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 lacZ gene, (b) a ‘minigenome’ derived from hepatitis C virus (HCV) and carrying 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 lacZ was controlled by ponasterone, but β-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 δ virus ribozyme. Northern blot analysis was consistent with accurate cleavage of the minigenome transcript by the hepatitis δ 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 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 et al., 2000; Lohmann 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 et al., 1987; Tsurimoto 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 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 et al., 1995; Myung 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 effective 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 element repeats upstream of a minimal promoter. Our results show that the tet-off system in baculovirus is an extremely effective delivery system for 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., 1999; Shibuya et al., 1999) and have already been shown to accommodate an entire HCV genome (Fipaldini et al., 1999). Furthermore, cytopathic effects on infected cells are limited, as compared to some other viral delivery systems, and potential risks associated with viral delivery of a full-length HCV genome are likely to be reduced in vectors, such as baculovirus, 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 upstream 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 2.8 × 10⁴ cells/cm² (HepG2), 1.4 × 10⁴ cells/cm² (HuH7) or 0.6 × 10⁴ 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 removed, 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 pBACVgRxR, part of the CMV promoter of pHVgRxR (Invitrogen) was amplified by PCR using primers VgRxR(P+REV) (5′-ATTATTAATGGCTTAC) and VgRxR(P-REV) (5′-AATAGGGGCGTACT-TGG). The fragment was digested with SstI and NotI and cloned, along with the Ndel–NotI fragment of pHVgRxR, into SplI-polished–NotI cut pBacMam2 (Novagen). pBACINDLacZ was generated by cloning the SalI–polished–NotI fragment of pNLDlacZ (Invitrogen) into the SplI-polished–NotI cut pBacMam2. To generate pBACINDLacZ++, the tetracycline-responsive promoter (P++) from pHVD10-3 (Gossen & Bujard, 1992) was amplified by PCR using primers tet-cmv(fwd) (5′-TTTCAAGAGTCCAGGAGGAGGCCTTCTGTC3′) and tet-cmv(rev) (5′-GGGGGTCCCGCCCGCTTTCAATAACAGG3′), and cloned into pCR-Blunt. P++ was excised using SplI and HindIII (present within the MCS of pCR-Blunt) and cloned into the HindIII–NotI fragment of pNLDlacZ into SplI–NotI cut pBacMam2. The TTA element was amplified from pHUD15-1 (Gossen & Bujard, 1992) using primers TTA(fwd) (5′-TAGATCTCGCCACCATGCTAGTAGATAGTAAAAG3′) and TTA(rev) (5′-GGCCGCCGCTCCCTACCC3′) and cloned directly into pBacMam2 using BglII and NotI to generate pBACITA.

