Efficient delivery and regulable expression of hepatitis C virus full-length and minigenome constructs in hepatocyte-derived cell lines using baculovirus vectors

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Baculovirus vectors have been used as efficient delivery vehicles for constitutive gene expression in a variety of mammalian cells. We have further developed the system to allow for regulable expression by placing the gene of interest under the control of an inducible promoter, and complementing it with a second baculovirus vector providing the control elements necessary for promoter activity. We have used this system to express (a) the \( \text{lacZ} \) gene, (b) a 'minigenome' derived from hepatitis C virus (HCV) and carrying \( \text{lacZ} \) or (c) the full-length HCV viral genome, in human hepatocyte cell lines in an inducible fashion. Control systems that rely on either the absence of tetracycline or presence of ponasterone to induce gene expression were tested. Expression of \( \text{lacZ} \) was controlled by ponasterone, but \( \beta \)-galactosidase activity was limited to 10–20% of cells. In contrast, the tetracycline-controlled expression system gave a low basal activity and was highly inducible in almost 100% of cells. Inducible expression was also obtained in almost 100% of cells infected with baculoviruses in which an HCV minigenome was placed downstream of the tetracycline-inducible promoter and upstream of either a hammerhead or hepatitis \( \delta \) virus ribozyme. Northern blot analysis was consistent with accurate cleavage of the minigenome transcript by the hepatitis \( \delta \) virus ribozyme. Finally, regulable transcript production and viral polypeptide processing could be demonstrated in HepG2 cells infected with baculoviruses bearing the full-length HCV genome. This system thus provides a novel tool for the analysis of HCV replication and host–cell interactions.

Introduction

It is estimated that 170 million people worldwide are chronically infected with hepatitis C virus (HCV), a positive-strand RNA virus of the family \( \text{Flaviviridae} \). Although the clinical consequences of acute infection are often mild or non-apparent, as many as 85% of infected individuals develop a chronic infection, frequently with severe long-term pathologies such as cirrhosis and hepatocellular carcinoma. A major hindrance to HCV research is the lack of an efficient and convenient culture system (Bartenschlager & Lohmann, 2000). Although this has in part been overcome by the development of HCV replicons (Blight \textit{et al.}, 2000; Lohmann \textit{et al.}, 1999), bicistronic RNAs that express both the HCV non-structural proteins required for replication and a selectable marker, there is still no ideal system for studying replication of the complete virus. One approach to overcoming this obstacle that has been successfully used for other viruses is to generate stable cell lines containing an infectious viral clone integrated into the genome (Sells \textit{et al.}, 1987; Tsurimoto \textit{et al.}, 1987). However, it appears that long-term expression of the HCV open reading frame, even at low basal levels when under the control of an inducible promoter, is cytotoxic (Moradpour \textit{et al.}, 1998). An alternative approach has been to use viral delivery systems in which cells are transfected with a plasmid carrying a cDNA clone under the control of a T7 promoter and then infected with a virus that expresses T7 polymerase. While this approach has met with some success (Mizuno \textit{et al.}, 1995; Myung \textit{et al.}, 2001), it is limited by the efficiency with which the plasmid can be transfected into cells and, in the case of hepatocyte-derived cell lines, this is often low. Recently, it was shown that the inefficiency of DNA transfection could be overcome in some instances by using recombinant fowlpox virus (FPV) to deliver...
an HCV minigenome under the control of a T7 promoter into cells co-infected with an adenovirus expressing T7 polymerase (Yap et al., 1998). Although this system improved the efficiency of delivery, it was not possible to control gene expression. Furthermore, it has yet to be determined whether an entire HCV genome can be stably maintained in FPV.

