Introduction of replication-competent hepatitis C virus transcripts using a tetracycline-regulable baculovirus delivery system

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We have developed a baculovirus delivery system that enables tetracycline-regulated expression of pol II-derived hepatitis C virus (HCV) transcripts in hepatocyte-derived cell lines (McCormick et al., 2002). As part of a study to determine whether such transcripts are replication competent, the transcription start site of the tetracycline-regulable promoter was mapped and three baculovirus transfer vectors containing a neoR-expressing culture adapted replicon cDNA were generated. These vectors either had the first nucleotide of the 5′ UTR positioned −2 (mkI) and +1 (mkII) with respect to the transcription start site, or included a hammerhead ribozyme at the 5′ end of the transcript (5′HH) that cleaves between the ribozyme–5′ UTR boundary. Transfection of all of the culture-adapted replicon constructs into Huh7 cells resulted in the formation of more neomycin-resistant colonies than seen with a polymerase knock-out replicon construct, although this was less pronounced in the mkI group. Furthermore, both the positive- and negative-strands of the replicon could be detected in all neomycin-resistant polyclonal cell lines except for those derived from transfection of the polymerase knock-out construct. Transduction of Huh7 cells with recombinant baculoviruses carrying the same expression cassettes improved replicon delivery, but the relative efficiency of the constructs remained the same. The baculovirus vectors were also used to introduce the replicon transcript into HepG2 cells. Expression of the culture-adapted but not the polymerase knock-out construct induced transcription of the β-interferon gene, a response that may contribute to this cell line being unable to maintain the replicon over long-term culture.

INTRODUCTION

Delivery of a functional viral genome into cells using a DNA/RNA layered approach has been successfully demonstrated for a number of RNA viruses, including members of the Picornaviridae (Yap et al., 1997), Flaviviridae (Varnavski et al., 2000), Togaviridae (Dubensky et al., 1996) and Coronaviridae (Almazan et al., 2000). These studies have involved either transcription of the viral genome by host cell DNA-dependent RNA polymerases, or relied on expression of exogenous polymerases, such as T7, within the cytoplasm of the cell. A layered delivery approach may prove particularly useful in cases where viruses are able to complete their full life-cycle, albeit at a low level that is ultimately self-limiting. One example is the study of hepatitis C virus (HCV) replication, which until recently was limited to detection of negative-strand production by RT-PCR (Lanford et al., 1994). Indeed, production of a full-length HCV genome in cells expressing T7 polymerase and transfected with a full-length HCV genomic cDNA has already been documented (Chung et al., 2001; Myung et al., 2001), with detection of both negative-strand RNA and genetic drift having been used to infer virus replication. A disadvantage of these delivery regimes is that they are limited by the efficiency with which cells can be transfected with the viral cDNA. We recently described a baculovirus delivery system where an inducible pol II promoter was used to drive expression of a full-length HCV genome in HepG2 cells (McCormick et al., 2002). Unlike T7-based systems, the HCV genome is efficiently introduced into the cell as a result of viral transduction. In addition, cells challenged with baculovirus show few signs of cytotoxicity. However, due to the nature of the delivery system, the transcripts produced are both capped and rely on ribozyme cleavage to remove the poly(A) tail. In order to establish whether such transcripts can replicate, we have investigated the ability of the baculovirus system to introduce a culture-adapted HCV replicon (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 1999) into cells. Our findings demonstrate that it is possible using baculovirus transduction to introduce functional replicons into cells, thus validating the use of this delivery system for studying replication of full-length and other subgenomic HCV
constructs. Furthermore, we show that when baculovirus transduction is used to introduce a functional replicon into HepG2 cells, an IFN-β response is induced. This response is not observed in Huh7 cells, the one cell line capable of maintaining the replicon, implying that an antiviral response may contribute to preventing establishment of the HCV replicon in other cell lines.

