Replication of a recombinant hepatitis E virus genome tagged with reporter genes and generation of a short-term cell line producing viral RNA and proteins

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Hepatitis E virus (HEV) replication has been demonstrated in HepG2 cells transfected with full-length in vitro transcripts of an infectious cDNA clone. This cDNA clone was modified to generate several subgenomic HEV replicons with fused reporter genes. In vitro-transcribed capped RNAs generated from these were transfected into HepG2 cells. Negative-strand RNA was detected, indicating the occurrence of replication. The replicon containing an in-frame fusion of HEV ORF2 with enhanced green fluorescent protein (EGFP) was positive for fluorescence, whereas no signal was observed when the replicase domain was deleted. An HEV ORF3–EGFP in-frame fusion did not yield fluorescence. Deletions introduced into ORF2 did not affect the replication competency of the viral RNA. To explore the possibility of using a reporter-gene assay to monitor the synthesis of plus- and minus-strand RNA, the EGFP gene fused to the encephalomyocarditis virus internal ribosome entry site (IRES) was inserted into partially deleted ORF2 of HEV, in both the sense [HEV–IRES–EGFP(+)] and antisense [HEV–IRES–EGFP(−)] orientations. HepG2 cells transfected with HEV–IRES–EGFP(+) and HEV–IRES–EGFP(−) vectors were positive for EGFP fluorescence. To quantify HEV replication, EGFP was replaced with Renilla luciferase (RLuc). HEV–IRES–RLuc(+) showed approximately 10-fold higher luminescence than HEV–IRES–RLuc(−). There was complete loss of activity when the helicase–replicase domain in HEV–IRES–RLuc(−) was deleted. A short-term HepG2 cell line containing the full-length viral genome in the pcDNA3 vector was established. Viral RNA and proteins (RdRp, pORF2 and pORF3) could be detected in the geneticin-resistant cells, even after the seventh passage. In the absence of a reliable cell-culture system to study HEV biology, these reporter replicons, as well as the cell line, bestow immense utility.

INTRODUCTION

Hepatitis E virus (HEV) is a water-borne pathogen that is responsible for large epidemics and sporadic cases of acute viral hepatitis (Khuroo, 1980; Wong et al., 1980; Purcell & Ticehurst, 1988; Bradley, 1990). Serological surveys show a low HEV seroprevalence in most parts of the world, including industrialized countries (Skidmore et al., 1991; Dawson et al., 1992; Skidmore & Sherratt, 1996; Wu et al., 1998; Ooi et al., 1999; Tokita et al., 2003). Recent data suggest that hepatitis E may be a zoonosis (Clayson et al., 1995; Meng et al., 1997, 1998; Hsieh et al., 1999; Garkavenko et al., 2001; Nishizawa et al., 2003).

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HEV replication is poorly understood, due to the absence of a reliable cell-culture system. In a rhesus macaque (Macaca mulatta) model, the presence of both positive- and negative-sense RNAs of HEV has been demonstrated in the liver (Nanda et al., 1994). HEV is related closely to alphaviruses, based on its genomic organization (Purdy et al., 1993). A sequence stretch in the negative strand of HEV is similar to the Sindbis alphavirus subgenomic promoter for transcription of the structural gene (Levis et al., 1990). No information is available regarding the subgenomic promoter function or the mechanism of translation of structural genes of HEV. The presence of one HEV genomic (~7.5 kb) and two subgenomic (~3.7 and ~2 kb) RNAs has been demonstrated in infected animal liver (Tam et al., 1991) and in cell culture (Xia et al., 2000). We have previously described the preparation of a full-length cDNA clone of an Indian strain (Hyderabad isolate) of HEV (Panda et al., 2000). Transfection of cultured HepG2 cells with in vitro-generated RNA transcripts from this clone was shown to produce negative-strand RNA and processed viral proteins, as well as infectious virions. Full-length cDNA clones of HEV have been described and in vitro-transcribed, capped RNA was reported to be infectious for chimpanzees (Emerson et al., 2001). However, in a follow-up study, Emerson et al. (2004) reported an HEV replicon expressing green fluorescent protein that demonstrated replication, albeit at a lower level, even in the absence of the 7mG cap structure. Neither of these studies described either negative-strand RNA synthesis or subgenomic RNA production, but rather depended on protein expression as an indicator of replication.

