INTRODUCTION

The hepatitis C virus (HCV) is a major aetiological agent of chronic hepatitis, often leading to liver cirrhosis and hepatocellular carcinoma (Choo et al., 1989; Hoofnagle, 2002; Zoulim et al., 2003). The HCV genome, flanked by conserved, non-translated regions (NTRs), encodes a polyprotein precursor, which is co- and post-translationally processed into three structural (core, E1 and E2) and at least six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. Translation initiation of the viral genome is controlled by an internal ribosome entry site (IRES) located mainly within the 5′-NTR. (Lindenbach & Rice, 2005; Penin et al., 2004; Rice, 2003)

Early computer-assisted analysis of the HCV genome has revealed the presence of an alternate open reading frame (ORF) overlapping the core gene at +1 nucleotide (core +1 ORF) (Ina et al., 1994; Smith & Simmonds, 1997). Recently, several studies have shown the presence of anti-core +1-specific B-cell and T-cell responses, suggesting the expression of the core +1 ORF during natural HCV infection (Bain et al., 2004; Branch et al., 2003; Komurian-

The primer sequences for the insertion of mutation N25 in the HCV-1a (H) sequence and also for N27–N29 and N30 are described in Supplementary Table S1 available with the online version of this paper.

Differences in the expression of the hepatitis C virus core +1 open reading frame between a nuclear and a cytoplasmic expression system

Niki Vassilaki, Katerina I. Kalliampakou and Penelope Mavromara

Molecular Virology Laboratory, Hellenic Pasteur Institute, 127 Vas. Sofias Avenue, Athens 11521, Greece

The hepatitis C virus (HCV) genome possesses an open reading frame (ORF) overlapping the core gene at +1 nucleotide (core +1 ORF). Initial in vitro studies suggested that the core +1 ORF is translated by a ribosomal −2/+1 frameshift mechanism during elongation of the viral polyprotein. Recent studies, however, based on transfection of mammalian cells with reporter constructs have shown that translation of the core +1 ORF is mediated from internal core +1 codons. To resolve the apparent discrepancies associated with the mechanism of core +1 translation, we examined the expression of the HCV-1 and HCV-1a (H) core +1 ORF in a cytoplasmic transcription system based on Huh-7/T7 cells that constitutively synthesize the T7 RNA polymerase in comparison to that in Huh-7 cells. We showed that the efficiency of both the −2/+1 and −1/+2 frameshift events operating at the HCV-1 core codons 8–11 is significantly enhanced in the Huh-7/T7 cytoplasmic transcription system and is dependent on the presence of the consecutive adenine (A) residues within core codons 8–11. In contrast, internal translation initiation at core +1 codons 85/87 occurs in both the nuclear and cytoplasmic transcription systems and is not repressed by the ribosomal frameshifting event. Finally, although core +1 codons 85/87 is the most efficient site for internal initiation of core +1 translation, it may not be unique, as additional internal core +1 codon(s) appear to drive translation at low levels.
In an effort to resolve the apparent discrepancies of the mechanism of core +1 expression, we established a novel cytoplasmic expression system based on Huh-7 cells that constitutively synthesizes T7 RNA polymerase. Following plasmid DNA transfection, this system supports T7-driven transcription in the cytoplasm, thus providing an environment for the expression of the transgene closer to that supporting the expression of the viral RNA during natural HCV infection. This may be of importance as nuclear transcription may be followed by post-transcriptional modifications or interactions with nuclear proteins that could affect the ribosome–RNA interactions involved during translation initiation. Here, we compared the expression of the core +1 ORF from the HCV-1 and HCV-1a (H) isolates in transiently transfected Huh-7/T7 and Huh-7 cells. Our results can be summarized as follows: (i) Huh-7/T7 cells, in contrast to Huh-7, efficiently support both the −2/+1 and −1/+2 frameshift events operating at the A-rich region of the HCV-1 core codons 8–11. (ii) The HCV-1a (H) isolate, which lacks the stretch of the 10 consecutive A residues at the core codons 8–11, failed to support the −2/+1 frameshift in Huh-7/T7 cells, indicating that the synthesis of the frameshifted core +1/F protein is directly related to the presence of the A-rich region. (iii) Translation initiation from internal core +1 codons 85/87 operates in the context of cytoplasmic transcription and most importantly concomitantly with ribosomal frameshifting within the A-rich region.