METHODS

Cells and viruses. Cell lines were maintained and baculoviruses were isolated and amplified as described previously (McCormick et al., 2002). The 5.1 replicon-containing Huh7 cell line and a stable polyclonal Huh7 cell line expressing neo<sup>+</sup> were generated as described previously (Macdonald et al., 2003). Cells were seeded at 3·0 ·10<sup>4</sup> cells cm<sup>−2</sup> (HepG2) or 2·0 ·10<sup>4</sup> cells cm<sup>−2</sup> (Huh7) for 20–24 h prior to a 4 h transduction with 1·25 ·10<sup>5</sup> p.f.u. ml<sup>−1</sup> of both BAC<sup>T</sup>A and another baculovirus, or transfection of plasmid DNA using Lipofectin (Invitrogen) to generate pT5U(mkII). The DNA was subsequently electroporated using a Bio-Rad Gene Pulser II (270 V, 950 m<sup>−</sup>) in vitro transduction is used to introduce a functional replicon into cells using Lipofectin (Invitrogen) according to the manufacturer’s recommendations. For transfection of T7-derived replicon transcripts Huh7 cells (400 μl of 1 ·10<sup>5</sup> cells ml<sup>−1</sup> in DEPC-treated PBS) were mixed with 2 μg of RNA, placed in a 0 ·4 cm cuvette and electroporated using a Bio-Rad Gene Pulsar II (270 V, 950 μF). For the IFN-β RT-PCR assays, polyinosinic-polyric acid [poly(I)-poly(C)] (Sigma) and yeast tRNA (Invitrogen) were transfected into cells using Lipofectin.

Generation of DNA constructs. The construction of pBAC<sub>H</sub>alacr<sub>77</sub>-lacZ(H<sub>0</sub>V) was previously described (McCormick et al., 2002). In order to generate pBAC<sub>H</sub>alacr<sub>77</sub>-lacZ(H<sub>0</sub>V)(mkII), the tetracycline-responsive promoter (Ptet) and the 5′ UTR by means of a two-step PCR using the primers tet-cmv(fwd), tet-cmv(rev)mk1, 5′-TGTACTCACCGGTTCCGC-3′ and yeast tRNA (Invitrogen) were transfected into cells using Lipofectin.

To produce in vitro RNA transcripts from the baculovirus transfer vectors, PCR was used to place a T7 promoter immediately upstream of the HCV 5′ UTR using the primers T7-5′UTR and 5′UTR(out). The resulting DNA fragment was digested with XbaI and AgeI, cloned into XbaI/AgeI-digested pT5U(HH)(mkII) and pBAC<sub>GND</sub>neoT7 and pBAC<sup>5.1</sup>neoT7 was transferred into pBAC<sub>H</sub>alacr<sub>77</sub>-lacZ(H<sub>0</sub>V)(mkII).

Table 1. Sequences of oligonucleotides used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>tet-cmv(fwd)</td>
<td>5′-TTCTAGAGCATGACGAGGCCGCCCCTTTGTCG-3′</td>
</tr>
<tr>
<td>tet-cmv(rev)mk2</td>
<td>5′-GGGGGCTGCGCGAATGCTTTGACATAAAGGAG-3′</td>
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<td>5′-GCTAACTCGCCGCGCCTGATGGG-3′</td>
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<td>5′UTR(out)</td>
<td>5′-TGGTTACTACCCGGTTCCGC-3′</td>
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<tr>
<td>tet-cmv(rev)BamHI</td>
<td>5′-GGGATGGCGGGCGGTTCCACTTAAACGAG-3′</td>
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<tr>
<td>5′UTR(fwd/HH)</td>
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<tr>
<td>5′UTR(out2)</td>
<td>5′-CCCCGGATCTACTTACCCGGTTCGG-3′</td>
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<tr>
<td>T7-5′UTR</td>
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<tr>
<td>Rist(NorI)</td>
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</tr>
<tr>
<td>lacZ5′RACE</td>
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<td>IFNbeta (+)</td>
<td>5′-GAACTTTAGACATCCCTGAGGAGATTAAACGCCG-3′</td>
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<tr>
<td>IFNbeta (-)</td>
<td>5′-GTTCTGCTGATCCACTCTGATGATGTC-3′</td>
</tr>
<tr>
<td>GAPDH (+)</td>
<td>5′-TGAAGGCTGCGAGGATTCAAGCGGATTGTG-3′</td>
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<tr>
<td>GAPDH (-)</td>
<td>5′-CATGTTGGGCGATGGTGCCAC-3′</td>
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orientations into PCR-Script (Stratagene). Single-stranded phagemid DNA was produced as described previously (Kunkel, 1985). For production of T7 transcriptions, the vectors were linearized with HindIII.