It has been shown for other positive-strand RNA viruses that full-length transcripts produced in the nucleus by Pol-II are difficult to amplify and package, whereas full-length transcripts produced in the cytoplasm (Almazan et al., 2000; Beard et al., 1999; Dubensky et al., 1996; Khromykh et al., 2001; Semler et al., 1984). This enables the utilization of various viral systems to deliver viral cDNA into cells, and could be adapted to facilitate inducible expression of the viral transcript. Hybrid baculovirus vectors that contain mammalian promoters have advantages in this respect as they are effective for gene delivery into hepatocyte-derived cell lines (Boyce & Bucher, 1996; Hofmann et al., 1995; Shoji et al., 1997) and have already been shown to accommodate an entire HCV genome (Fipaldini et al., 1997). Furthermore, cytopathic effects on infected cells are limited, as compared to some other HCV vectors, which are unable to replicate in mammalian cells. For this reason we have investigated the potential for controlled gene expression from baculovirus using two established methods, the tet-off system (Gossen & Bujard, 1992) and ecdysone/ponasterone-inducible (pon) system (No et al., 1996). The tet-off system relies on the presence of tetracycline (tet) to inhibit the interaction of the VP16 fusion protein, tTA, with a tet response element that is situated upstream of a minimal CMV promoter, thereby preventing transcription. In contrast, the pon system requires ponasterone for activation of transcription by allowing heterodimerization of a modified VP16–ecdysone receptor hybrid and the retinoid X receptor, thereby targeting the VP16 domain to a DNA sequence containing hybrid glucocorticoid–ecdysone response element repeats upstream of a minimal promoter. Our results show that the tet-off system in baculovirus is an extremely effective delivery system for controllably driving high levels of reporter gene expression and for the expression of both HCV minigenome and full-length HCV constructs.

### Methods

#### Cells and viruses
Mammalian cell lines were maintained in either MEM with 10% FCS, 2 mM glutamine, non-essential amino acids and antibiotics (HepG2 and HuH7) or DMEM with 10% FCS, 2 mM glutamine and antibiotics (COS-7, HeLa and 293 cells). Sf9 cells were maintained in TC100 with 10% FCS and antibiotics and used to isolate, amplify and titrate baculovirus clones using standard procedures (King & Possee, 1992). Recombinant virus was generated as described previously (Kitts & Possee, 1993). Concentrated virus stocks were obtained by clarification of the virus supernatant using a 0.45 μm filter, centrifugation at 80000 × g for 1 h at 4°C, and resuspension in PBS. For infection, cells were seeded at either 2 × 10^5 cells/cm² (HepG2), 4 × 10^5 cells/cm² (HuH7) or 8 × 10^5 cells/cm² (COS-7, HeLa and 293), 16–24 h prior to virus challenge. Unless otherwise stated, cells were infected with virus for 4 h in the appropriate cell growth medium (± either tetracycline hydrochloride (Sigma) or ponasterone (Invitrogen)) after which time the virus suspension was replaced, with fresh medium and the cells maintained for a further 48 h prior to harvesting. Preliminary work showed that reporter gene activity was more dependent on virus concentration than on the quantity of virus introduced; therefore this parameter (i.e. p.f.u./ml) rather than m.o.i. is used throughout this study.

#### Generation of baculovirus vectors
For construction of pBACVgR, part of the CMV promoter of pVGXgR (Invitrogen) was amplified by PCR using primers VgR(Pf) (5′-ATTTAAATGGCT-AGAGTCGGTTAC) and VgR(Pv) (5′-AAATAGGGGCGTACT-TGG). The fragment was digested with SmaI and NdeI and cloned, along with the NdeI–NotI fragment of pVGXgR, into SpI(polished)–NotI cut pBacMam2 (Novagen). pBACINDLacZ was generated by cloning the SpI(polished)–NotI fragment of pNLacZ (Invitrogen) into the SpI(polished)–NotI cut pBacMam2. To generate pBACINDLacZ, the tetracycline-responsive promoter (P_\text{tet}) from pUHD10-3 (Gossen & Bujard, 1992) was amplified by PCR using primers tet-cmv(fwd) (5′-TTTCTAGAGGATCCAGGACGACCCTTCCGTTC 3′) and tet-cmv(rev) (5′-GGGGGCTGGCGGCTCCGTAGACGAAGC-3′), and cloned into pCR-Blunt. P_\text{tet was excised using SpI and HindIII (present within the MCS of pCR-Blunt) and cloned with the HindIII–NotI fragment of pNLacZ into SpI–NotI cut pBacMam2. The TTA element was amplified from pUHD15-1 (Gossen & Bujard, 1992) using primers TTA(fwd) (5′-TAGATCTGAGTCACCTGCTAGATAGATTAGATAAAAG-3′) and TTA(rev) (5′-GCGGCGGCCGCCCCCTACCCAC3′) and cloned directly into pBacMam2 using BglII and NotI to generate pBACITA.

