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Transfection of a Differentiated Human Hepatoma Cell Line (Huh7) with In Vitro-Transcribed Hepatitis C Virus (HCV) RNA and Establishment of a Long-Term Culture Persistently Infected with HCV

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T7 RNA polymerase transcripts of a putative full-length cDNA clone of hepatitis C virus type 1 (HCV-1) were used to transfect a differentiated human hepatoma cell line, Huh7. The transfected genome replicated in cells, as evidenced by the appearance of progeny HCV RNA, detection of negative-strand viral RNA, and incorporation of [^{3}H]uridine into the viral genome. Incubation of naive Huh7 cells with conditioned medium from transfected cells resulted in a new HCV infection, suggesting the production of biologically active virus in the inoculum. Maintenance of the transfected cells under serum-free culture conditions resulted in the selection of persistently infected cells which displayed a distinctive cellular morphology. This is the first demonstration that HCV RNA produced from cloned HCV cDNA is infectious and replication competent. This approach should provide a valuable system for studying HCV replication, persistence, and pathogenicity.

Hepatitis C virus (HCV) is the major cause of parenteral non-A, non-B hepatitis worldwide (1). It is an enveloped virus containing a positive-strand RNA genome of approximately 9,500 nucleotides (nt) (5, 6). Its genome consists of a relatively long (341-nt) 5′ untranslated region (UTR), a large open reading frame encoding a polyprotein of >3,000 amino acids (9,033 nt), and a short (27- to 45-nt) 3′ UTR followed by a homopolymer tail of A (12) or U (13, 15, 20) residues. HCV resembles flaviviruses and pestiviruses in the organization of genes encoding the polyprotein (5, 6), and it is classified as a member of the *Flaviviridae* family (9). The 5′ UTR of HCV is highly conserved among all HCV isolates, and it shares significant sequence and secondary structures with those of pestiviruses (3, 12). This feature illustrates an evolutionary relationship of HCV to pestiviruses and suggests an important role of the 5′ UTR in viral replication. Recently, sequence elements that might possibly be important in the control of polyprotein translation have been identified. Tsukiyama-Kohara et al. (21) reported the detection of an efficient internal ribosome entry site within the 5′ UTR of HCV RNA which may mediate cap-independent translation of the polyprotein. Yoo et al. (22), however, identified the 5′-terminal hairpin as an inhibitor of translation and found that the translation mediated by the full-length 5′ UTR of HCV RNA is 5′ end dependent.

The HCV genome has a 5′ UTR very different from that of flaviviruses and a 3′ UTR different from that of pestiviruses, suggesting that HCV employs a unique strategy for viral gene expression and replication (11, 12). Currently, one of the major impediments to the structural analysis of the HCV genome and genetic analysis of viral replication has been the lack of a reliable cell culture system permissive for HCV replication. Experimental infection of chimpanzees has proven to be useful in studying HCV infectivity (8) and in testing the efficacies of candidate vaccines (4). However, the chimpanzee model, which is a required aid in the design of vaccines and therapeutics, is not a convenient system for the study of viral replication. Recently, advances in the development of an HCV cell culture system by Shimizu et al. (18) by infecting human T-cell lines and by Beach et al. (2) by infecting a porcine kidney cell line with chimpanzee or patient plasma have been reported. These systems, however, appear to be limited by a low efficiency of infection and replication. We have attempted to produce cell lines permissive for viral replication by infecting various primary liver cultures and cell lines with patient plasma, but the infections were transient and virus yields were low. As an alternative approach, we transfected a differentiated human liver cell line (Huh7) with HCV RNAs which were transcribed in vitro from a full-length cDNA clone of HCV-1 (17). We report here the establishment of a long-term, persistently infected Huh7 culture following RNA transfection. Although a similar approach has been successfully used with flaviviruses (16, 19), this is the first demonstration that HCV RNA produced from a recombinant cDNA clone is infectious. This approach will allow us to map the region(s) of the viral genome important for replication and further provides an opportunity to study the mechanism of viral pathogenicity in human hepatocytes.

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MATERIALS AND METHODS

Cells. Huh7 cells were grown in the presence of 7.5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with high levels of glucose (0.45%) supplemented with 10% fetal calf serum or in a defined serum-free medium (Ham's F-12 minimal medium supplemented with growth factors [epidermal growth factor, 50 ng/ml; glucagon, 1 ng/ml; insulin, 10 μg/ml; and somatostatin, 6.5 ng/ml], antibiotics [penicillin, 100 U/ml, and streptomycin, 100 μg/ml], and other