In order to extend our range of experimental tools to understand HEV replication, we modified the infectious cDNA clone (Panda et al., 2000) to generate: (i) HEV replicons carrying in-frame fusions of the ORF2 and ORF3 genes with enhanced green fluorescent protein (EGFP); (ii) dicistronic replicons carrying EGFP/Renilla luciferase (RLuc) reporter genes driven by the encephalomyocarditis virus internal ribosome entry site (EMCV-IRES); (iii) a short-term cell line carrying the full-length HEV genome that produces HEV RNA and viral proteins. These virus-based expression systems may be useful for understanding the factors that are involved in HEV replication.

METHODS

**Cells.** A human hepatoma cell line (HepG2) was sustained in maintenance medium [Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies), 100 U penicillin ml⁻¹, 10 µg streptomycin ml⁻¹ and 25 µg amphotericin B ml⁻¹ in 0.85% saline (Sigma)] at 37°C in 5% CO₂.

**Construction of plasmids.** The full-length HEV cDNA clone pSG1-HEV(I) (GenBank accession no. AF076239) was modified to introduce the EGFP reporter gene as an in-frame fusion with the ORF2 and ORF3 genes. The EGFP gene was amplified by PCR from pEGFP1 (Clontech) and inserted into the unique HindIII site at nt 5679 of pSG1-HEV(I) and pSG1-ORF2 (Jameel et al., 1996), to obtain an in-frame fusion of ORF2 with EGFP (Fig. 1). The HEV-ORF2-EGFP vector was digested with NruI, removing the fragment from nucleotide positions 4010 to 5141 and resulting in a replicase-deletion vector [HEV-ORF2Δ(4010–5141)].

The ORF3 termination codon was mutated and fused in-frame with EGFP as a carboxy-terminal fusion, resulting in HEV-ORF3-EGFP (Fig. 1). The ORF3-EGFP fusion cassette was amplified from the HEV-ORF3-EGFP template and cloned into vector pSG1 to serve as a subgenomic fusion control.

The full-length HEV cDNA clone pSG1-HEV(I) was further modified to introduce the IRES-EGFP/RLuc cassette in both the forward and reverse orientations to generate HEV–IRES–EGFP/RLuc(+) and HEV–IRES–EGFP/RLuc(−) expression vectors (Fig. 1), using conventional cloning, The region from nucleotide positions 5679 to 6818 of recombinant HEV was deleted to insert an 80 bp EcoRI–XhoI polylinker from vector pSG1 (Jameel et al., 1996) to produce pSG1-HEVΔ(5679–6818)MCS (Fig. 1). EGFP/RLuc was fused downstream of the IRES and cloned into the EcoRV site of pSG1-HEVΔ(5679–6818)MCS to produce HEV–IRES–EGFP/RLuc(+/−). Digestion of HEV–IRES–EGFP/RLuc(−) with NolI and deletion of the region encompassing the helicase and replicate domains (nt 2546–5679) resulted in the construct HEV–IRES–RLuc(−)Δ(2546–5679) (Fig. 1). The resulting clones were characterized by restriction-enzyme digestion and confirmed by sequencing using a model ABI 310 automated sequencer (Applied Biosystems). Primer sequences and cloning details are available on request.

For the generation of a cell line transfected stably with the HEV genome, vector pSG1-HEV(I) was digested with EcoRI and XhoI to release full-length HEV cDNA, which was ligated into equivalent sites in vector pCDNA3 (Invitrogen), generating the construct pCDNA3-HEV(I).

**RNA transcrption.** Plasmids were digested with XhoI (Amersham Biosciences) to produce DNA templates for run-off transcription. Linearized, purified plasmids were resuspended in 10 µl RNase-free water (0-5 µg µl⁻¹). mMESSAGE mMACHINE kits (Ambion) were used to in vitro-transcribe capped RNA in a 20 µl reaction with an additional 1-0 µl 30 mM GTP solution added. Following transcription, the DNA template was removed by DNase I treatment. The integrity of purified transcripts was determined by 1% formaldehyde agarose-gel electrophoresis; the RNA was then quantified by spectrophotometry (Pye Unicam 8800 UV/visible spectrophotometer; Philips) and stored at −80°C in aliquots for use within 24 h.

**DNA and RNA transfection.** Cells at a confluence of 60-80% were used for DNA transfection. Plasmid transfections were carried out by the liposome-induction method (Lipofectamine 2000; Invitrogen) in accordance with the manufacturer’s guidelines. For each 30 mm petri dish or slide flask (Nunc), 3-5 µg purified plasmid.
mixed with 10 µl Lipofectamine 2000 in 1 ml Opti-Mem (Life Technologies) was used. Cells were incubated in 5% CO₂ at 37°C, to be analysed at different time points.