The construction of the baculovirus-based vectors involved several steps. The first stage in the process comprised the following steps: (1) construction of a baculovirus vector for the expression of an HCV minigenome under the control of a T7 promoter (H77C clone (Yanagi et al., 1997)); (2) construction of a baculovirus vector for the expression of a T7 polymerase (pUHD10-3 (Gossen & Bujard, 1992)); (3) construction of a baculovirus vector for the expression of a T7 promoter (pUHD15-1 (Gossen & Bujard, 1992)); and (4) construction of a baculovirus vector for the expression of a T7 promoter (pUHD15-1 (Gossen & Bujard, 1992)).
Inducible HCV gene delivery by baculovirus

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Results

Comparison between tetracycline- and ponasterone-regulated reporter gene expression

Constitutive mammalian promoters can show a different level of activity when introduced into cells using baculovirus, compared to when they are transiently transfected as plasmid constructs (Boyce & Bucher, 1996). It was possible that this might also apply to the constitutive promoters used for driving the expression of control elements, or the promoters recognized by these control elements. For this reason, two inducible gene expression systems were introduced into baculovirus to compare their effectiveness. This required the generation of four baculovirus vectors. One of these, BACITA, expresses the tetr-controlled transactivator (tTA) which activates P(tet) in the absence of tet, but is prevented from binding and therefore activating the promoter when tet is present (Gossen & Bujard, 1992). A second vector, BACVGxR, expresses the modified VP16–ecdysone receptor hybrid and the retinoid X receptor, which interact as a heterodimer and activate P(pont) in the presence of pon (No et al., 1996). The remaining two viral vectors expressed the lacZ reporter gene under the control of P(tet) (BACINDLacZ(tet)) or P(pont) (BACINDLacZ(pont)).

The hepatoblastoma cell line HuH7 was co-infected with BACITA and BACINDLacZ(tet) or BACVGxR and BACINDLacZ(pont) and the levels of β-galactosidase activity were determined (Fig. 1). Under conditions where reporter gene expression was suppressed (+tet/−pon respectively) levels of β-galactosidase were very low and appeared similar for both delivery systems. However, when reporter gene activity was induced, β-galactosidase activity increased up to 2000-fold in cells that had been co-infected with BACITA and BACINDLacZ(tet), compared to only 20- to 50-fold in cells co-infected with BACVGxR and BACINDLacZ(pont). Furthermore, only low virus concentrations were required to obtain both detectable (≤ 2.5 × 10^6 p.f.u./ml of each virus) and saturable (2.5 × 10^7 p.f.u./ml of each virus) β-galactosidase expression with the tet system, whereas β-galactosidase activity was only detected at ≥ 1.25 × 10^7 p.f.u./ml with the pon system. Histochemical staining of the cells revealed that almost 100%
expressed detectable levels of β-galactosidase when co-infected with BACtTA and BACINDlacZ\textsuperscript{tet} in the absence of tet (Fig. 2a), as compared to at best 20% when co-infected with BACVgRxR and BACINDlacZ\textsuperscript{prom} in the presence of pon (data not shown). Cells co-infected with BACtTA and BACINDlacZ\textsuperscript{tet} in the presence of pon showed little or no staining (Fig. 2b).

One possible problem that might occur with both systems is transcription factor squelching due to overexpression of the HSV transactivating domain VP16, found in the tTA and VgEcR elements. However, cytotoxic effects in HuH7 cells resulting from baculovirus challenge with either of the two systems were only observed at total virus concentrations \( \geq 1.25 \times 10^6 \text{ p.f.u./ml} \), well beyond that necessary for maximal β-galactosidase activity in the tet system. The two systems also showed similar relative patterns of β-galactosidase activity when the transfer vectors were introduced into HuH7 cells by transfection, compared to when introduced by baculovirus (data not shown). Therefore, it is likely that the difference in activity between the two is a reflection of their ability to function in HuH-7 cells, rather than their ability to operate in the context of a baculovirus genome.