**METHODS**

**Plasmid construction and site-directed mutagenesis.** The HCV-1 and HCV-1a (H) CDNA sequences were obtained from plasmids path 10/17–38, path 11/36–27, kindly provided by Dr M. Beach (CDC, Atlanta, USA), and pDNA-C1, kindly provided by Dr G. Inchauspe (Department of Infectious Diseases, Transgene, Lyon, France). The basic characteristics of the plasmids used in this study are summarized in Table 1. Mutations were introduced by site-directed mutagenesis using the Quikchange kit (Stratagene) and confirmed by sequence analysis. The sequences of the priming oligonucleotides were used for the insertion of mutations N1, N3, N6, N19 and N25 in the HCV-1 sequence have been previously described (Vassilaki & Mavromara, 2003). The primer sequences for the insertion of mutation N25 in the HCV-1a (H) sequence as well as for N27–N29 and N30 are described in Supplementary Table S1 (available in JGV Online).

Plasmids pHPI-1699, pHPI-1690 and pHPI-1694 carry the CAT–IRES-core\(^{630}\)–LUC–98X bicistronic unit under the control of the T7 promoter of pGEM-3Zf(+) vector (Promega). This unit contains the IRES sequence and core nt 342–630 from HCV-1 (pHPI-1699 and pHPI-1690) or HCV-1a (H) (pHPI-1694) placed between the 98X sequence of the viral 3'-NTR placed downstream of the T7 promoter of pHPI-1699, with the T7-directed LUC expression normally peaks (data not shown), and at 48 h p.t. in Huh-7 cells. Quantification of the β-galactosidase protein was performed with the β-Galactosidase Assay system (Promega), as specified by the suppliers.

**Cells and transfection experiments.** Huh-7 (human hepatoma) and Huh-7/T7 were kindly provided by Dr R. Bartenschlager (University of Heidelberg, Germany). Cells were maintained in Dulbecco’s modified Eagle medium (Biochrom KG) supplemented with 10% fetal bovine serum (Gibco-BRL), non-essential amino acids (1×; Biochrom KG), 100 U penicillin ml\(^{-1}\), 100 µg streptomycin ml\(^{-1}\) and 2 mM l-glutamine, and specifically for Huh-7/T7 cells with Zeocin (5 µg ml\(^{-1}\); Invitrogen). Cells seeded in six-well plates (Nunc), at a confluence of 60–70% for Huh-7 and 80–90% for Huh-7/T7 cells, were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol.

**Quantification of LUC, CAT and β-gal.** Firefly LUC activity and CAT production were quantified as previously described (Vassilaki & Mavromara, 2003). LUC activity was measured at 24 h post-transfection (p.t.) in Huh-7/T7 cells, when the T7-directed LUC expression normally peaks (data not shown), and at 48 h p.t. in Huh-7 cells. Quantification of the β-galactosidase protein was performed with the β-Galactosidase Assay system (Promega), as specified by the suppliers.

**Immunoblotting.** Cells were treated with the proteasome inhibitor MG-132 (5 µM) for 12 h before harvesting, Huh-7 and Huh-7/T7 cells were harvested at 48 and 24 h p.t., respectively, and lysates were analysed as previously described (Kalamvoki & Mavromara, 2004).

**Confocal immunofluorescence microscopy.** Confocal immunofluorescence microscopy was performed as previously described (Kalamvoki & Mavromara, 2004).

**Antibodies.** For the production of the polyclonal antibody against the core +1 ORF, the peptide NK1, TYRSSAPLEALPGP(C) (core +1 aa 135–149), was conjugated to keyhole limpet haemocyanin and used to immunize rabbits according to a classical immunization protocol (Harlow, 1988). Antisera were collected 2 weeks after the last booster
**Table 1.** Summarized information for the LUC reporter constructs used in the transfection assays