For RNA transfection, approximately 1 x 10⁷ HepG2 cells in DMEM without FCS were electroporated with 10 µg in vitro-transcribed capped RNA in 0-4 cm cuvettes using a Gene Pulser II apparatus (Bio-Rad) set at 200 V and 1000 µF. After electroporation, the cell suspension was kept for 5 min at room temperature and then diluted into DMEM supplemented with 10% FCS and divided equally at subconfluent levels (2 x 10⁶ cells) into 30 mm petri dishes. For fluorescence analysis of the expressed proteins, approximately 2 x 10⁵ transfected cells were cultured onto 22 mm glass coverslips.

Cells were fixed with 4% paraformaldehyde in PBS (pH 7.5) at room

Fig. 1. Schematic representation of HEV replicons expressing reporter genes. Organization of the full-length HEV genome (GenBank accession no. AF076329) where numbers represent nucleotide positions and open boxes represent the HEV coding region, including non-structural ORF1 (homology domains are designated methyltransferase (Met), protease (Pro), helicase (Hel), RNA-dependent RNA polymerase/replicase (RdRp) and the structural region (ORF3 and ORF2)). For HEV-ORF2–EGFP, the 780 bp EGFP-coding region (solid box) was inserted in-frame with ORF2 at nucleotide position 5679 without removing any region of the full-length cDNA clone of HEV. The remaining ORF2 region after the EGFP stop codon is not translated (dashed box). HEV-ORF2–EGFPΔ(4010–5141) is identical to HEV-ORF2–EGFP except for the deletion (dashed box) from nucleotide positions 4010 to 5141, removing the replicase domain. HEV-ORF3–EGFP contains an in-frame fusion of ORF3 with EGFP in the full-length viral genome. pSG1–HEVΔ(5679–6818)MCS carries a major deletion in the structural region of ORF2 from nucleotide positions 5679 to 6818 and carries unique cloning sites, including EcoRV. Dichotomous HEV replicons with EMCV-IRES–EGFP/RLuc inserted in the partially deleted ORF2 in the forward [HEV–IRES–EGFP(+)] and reverse [HEV–IRES–EGFP(−)] orientations. The orientation of IRES–EGFP/RLuc defines the translation of EGFP/RLuc genes from either the plus (genomic sense) or minus (antisense) strands of dichotomous HEV replicon RNA, respectively. In HEV–IRES–RLuc(−)Δ(2546–5679), a deletion of HEV–IRES–RLuc(−) from nucleotide positions 2546 to 5679 (dashed box) removed the helicase and replicase domains. pSG1–ORF2–EGFP and pSG1–ORF3–EGFP represent the EGFP fusion controls for pORF2 and pORF3, respectively.
temperature for 10 min, washed with PBS and used for analysis. RNA transfection was also carried out by liposome induction using 2–4 μg RNA for each transfection, as described above.

**Immunofluorescence assay (IFA).** The fixed cells on coverslips were incubated at room temperature for 1 h in 1:500-diluted anti-ORF1 (anti-methyltransferase domain) or 1:1000-diluted anti-ORF3 or anti-ORF2 specific rabbit polyclonal sera in dilution buffer (0-5% BSA and 0.1% saponin in PBS). The cells were washed and further incubated with 1:200-diluted goat anti-rabbit IgG conjugated with fluorocein or Texas red (DakoPatts) at room temperature for 30 min. The monolayer was washed with PBS, mounted with mounting medium and observed under a fluorescence microscope (Eclipse E-600).

**Immunofluorescence assay by confocal microscopy.** Fixed cells were permeabilized with 100% methanol at –20 °C for 3 min. Cells were then rehydrated with PBS for 30 min at room temperature. Blocking was done with 0.5% BSA in PBS for 2 h followed by 5% normal goat serum in PBS-T (PBS/0.5% TWEEN 20) for 1 h. Cells were incubated with a 1:1000 dilution in PBS-T of rabbit anti-pORF2/pORF3 primary antibody, washed three times with PBS-T and incubated with a 1:1000 dilution in PBS-T of goat anti-rabbit IgG Alexa 546 (red)-conjugated secondary antibody. The cells were mounted in 50% glycerol in PBS on a glass slide and sealed. Fluorescence images were collected by using a 63 × 0.75 oil immersion objective at 1024 × 1024 resolution format on a Leica TCS-SP2 confocal microscope. For EGFPP excitation, Ar/Kr 488 nm LASER lines were used, whereas for the excitation of Texas red/Alexa 546, Gre/Ne LASER lines were used. Cells were scanned in the sequential mode and images were processed by using Leica confocal software and Adobe Photoshpf version 7.0.