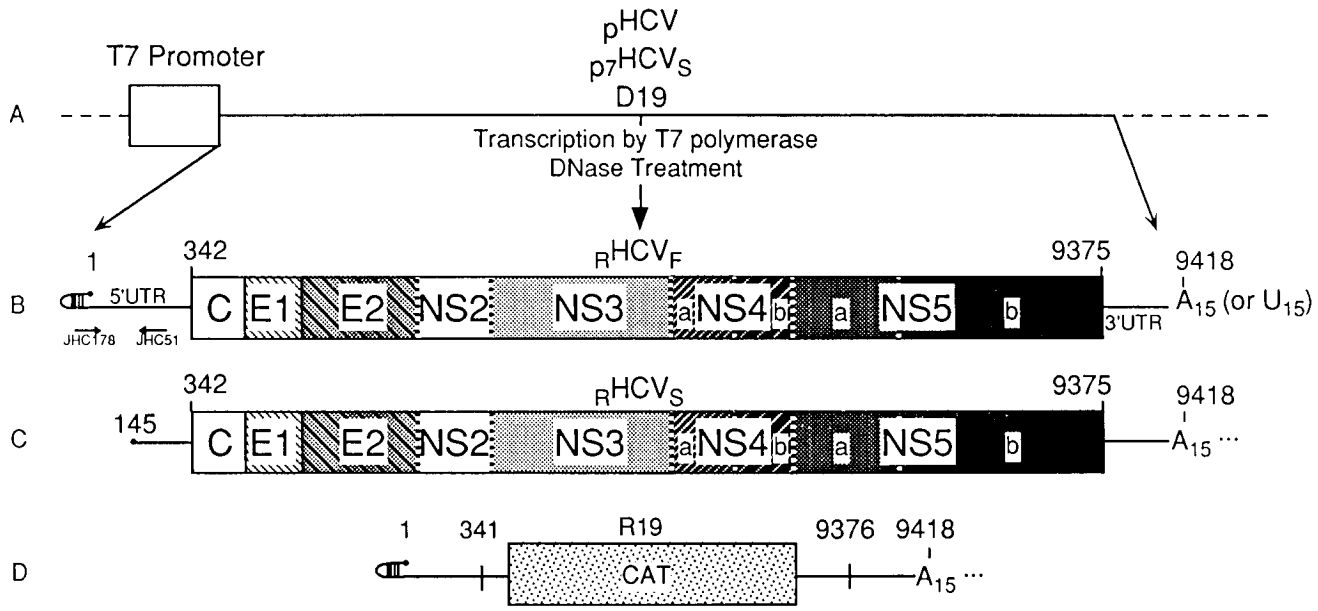

FIG. 1. Transcription vectors and synthetic RNAs. (A) Three transcription vectors, $_{p73}HCV_F$, $_pHCV_S$, and D19, schematically shown as one diagram. Each DNA was transcribed into capped full-length HCV RNA ($_RHCV_F$) (B), subgenomic HCV RNA ($_RHCV_S$) (C), and HCV-CAT RNA (R19) (D), respectively. The positions of nucleotides in the HCV-1 genome are shown as determined by Han et al. (12). Positions of the two PCR primers, JHC178 and JHC51, are indicated (B). $_RHCV_F$ and R19 contain the complete 5′ leader region which is truncated in $_RHCV_S$. All RNAs have the HCV 3′ leader region followed by a poly(A) tail.

supplements [glutamine, 2 mM; hydrocortisone, 3.5 μM; linoleic acid, 0.5 μg/ml; transferrin, 5 μg/ml; $CuSO_4 \cdot 5H_2O$, 1×10^{-7} M; H_2SeO_3, 3×10^{-8} M; and $ZnSO_4 \cdot 7H_2O$, 5×10^{-8} M]).

RNA preparation and transfection. Full-length ($_RHCV_F$) and subgenomic ($_RHCV_S$) HCV RNAs were transcribed by T7 RNA polymerase from expression vector pHCV (17) and $_{p7}HCV_S$, respectively, using reagents purchased from Promega (Fig. 1). Briefly, 0.2 μg of linearized template DNA was incubated with 60 U of T7 polymerase in a 100-μl reaction buffer, treated with DNase, and extracted with phenol-chloroform as suggested by the manufacturer. RNAs were aliquoted and stored at −80°C until use. For a typical transfection, 2 μg of RNA was mixed with 15 μg of Lipofectin (Bethesda Research Laboratories) and incubated with 5×10^5 Huh7 cells as previously described (22).

Labeling of HCV RNA and RNA-RNA slot blot hybridization. HCV RNAs were in vitro labeled by adding [^{3}H]UTP in the in vitro transcription reaction mixtures. HCV RNAs were labeled de novo by incubating cells with 0.1 mCi of [^{3}H]uridine (Amersham) in defined medium containing actinomycin D (2 μg/ml) for 8 h. The labeled cells were harvested, treated with 0.1% Nonidet P-40 in phosphate-buffered saline at 0°C for 5 min, and centrifuged at 10,000 × *g* for 5 min, and the supernatants were collected. RNAs were extracted by the proteinase K-phenol method. Each labeled RNA was hybridized as a probe to 1 μg of unlabeled positive- or negative-strand HCV RNA which was immobilized on a cellulose nitrate filter. The negative-strand HCV RNAs were transcribed by T3 polymerase from $_{p73}HCV_F$, a cloned full-length HCV cDNA, in Bluescript II SK+ vector (Stratagene). Hybridization was carried out in a solution of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt solution, 50 mM phosphate buffer (pH 7), 0.1% sodium dodecyl sulfate (SDS), 50 μg of yeast RNA per ml, and 10 μg of poly(A) per ml at 50°C overnight. Each filter was washed once with 2× SSC–0.1% SDS at 20°C for 15 min and further washed twice in 0.1× SSC–0.1% SDS at 65°C for 15 min.