**Characterization of the tet delivery system**

As control of baculovirus-derived gene expression was best facilitated using the tet promoter, this delivery system was characterized further. Temporal analysis showed that β-galactosidase expression was detectable 7 h after initiating infection of HuH7 cells; maximal levels were achieved between 24 and 48 h and remained high for at least 5 days (data not shown). Subsequent experiments were analysed at 48 h following infection. To ensure that β-galactosidase expression was a direct result of tTA binding to P\textsubscript{tet}, HuH7 cells were infected with either BACtTA or BACINDlacZ\textsuperscript{tet} in the presence or absence of tet, and the levels of β-galactosidase activity compared to cells co-infected with both vectors. As expected, infection with either BACtTA or BACINDlacZ\textsuperscript{tet} alone, irrespective of the presence of tet, did not result in significant expression of β-galactosidase (data not shown). Only when cells were infected with both viruses in the absence of tet were high levels of β-galactosidase activity observed.

An important aspect of an inducible delivery system is the ability to control the level of gene expression. It is clear that this can be done to a limited degree by altering the concentration of baculovirus used to infect the cell (Fig. 1). However, an advantage of the tet system is that it should also allow gene expression to be more finely regulated. To examine this, HuH7 cells were co-infected with BACtTA and BACINDlacZ\textsuperscript{tet}, and then allowed to express β-galactosidase in the presence of various concentrations of tet. At concentrations of 0.01 µg/ml or less, little or no suppression of β-galactosidase was observed, but tet concentrations above this level resulted in reduced β-galactosidase expression such that between 0.4 and 1.0 µg/ml, inhibition was maximal (data not shown).

To determine the extent to which this system could be used for gene delivery, a number of other hepatocyte- and non-hepatocyte-derived cell lines were co-infected with BACtTA and BACINDlacZ\textsuperscript{tet} and levels of β-galactosidase measured (Fig. 3). The hepatocellular carcinoma cell line HepG2 expressed β-galactosidase in the absence of tet at levels greater than HuH7 cells, with almost 100% of cells staining with X-Gal (Fig. 3). The hepatocellular carcinoma cell line HepG2 expressed β-galactosidase in the absence of tet at levels greater than HuH7 cells, with almost 100% of cells staining with X-Gal (Fig. 2c). However, it also showed higher background levels in the presence of tet (Fig. 2c) such that β-galactosidase expression was only induced 500-fold in the absence of tet. Two other cell lines tested, COS-7 and 293 cells, showed an intermediate response, with approximately 20% and 50% of the cells positive for β-galactosidase expression in the absence of tet (data not shown) and levels of expression being 2% and 10% of that seen for HuH7. No detectable β-galactosidase expression was seen in either of these cell lines in the presence of tet. Finally, one cell line, HeLa\textsuperscript{SH10}, repeatedly failed to express β-galactosidase when infected with BACtTA and BACINDlacZ\textsuperscript{tet}.

**Controllable expression of an HCV minigenome**

The efficiency and controllability of the tet-regulated baculovirus delivery system for foreign gene expression in hepatocyte-derived cell lines marked it out as a candidate system for studying HCV–hepatocyte interactions. To determine whether it would be feasible to use this system to drive efficient expression of an HCV-like transcript, two HCV minigenome constructs were generated that carried the lacZ
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Fig. 2. Visualization of β-galactosidase expression. HuH7 (a, b, e, f) or HepG2 (c, d, g, h) cells were co-infected with 1–25 × 10^7 p.f.u./ml of both BACtTA and BACINDlacZtet in the presence (a, c) or absence (b, d) of 5 µg/ml tet, or co-infected with 1–25 × 10^7 p.f.u./ml of both BACtTA and either BACAH77lacZ(HH)tet (e, g) or BACAH77lacZ(HδV)tet (f, h) in the absence of tet. β-Galactosidase expression was visualized using X-Gal.

