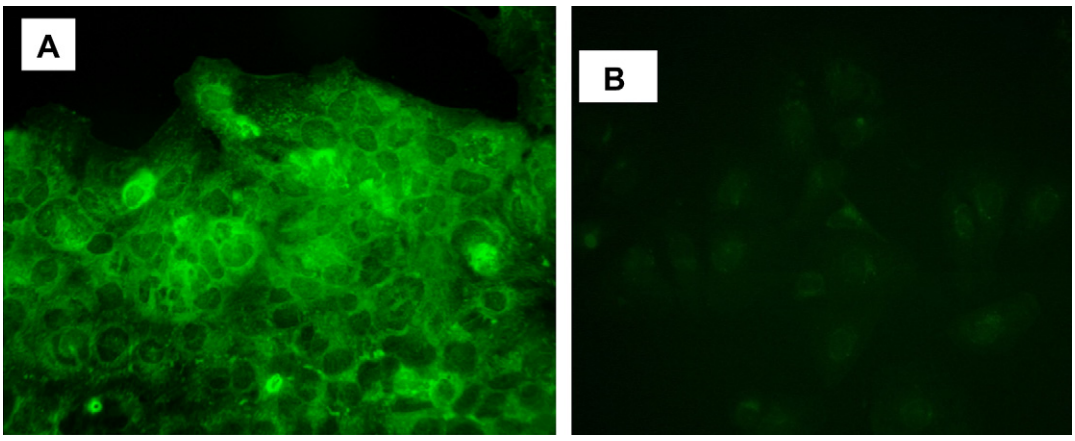

Figure 2 Expression of NS3 in RNA replicon transfected cells. (A) Rep 4. (B) Untransfected Huh7.

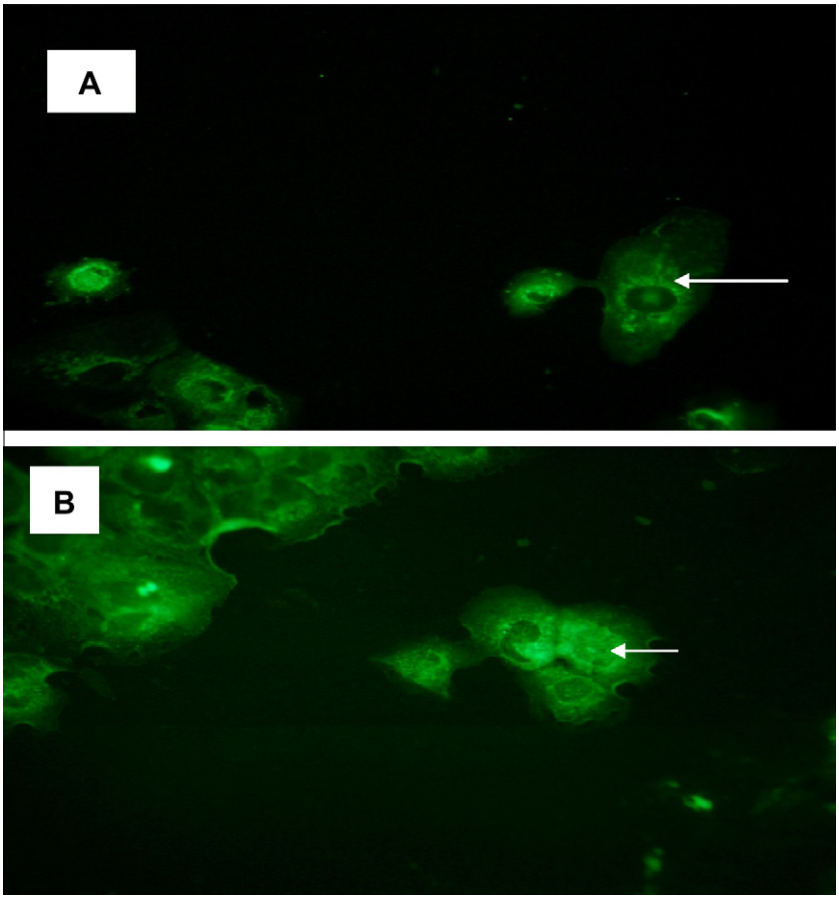

Figure 3 Localization of HCV NS3 into the cells. (A) Perinuclear localization of NS3 and no nuclear translocation was there as indicated by the arrow. (B) Translocation of HCV NS3 to the nucleus as shown by the arrow. However, perinuclear localization is clearly also seen as granules.

The transfection of Huh7 cells with replicon RNA was performed in two ways, electroporation and lipofectin. Both procedures had to be performed as rapidly as possible to avoid RNA degradation. The results showed that both lipofectin transfection and electroporation were successful in transforming cells with replicon RNA. With the transfection by electroporation, it was also important that a high concentration of cells was used since a large number of the cells died during the electrical pulse.

Twenty-four hours after adding G418, untransfected cells started to die. After few days, small colonies of transfected

cells were scattered throughout the cell culture flasks. About 3 weeks later, the colonies had proliferated and had become confluent. Subsequent to this, the transformed cells could be propagated in new culture flasks.

Although transfection and selection of G418 resistant cells was successful, confirmatory tests were performed to show the presence of replicon RNA. Northern blot analysis of RNA from the replicon cell lines using a DNA probe corresponding to the NS3 region of HCV-1b showed the presence of labelled bands corresponding to the predicted size of the replicon RNA.