Detection of HCV RNA by RT-PCR. RNA was converted into cDNA by using JHC51 (CCCAACACTACTCGGCTA; nt 269 to 251 [12]) for the detection of positive-strand RNA or by using JHC178 (ACCATGAATCACTCCCCT; nt 29 to 46) for negative-strand RNA detection. The cDNAs were amplified for 35 cycles by using JHC51 and JHC178 as previously described (10, 12). Occasionally, the PCR products of weak signal from cellular RNA were reamplified by using JHC184 (CCCCTGTGAGGAACTACT; nt 42 to 59) and JHC418 (GCG GGGGCACGCCCAAA; nt 242 to 224) as nested primers. The amplified HCV DNAs (240 bp by single-stage PCR or 200 bp by nested PCR) were detected by Southern blot hybridization with ^{32}P-labeled Alx 89 (CCATAGTGGTCTGC GGAACCGGTGAGTACA; nt 138 to 167) as a probe. Quantitative strand-specific reverse transcription-PCR (RT-PCR) was optimized by using two internal control HCV RNAs which represent positive- and negative-strand HCV species. These RNAs correspond to nt 29 to 269 within the 5′ UTR of HCV-1 (12) and contain a 60-nt foreign sequence insertion at nt 171. The negative-strand control RNA has a binding site for JHC178, and the positive-strand RNA has a binding site for JHC51. For quantitative and strand-specific PCR, a known number of relevant control RNAs were coamplified with HCV sample RNA. HCVs titers were estimated by a comparison to the band intensity generated from internal control RNA by Southern blot analysis.

In situ PCR. Cells were fixed in 10% buffered formalin overnight, cytospun on silane-coated slides at 90 rpm for 2 min, and then washed in RNase-free water. The cells were analyzed for the presence of HCV RNA, using a published protocol (14). Briefly, cells were treated with pepsin and DNase as described; that treatment was followed by the initiation of cDNA synthesis by adding 10 μl of a solution containing 1 μM JHC 51 and reverse transcriptase (Perkin-Elmer, Norwalk, Conn.). The resulting cDNAs were subjected to amplification by adding PCR mixtures containing a 1 μM concentration (each) of JHC51 and JHC178. The PCR products were detected by using an antidigoxigenin-alkaline phosphatase conjugate and the chromagen nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) (BCIP). The counterstain nuclear fast red was used to stain nuclei.

RESULTS

In vitro synthesis of HCV RNA. Three HCV RNAs of positive polarity were transcribed from transcription vectors by T7 polymerase in the presence of a cap analog (Fig. 1A). Full-length HCV ($_RHCV_F$) and chloramphenicol acetyltransferase, (CAT) (R19) transcripts were initiated at the 5′-terminal G residue, and the subgenomic transcript ($_RHCV_S$) was initiated at nt 145 in the HCV-1 map (Fig. 1) (12). All three RNAs were terminated after a poly(A) tail at the 3′ terminus. A portion of each transcript reaction mixture was denatured with formaldehyde and analyzed on a 1.5% agarose gel (Fig. 2A). Analysis by Northern (RNA) blot hybridization revealed that approximately 80% of each RNA was of the expected full-length size (data not shown). Approximately 1 μg of capped RNA was transcribed from 0.2 μg of template DNA in a 100-μl T7 RNA polymerase reaction mixture. Each reaction mixture was treated with DNase, and the absence of DNA was confirmed by direct PCR without a reverse transcriptase reaction.

Transfection of the synthetic HCV RNA into Huh7 cells. A previous translation study in which the 5′ UTR of HCV RNA was linked to a CAT reporter gene showed that the full-length HCV leader is a relatively inefficient template for translation

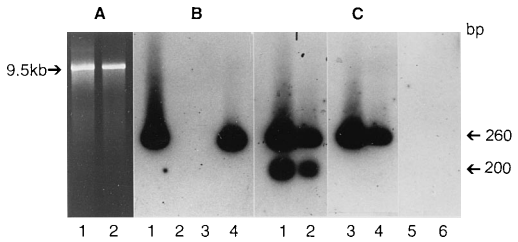

FIG. 2. Analysis of HCV RNA. (A) Gel analysis of HCV RNA transcribed by T7 polymerase in vitro. Approximately 0.1 μg each of $_{R}HCV_{F}$ (lane 1) and $_{R}HCV_{S}$ (lane 2) was denatured with formaldehyde, electrophoresed in a 1.5% agarose gel, and stained with ethidium bromide. (B) Amplification of control HCV RNA by strand-specific RT-PCR. Ten thousand molecules of positive-strand internal control RNA (10) (lanes 1 and 2) and negative-strand control RNA (lanes 3 and 4) were mixed with 1 μg of Huh7 cell RNA and amplified by positive-strand (lanes 1 and 3)- or negative-strand (lanes 2 and 4)-specific RT-PCR. (C) Analysis of RNA from transfected cells by quantitative strand-specific RT-PCR. Sixteen days after transfection, cytoplasmic RNA was extracted from approximately 10^5 Huh7 cells that were cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$ (lanes 1 and 2) or from cells transfected with R19 alone (lanes 3 to 6). These cellular RNAs were mixed with 10^4 molecules of positive (lanes 1 and 3) and negative (lanes 2 and 4) internal control RNAs. These RNAs with control RNAs (lanes 1 to 4) or without control RNAs (lanes 5 and 6) were analyzed by positive-strand-specific (lanes 1, 3, and 5) or negative-strand-specific (lanes 2, 4, and 6) RT-PCR. The mobilities of RNA and PCR products are shown on the left and right, respectively.