**G418-resistant colony formation assays.** For polII-directed delivery of the replicon, Huh7 cells were allowed to express the replicon transcript for 24 h and then seeded into six-well plates along with naive Huh7 cells such that the final cell density was $2 \times 10^5$ cm$^{-2}$. Cell cultures were maintained in the presence of 5 μg tetracycline (Sigma) ml$^{-1}$ with the addition of 1 mg G418 ml$^{-1}$ after 48 h. Selection was maintained for 14 days with a change of medium twice a week before cell colonies were counted. Under circumstances where the replicon was directly introduced by RNA transfection, the assay was essentially the same except that the cells were seeded immediately after transfection and tetracycline was omitted from the selection medium. The efficiency with which the replicon was introduced into cells was measured as the percentage of viable cells that formed G418-resistant colonies.

**Northern blot analysis.** RNA was harvested using Trizol (Invitrogen), electrophoresed through a MOPS/formaldehyde gel and transferred to Bright-Star Nylon Plus membrane. Biotinylated probes and markers were generated using Biotin-Chem-Link reagent (Roche). Hybridization was performed overnight at 42°C in Ultrahyb (Ambion) and bound probe was detected using Bright Star Detection Kit (Ambion).

**In vitro synthesis of RNA transcripts.** Transcripts were produced using T7 polymerase (New England Biolabs), treated with RQ1-DNase (Promega), extracted with acid-phenol/chloroform and chloroform and spun through a mini QuickSpin Column (Roche). RNA integrity was confirmed by MOPS/formaldehyde gel electrophoresis.

**RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE).** This was performed using the First Choice RLM-RACE kit (Ambion) according to the manufacturer’s recommendations. In addition to those provided, primers used during the PCR and nested PCR steps included lacZ$^5$ RACE and 5' UTR (Ambion). In order to determine the transcription start sites, PCR products were cloned into pCR-Blunt and four clones from each RLM-RACE reaction sequenced.

**Detection of IFN-β by RT-PCR.** RNA was harvested using Trizol, treated with RQ1-DNase, extracted with acid-phenol/chloroform and chloroform, and ethanol precipitated. Reverse transcription was performed on 1 μg cellular RNA at 42°C using Superscript (Invitrogen) according to manufacturer’s recommendations. cDNA (2-5 μl) was used in a 25 μl PCR reaction containing 1-25 units HotStarTaq DNA polymerase (Qiagen), 1 × PCR buffer, 2mM MgCl$_2$, 0-8 mM dNTPs and 0-25 μM of each primer. For detection of IFN-β and GAPDH primer pairs used were IFNb(+) and IFNb(−), and GAPDH(+ ) and GAPDH(−), respectively. The parameters of the reaction were as follows: 95°C for 15 min; 22 (GAPDH) or 35 (IFN-β) cycles of 94°C for 30 s, 72°C for 90 s; 72°C for 10 min.