Fig. 3. Activity of the tet-delivery system in various cell lines. Cells were either mock infected (hatched) or challenged with 1–25 × 10^7 p.f.u./ml of both BACtTA and BACINDlacZtet in either the presence of 5 µg/ml tet (vertical lines) or absence of tet (horizontal lines). Values represent mean ± SD of one of three separate experiments.

reporter gene flanked by the 5’ and 3’UTR (Fig. 4a) such that translation of a fusion protein of 12 residues of Core fused to β-galactosidase was mediated by the HCV IRES. Ptet was placed immediately upstream of the 5’UTR such that the first nucleotide of the genome corresponded to the previously mapped transcriptional start site (Akrigg et al., 1985; Stenberg et al., 1984) – transcripts from Ptet will therefore be predicted to start at the authentic 5’ end of the HCV genome. The 3’UTR was followed by either an HH or a HδV ribozyme sequence and poly(A) signal, generating BACAH77lacZ(HH)tet and BACAH77lacZ(HδV)tet respectively. Southern blot analysis showed that both baculovirus constructs were intact (data not shown). Northern blot analysis of RNA extracted from HepG2 cells infected with both viruses, using a probe complementary to both the lacZ gene and the region between the ribozyme and poly(A) tail (Fig. 4b), revealed a major band of the predicted size (~ 4 kb) for the minigenome transcript for both constructs. The presence of a ~ 0.5 kb species was consistent with ribozyme cleavage at the predicted site at the end of the 3’UTR. Interestingly, this species was only observed in cells infected with BACAH77lacZ(HδV)tet, suggesting inefficient cleavage by the HH ribozyme.

HuH7 or HepG2 cells were co-infected with BACtTA and either of the above vectors or BACINDlacZtet (Fig. 5) to
Fig. 4. The HCV minigenome. The schematic (a) depicts the structure of the BACΔH77lacZ(HH)tet and BACΔH77lacZ(HδV)tet constructs and indicates the predicted size of the minigenome transcript, when ribozyme cleavage is either present or absent. For analysis of transcript production, a Northern blot was first probed with a sequence complementary to the lacZ gene and the region of the HCV minigenomes immediately proceeding the ribozyme (b) and then stripped and re-probed with GAPDH (c). Each lane contains 10 µg total RNA extracted from HepG2 cells that had either been mock infected (lane 1), infected with 1.25 × 10² p.f.u./ml of both BACtTA and either BACINDlacZtet, BACΔH77lacZ(HH)tet or BACΔH77lacZ(HδV)tet in the presence or absence of 5 µg/ml tet and levels of β-galactosidase determined. Graphs represent the mean ± SEM of three separate experiments. Values indicate the percentage of reporter gene activity in cells infected with BACΔH77lacZ(HH)tet or BACΔH77lacZ(HδV)tet compared to BACINDlacZ in the absence of tet.

Fig. 5. Expression of β-galactosidase from the HCV minigenome constructs. HuH7 (a) or HepG2 (b) cells were co-infected with 1.25 × 10⁷ p.f.u./ml of both BACtTA and either BACINDlacZtet, BACΔH77lacZ(HH)tet or BACΔH77lacZ(HδV)tet in the presence or absence of 5 µg/ml tet and levels of β-galactosidase determined. Importantly, this difference does not appear to be due to differences in the percentage of cells expressing the minigenome transcript, as X-Gal staining indicated that almost 100% of both cell lines expressed β-galactosidase (Fig. 2e, f, g, h).

Generation and characterization of baculovirus clones carrying the full-length HCV genome

The results obtained with the HCV minigenome were consistent with cleavage of Pol-II-derived transcripts by the HδV ribozyme immediately after the X-region of the 3'UTR,