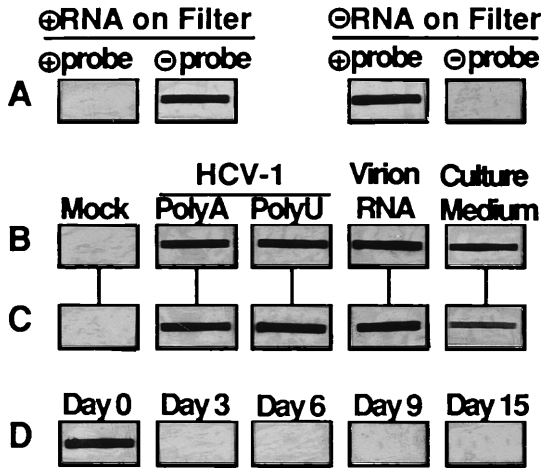

FIG. 3. Incorporation of [^{3}H]uridine into HCV RNA in transfected Huh7 cells detected by RNA-RNA slot blot hybridization. (A) Test of hybridization specificity. One microgram of unlabeled positive- or negative-strand HCV RNA immobilized on a cellulose nitrate filter was hybridized with in vitro [^{3}H]UTP-labeled HCV RNA as a probe. + probe, labeled positive-strand HCV RNA; − probe, labeled negative-strand HCV RNA. (B) Detection of [^{3}H]uridine incorporation into positive-strand HCV RNA in cells cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$. One microgram of unlabeled negative-strand HCV RNA was immobilized on a cellulose nitrate filter and hybridized with the [^{3}H]uridine-labeled cytoplasmic RNA isolated from 10^7 cells which were transfected or infected with the indicated source as described in Materials and Methods. PolyA, HCV-1 RNA with a poly(A) tail; PolyU, HCV-1 RNA with a poly(U) tail; Virion RNA, RNA phenol extracted from 1 ml of chimpanzee (Rodney) plasma; Culture Medium, medium from day 16 posttransfection conditioned with $_{R}HCV_{F}$ and $_{R}HCV_{S}$. (C) Detection of [^{3}H]uridine incorporation into negative-strand HCV RNA in cells. One microgram of unlabeled positive-strand HCV RNA was immobilized on a filter and hybridized with the same cytoplasmic RNA as described for panel B. (D) Detection of [^{3}H]uridine incorporation into negative-strand HCV RNA and stability of subgenomic HCV RNA in Huh7 cells. The same cellulose nitrate filter as described for panel C was hybridized with [^{3}H]uridine-labeled cytoplasmic RNA isolated from cells which were transfected with $_{R}HCV_{S}$ along with in vitro [^{3}H]UTP-labeled $_{R}HCV_{S}$ as a tracer.

(22). This was confirmed by Selby et al. (17), using full-length HCV constructs as templates in a vaccinia virus-based expression system. Our recent study confirmed an earlier observation that subgenomic HCV RNA ($_{R}HCV_{S}$) (Fig. 1C) lacking the 5′-terminal sequence of 144 nt was more efficient in translation of the polyprotein (data not shown and reference 22). On the basis of these observations and to ensure successful initiation of an infectious cycle, both $_{R}HCV_{F}$ and $_{R}HCV_{S}$ were cotransfected into Huh7 cells with Lipofectin. Huh7 cells were grown in a defined serum-free medium or in a serum-containing medium. A few days after transfection, the morphology of transfected cells was unchanged, and no plaques were detectable in a standard plaque assay used for cytolytic viruses.

Biological activity of transfected HCV RNA. In the absence of a successful plaque or focus assay, we sought to determine by PCR assays whether transfected cells could support replication of the viral genome. Initial attempts to demonstrate progeny HCV genomes were complicated by the presence of a large quantity of input RNA in the extracted RNA samples. However, HCV RNA levels detectable in the cell culture medium progressively decreased, became undetectable at day 9 posttransfection, and reappeared at day 12 (see below). Therefore, to circumvent the problem with the initial inoculum, we extracted cellular RNA at day 16 and performed strand-specific RT-PCR for both positive- and negative-strand HCV RNAs. As shown in Fig. 2C, we detected positive- and negative-strand HCV RNAs in cells cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$ (lanes 1 and 2, 200-bp band). In contrast, HCV RNA represented by a 200-bp PCR product was undetectable in cells transfected with R19 in which the coding region of the HCV polyprotein was replaced with that of the bacterial CAT protein (Fig. 2C, lanes 3 to 6). Our RT-PCR was strand specific, as evidenced by the results shown in Fig. 2B. When we assayed 10,000 molecules of positive-strand control HCV RNA in the presence of RNA from untransfected Huh7 cells, a signal (260 bp) was detected only by the positive-strand-specific RT-PCR assay (Fig. 2B, lane 1) and not by the negative-strand-specific RT-PCR assay (Fig. 2B, lane 2). Conversely, when we assayed negative-strand control RNA, the signal was obtained only by negative-strand-specific RT-PCR assay (Fig. 2B, lanes 3 and 4). These data suggest that progeny HCV RNAs were produced from cells cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$.

Replication of transfected viral RNA was confirmed by detection of specific de novo incorporation of [^{3}H]uridine into HCV RNA (Fig. 3). In this experiment, Huh7 cells were cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$ and labeled with [^{3}H]uridine in the presence of actinomycin D at day 16 posttransfection. As control experiments, cells were also transfected with virion RNA isolated from infectious plasma (Fig. 3B and C) or $_{R}HCV_{S}$ alone (Fig. 3D). From these radiolabeled cells, cytoplasmic RNAs were extracted and then hybridized to cellulose nitrate filters on which unlabeled positive- or negative-strand HCV RNA was immobilized. After the RNA-RNA slot blot hybridization, filters were washed under highly stringent conditions. As shown in Fig. 3B and C radioactivity was detectable on both filters, indicating that both strands of HCV RNA were synthesized in cells transfected with virion RNA or cotransfected with $_{R}HCV_{F}$ and $_{R}HCV_{S}$. In contrast, [^{3}H]uridine incorporation was not detectable in Huh7 cells transfected with $_{R}HCV_{S}$ alone (Fig. 3D). This result shows that de novo synthesis of HCV RNA has occurred and that it resulted from the transfection of full-length HCV RNA into Huh7 cells.