<table>
<thead>
<tr>
<th>Plasmid (parental vector)</th>
<th>Promoter</th>
<th>Isolate of core/core + 1 sequence</th>
<th>Bicistronic cassette</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHPI-1333*</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>–</td>
</tr>
<tr>
<td>pHPI-1332*</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>–</td>
</tr>
<tr>
<td>pHPI-1335*</td>
<td>CMV/T7</td>
<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>–</td>
</tr>
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<td>CAT–IRES-core + 1.630–LUC</td>
<td>–</td>
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<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N19 (A&lt;sup&gt;367&lt;/sup&gt;N67, A&lt;sup&gt;369&lt;/sup&gt;N69, A&lt;sup&gt;373&lt;/sup&gt;N73 →C)</td>
</tr>
<tr>
<td>pHPI-1342 (-1333)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1 (G&lt;sup&gt;473&lt;/sup&gt;N473 →A, W&lt;sup&gt;473&lt;/sup&gt;N473 →stop in core + 1 ORF)</td>
</tr>
<tr>
<td>pHPI-1401 (-1333)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 (ATG&lt;sup&gt;598&lt;/sup&gt;N598 →GAG, M&lt;sup&gt;598&lt;/sup&gt;N598 →Gly, M&lt;sup&gt;623&lt;/sup&gt;N623 →stop in core + 1 ORF)</td>
</tr>
<tr>
<td>pHI-1345 (-1335)</td>
<td>CMV/T7</td>
<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1</td>
</tr>
<tr>
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<td>CMV/T7</td>
<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25</td>
</tr>
<tr>
<td>pHPI-1544 (-1401)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N19</td>
</tr>
<tr>
<td>pHPI-1539 (-1401)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N1</td>
</tr>
<tr>
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<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N1</td>
</tr>
<tr>
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<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N28 (N28: CTA&lt;sup&gt;355&lt;/sup&gt;N355 →TAG, L&lt;sup&gt;27&lt;/sup&gt;N27 →stop in core + 1 ORF)</td>
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<tr>
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<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N27 (N27: C&lt;sup&gt;351&lt;/sup&gt;N351 →A, S&lt;sup&gt;355&lt;/sup&gt;N355 →stop in core + 1 ORF)</td>
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<tr>
<td>pHPI-1755 (-1401)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N29 (N29: CTA&lt;sup&gt;351&lt;/sup&gt;N351 →TAG, L&lt;sup&gt;39&lt;/sup&gt;N39 →stop in core + 1 ORF)</td>
</tr>
<tr>
<td>pHPI-1542 (-1401)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25 + N31 (N31: C&lt;sup&gt;351&lt;/sup&gt;N351 →T, R&lt;sup&gt;38&lt;/sup&gt;N38 →stop in core + 1 ORF)</td>
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<td>pHPI-1531 (-1333)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-9As-core + 1.630–LUC(N) Deletion of an adenosine (A) at core codons 8–11</td>
<td></td>
</tr>
<tr>
<td>pHPI-1532 (-1332)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-9As-core + 1.630–LUC(N) Deletion of an adenosine (A) at core codons 8–11</td>
<td></td>
</tr>
<tr>
<td>pHPI-1533 (-1331*)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-9As-core&lt;sup&gt;650–LUC&lt;/sup&gt;N1 Deletion of an adenosine (A) at core codons 8–11</td>
<td></td>
</tr>
<tr>
<td>pHPI-1536 (-1532)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-9As-core&lt;sup&gt;650–LUC&lt;/sup&gt;N6 Deletion of an adenosine (A) at core codons 8–11</td>
<td></td>
</tr>
<tr>
<td>pHPI-1537 (-1532)</td>
<td>CMV/T7</td>
<td>HCV-1</td>
<td>CAT–IRES-9As-core&lt;sup&gt;650–LUC&lt;/sup&gt;N6 Deletion of an adenosine (A) at core codons 8–11</td>
<td></td>
</tr>
<tr>
<td>pHPI-1690 (-1689)</td>
<td>T7</td>
<td>HCV-1</td>
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<td>N1</td>
</tr>
<tr>
<td>pHPI-1691 (-1689)</td>
<td>T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1</td>
</tr>
<tr>
<td>pHPI-1692 (-1690)</td>
<td>T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25</td>
</tr>
<tr>
<td>pHPI-1693 (-1690)</td>
<td>T7</td>
<td>HCV-1</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1 + N25</td>
</tr>
<tr>
<td>pHPI-1694 (-1690/1335)</td>
<td>T7</td>
<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N25</td>
</tr>
<tr>
<td>pHPI-1695 (-1694)</td>
<td>T7</td>
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<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1</td>
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<td>pHPI-1696 (-1694)</td>
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<td>N25</td>
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<td>HCV-1a (H)</td>
<td>CAT–IRES-core + 1.630–LUC</td>
<td>N1 + N25</td>
</tr>
</tbody>
</table>

*Previously described (Vassilaki & Mavromara, 2003).

and anti-core + 1 polyclonal antibody was purified by a slightly modified affinity chromatography method based on CNBr-activated Sepharose 4B beads (Harlow, 1988). The antibody was used in Western blotting at a concentration of 1 μg ml<sup>–1</sup>. The goat polyclonal antibody against the bacteriophage T7 RNA polymerase protein was a generous gift from Dr W. Studier (Brookhaven National Laboratory, Upton, NY, USA) and used in immunofluorescence analysis at a dilution of 1 : 500.

**RESULTS**

To investigate the expression of the core + 1 ORF in a mammalian cytoplasmic transcription system, we used the Huh-7/T7 cell line that constitutively expresses T7 RNA polymerase. The cytoplasmic localization of T7 RNA polymerase in Huh-7/T7 cells was confirmed by immunofluorescence analysis (data not shown).