**RESULTS**

**Defining the 5’ end of the replicon transcripts**

An important aspect of maximizing the infectivity of artificially synthesized positive-strand viral RNA transcripts is to ensure that the sequence of the 5’ end accurately reflects that seen in the virus genome (Herold & Andino, 2000). This can be accomplished by either positioning the viral cDNA precisely with respect to the promoter, or by including a ribozyme that cleaves to produce the correct 5’ end. We decided to assess both approaches, as it was unclear whether the cap structure at the end of a polII-derived replicon transcript would inhibit replication. To define the 5’ end of the replicon transcript by positioning it with respect to the tetracycline-regulable promoter (P$_{tet}$), it was first necessary to locate the transcription start site. Therefore, HepG2 and Huh7 cells were co-transduced with BACtTA and BACAH77lacZ(HoV)$^{5b}(mkI)$, a β-galactosidase-expressing HCV minigencode construct where the first nucleotide of the 5’UTR was positioned to coincide with the predicted transcription start site (Fig. 1a) (Akrigg et al., 1985; Gossen & Bujard, 1992). Twenty-four hours after transduction, the cellular RNA was subjected to RLM-RACE, resulting in the production of a PCR product of about the expected size. However, the sequence of this PCR product revealed that the HCV minigencode lacked the first two nucleotides of the 5’UTR. A further construct, BACAH77lacZ(HoV)$^{5b}(mkII)$, was created where the 5’UTR was repositioned a further two nucleotides downstream from the TATA box. RLM-RACE analysis of RNAs from HepG2 and Huh7 cells transduced with this second construct confirmed that the transcription start site coincided with the first nucleotide of the 5’UTR.

As well as mapping the transcription start site for P$_{tet}$ it was also necessary to establish whether a cis-acting hammerhead ribozyme could cleave itself from the HCV 5’UTR. Therefore, a short RNA transcript, including the entire hammerhead ribozyme and the first 167 nucleotides of the 5’UTR, was synthesized in vitro from p5U(HH). Gel analysis demonstrated that the majority of the transcripts had cleaved to produce two RNA fragments of the expected size (data not shown).

**Delivery of functional replicons by DNA transfection**

A series of vectors containing a culture-adapted replicon [pBACrep5.1neo(mkI), pBACrep5.1neo(mkII)] and pBACrep5.1neo(5’HH)] was generated, along with a control polymerase knock-out replicon (pBACrepGNDneo) (Fig. 1b). As a consequence of the cloning strategy, the first 160 bp of the 5’UTR and the SL1 of the 3’UTR X region are derived from the HCV 77 genome (Yanagi et al., 1997), resulting in five and two nucleotide substitutions in the 5’UTR and 3’UTR respectively. Previous work (Gu et al., 2003; Ikeda et al., 2002; Luo et al., 2003; Yanagi et al., 1998) indicates that the HCV replicon is likely to tolerate these small changes. However, to confirm this, a fifth vector (pBA-Crep5.1neo) that allowed in vitro transcription of this modified replicon was generated, along with an appropriate negative control vector (pBACrepGNDneo) (Fig. 1c). Transfection of in vitro transcripts derived from either pBACrep5.1neo or the original culture-adapted replicon plasmid pFK-I389neo/NS3-3/5 resulted in similar numbers of G418-resistant colonies (Fig. 2), indicating that the nucleotide substitutions within the 5’ and 3’UTRs did not affect replication. As expected, the efficiency of colony
formation by the control transcript derived from pBACrepGNDneo59 was negligible.

To ascertain whether a functional replicon could be delivered via a polII promoter, Huh7 cells were co-transfected with pBACTA and either pBACrep5.1neo(mkI), pBACrep5.1neo(mkII), pBACrep5.1neo(59HH) or pBACrepGNDneo. After introduction of tetracycline to shut-off transcription from Ptet, and G418 to select for neoR expression, there were consistently higher numbers of colonies formed in those groups transfected with the culture-adapted replicon constructs compared to the polymerase knock-out control, the order being mkII > 59HH > mkI > GND (Fig. 3a). Colonies formed during selection were subsequently grown as polyclonal cell lines for Northern analysis to look for the presence of both genomic and negative-strand replicon transcripts. The single-stranded DNA probes used to detect positive- (Fig. 3b) or negative- (Fig. 3c) strand transcripts showed approximately a 20 and 50-fold greater specificity towards their respective RNAs compared to the complementary sequence. Consistent with the introduction of a replication-competent replicon, both probes detected an 8 kb transcript in the cell lines derived after transfection with the mkI, mkII and 59HH constructs but failed to identify a similar transcript in cell lines derived after transfection of pBACrepGNDneo and naïve Huh7 cells. In the cell lines containing the replicon it was estimated that there was approximately 10-fold more positive-strand than negative-strand transcript (up to approximately 108 and 107 copies per 5 mg cellular RNA respectively), values which are comparable with previous reports (Lanford et al., 2003; Lohmann et al., 1999). One further observation was the detection of a transcript much larger than 8 kb, most readily seen using the negative-strand specific probe (Fig. 3c).