**Strand-specific anchored RT-PCR.** Cells were harvested and lysed in TRIzol reagent (Invitrogen Life Technologies) at various time points post-transfection. Total RNA was isolated by chloroform extraction and 2-propanol precipitation, followed by a wash in 70% ethanol. The RNA pellet was air-dried, resuspended in diethyl pyrocarbonate-treated water and quantified spectrophotometrically. For strand-specific RT-PCR, reverse transcription was carried out by using 2 μg total cellular RNA with 200 U Superscript RT-II enzyme (Invitrogen Life Technologies) into cDNA and either a sense or an antisense primer.

For antisense-strand detection, RNA was reverse-transcribed by using a forward primer (5'-GCGCCCCCTTTTTGATGA-3') and, for sense-strand detection, with the reverse primer (described below). Following cDNA synthesis, the RNA in the reaction mixture was degraded by digestion with 2 U RNase H (Amersham Biosciences) and 1 μg RNase A (Promega). The cDNA was used for anchored PCR; amplification was carried out by using poly(G17) as the forward primer and 5'-CAAGGACGGCGGAACGGAACGCAG-3' as the reverse primer. Mock-transfected cells and RNA without reverse transcriptase were included as negative controls. The amplified products were analysed by electrophoresis on an agarose gel and photographed with a gel-documentation system (UVP). HEV-infected rhesus macaque (M. mulatta) bile RNA with a viral titre of >1 × 10^12 particles ml^-1 was used for standardization of the experiment (data not shown). The minus strand was undetectable with 100 ng RNA.

A comparative analysis of capped and uncapped full-length HEV transcripts was carried out by running parallel experiments. Briefly, 4 μg capped or uncapped RNA was transfected into HepG2 cells. RNA was extracted and reverse-transcribed into cDNA by using a strand-specific forward or reverse primer, as described. The cDNA was amplified by real-time PCR using SYBR green PCR mix (Applied Biosystems). The reaction was carried out with 2 × SYBR green PCR mastermix in a 25 μl volume. The samples were aliquoted into a MicroAmp Optical 96-well reaction plate (Perkin Elmer Applied Biosystems) and sealed. Each reaction was done in triplicate in the Perkin Elmer ABI Prism 7700 Sequence Detection system (Applied Biosystems). A 1000-fold serial dilution of in vitro-transcribed full-length HEV RNA was used as the standard for quantification.

**In vitro coupled transcription and translation.** In vitro synthesis of the desired polypeptide was carried out by using a coupled transcription and translation system (Promega) according to the manufacturer’s guidelines. The protein was synthesized in the presence of [35S]labelled methionine-cysteine [Promix; specific activity ~37 TBq (1000 Ci) mmol^-1; BRIT, Mumbai, India] in a volume of 25 μl. The reaction was carried out at 30 °C for 90 min. The translated protein was analysed by SDS-PAGE followed by autoradiography.

**Luciferase assay.** RLuc expression was detected in HepG2 cells co-transfected with the expression vectors/replicons carrying RLuc. A firefly luciferase reporter vector (Sriram et al., 2003) was used as an internal equilizer. Briefly, growth medium was removed from the transfected cells and the monolayer was rinsed with PBS. RLuc and firefly luciferase activity was determined by using the Dual Luciferase Assay system (Promega) according to the manufacturer’s instructions. All assays were done in triplicate and the results reported were reproducible and expressed as means ± 50% DMSO and 20% FCS in liquid nitrogen or propagated in maintenance medium containing 250 μg G418 ml^-1.

**Western blot analysis and immunoprecipitation.** Cellular proteins, isolated from genetically-resistant clones by boiling in 1 × SDS gel-loading buffer, were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane (Hybond-C Extra, 0.45 μm; Amersham Biosciences) by using a semi-dry Western blot apparatus (Sigma-Aldrich) as recommended by the manufacturer. The nitrocellulose membrane was blocked (5% skimmed milk powder and 0.05% TWEEN 20 in PBS) and incubated with the primary antibody at a dilution of 1:1000 in blocking solution (rabbit anti-pRdRp/anti-pORF2/anti-pORF3 antibody). After three washes with PBS containing 0.05% TWEEN 20, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (DakoPatts) at a dilution of 1:500 for 1 h at room temperature. The membrane was washed three times with PBS and 6 mg diaminobenzidine substrate dissolved in 9 ml 0.01 M Tris/HCl (pH 7.6), 10 μl 30% hydrogen peroxide was added. It was incubated at room temperature until the coloured bands developed.

Immunoprecipitation of the [35S]methionine–cysteine-labelled proteins was carried out as described previously (Panda et al., 2000). Images of the autoradiographs were generated by using a gel-documentation system (UVP).