To assess expression of core + 1 ORF, we chose the LUC tagging approach, and translation of the core + 1 ORF was monitored by measuring the enzymic activity resulting from the expression of a chimeric core + 1–LUC protein containing the first 95 aa of core + 1 (nt 342–630) fused to the LUC gene. This approach allows the sensitive enzymic detection of the core + 1 translation product and can be
used to estimate the relative expression levels of the core + 1 ORF in Huh-7 and Huh-7/T7 cells.

Initially, we used a series of previously described constructs (Vassilik & Mavromara, 2003) that carry the CAT–IRES-core + 1-530–LUC bicistronic cassettes under the control of both HCMV and T7 promoters. The T7-driven CAT–IRES-core + 1–LUC bicistronic transcripts produced in the cytoplasm are uncapped and permit translation only of the core + 1-LUC gene as this is under the control of the HCV IRES element. On the other hand, CAT is produced only from cap modified transcripts derived through nuclear transcription under the control of the HCMV promoter and, when expressed, it serves as a control for transfection efficiency. Additionally, to assure conditions for exclusive cytoplasmic transcription of the core + 1-LUC gene, we also proceeded with the construction of a new series of plasmids that carry the bicistronic CAT–IRES-core + 1–LUC cassette under the T7 promoter alone (see Fig. 4a).

The efficiency of the −2/+1 frameshift event within the HCV-1 core codons 8–11 is substantially enhanced in Huh-7/T7 cells

To assess the efficiency of frameshift and internal translation initiation within the core + 1 ORF, we introduced the following mutations: N19 that disrupts the A-rich slippery site within codons 8–11 of HCV-1 (Fig. 1a(ii)), N1 that introduces a stop codon at the core +1 codon 43 (W43 to TGG) [Fig. 1a(i)] and N3 that converts the initiator AUG of the polypolymer into a stop codon [Fig. 1a(i)], which are expected to abolish expression of the core +1–LUC resulting from ribosomal frameshift. On the other hand, mutation N25 that converts both ATG codons at positions 85/87 to glycine codons [Fig. 1a(i)] is expected to abolish the expression of the core +1–LUC that results from internal translation initiation at codons 85/87.

As shown in Fig. 1b(i), in agreement with previous studies, in Huh-7 cells, mutations N19 (pHPI-1383) and N1 (pHPI-1342) had no significant effect on the synthesis of the core +1–LUC protein, whereas mutation N25 (pHPI-1401) substantially inhibited core +1–LUC expression, suggesting that translation of the core +1 ORF is mediated mainly from internal translation initiation at core +1 codons 85/87 (Vassilik & Mavromara, 2003). In contrast, in Huh-7/T7 cells, both the N19 (pHPI-1383) and N1 (pHPI-1342) mutations severely affected LUC activity [Fig. 1b(ii)], which was about 20% of the wild-type level, suggesting that the frameshift event at codons 8–11 of the HCV-1 polypolymer is significantly favoured in a mammalian expression system that supports cytoplasmic transcription. In addition, N25 caused a significant reduction of the levels of LUC activity in Huh-7/T7 cells, which were 70% of those exhibited from the wild-type plasmid, suggesting that translation initiation at internal codons 85/87 can occur simultaneously with the frameshifting event.

As none of the above single mutations resulted in background levels of LUC activity in Huh-7 or Huh-7/T7 cells, we sought to examine the effect of combined mutations on the core +1–LUC expression. As shown in Fig. 1b(ii), in Huh-7/T7 cells both the N25+N19 (pHPI-1544) and N25+N1 (pHPI-1539) double mutations severely inhibited core +1–LUC expression, suggesting that frameshift at codons 8–11 and internal initiation at codons 85/87 are the main mechanisms involved for core +1 expression in Huh-7/T7 cells. On the other hand, in Huh-7 cells [Fig. 1b(i)], the double N25+N19 (pHPI-1544) and N25+N3 (pHPI-1542) mutations produced LUC levels similar to those yielded by N25 alone (pHPI-1401), a result consistent with the lack of efficient frameshifting in Huh-7 cells. Interestingly, however, the double N25+N1 mutation (pHPI-1539) caused a reduction of the LUC activity levels as compared with those of N25 alone, suggesting the presence of a second, low efficiency, internal translation initiation site for core +1 upstream of codon 43, which is the codon that is converted to a termination codon in the N1 mutation.