HCV-1 RNA appears to have a 3′ poly(A) tail (12), whereas HCV RNAs of other isolates may have poly(U) tails at the 3′ ends of the genome (13, 15, 20). We therefore tested whether this difference in homopolymer tails has a detectable effect on viral replication. For this experiment, we constructed a mu-

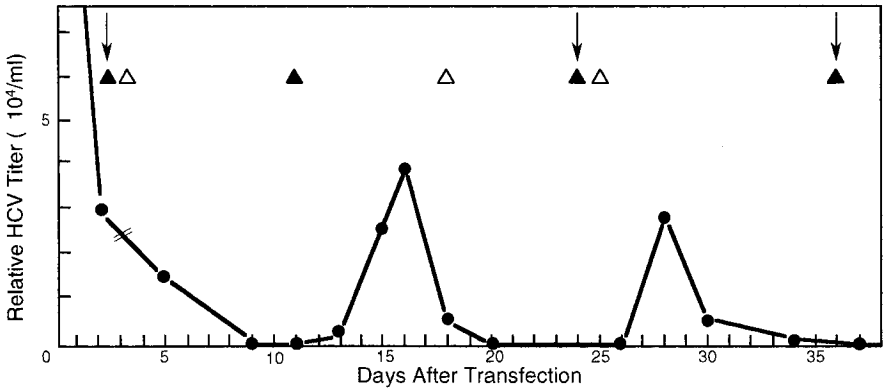

FIG. 4. Profile of HCV RNA accumulation in Huh7 culture medium. Cells (5×10^5) were cotransfected with 1 μg each of $_RHCV_F$ and $_RHCV_S$ and cultured in serum-containing medium (closed triangle) and serum-free medium (open triangle). Media were changed every 3 or 4 days. Cells were split at the time points indicated by the arrows. The culture medium at each time point was analyzed for positive-strand HCV RNA by a semiquantitative RT-PCR (closed circle).

tated HCV-1 clone in which the poly(A) tail was substituted with poly(U) (Fig. 1B). We repeated the transfection experiment, and the replication of this modified RNA was compared with that of poly(A)-tailed RNA by measuring [^{3}H]uridine incorporation. As shown in Fig. 3B and C, we could not detect a significant difference of replication activity between the two RNAs as judged by the radioactivity intensities on hybridized filters.

We next tested whether culture supernatants collected from transfected cells which were positive for replication intermediates were infectious. To this end, a PCR-positive culture supernatant (16 days posttransfection) was centrifuged (10,000 $\times$ *g* for 5 min) and filtered through a membrane (Millipore; pore size, 0.22 μm), prior to inoculating fresh Huh7 cells and monitoring the incorporation of [^{3}H]uridine. As shown in Fig. 3B and C, incorporation of [^{3}H]uridine into both strands of HCV RNA was detectable in the infected cells. We repeated this reinfection process for four passages, and each time we were able to detect the viral genome by PCR in the resultant culture supernatants (data not shown).

Profile of HCV accumulation in culture media after transfection. Following HCV RNA transfection into Huh7 cells, we noticed that the level of HCV accumulation in the media varied dramatically in response to different culture conditions. HCV replication was lost if the transfected cells were maintained in rich medium (DMEM) supplemented with 10% fetal calf serum. Under this condition, replication of transfected HCV RNA was suggested by the detection of HCV RNA in culture medium, but replication became undetectable at approximately 2 weeks after transfection, during which time cells were split twice. On the other hand, if the cells were maintained continuously in defined medium lacking serum, they died between 20 and 30 days posttransfection. Therefore, in order to keep the cells viable and maintaining HCV replication, we maintained them in serum-free media in most of the culture periods, but cells were fed in serum-containing media after every cell split and when they displayed unhealthy morphologies. Transfection of Huh7 cells with a large amount of synthetic HCV RNA followed by an initial maintenance in defined medium resulted in a decline in cell density. Following transfection, initially large but decreasing amounts of HCV RNAs were detected in the culture medium by PCR assays (Fig. 4). This RNA, which was presumably derived from the inoculum, became undetectable between 9 and 12 days after transfection. As cells were allowed to recover in rich medium, HCV RNA typically reappeared in the culture medium 3 to 5 days after the initial eclipse period, which coincided with the onset of cell growth. The peak in HCV titer which occurred 2 weeks after transfection was followed by a sharp decline and the second eclipse period as cells entered stationary phase. Upon passaging and maintaining these cells at day 24, the pattern of HCV accumulation in the medium was repeated with diminished but persistent HCV titers. However, HCV RNA was undetectable in culture medium collected from cells after 14 days posttransfection with $_RHCV_S$ alone (data not shown). These data suggest that HCV replication in cells fluctuates and may be affected by culture conditions that influence cell division.