compare the efficiency of IRES-driven versus cap-dependent translation. For BACΔH77lacZ(HH)tet, β-galactosidase activity was approximately 13% of BACINDlacZtet in both cell lines. In contrast, while β-galactosidase levels were also low in cells infected with BACΔH77lacZ(HδV)tet, there was a significant difference between the two cell lines, activities being 18.7% and 8.6% of those seen in HuH7 and HepG2 cells, respectively.
indicating that this delivery system had the potential to generate correctly processed, full-length HCV transcripts. To this end, baculovirus transfer vectors were constructed carrying the entire sequence of the infectious HCV 1a clone H77C (Yanagi et al., 1997) under the control of Ptet, flanked at the 3’ end by either the HH (pBACH77(HH)tet) or the HδV (pBACH77(HδV)tet) ribozymes (Fig. 6a). A degree of genome instability was observed in baculoviruses bearing the full-length HCV cDNA: 50% of recovered viruses had large deletions both within the HCV ORF and at the 3’ end of the construct as judged by Southern blotting of viruses analysed after one round of amplification (data not shown); 75% of full-length clones remained stable through three rounds of amplification (Fig. 6b). Expression of HCV proteins could be demonstrated in both HuH7 and HepG2 cells; however, as shown for the minigenome constructs (Fig. 5), expression was more readily detected in HepG2 and thus subsequent characterization of HCV transcription and translation was restricted to these cells. As the HδV ribozyme was proven to be active (Fig. 5), results are presented for HepG2 cells co-infected with BACtTA and BACH77(HδV)tet. At 48 h post-infection, two tet-regulable transcripts of ~1.5 kb and 9 kb that hybridized to a 5’UTR probe were detected (Fig. 7). Consistent with the larger transcript being full-length, a probe derived from the 3’ end of the genome (within NS5B) also detected the 9 kb transcript but failed to detect the 1.5 kb transcript (data not shown). Furthermore, Northern analysis at 12 h post-infection showed that the 1.5 kb transcript was much less abundant (data not shown) and thus is likely to result from a time-dependent accumulation of transcript degradation products.
Discussion

The inability to culture HCV remains a major obstacle to the study of this virus. Approaches to resolve this problem have included the use of cell culture-adapted replicons (Blight et al., 2000; Lohmann et al., 1999), and expression of all or some of the proteins encoded by the virus. However, until the recent demonstration of the use of the adenovirus–T7 system to drive production of full-length HCV transcripts (Myung et al., 2001), there were no published reports on the introduction of full-length infectious HCV clones into cultured cells. One report had shown that a baculovirus could be used to introduce a full-length HCV clone into the HuH7 cell line (Fipaldini et al., 1999); in this instance expression was from a constitutive Pol-II promoter and the clone had not been proven to be infectious. Moreover, the proportion of cells showing detectable expression of HCV polypeptides was only 11%, and increased to 27% when dexamethasone was used to increase transcript production. Current systems that rely on viral delivery of T7 are also restricted by the efficiency with which HCV cDNAs can be transfected into cells, which in the case of hepatocyte and hepatocyte-derived cell lines is often low. The dual baculoviral tet-delivery system described here permits both the regulated production of the HCV transcript and efficient delivery into almost all cells within a culture. Therefore it appears to be the most effective delivery system for the full-length HCV genome reported to date.

Numerous reports have described exogenous gene expression from a viral genome under the control of inducible promoters including the same tet (Corti et al., 1999; Harding et al., 1998; Hofmann et al., 1996; Hwang et al., 1996; Iida et al., 1996; McVoy & Mocarski, 1999; Neering et al., 1996; Paulus et al., 1996; Yoshida & Hamada, 1997) and pon (Hoppe et al., 2000; Johns et al., 1999) systems used in this study. Consistent with these previous studies we found that the pon system did function to a limited extent in HuH7 cells, but the differences in the level of induced versus uninduced expression were at best 50-fold compared to 1000- to 2000-fold for the tet system. In addition, basal levels of β-galactosidase activity were also comparable between the two systems. The possibility that the difference between the two systems was solely due to changes in promoter activities as a result of using baculovirus seem unlikely as transient transfection of HuH7 cells with the transfer vectors produced similar results (data not shown). Nor was this difference attributable to cell type as the pon baculovirus delivery system also had low activity in HepG2 cells and was effectively inactive in COS-7 and 293 cells (data not shown). We therefore chose to develop the tet delivery system, although it is possible that alterations to the pon system, such as replacing the VgRxR element with a chimeric Drosophila/Bombyx ecdysone receptor (Hoppe et al., 2000), might improve responsiveness.