To further map this site, we designed four additional mutations that introduced termination codons downstream of codon 43 (N1) in the background of the N25 mutant [Fig. 1a(i)]. Specifically, mutation N31 converts core +1 codon 48 (R48, CGA) into a stop codon, whereas N28, N27 and N29 convert the fourth (L4, CTA), twentieth (S20, TCG) and forty-first (L41, CTA) core +1 codons, respectively, to termination codons. As shown in Fig. 1b(i), the N25+N31 mutant (pHPI-1755) produced very low LUC levels, similar to those yielded from the N25+N1 double mutant (pHPI-1539), whereas mutations N28 (pHPI-1799), N27 (pHPI-1540) and N29 (pHPI-1543) had no significant effect on the expression of the HCV-1 N25 mutant. This provides strong evidence of an additional, low efficiency internal initiation event at core +1 codons 42 or 43.

Taken together, these results indicate that cytoplasmic transcription of core +1 ORF in cultured cells (Huh-7/T7) is associated with significant efficiency of the −2/+1 frameshift event at the 10 A residue slippery site (codons 8–11) of the HCV-1 isolate. This is in contrast to the expression of core +1 ORF from the same plasmid DNA in Huh-7 cells that support nuclear transcription. Interestingly, internal initiation at core +1 codons 85/87 can direct translation of the HCV-1 core +1 ORF in Huh-7/T7 cells and it is not excluded by the frameshift event. Furthermore, an additional internal initiation site at the core +1 codon 42 or 43 is likely to account for core +1 translation in Huh-7 cells, albeit at low levels.

The A-rich sequence at codons 8–11 of HCV-1 core coding region serves as a dual translational frameshift signal in Huh-7/T7 cells

Previous studies have suggested that the A-rich sequence located at core codons 8–11 of HCV-1 can support both +1−2 and −1/+2 frameshift events (Choi et al., 2003;
Varaklioti et al., 2002). Thus, we also assessed the efficiency of the $2^{1}/2^{1}$ frameshift in Huh-7/T7 cells in comparison to Huh-7. To this end, we used a second series of constructs carrying an A deletion in the 10 A residue sequence of HCV-1 core codons 8–11 of the cassettes core630 +1630 LUC (pHPI-1331), core+1630 LUC (pHPI-1333) and core$^{-1}$630 LUC (pHPI-1332) (Fig. 2a). This deletion fuses the first 10 codons of the core protein coding sequence to the core+1 ORF. Thus, the resulting 9As-core$^{+1630}$/LUC(0) cassette (pHPI-1531) resets core$^{+1}$LUC from the +1 to the 0 frame relative to the initiator ATG and represents the positive control. On the other hand, the 9As-core$^{-1}$630/LUC(+1) cassette (pHPI-1532) resets core$^{-1}$LUC from the -1 to the +1 frame relative to the initiator ATG and thus LUC could be expressed only by a $-1/+2$ frameshift. This means that LUC expression from this construct would be indicative of the $-1/+2$ frameshift event. Lastly, the 9As-core$^{+1630}$/LUC(-1) cassette (pHPI-1533) resets the LUC gene from the 0 to the $-1$ frame of core sequence, which contains multiple stop codons, thus serving as a negative control.

As shown in Fig. 2b(i), the expression of the 9As-core$^{+1630}$/LUC(+1) cassette (pHPI-1532) in Huh-7/T7 cells yielded significant levels of LUC activity, supporting a $-1/+2$ frameshift event within the HCV-1 core coding sequences. As expected, only background levels of LUC activity were produced in Huh-7 cells [Fig. 2b(ii)]. To further confirm these results, we separately introduced in the 9As-core$^{+1630}$/LUC vector the nonsense mutation N6 which converts the twenty-fifth codon of core (0) frame at nt 414 (P25, CCG) to a TAA stop codon (pHPI-1537), and mutation N1 which
changes the forty-third codon of core +1 frame at nt 472 (W43) to a termination codon (pHPI-1536) (Fig. 2a). As shown in Fig. 2b(iii), the N6 mutation abolished the synthesis of LUC in the Huh-7/T7 cell line, whereas the N1 mutation had no significant effect, which is consistent with the presence of a −1 frameshift event which would allow the ribosome to shift from the core +1 to the core (0) frame. Taken together, these data indicate that both the −1/+2 and −2/+1 frameshifts occurring at the A-rich region of HCV-1 core codons 8–11 are substantially enhanced in the cytoplasmic transcription system.