**RESULTS**

**Detection of full-length HEV replication.**

In vitro-generated transcripts of full-length HEV were shown to produce negative-strand RNA as well as virally...
encoded proteins in transfected HepG2 cells in our previous report (Panda et al., 2000). Expression of processed non-structural pORF1 and pORF3 and structural pORF2 virally encoded proteins was shown previously by using immunofluorescence and immunoprecipitation. In the present study, a comparison between HEV replication with either uncapped or capped full-length HEV transcripts was carried out. A 10-fold greater amount of the minus strand was detected with capped transcripts (Table 1).

Therefore, capped RNA transcripts generated from full-length HEV were used in further experiments. IFA was performed with anti-ORF1/anti-ORF3/anti-ORF2 specific rabbit polyclonal sera and anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) as primary and secondary antibodies, respectively, to monitor viral protein synthesis (Fig. 2). The expression of virally encoded proteins increased from 24 to 72 h post-electroporation. Almost no cytopathic effect was observed in the IFA-positive cells at any time after transfection and the mock-transfected cells were negative for staining (Fig. 2). To confirm that the transfected RNA was replicating, synthesis of both positive and negative strands of HEV RNA was initially determined by strand-specific anchored RT-PCR of total cellular RNA, with HEV-specific primers (Fig. 3a). The strand specificity of the anchored RT-PCR assay was tested with HEV-infected rhesus macaque (M. mulatta) bile (data not shown). Minus-strand RNA synthesis increased from the time of transfection until 6 h, remained constant and decreased by 72 h (Fig. 3a). In addition, cells transfected with capped RNA from a deletion construct [pSG1-HEV D(4010–5141)] that had its replicase domain removed did not show any antisense RNA synthesis (Fig. 3b).

HEV replicons expressing EGFP fused in-frame with ORF2

Several versions of capped replicons were prepared from the replication-competent cDNA clones of HEV (Fig. 1). The RNA generated contained the authentic viral 3′ non-coding sequence, followed by five A residues and an extra, non-viral G residue. More than 95% of the in vitro transcripts from full-length HEV and of other replicons were of the expected length, as analysed by 1% formaldehyde agarose-gel electrophoresis (data not shown).

Characterization of the HEV replicon expressing EGFP fused in-frame with ORF2

The HEV-ORF2–EGFP fusion resulted in a replicon of 7944 nt (Fig. 1). The fusion junction had eight additional nucleotides upstream of the EGFP start codon, inserting a 3 aa (Phe–Gly–Thr) spacer at the ORF2–EGFP junction. The 5′ NCR, ORF1, ORF3 and the 3′ NCR in this replicon were intact, with part of the amino terminus of ORF2 (176 aa) fused to EGFP. HepG2 cells transfected with capped HEV-ORF2–EGFP RNA were positive for

Table 1. Comparative analysis of capped and uncapped HEV transcripts using real-time RT-PCR

Levels of both plus- and minus-strand RNA were quantified by real-time RT-PCR in cells transfected with either capped or uncapped full-length HEV transcripts after 48–72 h. A standard curve was generated from a 1000-fold serial dilution of full-length HEV in vitro RNA (R constant, 0.99). Values (RNA copies ml⁻¹) of replicates and means from parallel experiments are reported.

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Fig. 2. Indirect immunofluorescence microscopy of HepG2 cells 48 h after transfection with capped full-length HEV genome (a–c) or mock-transfected (d–f). Cells on coverslips were fixed with 4% paraformaldehyde, stained with anti-ORF1 (a, d), anti-ORF3 (b, e) or anti-ORF2 (c, f) rabbit polyclonal sera and subsequently with FITC-conjugated goat anti-rabbit IgG.
fluorescence (Fig. 4a). Total cellular RNA from these cells was positive for the minus strand (Fig. 4b). Deletion of the replicase region of HEV-ORF2–EGFP (Fig. 4c) resulted in loss of the EGFP signal, as well as the minus strand (Fig. 3b). This confirmed that expression of the fusion protein was dependent on virus replication.

In vitro transcription-coupled translation of pSG1-ORF2–EGFP resulted in the expected 46 kDa fusion protein (Fig. 4d), which could be immunoprecipitated by anti-ORF2 polyclonal sera (Fig. 4d). HepG2 cells transfected with pSG1-ORF2–EGFP fusion control showed positive EGFP expression (data not shown). The fusion protein could be detected in HEV-ORF2–EGFP RNA-transfected cells by IFA using a Texas red-conjugated secondary antibody. Superimposition of EGFP-positive cells and Texas red-stained cells confirmed the presence of the fusion protein (Fig. 4e).