Fig. 1. Schematic of the constructs used. (a) β-Galactosidase-expressing BACΔH77lacZ(ΔI3V)tet(mkI) and BACΔH77lacZ(ΔI3V)tet(mkII). Included are the nucleotide sequences of the minimal CMV-IE promoter (lower-case) and the start of the HCV 5’UTR (bold, upper-case). Baculovirus transfer vectors that transcribe HCV replicon transcripts from both the Ptet and the T7 promoter are depicted in (b) and (c) respectively. The shaded areas within the 5’ and 3’UTR represent regions derived from the H77 infectious clone of HCV genotype 1a. The figure also denotes nucleotide and amino acid differences between the various constructs.
Baculovirus delivery of the HCV replicon

Fig. 2. Replicon transcripts in the baculovirus transfer vectors are functional. Huh7 cells were transfected with the original culture-adapted replicon RNA transcript (FK5.1), an RNA transcript of the culture-adapted replicon cDNA cloned into the baculovirus transfer vectors (BAC5.1), or a polymerase knock-out control RNA transcript (BACGND). Transfected cells were then subjected to selection with G418 over 2 weeks and neoR colonies counted. The results shown are the mean ± SEM of three separate experiments.

Fig. 3. Delivery of replicons by DNA transfection. (a) Huh7 cells were co-transfected with pBACtTA and pBACrepGNDneo, pBACrep5.1neo(mkI), pBACrep5.1neo(mkII) or pBACrep5.1neo-(5’HH) and allowed to express the polII-derived replicon transcripts for 24 h. Tetracycline was then used to switch off Ptet and G418 introduced into the medium to select for neoR colony formation. The graphs show the mean ± SD of a representative experiment that had been performed in triplicate. Northern analysis was also performed on 5 µg of cellular RNA derived from these G418-resistant cells using an M13 single-stranded DNA probe complementary to either (b) the positive- or (c) the negative-strand of the replicon [lane 9, naïve cells; lanes 10–13, cells selected after transfection with pBACrepGNDneo, pBACrep5.1neo(mkI), pBACrep5.1neo(mkII) and pBACrep5.1neo-(5’HH) respectively]. Included was a series of 10-fold dilutions (10⁵–10⁸) of an in vitro transcript representing nucleotides 2537–3431 of the culture-adapted replicon (lanes 1–4) or the complementary strand of this sequence (lane 5–8). (d) A duplicate blot was probed with a GAPDH probe as a control for RNA loading with the order of cellular RNA samples being the same as in the previous two blots.
clones carrying the replicon constructs (G1, 1.5, 2.5 and H1). After selection with G418 in the presence of tetracycline, the number of colonies observed as a result of transduction with the different constructs followed the same order as seen when the related transfer vectors had been transduced into Huh7 cells (mkII > 5’HH > mkI > GND) (Fig. 5). However, the efficiency with which the culture-adapted replicon was delivered into the Huh7 was approximately 10-fold greater than seen by DNA transfection, resulting in up to 0.8% of cells proceeding to form G418-resistant colonies.

Northern analysis was also used to follow the fate of the polII-derived replicon transcript in Huh7 cells in the absence of G418 selection (Fig. 6). In this instance, Huh7 cells were allowed to recover for 12 h after transduction before tetracycline was introduced into the medium. After either 11 or 9 days, viable cells were transferred into 25 cm² flasks and were maintained in the presence of G418 and tetracycline until they reached confluency (the number of days between transduction and harvesting of the cells for the two experiments shown in italic and bold font respectively). Five µg of cellular RNA from each cell line was analysed for the presence of a replicon transcript by Northern blot using a dsDNA probe directed towards the NS5B coding region (nucleotides 6266–7100 of pFKlSSneo/NS3-3’/5; upper panel) and a duplicate blot was probed with GAPDH probe as a control for RNA loading (lower panel). Those clones selected for inclusion in the remainder of the study (G1, 1.5, 2.5 and H1) are indicated (asterisks).