The A-rich sequence at codons 8–11 of the HCV-1 core coding region is critical for the −2/+1 frameshift event in Huh-7/T7 cells

HCV-1 is characterized by the presence of an A-rich sequence (364-AAAAAAAAAA-373) within the core codons 8–11 of the polyprotein. In contrast, this sequence is disrupted by a G and a C at nucleotide positions 367 and 373, respectively, in the majority of the HCV isolates (AAAGAAAAAC). Thus, it was of interest to analyse the expression of the core +1 ORF from another HCV isolate that lacks the A-rich sequence (Fig. 3a). For this, a series of bicistronic constructs was made carrying the wild-type and mutated core +1 sequences of the HCV-1a (H) isolate fused to the LUC gene, and their expression was analysed comparatively in Huh-7 and Huh-7/T7 cells as above. As shown in Fig. 3(b), the N25 mutation (pHPI-1546) caused a substantial reduction in the synthesis of core +1-LUC protein in both the Huh-7 [Fig. 3b(i)] and Huh-7/T7 [Fig. 3b(ii)] cells, as compared with those exhibited from the HCV-1a (H) wild-type plasmid (pHPI-1335), indicating that the internal translation initiation at core +1 codons 85/87 of the HCV-1a (H) occurs with significant
efficiency in both Huh-7 and Huh-7/T7 cells. On the other hand, the N1 mutation (pHPI-1345) had only a slight effect on core +1–LUC expression in Huh-7/T7 cells [Fig. 3b(ii)], indicating that, in contrast to HCV-1 isolate, a frameshift event within core codons 8–11 of HCV-1a (H) is not favoured in the Huh-7/T7 cells. Furthermore, the double N25 + N1 mutation (pHPI-1698) almost completely abolished the synthesis of core +1–LUC protein in both Huh-7 and Huh-7/T7 cells [Fig. 3b(i, ii)]. As the HCV-1a (H) N25 mutant does not support frameshift at core codons 8–11 nor internal initiation at core +1 codons 85/87, the difference that is observed in the levels of core +1–LUC expression between the mutants N25 and N25 + N1 supports the function of the additional, weak internal initiation site located upstream of codon 43 that has been suggested for the HCV-1 isolate.

Collectively, these results showed that the frameshift mechanism at core codons 8–11 is directly related to a stretch of 10 consecutive A residues, which is present only in a limited number of HCV isolates (2 of 721 sequences available at the hepatitis virus database http://s2as02genes.nig.ac.jp/). On the other hand, internal translation initiation at core +1 codons 85/87 operates at similar levels for the two HCV-1 and HCV-1a (H) isolates in both Huh-7 and Huh-7/T7 cells.

Finally, we directly compared the expression levels of the core +1–LUC fusion protein derived from HCV-1 and HCV-1a (H) in Huh-7 and Huh-7/T7 cells. As shown in Fig. 3c(ii), in Huh-7/T7 cells, the LUC activity yielded from HCV-1a (H) core +1–LUC (pHPI-1335) is about 35 % of that exhibited from HCV-1 core +1–LUC (pHPI-1333), which is consistent with the undetectable levels of the frameshifted core +1–LUC product from HCV-1a (H). On the contrary, in Huh-7 where -2/+1 frameshift is not favoured, HCV-1a (H) produces core +1–LUC at similar levels to that from HCV-1 [Fig. 3c(i)].

**Internal translation initiation at codons 85/87 is functional in a strictly dependent cytoplasmic transcription system**

We next sought to analyse the expression of the core +1 ORF under conditions that exclusively direct cytoplasmic transcription. To this end, we constructed a new series of bicistronic CAT–IRES-core +1–LUC cassettes carrying the wild-type or mutated core +1 sequences from HCV-1a...
(H) or HCV-1 isolates under the transcriptional control of the T7 promoter alone (Fig. 4a). The 98X sequence of the viral 3′-NTR was added at the 3′-ends of the IRES-core-LUC cassettes in order to increase stability (Fang & Moyer, 2000) and translation efficiency (Song et al., 2006) of the resulting transcripts, as there is no poly(A) tail.

As shown in Fig. 4(b), the N1 mutation (stop codon at the core +1 codon 43) strongly affected the LUC activity levels derived from HCV-1 (pHPI-1691), which were about 18% of that of the wild-type (pHPI-1690), whereas no significant effect on the LUC activity yielded from HCV-1a (H) (pHPI-1695) was detected. On the other hand, the insertion of the N25 mutation (conversion of both ATG codons 85/87 to glycine codons) in both the HCV-1 and HCV-1a (H) core +1–LUC constructs (pHPI-1692 and pHPI-1696, respectively) caused a significant decrease in the LUC activity levels (Fig. 4b). These data confirm that efficient frameshift is limited to the HCV-1 isolate. Furthermore, they show that internal translation initiation at codons 85/87 operates at significant levels in the cytoplasmic transcription environment of Huh-7/T7 cells, for both HCV-1 and HCV-1a (H) isolates, and can occur concomitantly with the frameshift event at core codons 8–11. As the magnitude of the decrease of the LUC activity in the presence of N25 is about 30% for both isolates, we can conclude that the internal initiation event occurs at similar efficiencies for both HCV-1 and HCV-1a (H).