**Fig. 4.** Characterization of an HEV replicon carrying an ORF2–EGFP in-frame fusion. (a) HepG2 cells were transfected with in vitro-capped RNA generated from HEV-ORF2–EGFP and visualized 72 h after transfection under a confocal microscope. (b) Total cellular RNA (2 μg) isolated 6, 24, 48 and 72 h after transfection with capped HEV-ORF2–EGFP RNA was subjected to anchored RT-PCR analysis. Upper panel: strand-specific RT-PCR of the HEV plus strand at 6 (lane 1), 24 (2), 48 (3) and 72 (4) h post-electroporation. Plain cells (5), reactions without reverse transcriptase (6) and a no-template control (7) served as negative controls; M, DNA molecular size marker (Promega). Minus-strand HEV RNA was detected 6, 24, 48 and 72 h after electroporation with identical negative controls. (b) Similarly, cells transfected with a polymerase-defective HEV RNA, pSG1–HEVΔ(4010–5141), were tested for both plus- and minus-strand synthesis from total cellular RNA, isolated at 6 (lane 4), 24 (5) and 48 (6) h after electroporation with similar negative controls (1–3).
Analysis of HEV replicon with the ORF3–EGFP in-frame fusion

EGFP was fused to ORF3 in full-length HEV by mutating its stop codon. Transfection of HEV-ORF3–EGFP capped RNA into HepG2 cells did not yield any EGFP fluorescence (Fig. 5a), although the minus strand could be detected in the transfected cells (Fig. 5b).

However, positive fluorescence was obtained upon transfection of the subgenomic fusion control pSG1-ORF3–EGFP (Fig. 5c), of which coupled transcription and translation resulted in a protein of ~40 kDa that could be immunoprecipitated by an ORF3-specific antibody (Fig. 5d). ORF3–EGFP fusion protein showed a typical distribution of EGFP at localized foci. Fusion protein was detected and confirmed by overlapping the EGFP-positive cells with immunofluorescence detection of pORF3 (Fig. 5e).

In the absence of information on the role of ORF3 in virus biology, we chose ORF2 as the target for deletion without affecting the replication competency of the recombinant clone of HEV.

HEV carrying a deletion in the structural region of its genome

For the construction of bicistronic replicons of HEV, a segment of the HEV genome encompassing nucleotide positions 5679 to 6818 was deleted from the full-length HEV [pSG1-HEVΔ(5679–6818)] (Fig. 1). This leaves the terminal part of ORF2 and the 3’ NCR intact, as this region folds into stem–loop structures that bind specifically to RdRp (Agrawal et al., 2001). It has been shown that initiation of replication in sense-strand RNA viruses requires this replicase complex and interactions with host-cell proteins for initiating negative-strand RNA synthesis (Song & Simon, 1995). The replication potential of the recombinant vector with a partial deletion in ORF2 was determined by strand-specific RT-PCR analysis of total cellular RNA isolated from pSG1-HEVΔ(5679–6818)-transfected HepG2 cells with HEV-specific primers. Both positive and negative strands were detected, whereas no amplification was observed in negative controls (Fig. 6a). This indicates that pSG1-HEVΔ(5679–6818), produced by the targeted deletion in the HEV structural region, was capable of replication. Hence, the pSG1-HEVΔ(5679–6818) construct was evaluated for the possibility of being used as a reporter-gene vector to detect the production of the plus and minus strands of HEV RNA. The reporter genes EGFP and RLuc, driven by EMCV-IRES, were utilized for this purpose.
the IRES–EGFP cassette in both orientations into pSG1-HEVΔ(5679–6818) was carried out in order to monitor the synthesis of plus and minus strands of the replicon during replication, via expression of EGFP driven by the IRES. Only about 5–10% of the transfected cells were positive for fluorescence with both HEV–IRES–EGFP(+) (Fig. 6b) and HEV–IRES–EGFP(–) constructs (Fig. 6c). As a defective control, HEV–IRES–EGFP(–) vector with a deletion in the helicase and replicase domains used for transfection did not show any fluorescence (Fig. 6d). Minus-strand HEV RNA could be detected in test cells (Fig. 6e), implying that the sense-strand viral RNA was generated in the HEV–IRES–EGFP(–) vector in the transfected HepG2 cells, followed by production of antisense RNA, hence supporting the occurrence of HEV replication. Due to the limited percentage of cells showing fluorescence for EGFP, it offered little help in serving as a quantitative assay for the detection of HEV replication. Therefore, the RLuc reporter was used to replace EGFP, making quantification possible.