Recently, attention has focused on the innate antiviral response in replicon-containing HepG2 cells. It has been demonstrated that a replicon containing a mutation disrupting the interaction between NS5A and PKR activates transcription factors IRF-1 and IRF-3 that are associated with a cellular antiviral response. It has been shown that another replicon lacking this interaction was able to trigger an antiviral response in Huh7 cells. However, it was first necessary to show that the culture-adapted replicon used in our study did not trigger an antiviral response in Huh7 cells. Analysis of the levels of IFN-β transcript by RT-PCR demonstrated that low basal levels were present in a Huh7 polyclonal cell line containing the 5.1 replicon as was also seen in naïve Huh7 cells and a Huh7 cell line expressing neoR (Fig. 7a). In contrast, Huh7 cells infected with Semliki Forest virus showed up-regulation of IFN-β transcription.
RT-PCR was then used to analyse the IFN-β response in both HepG2 and Huh7 cells when co-transduced with BACtTA and either BACrep5.1neo(mkII) (clone 2.5) or BACrepGNDneo (clone G1), and when transfected with poly(I)–poly(C) (Fig. 7b). Transfection of the cells with poly(I)–poly(C) triggered production of IFN-β in HepG2 but not Huh7 cells, confirming that a typical antiviral response to dsRNA occurs in the former but not the latter cell line. As expected, mock transfection either in the presence/absence of yeast tRNA did not trigger an IFN-β response. Transduction of both cell lines with a β-galactosidase-expressing baculovirus under the control of Ptet (BACINDlacZtet) failed to elicit a significant IFN-β response, demonstrating that baculovirus transduction by itself does not trigger an antiviral response. In contrast, the culture-adapted replicon did trigger a detectable increase in IFN-β transcription in HepG2 but not Huh7 cells. As the polymerase knock-out construct failed to elicit an IFN-β response, the response seen in BACrep5.1neo(mkII)-transduced HepG2 cells was not simply due to expression of non-structural proteins and so seems likely to involve the formation and subsequent detection of dsRNA. However, under the conditions used in the above experiment there would have been significantly more polIII-derived replicon transcript present in transduced HepG2 cells compared to both transduced Huh7 cells and also replicon-containing Huh7 cell lines (compare Fig. 6 and Fig. 7d). Therefore, it is possible that while the IFN-β response specifically requires the presence of a replication-competent replicon transcript, it is dependent on higher levels of this transcript than would be attainable in a replicon-containing cell line. For this reason, the IFN-β response was examined in HepG2 cells co-transduced with BACtTA and either BACrep5.1neo(mkII) (clone 2.5) or BACrepGNDneo (clone G1), and then maintained in medium containing various concentrations of tetracycline in order to modulate the level of polIII-derived replicon transcript (Fig. 7c). In the absence of tetracycline, when Ptet is fully activated, increased IFN-β transcription occurred after transduction with BACrep5.1neo(mkII) but not BACrepGNDneo, as observed previously. A detectable increase in IFN-β transcription was also seen when the BACrep5.1neo(mkII)-transduced HepG2 cells were maintained in low concentrations of tetracycline (0.02 and 0.04 μg ml⁻¹). However, when higher concentrations of tetracycline were used there was no detectable increase in IFN-β transcription, and neither was there an increase in IFN-β transcription under any of the concentrations of tetracycline used in cells transduced with BACrepGNDneo. The level of polIII-derived replicon transcript was then assessed alongside cellular RNA from a replicon-containing Huh7 cell line using Northern blot analysis (Fig. 7d). This confirmed that for any given concentration of tetracycline, both BACrep5.1neo(mkII)- and BACrepGNDneo-transduced cells contained similar levels of replicon (8 kb) transcript. Two other features were also apparent. The first was the detection of a low abundance high molecular mass RNA species (>8 kb) in HepG2 cells transduced with the culture-adapted but not the polymerase knock-out replicon construct when the cells were maintained in the absence of tetracycline. This band migrated at the same position as the
large transcript seen in a replicon-containing cell line, an RNA species earlier shown to hybridize to both negative- and positive-sense probes. In addition, baculovirus-transduced HepG2 cells maintained in the presence of 0.04 μg tetracycline ml⁻¹, conditions where an IFN-β response was still observed, had a similar level of replicon transcript compared to the replicon-containing Huh7 cell line. It is therefore unlikely that the IFN-β response observed is simply due to overexpression of the replicon in HepG2 cells.