**Detection of core+1/S and core+1/F proteins in Huh-7/T7 transfected cells**

To characterize the native products of core +1 protein that are produced in the cytoplasmic transcription system, Huh-7/T7 cells were transfected with the plasmid pHPI-1747 that carries the HCV-1 IRES and nt 342–825 of the core/core +1 sequence fused to the myc epitope sequence (EQKLISEEDL) at the core +1 frame, under the control of the T7 promoter (Fig. 5a). The cells were treated with MG-132. Both the fusion of the core +1 ORF to the myc epitope and the treatment of the transfected cells with MG-132 are conditions that increase the stability of the core +1 protein and facilitate its detection (data not shown). As shown by Western blotting using an antibody raised against a carboxy-terminal core +1 epitope (anti-NK1), pHPI-1747 yielded expression of the 13 kDa myc-tagged

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**Fig. 4.** Expression of the HCV-1 and HCV-1a (H) core +1 ORF in Huh-7/T7 cells based on T7-driven transcription. (a) CAT–IRES–core–LUC bicistronic cassettes previously described (Vassilaki & Mavromara, 2003), followed by the 98X sequence of the viral 3′-NTR, were cloned downstream of the T7 promoter. Specifically, the LUC gene is fused to the +1 frame relative to the preceding core coding sequence of HCV-1 in pHPI-1690 and of HCV-1a (H) in pHPI-1694. Mutations N1 and N25 were inserted into the core +1–LUC cassette of pHPI-1690 (HCV-1) and pHPI-1694 [HCV-1a (H)] (indicated by arrows), separately or simultaneously, resulting in plasmids pHPI-1691 (HCV-1 +N1), pHPI-1692 (HCV-1 +N25), pHPI-1695 [HCV-1a (H) +N1] and pHPI-1696 [HCV-1a (H) +N25] or pHPI-1693 (HCV-1 +N25 +N1) and pHPI-1697 [HCV-1a (H) +N25 +N1]. (b) Cultures of Huh-7/T7 cells were transiently co-transfected with one of the above WT or corresponding mutated constructs (1.5 μg DNA per well) and the β-galactosidase (β-gal) reporter vector pA-EUA2+ lacZ (0.5 μg DNA per well). Cells were lysed at 24 h p.t. and the ratio of LUC to β-gal activity was determined for each core–LUC expressing vector. The LUC/β-gal ratio derived from the HCV-1 core +1–LUC construct, pHPI-1690, was set at 100%. Bars represent the means obtained in two separate experiments in duplicate.
core + 1 short protein (core + 1/S–myc) [Fig. 5b(i), lane 1], predicted to be translated by internal initiation at core + 1 codons 85/87, as well as expression of a larger form of the core + 1 protein with an apparent molecular mass of 22 kDa, which is predicted to be produced by the +1 frameshift event at core codons 8–11 (core + 1/F–myc). On the other hand, the same HCV-1 IRES-core + 1-myc sequence cloned under the control of the HCMV promoter (pHPI-1705) supports the synthesis of the 13 kDa core + 1/S–myc protein in Huh-7 cells only [Fig. 5b(ii), lane 1]. These data are comparable to those observed before, indicating that, in contrast to Huh-7, in the Huh-7/T7 cells both the core + 1/S–myc and core + 1/F–myc proteins are concomitantly expressed at detectable levels from the HCV-1 isolate.

**Fig. 5.** Detection of both myc-tagged core + 1/F and core + 1/S proteins in transiently transfected Huh-7/T7 cells. (a) Schematic representation of the myc fusion constructs used in this study. In plasmid pHPI-1747, the myc epitope sequence is placed under the control of the HCMV promoter, whereas in pHPI-1705 the same HCV-1 core + 1-myc sequence is placed under the control of both HCMV and T7 promoters. (b) The positions of core + 1/F–myc and core + 1/S–myc proteins, at about 22 and 13 kDa, are indicated by the open and filled arrowheads, respectively. The positions of the molecular mass markers are shown on the left.