The construction of the baculovirus vectors involved several steps. The first stage in generating the 5′ end of the constructs required coupling the ponasterone-inducible promoter (Ppon) to the start of the HCV 5′UTR (H77C clone (Yanagi et al., 1997)). This was done by means of a two-step PCR reaction involving 5′plND (5′-GCATCGGGGAGATCTCGCGGCC3′), 3′plND (5′-GGGGGTCCCGCCCGCTTTCAATAACAGG3′), 5′HCVlig (5′-GGGGCCGACCACCTGATAGTAAAAG3′) and 5′UTR(out) (5′-TGTACTACCGGTTCGCC3′) primers; the resulting fragment was cloned into pCR-Blunt to give pEC5UTR. At the 3′ end of the construct, the majority of the HCV 3′UTR was fused to a hammerhead (HH) ribozyme by PCR using the primers 3′UTR(out) (5′-CGTGAATACAGCTACACTATAGGGGTATTACACTCC3′) and 3′HH-UTR (5′-GAATTACATCCATCTTCCTGCAGAAGCCAATAGCTTCGAGAGG3′) primers, and cloned into pCR-Blunt to generate p3′UTR(HH). The 5′UTR was also fused to part of the positive-strand hepatitis δ virus (HδV) ribozyme by PCR using the 3′UTR(out) (5′-GGCGCCAAGGAGGAGTCTGAGAAGCGG3′) primers. The resulting DNA fragment was cloned into a pBluescript SK-derived vector containing the full-length HδV ribozyme through XhoI and NotI sites, to give p3′UTR(HδV). To remove an AgeI site in pBacMam2 that would have interfered with subsequent cloning steps, the plasmid was cut with AgeI, polished, and religated to give pBacMam2*. Fragments representing both the 5′ and 3′ ends of the HCV construct were then excised from pEC5′UTR and either p3′UTR(HδV) or p3′UTR(HH) using SplI and XhoI and cloned as a three-way ligation into SplI–EcoRI cut pBacMam2* to generate pEC5′/3′(HH) and pEC5′/3′(HδV) respectively. Due to cloning difficulties, the H77C ORF was inserted into these baculovirus transfer vectors in two sequential steps, first by cloning a Ndel–AlaI fragment (to give pEC5′/3′(HH)) and pEC5′/3′(HδV)) followed by the AgeI–NotI fragment to give pBAC177(HH) and pBAC177(HδV). The former two plasmids were also used to derive the HCV minigenome constructs.
In this instance the lacZ gene was excised from pINDlacZ using HindIII and NotI, and cloned into EcoRI–NotI cut SK-HCV 5′UTR (a gift from Professor S. Lemon), a construct consisting of the first 380 nucleotides of the HCV N2 strain, using lacZ linker (+) (5′ AATTAAGGCGG-TAGTGGTTGATCCA 3′) and lacZ linker (−) (5′ AGCTTGGAT-CCACACTACCCCCCAT 3′) oligonucleotides to link to the EcoRI and HindIII sites. An AgeI–NotI fragment was excised from this clone and cloned into AgeI–NotI cut pECS'/3'αl(HH) and pECS'/3'αl(HO) to give pBACAH77lacZ(HH)mm and pBACAH77lacZ(HO)mm.

When it was established that the tet-off system was more effective in baculovirus, Ppon was exchanged for the tetracycline promoter (Ptet) as follows. Ppon was coupled to the start of the HCV 5′UTR by means of a two-step PCR reaction involving tet-cmv(fwd) (5′ TTCTAGAGGCTC-CCAGGCCCTTCCTGTC 3′), tet-cmv(rev) (5′ GGGGGCTGGCCG- GTTCACTAAACGAGC 3′) primers and cloned into pCR-Blunt to give ptetU. In addition, a fragment of the ORF1629 essential gene from pbacMam2 was obtained by PCR using 1629(fwd) (5′ TATTTAATTTCAATGATAA-AG 3′) and 1629(rev) (5′ TTCTAGACTGATCCGGGTTATTAG 3′) primers and cloned into pCR-Blunt to give ptetD. The Ptet/5′UTR DNA fragment was then excised from ptetU using XhoI and cloned into XhoI cut p1629 to generate p1629-TSU. The 1629-TSU gene fragment was excised using SaaI and AgeI, and cloned into the relevant SaaI–AgeI cut vectors to give pBACH77(TSU)(HH)mm, pBACH77(TSU)(HO)mm, pBACAH77lacZ(TSU)(HH)mm and pBACAH77lacZ(TSU)(HO)mm.

### Analysis of β-galactosidase expression.

Cells were washed in PBS, lysed in RLB (Promega) and a colorimetric assay with β-nitrophenyl-β-d-galactosidase was used to measure the level of β-galactosidase activity (Nielsen et al., 1983). Protein was quantified using the BCA protein assay reagent (Pierce). Levels of β-galactosidase activity were expressed as units (μmol β-nitrophenyl β-d-galactopyranoside cleaved/min) per mg protein.

For histochemical detection of β-galactosidase activity, cells were washed twice with PBS, fixed with 0.25% glutaraldehyde for 15 min, and stained with a solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal), 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$, and 2 mM MgCl$_2$ in PBS for 2 h.

### Southern blot analysis of recombinant baculovirus clones.

Crude viral DNA was obtained from S90 cells that had been infected with virus supernatant 18 h previously, using a standard procedure (King & Possee, 1992). Digested DNA was run on a 0.8% TBE agarose gel and, along with a biotinylated DNA marker, and transferred under alkaline conditions to Bright-Star Nylon Plus membrane (Ambion) according to the manufacturer’s recommendations. 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 a Bright-Star Detection Kit (Ambion).