Identification and properties of Huh7 cell lines persistently infected with HCV. Although continuous plating of transfected cells in serum-free media after transfection generally resulted in cell death, we noticed the presence of surviving cells, some of which showed a distinctive morphology at approximately 2 months after transfection (Fig. 5B). When maintained alternately in serum-free and serum-containing media, these cells gave rise to visible colonies (Fig. 5C) which were not observed in mock-transfected cultures (Fig. 5D and E). Cells within the colony were morphologically distinct from those of uninfected Huh7 monolayers in that they were aggregated (Fig. 5F). Some of these colonies were expanded and maintained principally in the serum-free medium for up to 4 months after transfection. During this period, fluctuating titers of HCV RNA ranging from a few copies to 10^4 genome copies per ml were detected in the medium. At various times, HCV was undetectable in both the culture medium and the cells, but these cells appeared to be persistently infected with HCV, since at later times, HCV RNA was detected in the media and cells. When cells in the colony were analyzed by in situ PCR hybridization (Fig. 6), HCV RNA was detected in approximately one half of the cells in the colony. The viability of HCV-infected Huh7 cells in colonies was considerably reduced compared with that of the mock-transfected control cells. These cells were sensitive to trypsin treatment, pH, and mechanical disruption.

Transfection of Huh7 cells with $_RHCV_S$ with and without the 5′ cap. After we confirmed infection of Huh7 cells by cotransfection with $_RHCV_F$ and $_RHCV_S$, we tested whether $_RHCV_F$ alone could be infectious when it was transfected to Huh7 cells with or without a cap (Table 1). In this experiment, we also transfected Huh7 cells with capped $_RHCV_S$ alone or cotransfected with capped $_RHCV_F$ and $_RHCV_S$ for comparison. We cultured these transfected cells for 25 days, during which time cells were split twice. When we analyzed these RNAs by

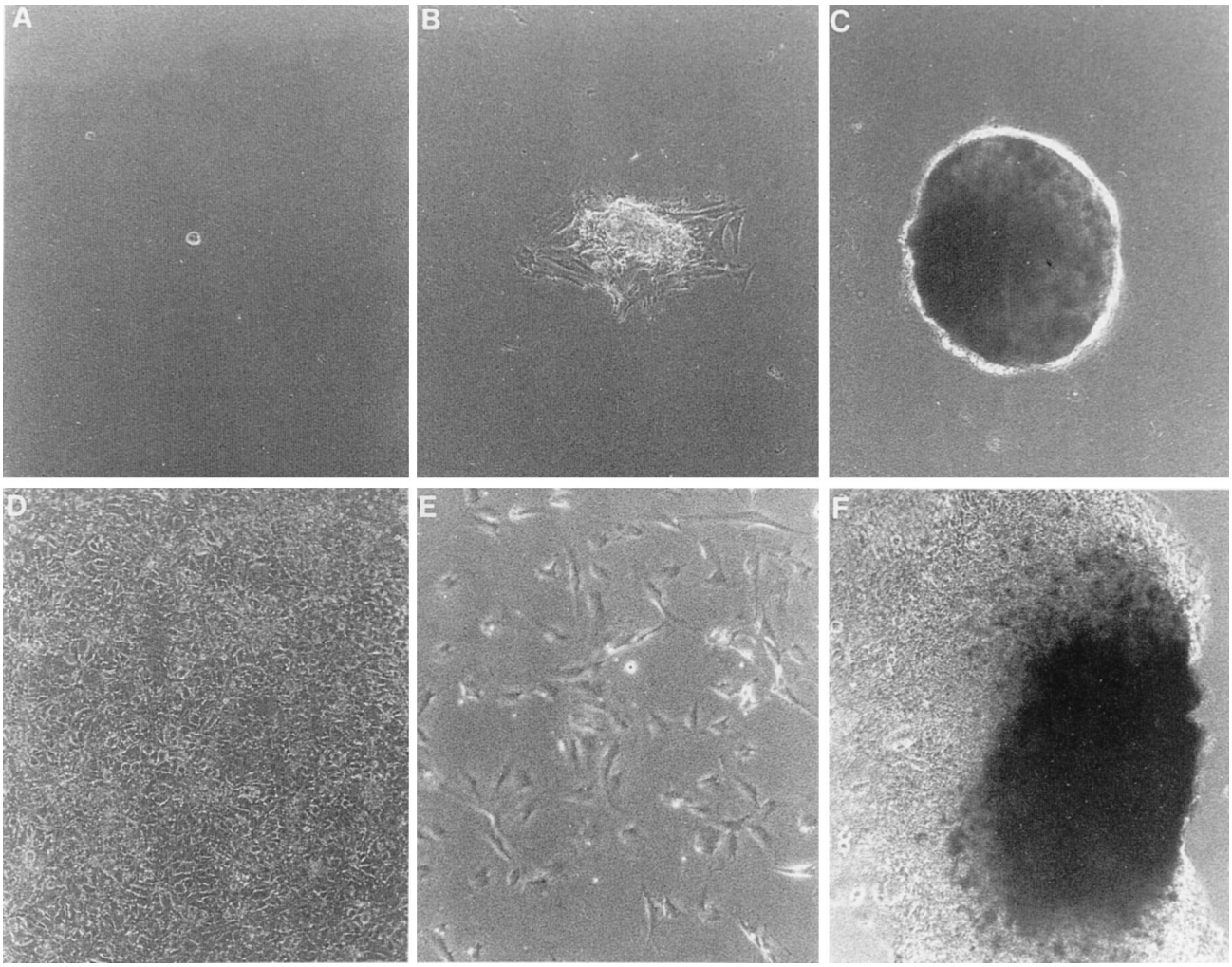

FIG. 5. Long-term culture of Huh7 cell lines transfected with HCV RNA. (A) Surviving Huh7 cell post-RNA transfection. (B) Representative growth pattern of progeny Huh7 cells at low density. (C) Established clone at 40 days after transfection. (D) Confluent monolayer of mock-transfected Huh7 cells. (E) Mock-transfected Huh7 cells at low density. (F) Morphology of a clone at a higher magnification (×200).