**DISCUSSION**

We showed here, that the expression of the HCV core + 1 ORF in transfected cell culture differs between Huh-7 and Huh-7/T7 cells, which support nuclear and cytoplasmic transcription, respectively, and it is affected by the sequence of the A-rich core codons 8–11. Direct comparison of core + 1 ORF expression in these cells showed that translational frameshift at the core codons 8–11 operates efficiently in Huh-7/T7 and not in Huh-7 cells and only when a stretch of 10 consecutive A residues constitute core codons 8–11, as is the case in the HCV-1 isolate. The HCV-1a (H) isolate that has a disrupted A-rich sequence at the fourth (A-to-G) and tenth (A-to-C) positions does not produce a frameshifted core + 1 protein. In contrast, internal translation initiation at the core + 1 ATG codons 85 and/or 87 occurs at significant levels in either Huh-7 or Huh-7/T7 cells by both HCV-1 and HCV-1a (H) isolates. We also showed that, under certain conditions, the non-ATG core + 1 codon 42 or 43 has the potential to drive translation, albeit at low levels. Notably, another ATG codon located at a similar context with codon 85 (Y^9, G^+^) but at a downstream site into the core + 1 coding sequence, at nt 799, does not serve as the start site (data not shown).

The reason for the significant difference in the frameshift efficiency observed between Huh-7/T7 and Huh-7 cells remains unclear. As previous studies from our laboratory (Varaklioti et al., 2002) as well as from another group (Xu et al., 2001) have independently shown that in vitro synthesis of the core + 1/F protein from a T7-driven transcript is not a result of transcriptional slippage of the T7 RNA polymerase within the A-rich region, our data suggest that the subcellular context of transcription rather than differences in the RNA polymerases used is responsible for the differences in the frameshift efficiency between Huh-7 and Huh-7/T7 cells. It is of interest to note that a role of the ribosomal peptidyltransferase (Meskauskas et al., 2003, 2005) and of the ribosome-tethered molecular chaperones (Muldoon-Jacobs & Dinman, 2006) on the frequency of the programmed ribosomal frameshifting has been shown recently. Thus, it is likely that, depending on the site of transcription, different cellular factors or events may be associated with specific structural elements of the core/core + 1 RNA and thus modulate diversely the translation of the core + 1 ORF. Notably, conserved RNA structural elements (stem–loop V and VI) predicted within codons 16–56 may stimulate frameshift within the A-rich region of HCV-1 isolate (Smith & Simmonds, 1997). Differences in the transcription context could also possibly explain the observation that, in contrast to Huh-7/T7 cells, in our previous expression studies in RRL, where T7 polymerase was also used for transcription, only frameshift but not internal initiation was detectable (Vassilaki & Mavromara, 2003).

On the other hand, our results indicate that internal initiation at core + 1 codons 85/87 directs translation of the core + 1 ORF independently of the presence of the A-rich region within core codons 8–11 [of both HCV-1 and HCV-1a.
(H) isolates), in both Huh-7 and Huh-7/T7 cells, suggesting that the expression of the core +1/S protein is not related to events resulting from the site of transcription. Interestingly, core +1/S expression is not suppressed by the synthesis of the core +1/F protein, suggesting that the expression of the two forms of core +1 protein is not mutually exclusive at the level of translation. Notably, Huh-7/T7 cells is the only expression system observed so far that is capable of supporting the synthesis of both the core +1/F and core +1/S proteins.

A comparative analysis of 117 HCV core sequences from genotypes 1a, 1b, 2a–c, 2k, 3a, 3b, 4a–f, 5a, 6a, 6d, 6e, 6h and 6k, from the GenBank database, revealed that 66 variants contain both ATG\(^\text{G85}\) and ATG\(^\text{G87}\), 23 carry only ATG\(^\text{G85}\) or ATG\(^\text{G87}\), and five lack both ATGs. Additionally, the Kozak context of the ATG\(^\text{G87}\) site, which is close to the optimal one, is also well conserved among the different genotypes. This high level of conservation is consistent with these ATGs having a functional role during core +1 translational initiation. Previous expression studies in HCV-1 have shown that both ATGs are involved in the initiation of core +1 translation and are able to substitute for each other (Vassilaki & Mavromara, 2003). However, the mechanism responsible for internal translation initiation is not clear yet. On the other hand, as the sequence of 10 As at core codons 8–11 is underrepresented in the majority of the reported HCV genomic sequences (http://s2as02.genes.nig.ac.jp/), it is intriguing to speculate that internal translation initiation plays an important role in core +1 expression in mammalian cells.

**ACKNOWLEDGEMENTS**

We are grateful to Dr R. Bartenschlager for kindly providing us with the Huh-7/T7 cell line. We also thank our colleagues from the Molecular Virology Laboratory for helpful discussions and Shirin Khalili for reading the manuscript.

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