### Northern blot analysis of baculovirus-derived transcripts.

RNA was harvested from cells using Trizol (Life Technologies), separated on a MOPS–formaldehyde gel and transferred to Bright-Star Nylon Plus membrane using standard procedures. Biotinylation of probes and RNA markers, and hybridization and detection of the probes were performed as for Southern blots.

### Immunofluorescence.

HepG2 cells seeded on Lab-Tek II chamber slides (Life Technologies) pre-coated with E-C-L (Upstate Biotechnologies) were infected with 6 × 10^8 p.f.u./ml BACITA and BACH77 (HO)mm for 4 h and allowed to recover for 24 h. To detect expressed antigen, cells were fixed with 1% formaldehyde in PBS for 1 h, permeabilized with 0.2% Triton X-100 (Merck) for 5 min, and incubated with sheep anti-NS5A sera (raised against E. coli expressed His-tagged NS5A). Bound antibody was detected with a donkey anti-sheep FITC conjugate (Sigma).

### Western blot assays.

Cell lysates were separated by SDS–PAGE and transferred to PVDF membrane (Millipore). Membranes were blocked with 5% (w/v) low-fat dried milk, 0.1% Tween 20 (Merck) in Tris-buffered saline and incubated with either sheep anti-NS3 sera (Aozubala et al., 2001), sheep anti-NS5A, sheep anti-NS5B sera (raised against E. coli expressed His-tagged NS5B) or the murine anti-Core mAb0126 (Bio-Genes, 1:1000). Bound antibody was detected with an appropriate HRP-conjugated secondary antibody (Sigma) and ECL reagent (Amer sham Pharmacia).

### 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 (Boycie & 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 tet-controlled transactivator (tTA) which activates Ptet 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, BACVgRxR, expresses the modified VP16–ecdysone receptor hybrid and the retinoid X receptor, which interact as a heterodimer and activate Ptet in the presence of pon (No et al., 1996). The remaining two viral vectors expressed the lacZ reporter gene under the control of Ptet (BACINDlacZtet) or Pon (BACINDlacZpon).

The hepatoblastoma cell line HuH7 was co-infected with BACITA and BACINDlacZtet or BACVgRxR and BACINDlacZpon 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 activity 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 BACINDlacZtet, compared to only 20- to 50-fold in cells co-infected with BACVgRxR and BACINDlacZpon. 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 tett 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 BACINDlacZtet in the absence of tet (Fig. 2a), as compared to at best 20% when co-infected with BACVgRxR and BACINDlacZpro in the presence of pon (data not shown). Cells co-infected with BACtTA and BACINDlacZtet in the presence of tet 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 I TA 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 >1.25 × 10⁷ 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 as a direct result of TTA binding to Ptet, HuH7 cells were infected with either BACtTA or BACINDlacZtet 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 BACINDlacZtet 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 BACINDlacZtet, 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 BACINDlacZtet 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. 2d). 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, HeLaSH110, repeatedly failed to express β-galactosidase when infected with BACtTA and BACINDlacZtet.

**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
Fig. 2. Visualization of β-galactosidase expression. HuH7 (a, b, e, f) or HepG2 (c, d, g, h) cells were co-infected with $1 \times 10^7$ 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 $1 \times 10^7$ p.f.u./ml of both BACtTA and either BACΔH77lacZ(HH)tet (e, g) or BACΔH77lacZ(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 $1 \times 10^7$ 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. $P_{tet}$ 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 $P_{tet}$ 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 BACΔH77lacZ(HH)tet and BACΔH77lacZ(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 BACΔH77lacZ(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 × 10⁷ p.f.u./ml of 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.