quantitative RT-PCR, we detected approximately 7×10^4 copies of HCV RNA from transfection with capped $_R$HCV$_F$, 3×10^4 from uncapped $_R$HCV$_F$, and 6×10^5 from cotransfection with capped $_R$HCV$_F$ and $_R$HCV$_S$. In contrast, HCV RNA was undetectable from transfection with $_R$HCV$_S$ alone. Although the specific infectivities of these RNAs shortly after transfection in cells are not known, these results suggest that both capped and uncapped $_R$HCV$_F$ are infectious and that cotransfection with $_R$HCV$_S$ increases transfection efficiency.

DISCUSSION

We have synthesized putative full-length HCV-1 RNA from a cDNA clone transcribed in vitro, using T7 RNA polymerase, and have then transfected it into Huh7 cells. Several lines of evidence show that this synthetic RNA was infectious and replication competent. First, the transfected cells were shown to produce both positive-strand RNAs as well as presumed replication intermediate negative strands of the HCV genome. The latter were detected by using a PCR assay for negative strands which were shown to be specific, since cells transfected with the control R19 template containing the intact 5′ UTR (Fig. 1) failed to produce negative strands detectable with this assay (Fig. 2). Second, newly synthesized positive and negative strands were demonstrated in these transfected cells by monitoring the incorporation of [^{3}H]uridine (Fig. 3). Third, long-term cultures of transfected cells were shown to harbor HCV, using in situ PCR methods (Fig. 6). Fourth, cell media derived from transfected cells were capable of infecting fresh Huh7 cells in the absence of the transfectant Lipofectin, suggesting that infectious virus was secreted from the transfected cells (Fig. 3).

The truncated synthetic HCV RNAs, $_R$HCV$_S$ and R19, failed to establish viral replication following transfection into Huh7 cells, while cotransfection with synthetic RNAs as well as a transfection with virion RNA was shown to be infectious (Fig. 3). These data indicate that the full-length HCV RNA is required for viral replication. In most of our transfection experiments, we used cotransfection to increase the detection sensitivity of replication and to ensure initiation of an infectious cycle. However, both capped $_R$HCV$_F$ and uncapped $_R$HCV$_F$ appear to be infectious, as tested by transfection. It is not known whether HCV RNA is capped at the 5′ terminus or bound with a virus-encoded protein, as is the case for poliovirus, or is in some other form. Although we did not investigate

FIG. 6. Detection of HCV RNA in infected cells by in situ PCR. In situ PCR analysis was performed on uninfected Huh7 cells (A), infected cells in a colony (B), and the same cells as in panel B but treated with RNase A solution (0.1 mg/ml) at 37°C for 30 min prior to the PCR analysis (C). Cells with no detectable viral RNA are pale because of counterstaining with nuclear fast red, which stains the nucleus (small arrowhead) but not the cytoplasm (large arrowhead). The positive staining is dark because of the action of the antidigoxigenin-alkaline phosphatase conjugate on the chromagen NBT-BCIP.

in detail the possible role of this cap on replication, capped HCV RNA yielded a higher HCV titer than the uncapped HCV RNA following transfection. It might be possible that the presence of the 5′ cap increases the stability of transfected RNA in cytoplasm. However, a possible role for a 5′ cap in infection remains to be established.

The genome of HCV-1 RNA appears to have a 3′ poly(A) tail, while other isolates may have poly(U) tails. In our experiment, both RNAs were infectious, and qualitatively, we could not detect a significant difference in the efficiency of viral replication between the two RNAs. Therefore, the homopolymer tail may be an element which stabilizes HCV RNA in cells but may not be the sole determinant of the efficiency of viral replication. It is possible that other sequence heterogeneities observed within the 3′ UTR of different HCV strains influence viral replication (11).

After transfection, we monitored cells and culture media for the presence of HCV RNA for up to 6 months. Throughout the observation period, we could not detect plaque formation in transfected cells by using conventional plaque assays, which suggests that HCV infection is not lytic. Currently, it is uncertain whether HCV infection is directly cytotoxic in Huh7 cells, although we observed that transfected cells with the altered morphology exhibited the reduced viability and increased sensitivity to trypsin, pH, and mechanical manipulation. Further analyses are necessary to clarify this issue.

TABLE 1. Detection of HCV RNA in Huh7 cells after transfection[a]

Transfected RNA	Cap	HCV titer
${}_{R}HCV_{S}$	+	Undetectable
${}_{R}HCV_{F}/{}_{R}HCV_{S}$	+/+	6×10^5
${}_{R}HCV_{F}$	+	7×10^4
${}_{R}HCV_{F}$	−	3×10^4

[a] Huh7 cells (10^6) were transfected with 4 μg of each indicated RNA and were cultured serum free, except for cell splits. After cells were split a second time, they were fed with serum-containing medium for 3 days before they were harvested. RNAs from approximately 10^6 cells were assayed 25 days after transfection by quantitative RT-PCR for the presence of genomic HCV RNA.