Tenfold-higher luciferase activity was observed in cells receiving the positive-orientation construct HEV–IRES–RLuc(+), in comparison to the negative-orientation construct HEV–IRES–RLuc(–) (Fig. 6f). These results indicated the relative abundance of plus-sense HEV RNA in comparison to the minus-sense viral RNA in the transfected cells and is in agreement with our earlier report (Panda et al., 2000). A negative control similar to that used for EGFP replicons yielded no RLuc expression. Therefore, this transient-transfection reporter assay can be used for relative quantitative analysis of HEV replication.

**pcDNA3-HEV(I) allows short-term selection of cells persistently expressing viral proteins**

HepG2 cells were transfected with a plasmid carrying the neomycin-resistance gene (neo) and full-length HEV under the control of the cytomegalovirus promoter. Culturing the cells in the presence of the antibiotic geneticin (G418) eliminated cells that did not harbour the plasmid and allowed the selected clones to propagate. The selected clones were tested for viral RNA by RT-PCR with HEV-specific primers (Fig. 7a). The cells, as well as the culture supernatant, were positive for HEV RNA even after the seventh passage (Fig. 7b). Viral proteins were detected by Western blot analysis for pORF2 (~72 kDa), pORF3 (~13.5 kDa)
and replicase (~37 kDa) (Fig. 7c). The band was diffuse in case of pORF3. Similarly, immunoprecipitation analysis carried out for the structural proteins revealed the presence of both pORF2 (~72 kDa) and pORF3 (~13.5 kDa) (Fig. 7d).

These results indicate that the G418-resistant HepG2 cells continuously produced HEV RNA and proteins without affecting the apparent morphology of the cells. These cells can be used for analysis of the transcriptional- and translational-control systems of the virus.

**DISCUSSION**

*In vitro*-transcribed uncapped RNAs generated from a full-length cDNA clone of HEV have been shown to produce negative-strand RNA (Panda *et al.*, 2000), viral proteins and infectious virions (Emerson *et al.*, 2001; Panda *et al.*, 2000). As it has been demonstrated that the HEV genome is capped (Kabrane-Lazizi *et al.*, 1999), we generated full-length HEV transcripts with a 5’ cap and tested them by transfection into HepG2 cells. A comparative analysis with 4 μg transcript demonstrated a 10-fold advantage in the case of capped RNA (Table 1). Immunofluorescence analysis (Fig. 2) and strand-specific RT-PCR (Fig. 3) demonstrated the replication of wild-type viral RNA in the transfected HepG2 cells. HEV-specific negative strand was detected in the transfected cells by an anchored RT-PCR developed in our laboratory. This also agrees with recent observations by IFA in a cell-culture system (Emerson *et al.*, 2004). In this study, the methodology was partially modified and optimized with 2–4 μg capped transcripts instead of 20 μg uncapped RNA, which was used previously (Panda *et al.*, 2000).

Lower transfection efficiency limits the use of Northern hybridization in these studies, although we have detected both the positive and negative strands of HEV RNA by slot-blot hybridization in our previous study (Panda *et al.*, 2000). Early protein synthesis in HEV occurs within first

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subgenomic RNAs at 6 h following the presence of replicase up to 36 h (Panda et al., 2000) and subgenomic RNAs at 6.5–7.5 h post-infection (Xia et al., 2000).

In the absence of information on the subgenomic promoter sequences or the mechanism of translation of the internal ORFs of HEV, our initial approach was to determine the region of the HEV genome that could be manipulated without affecting the replication competency of the virus. Therefore, we attempted to insert the reporter gene EGFP as an in-frame fusion with both ORF2 and ORF3. EGFP was expressed successfully when fused to amino-terminal ORF2, but no signal was observed when a deletion was introduced upstream of the ORF2–EGFP sequence, removing a major region spanning the replicase as well as the non-coding spacer between ORF1 and ORF2 (Fig. 4). Loss of EGFP expression from the deleted ORF2–EGFP replicon could be attributed to the absence of the replicase domain and possible lack of the cis-acting upstream sequences. Primer-extension analysis data that had mapped the 5′ end of the large subgenomic RNA to the initiative region within RNA polymerase region, and the small subgenomic RNA to the intergenic region between ORF1 and ORF2 (Xia et al., 2000), indicated that the expression of ORF2 is probably mediated by subgenomic RNA.