During the propagation of Huh7 cells transfected with replicon RNA, it was noticed that they grew slower than untransfected Huh7 cells grown in the absence of G418. This may due to the presence of HCV RNA replicating in transfected cells which may reduce the capacity of the cells to replicate as efficiently (Bartenschlager et al., 2003). Also when comparing the growth rate of the transfected cells, it was noticed that cells transfected with S1179I (Rep 4) grew slower than those transfected with A1174T (Rep 3). One possibility was that the Rep 4 cells might contain more replicon RNA than those transfected with the Rep 3 cells. Comparisons of the quantities of HCV RNA in replicon cell lines confirmed that there was a greater copy number of RNA replicon molecules in the Rep 4 cells (Fig. 1). It is therefore possible that more replicon RNA was used in the transfection to produce Rep 4 cells even though it had been intended that both transfections should have used the same quantity of RNA. It has previously been reported that replicon cell lines that contain smaller quantities of replicon RNA usually grow faster than those that contain higher concentrations and this could explain the different growth rates of Rep 3 and Rep 4 cells (Lohmann et al., 1999; Bartenschlager et al., 2003). Another possible reason for the different growth rates of Rep 3 and Rep 4 cells, which has been described by several authors, could be the appearance and selection of mutations in the replicons which affect replication efficiency. These mutations could be found in any area of HCV non-structural region but they mainly cluster in certain regions of NS5A (Lohmann et al., 2003). A second cluster of adaptive mutations has also been found at the carboxy-terminus of the NS3 serine proteinase domain and at the amino-terminus of the NS3 helicase domain (Blight et al., 2003; Lohmann et al., 2003). However, a comparison of the sequences of the Rep 4 and Rep 3 replicon RNAs derived from the established cell lines was not carried out in this study. One other possible reason for the differences in growth rate of the cell lines could be due to the permissiveness of the host cells. It has been reported that replicons start off replicating slowly in non-permissive or semi-permissive cells however, after serial passage highly permissive cells, in which the replicons replicate more efficiently, can be selected (Blight et al., 2003; Murray et al., 2003).

It was shown previously that RNA replicon cell lines have to be grown in the presence of selective antibiotic continuously to maintain the replicon. By contrast, in the absence of the selective antibiotic, a gradual reduction of the replicon RNA should take place (Pietschmann et al., 2001; Bartenschlager et al., 2003). To examine the effect of growing Rep 4 cells in the absence of G418, cells were passaged for three months without antibiotic selection and in parallel to these, other cells were passaged with antibiotic selection. RNAs were extracted from the cells grown under both conditions and the levels of replicon RNA were measured by Real-Time PCR. The results showed that there was a large reduction in the quantity of replicon RNA (approximately 92%) in the cells passaged in the absence of G418. This reduction of replicon RNA highly dependent on the way cells were passaged. When cells were kept confluent for extended periods, replicon levels dropped rapidly, whereas a more constant amount of HCV RNA was found in cells passaged while pre-confluent (Pietschmann et al., 2001). A possible explanation for this is that the level of replicon RNA replication in confluent cells may be reduced compared to actively growing pre-confluent cells. Therefore, RNA degradation is no longer compensated by *de novo* RNA synthesis. Consequently, the drop in the amount of replicon RNA is highest in cells that are left in a confluent state for prolonged times (Pietschmann et al., 2001; Bartenschlager et al., 2003). As a result of these findings, the replicon cell lines in our laboratory were regularly passaged at 70% confluence.

The expression of HCV viral antigens in Rep 4 cells was also demonstrated by staining for the presence of NS3 with an anti-NS3 antibody (NS3–7) which showed both cytoplasmic and nuclear staining (Errington et al., 1999). Overall the evidence shows that the transfection of Huh7 cells with HCV RNA replicons and the selection of transfected replicon cell lines using G418 were successful.

Evidence from many studies has indicated that HCV NS3 is likely to have important effects on host cells and on the replication of HCV RNA (Booth, 1998). In previous studies, antibodies against NS3 were used to demonstrate its expression in tissue culture cells transfected with the NS3 coding sequence and in HCV infected human hepatocytes (Errington et al., 1999). It was found that NS3 was present in the cytoplasm in most of the cells and in some of the cells NS3 was also in the nucleus. The NS3 staining, in both the cytoplasm and the nucleus, was granular in pattern which may indicate a possible association of NS3 with sub-cellular structures (Errington et al., 1999).

Although most of the replication processes of HCV virus take place in the cytoplasm of the cell, the detection of NS3 protein in the nucleus (Errington et al., 1999) suggests that it may have other functions in addition to those of RNA replication and polyprotein processing. The presence of NS3 in the nucleus may be due to its association with cellular proteins. This may explain its association with the cellular tumor suppressor protein p53 in the nucleus (Ishido et al., 1997; Booth, 1998). It has also been shown that NS3 contains an amino acid sequence similar to one found in the regulatory subunit of protein kinase A (PKA) (Muramatsu et al., 1997). It was shown that NS3 alters PKA localization to the nucleus in transfected cells (Borowski et al., 1996). This suggests the possibility that the presence of NS3 in the nucleus could interfere with host cell signaling processes which in turn could favor viral persistence.

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