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. 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.
Inducible HCV gene delivery by baculovirus

Fig. 6. Demonstration of intact full-length HCV constructs in baculovirus vectors. The schematic (a) depicts the structure of the full-length HCV constructs including restriction sites, and indicates the region of the construct complementary to the probe used for Southern blot analysis. For Southern blot analysis (b) DNA from PAK6 (the parental baculovirus clone used to generate recombinant virus) (lane 1), BACH77(HδV)tet (lane 2) and the plasmid transfer vector pBACH77(HH)tet (lane 3) were digested with the restriction enzymes NcoI, BglII, HindIII, or SalI. Arrows indicate the location of the DNA fragments generated from digestion of the pBACH77(HH)tet transfer vector, next to which are marked their predicted sizes (in kb).

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.
BACtTA/BACH77(HsV)tet co-infected HepG2 cells were analysed by Western blotting to determine whether the HCV polypeptide was appropriately processed. As shown in Fig. 8(a), tet-regulable expression of Core, NS3, NS5A and NS5B was observed, consistent with expression and proteolytic processing of the complete polypeptide. Furthermore, both the basal- and hyper-phosphorylated forms of NS5A (p56/p58) were observed. The 46 kDa protein consistently detected with the anti-NS3 sera may be related to previously described cleavage products of NS3 (Shoji et al., 1999; Yang et al., 2000).

Finally, immunofluorescence was used to determine the percentage of cells expressing the HCV construct. As observed for β-galactosidase expression with the HCV minigenome construct (Fig. 2h), almost all of the cells were positive with either NS5A (Fig. 8b) or NS3 antisera (data not shown). The cytoplasmic localization of these two antigens was also consistent with previous reports.

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 polyproteins 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. 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 × 10⁶ p.f.u./ml BACtTA and 1.25 × 10⁷ p.f.u./ml BACINDlacz₂nd in the presence (lane 2) or absence (lane 3) of 5 µg/ml tet, or co-infected with 6.25 × 10⁶ p.f.u./ml BACtTA and 1.25 × 10⁷ 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 NSSB 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 × 10⁶ 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_{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 HoV 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(HoV)\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 HoV 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 is of interest that they also appeared to differ in their ability to drive expression of β-galactosidase. Reporter gene activity in BACAH77(HH)\textsuperscript{tet}-infected cells was approximately 13% of that seen in the BACINDlacZ\textsuperscript{tet}-infected controls, both for HepG2 and HuH7 cells. In contrast, reporter gene activity showed a 2-fold difference when comparing HuH7 (18–7%) and HepG2 (8–6%) cells infected with BACAH77(HoV)\textsuperscript{tet}, these levels being higher and lower respectively than with the cells infected with BACAH77(HH)\textsuperscript{tet}. It is likely that the ability to modulate IRES activity is necessary for HCV to switch between translation and replication as has been postulated for other positive-strand RNA viruses (Borman et al., 1994; Gamarnik & Andino, 1998). Domains within the HCV genome that have been implicated in modulation of IRES activity include the 3′ UTR and particularly the X-region (Hoppe et al., 2000; Ito et al., 1998; Michel et al., 2001; Murakami et al., 2001). However, probably as a result of using different constructs, it is still unclear as to whether the 3′ UTR and regions therein specifically enhance HCV IRES activity, generally enhance all forms of translation or suppress IRES activity. Although our results are complicated by the fact that the minigenome transcripts will be capped and may differ in their stability within the cell (even though no evidence of this was seen by Northern blot analysis), they do raise the possibility that 3′ UTR modulation of HCV IRES activity could also be cell line dependent.

Observations made using the full-length HCV bacculovirus vectors were generally in agreement with those of other investigators who have expressed the entire HCV ORF in vivo (Fipaldini et al., 1999; Grakoui et al., 1993; Mizuno et al., 1995; Moradpour et al., 1998). Although the additional 1.5 kb transcript detected by Northern blotting has not been previously described the observation that at early times after infection this product is less abundant than the full-length transcript suggests that it is a degradation product that accumulates over time. It is unlikely that the HCV proteins are translated from this (or other) degraded RNAs because authentically processed proteins were observed both at 12 h (data not shown) and 48 h (Fig. 8a). As we wish to use this system for HCV replication studies it will be important to confirm that the 5′ and 3′ ends of the transcripts correspond to 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.

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 bacculovirus 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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