EGFP fluorescence was absent in case of the HEV–ORF3–EGFP construct. ORF3 encodes a phosphoprotein and has been suggested to be a regulatory molecule in signal transduction (Zafrullah et al., 1997; Korkaya et al., 2001). The ORF3 reading frame was intact with its termination codon mutated, such that EGFP could be translated as a carboxy-terminal fusion protein. This was confirmed by sequencing, as well as by expression of EGFP from the subgenomic ORF3–EGFP fusion control (Fig. 5c). The ORF3–EGFP protein showed a typical distribution pattern, unlike the uniform cytoplasmic expression of the ORF2–EGFP fusion product. This kind of distribution could be attributed to the cytoskeletal association of ORF3–EGFP, as has been described previously (Zafrullah et al., 1997). In a recent report, EGFP has been expressed from an HEV replicon with fused ORF2–EGFP. The amino-terminal methionine of ORF2 was fused to EGFP, thereby removing most of the ORF2 and ORF3 proteins (Emerson et al., 2004). The reason that the ORF3–EGFP fusion in the context of full-length HEV replicon did not express the reporter is unknown, although the minus strand was detected, indicating the occurrence of replication, and the control expression with the subgenomic construct rules out misfolding. Any abnormality in generation of the RNA producing this protein needs further in-depth analysis, as does other transcriptional regulation of HEV.

Encouraged by EGFP expression from the ORF2–EGFP replicon, a major deletion was introduced into the structural ORF2 of the HEV cDNA clone with the 5′ NCR, ORF1, ORF3 and 3′ NCR intact and unique cloning sites were added by inserting multiple cloning sites. Both HEV–IRES–EGFP(+) and HEV–IRES–EGFP(−) constructs, with the reporter in either orientation, were positive for fluorescence, indicating the generation of plus and minus strands of HEV RNA, respectively (Fig. 6). The presence of sense and antisense RNA has been confirmed in the vector-transfected cells. Due to very low levels of expression, the relative expression levels produced from the forward- and reverse-orientation IRES–EGFP constructs could not be compared. Therefore, EGFP was replaced by the RLuc reporter gene in the same constructs. Tenfold-higher luminescence with HEV–IRES–RLuc(+)–transfected cells than those transfected with HEV–IRES–RLuc(−) (Fig. 6f) is well in agreement with the higher detectable levels of the positive strand than the negative strand (Panda et al., 2000). Expression from the antisense orientation-expressing cassette HEV–IRES–EGFP/RLuc(−) is in contradiction to the earlier reported cases of Kunjin virus (Khromykh & Westaway, 1997) and West Nile virus (Shi et al., 2002), where no expression was observed when IRES–reporter gene was inserted in the reverse orientation. We believe that this expression may be due to the presence of a polyadenylation signal (SV40pA) in our insertion cassette after the reporter gene, which does not base pair and might result in the unwinding of the negative strand, hence facilitating IRES-mediated reporter-gene expression. In addition, there might be a difference in the helicases of HEV and Kunjin virus.

Similar reporter replicons have been described for members of the genus Alphavirus (Liljestrom & Garoff, 1991; Xiong et al., 1989) and flaviviruses, such as West Nile virus (Shi et al., 2002) and Kunjin virus (Khromykh & Westaway, 1997). These replicons can be utilized for studying adaptive mutations, thereby providing replicons with better replication efficiency. The advantage of these dicistronic constructs is the separation of RNA replication from virion assembly and maturation. Hence, they may permit accurate mapping of the protein and RNA motifs that are involved directly in HEV replication.

These RNA replicons do not encode structural proteins; thus, they are incapable of generating infectious particles and the level of heterologous product synthesized in transfected cells is related directly to the transfection efficiency of the recombinant RNA. Under the described conditions for RNA transfection using cationic liposomes or electroporation for these vectors, their usefulness is limited for high-level production or experiments where expression in every cell is required. Either packaging cell lines or vectors that can deliver at 100% efficiency must be utilized (Polo et al., 1999).

With only a small percentage of cells being transfected with HEV–reporter replicons, we attempted to establish a cell line containing the full-length virus genome. Transfection of cells with an HEV-carrying plasmid (expressing neomycin phosphotransferase) allowed us to select G418-resistant cells maintaining the pCDNA3-HEV vector. The G418-resistant cells showed viral RNA and proteins (replicate,
ORF2 and ORF3. Viral RNA could be detected in the culture supernatant until the seventh passage (50 days). Virally encoded proteins of the expected sizes were obtained for both ORF2 and ORF3 (Fig. 7), but analysis of RdRp revealed a smaller protein of approximately 37 kDa. Similar patterns of protein expression were obtained in transient-transfection studies with uncapped transcripts of full-length HEV (Panda et al., 2000). The selected G418-resistant cells should serve as a useful tool to define the components of the replication complex.

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