DISCUSSION

When this work was initiated it was unclear whether polII could produce a replication-competent HCV transcript. Recently, HCV replication has been reported in Huh7 and HepG2 cells containing an integrated cDNA copy of a full-length HCV genome under the control of a tetracycline-regulable promoter (Lim et al., 2002). In these cell lines the transcription start site was positioned six nucleotides upstream of the start of the 5' UTR, and it was unclear how far the polII transcript extended beyond the 3' UTR. Moreover, because the full-length HCV genome undergoes limited, if any, replication in tissue culture, evidence for replication relied on detection of negative-strand production as a surrogate marker. By using the HCV replicon in combination with Huh7 cells which, unlike full-length HCV, is capable of self-sustaining replication, we have confirmed that polII-derived HCV transcripts are replication competent. Artificially synthesized RNA transcripts from the cDNA of other positive-strand RNA viruses require that both the 5' and 3' ends correspond to those found in the viral genome in order to maximize infectivity (Herold & Andino, 2000). Therefore, it was not unexpected that correct positioning of the 5' UTR with respect to the transcription start site was required to maximize the delivery of functional replicon transcripts into Huh7 cells. However, our results also indicate that the presence of a 5' cap structure does not impair the ability of the polII-derived replicon transcript to replicate. In fact, transcripts possessing a 5' cap were slightly more effective at establishing the replicon in Huh7 cells compared to transcripts where the 5' end was defined using

![Fig. 7](http://www.microbiologyresearch.org)
a hammerhead ribozyme. The reason for this has not been investigated further but could stem from incomplete cleavage by the ribozyme or the fact that the cleaved transcript will have a 5'-hydroxyl group as opposed to a 5'-phosphate group.

Despite the use of baculovirus transduction, the efficiency of replicon delivery into Huh7 cells was still approximately 10-fold less than seen using RNA transfection, maybe because of the low levels of pollII-derived transcript present within the cell. Therefore, it was surprising to find between 100- to 1000-fold higher levels of replicon transcript and HCV non-structural protein expression in HepG2 compared to Huh7 cells, particularly as there is only a 2- to 3-fold difference in the level of reporter gene expression between HepG2 and Huh7 cells transduced with β-galactosidase expressing baculovirus BACINDlacZ6st (McCormick et al., 2002). This higher level of expression has allowed us to detect non-structural protein expression by immunofluorescence and demonstrate that the majority of HepG2 cells do express the replicon construct (data not shown). We have not yet investigated whether the levels of pollII-derived replicon transcript can be further increased in Huh7 cells. However, should the replicon cDNA contain cryptic or dysfunctional RNA splicing signals, an approach such as the introduction of an HIV rev-response element into the pollII transcript in combination with expression of Rev (Malim et al., 1989) might improve transcript production.

An unexpected problem encountered in this study was the stability of the replicon cDNA in the baculovirus genome. Despite Southern blot analysis indicating that the constructs were intact, Western blot and later a functional replicon screen indicated that this was not the case. Even when a functional screen had been used to select replicon clones, sequencing these clones revealed point mutations that in one instance led to a change in the HCV non-structural open reading frame. As the transfer vectors used to generate these viruses do not contain point mutations, it seems likely that these mutations arose during integration into the virus genome or in the initial rounds of virus replication prior to cloning, presumably due to low fidelity of the baculovirus DNA polymerase. For this reason we have transferred our replicon constructs into the FAST-BAC system (Luckow et al., 1993). In this system, propagation of the baculovirus genome, recombination and subsequent cloning are all performed in E. coli and so rely on bacterial DNA polymerase, thereby reducing the chances of a point mutation arising prior to cloning of the virus. Initial results indicate that these FAST-BAC constructs enter and express the culture-adapted replicon constructs in HepG2 and Huh7 cells. Furthermore, there is no discernible clone-to-clone variation in their ability to deliver a functional replicon into Huh7 cells, indicating the absence of point mutations in the consensus sequence of these clones.