Our long-term goal in developing the baculovirus tet delivery system was to establish an efficient delivery system

Fig. 7. Analysis of transcript production from BACH77(H77) tet-infected cells. A Northern blot containing 2 μg per lane of total RNA extracted from HepG2 cells was first hybridized with a probe complementary to the 5′ UTR (nucleotides 104–416 of the H77C genome) (a) and then stripped and re-hybridized to a GAPDH probe (b). Groups included cells that were either mock infected (lane 1), co-infected with 6.25 × 10^5 p.f.u./ml BACtTA and 1.25 × 10^5 p.f.u./ml BACINDlacZtet in the presence (lane 2) or absence (lane 3) of 5 μg/ml tetr, or co-infected with 6.25 × 10^5 p.f.u./ml BACtTA and 1.25 × 10^5 p.f.u./ml BACH77(H77) tet in the presence (lane 4) or absence (lane 5) of 5 μg/ml tetr. Indicated on the blot is the position of the full-length transcript (arrow) as well as a major 1.5 kb transcript (arrowhead).
Fig. 8. Detection of HCV protein expression in BACH77(HδV)tet-infected HepG2 cells. Western blotting using antibodies raised to a variety of HCV proteins (a) was performed on lysates from cells that had either been mock infected (lane 1), co-infected with 6.25 × 10^6 p.f.u./ml BACtTA and 1.25 × 10^7 p.f.u./ml BACINDlacZtet in the presence (lane 2) or absence (lane 3) of 5 µg/ml tet, or co-infected with 6.25 × 10^6 p.f.u./ml BACtTA and 1.25 × 10^7 p.f.u./ml BACH77(HδV)tet in the presence (lane 4) or absence (lane 5) of 5 µg/ml tet. Included in each blot was a relevant positive control (lane 6). For Core this was a HuH7 lysate infected with a baculovirus expressing all the HCV structural proteins. The three other positive controls were full-length NS3 and NS5A recombinant proteins containing a 6 × His tag, and a recombinant His-tagged NS5B protein lacking the carboxy-terminal 20 residues. Indicated on the blots are the position of the full-length HCV proteins (arrows) and the 46 kDa NS3 cleavage product (arrowhead). For immunofluorescence (b) cells were challenged with 6.25 × 10^6 p.f.u./ml of both BACtTA and BACH77(HδV)tet and then allowed to recover for 24 h in the presence or absence of 5 µg/ml tet before processing for detection of NS5A expression.
for the complete HCV genome. To this end we first generated viruses in which P\textsubscript{tet} was used to drive expression of HCV minigenomes, such that the lacZ gene was flanked by the HCV 5′ and 3′ UTR and either an HH or HvV ribozyme was placed immediately downstream of the 3′ UTR. The expression of β-galactosidase in almost all infected HuH7 or HepG2 cells together with the detection of an appropriate size transcript indicated that the minigenome RNA transcript was exported from the nucleus. Cleavage of the poly(A) tail from the transcript could also be detected in cells infected with the BACAH77(HvV\textsuperscript{tet}) vector but not in the related HH vector. Failure to detect cleavage of the BACAH77(HH)\textsuperscript{tet} transcript could reflect low ribozyme activity or a reduced half-life of the cleaved product. It is perhaps pertinent to note that the HvV but not HH ribozyme has been shown to cleave RNA transcripts at the 3′ boundary of the 3′ UTR in vitro (J. Avis, personal communication). Given this possible difference between the two minigenome constructs, it was of interest that they also appeared to differ in their ability to drive expression between the two minigenome constructs, it was of interest that

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\item [\textbullet] the authentic ends of the HCV genome. Studies are under way to confirm this, as well as to investigate whether these capped transcripts will function as templates for negative-strand production (and subsequent positive-strand production) by the HCV replication complex.
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In summary, we have developed an efficient viral delivery system for introducing the HCV genome into hepatocyte-derived cell lines. This should allow the effects of HCV RNA and polyprotein expression to be studied in a variety of cell lines without undertaking the laborious task of generating stable cell lines. Furthermore, given the cell tropism shown by recombinant baculovirus vectors it may be possible to introduce full-length HCV transcripts into the cognate host of the virus – primary human hepatocytes. We are currently in the process of establishing this, as well as generating defective mutant HCV constructs as controls to determine whether virus replication and particle formation occur.

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\section*{References}


from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences, USA* 94, 8738–8743.


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