However, a correlation appears to exist between the morphology change and persistent HCV infection in Huh7 cells. When we tested colonies by PCR assays for the presence of HCV RNA, we found that 7 of 10 colonies were PCR positive; the remaining 3 became positive at later times. In addition, analysis of colony cells by in situ PCR revealed that about 50% of cells harbored HCV RNA. The patterns of virus production in media and cells in infected colonies were still variable, having intermittent eclipse periods during which HCV RNA was undetectable. Following this period, the reappearance of virus coincided with cell division. Our preliminary results suggest that virus production in the medium could be induced by various stimuli such as freezing and thawing of the cells, heat shock, and phorbol ester treatments (10a). More work is needed to understand the mechanism of persistent HCV infection, the episodic pattern of viremia, and viral induction.

HCV is a low-titer virus, yet its infection in patients is generally persistent and leads to chronic hepatitis at a high rate which can predispose to the development of hepatocellular carcinoma (7). HCV infection often produces low viral titers with long latent periods, but it can manifest clinical symptoms at a later stage, the severity of which correlates with viral titer (10). Our culture system mimics several aspects of HCV infection in patients, especially in viral persistence and low titers. It appears that there exists a mechanism(s) which limits HCV replication in infected hepatocytes, both in our culture system and in patients. Currently, HCV titers in our infected cells are low, and we have been unable to stimulate our clones to produce HCV titers at a level comparable to those of circulating HCV seen in patients with chronic active hepatitis. Finding conditions to induce our Huh7 clones to produce high HCV titers may be relevant to elucidating the mechanism of viral induction in patients. In this regard, our current culture system may be useful in studying the mechanism of HCV pathogenicity in general. Our ability to produce infectious HCV in transfected cells now makes genetic analysis of the viral genome possible and may also be useful in developing a cell culture-adapted HCV strain. This will facilitate future HCV research and will contribute to the development of an HCV vaccine.

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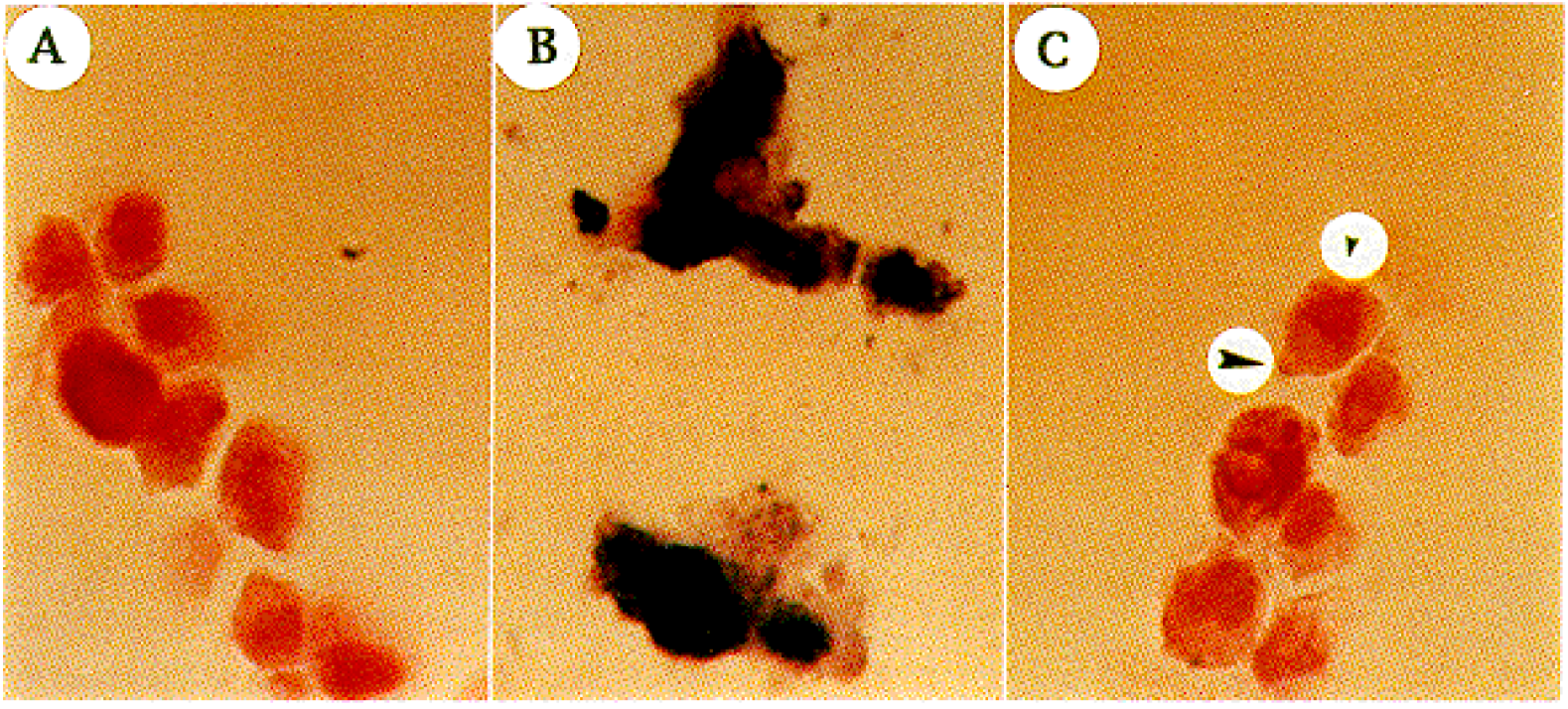
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