An observation made during this study was the presence of a high molecular mass RNA species detected by HCV-specific probes in replicon-containing cell lines. The existence of this RNA species has not been commented on in previous studies, although in the original paper first describing the HCV replicon (Lohmann et al., 1999), a transcript with an apparent size considerably greater than 8 kb is visible by Northern analysis. In addition, a study on the replication of an HCV-related pestivirus, bovine viral diarrhoea virus (BVDV), also observed a similar RNA species, which was considered to be a combination of replicative forms and replicative intermediates (Tomassini et al., 2003). Our data are consistent with this conclusion, as both positive- and negative-strand-specific probes hybridize to this RNA species, and it is seen in HepG2 cells transduced with baculovirus carrying the culture-adapted but not polymerase knock-out replicon. We are further characterizing this RNA species.

Although the replicon system is the best model available to study HCV replication, it is still not possible to examine many aspects of the virus life-cycle. For example, there is as yet no ideal way to study viral particle assembly and entry as replicon cell lines do not form infectious viral particles, even when the replicating RNA expresses the entire HCV genome (Pietschmann et al., 2002). The reason for this failure has not been identified and it has been speculated that the lack of particle formation may stem from a defect in Huh7 cells. The reliance on Huh7 cells also complicates the study of the antiviral response to both full-length and subgenomic HCV replicons, particularly as Huh7 cells have recently been shown to be unable to produce IFN-β in response to dsRNA (Lanford et al., 2003). A recent finding that a number of other cell lines are able to maintain the replicon (Zhu et al., 2003) may help address some of these issues. However, our finding that the presence of a functional replicon in HepG2 cells, unlike in Huh7 cells, does activate transcription of IFN-β highlights the problem of using Huh7 cells, particularly when studying the innate antiviral response of the cell to genomic and subgenomic HCV RNAs. Indeed, it seems to be the ability to respond to dsRNA that accounts for the discrepancy between the antiviral response to HCV replicons in HepG2 cells compared to Huh7 cells, as expression of the polymerase knock-out replicon construct in HepG2 cells did not trigger IFN-β transcription. While it is tempting to speculate that IFN-β production in HepG2 cells might prevent long-term establishment of replicons in this cell line, this seems unlikely as replicon-containing Huh7 cell lines exist that constitutively express IFN-β (Fredericksen et al., 2002). However, as replicons can be cured with IFN-β treatment (Cheney et al., 2002) and constitutive expression of IFN-β normally suppresses replicon replication (Pflugheber et al., 2002), it is possible that the production of IFN-β in HepG2 cells is a contributory factor in preventing establishment of replicons in this cell line. The interferon response observed in HepG2 cells containing the HCV replicon contrasts with a lack of a response seen in cells infected with BVDV (Baigent et al., 2002). In this instance the non-cytopathic strain of the virus is able to prevent IFN-β production in response to dsRNA by blocking IRF3 function. A similar function has also
recently been ascribed to NS3/4A of HCV, and therefore, while the presence of the HCV replicon in HepG2 cells did not block an IFN-β response completely, it remains possible that the response seen was attenuated. It is also possible that expression of the full-length HCV genome, as compared to the NS3–NS5B component expressed by the replicon, may further contribute in down regulation or blockage of IFN-β production. Baculovirus delivery of various HCV constructs along with a more quantitative assay for IFN-β production should allow these questions to be addressed. Indeed, production of replication-competent HCV transcripts from a polII promoter may be a superior approach to answering these questions as we have observed that unlike baculoviral transduction, transfection of T7 transcripts into mammalian-cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 89, 8979–8988.

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**REFERENCES**


