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Evidence for *in vitro* replication of hepatitis C virus genome in a human T-cell line

(cDNA/PCR)

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ABSTRACT **A human T-cell line, MOLT-4, either uninfected or infected with murine retroviruses, was tested for its susceptibility to hepatitis C virus (HCV) infection. The cell cultures were inoculated with a serum containing HCV and then examined for the presence of viral sequences by cDNA/PCR. In murine retrovirus-infected MOLT-4 (MOLT-4 Ma) cells, intracellular minus-strand viral RNA, a putative replication intermediate, was first detected 3 days after inoculation, and the maximum signal was seen on day 7. When the cells were continuously subcultured in fresh medium, HCV sequences were intermittently detected in cells over a period of 3 weeks. In MOLT-4 cells free of retroviruses, replication of minus-strand HCV RNA appeared less efficient than in MOLT-4 Ma cells. The presence of minus-strand viral RNA in MOLT-4 Ma cells inoculated with HCV was confirmed by *in situ* hybridization with a strand-specific RNA probe. Immunofluorescence tests with antibodies specific for HCV core and NS4 antigens showed that MOLT-4 Ma cells were positive for viral antigen 7 days after inoculation. Thus, it appears likely that the HCV genome can replicate in the human T-cell line MOLT-4.**

The genome of hepatitis C virus (HCV), the major cause of transfusion-associated non-A, non-B hepatitis, has been cloned (1). The virion was demonstrated by hybridization analysis to contain a plus-strand RNA of ≈10 kilonucleotides (2), and comparative sequence analysis indicated that the virus is distantly related to the flaviviruses and pestiviruses (3, 4). Synthesis of cDNA by reverse transcription of viral RNA and amplification by PCR with primers based on the nucleotide sequences of HCV cDNA clones have been useful for detecting the HCV genome (5). Immunodiagnostic assays for antibodies against HCV proteins have also been developed (6). The studies with chimpanzees have provided evidence that the virus is probably enveloped (7) and is ≈30–60 nm in diameter (8). Additional research, however, has been hampered because of the limited availability of chimpanzees and the relatively low titer of virus in clinical samples. To date, little is known about the replication and pathogenesis of this virus, and the virion has not yet been visualized with certainty. The development of *in vitro* systems for propagating HCV may be critical for the solution to these problems. Specifically, an *in vitro* system that would permit the development of an assay for virus infectivity remains a pressing need. Applying the recently developed assays for detecting the virus, we initially studied established liver cell lines for their ability to support HCV replication, but these attempts were unsuccessful. Previously, Hellings *et al.* (9, 10) reported the transmission of non-A, non-B hepatitis (presumably hepatitis C) to chimpanzees that were inoculated with leukocytes, most probably T lymphocytes, derived from either acutely or chronically infected patients or chimpanzees. In addition, the lactate dehydrogenase-elevating virus, another unclassified enveloped RNA virus, has recently been found to replicate more efficiently in mouse cells when they are superinfected with murine leukemia virus (11). Stimulated by these observations, we tested a human T-cell line, MOLT-4, either uninfected or infected with murine retroviruses, for its ability to support HCV replication. In this report, we describe the detection of minus-strand HCV RNA, believed to be involved in the replication of the viral genome, by cDNA/PCR, and viral antigens by immunofluorescence in MOLT-4 Ma cells inoculated with serum or plasma containing the virus.

MATERIALS AND METHODS

Inoculum. The principal inoculum was a serum obtained from a chimpanzee (*Pan troglodytes*) infected with the fifth chimpanzee passage of the F strain (12) of HCV. The serum was taken 8 weeks after inoculation, which was 5 weeks after the first detection of characteristic hepatic changes (13). In some experiments, human plasma H, containing $10^{6.5}$ 50% chimpanzee infectious doses (CID_{50}) per ml of HCV (12), served as the inoculum.

Human T-cell Line. MOLT-4 cells (14) and MOLT-4 cells containing the sarcoma virus–amphotropic murine leukemia virus complex (MOLT-4 Ma) were used. The MOLT-4 Ma line was established by cocultivation of MOLT-4 cells with S+L-mink cells infected with the amphotropic murine leukemia virus (15). The nonadherent MOLT-4 cells were then separated from the adherent mink cells by gentle pipetting, and several subcultures using the same procedures rid the MOLT-4 culture of the glass-adherent mink cells. The cells were maintained in RPMI 1640 medium/8% heat-inactivated fetal calf serum.

Virus Inoculation. Two milliliters of a cell suspension (3×10^6 cells per ml) was mixed with 1 ml of the undiluted inoculum and incubated for 2 hr at 37°C. Cells were then diluted to 1×10^5 per ml with medium, distributed into culture bottles treated with poly(L-lysine), and incubated overnight at 37°C. An equal volume of fresh medium was then added to each bottle, and incubation was continued. Cell suspensions were harvested at various times during the culture period, separated into supernatants and cell pellets, and prepared for cDNA/PCR. Samples for examination by *in situ* hybridization and immunofluorescence were harvested 7 days after virus inoculation.

cDNA/PCR. Nucleic acids were prepared from a 100-μl portion of culture supernatant or a pellet of ≈1×10^6 cells.

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Abbreviation: HCV, hepatitis C virus.

Nucleic acids present in 100 μl of HCV-containing serum or normal serum were also processed. Liver RNA was isolated by the guanidinium thiocyanate/cesium chloride method (16).

Whole nucleic acids extracted from serum or cells were used for cDNA synthesis and subsequent double PCR employing nested primers, as described by Kaneko *et al.* (17). After cDNA synthesis, the reaction mixture was diluted with H_2O, boiled for 5 min, cooled on ice, and incubated with 0.1 μg of RNase A at 37°C for 30 min to remove the residual RNA. The cDNA was then amplified in an automatic thermocycler. Specificity of the amplified DNA was confirmed by Southern blot hybridization with a ^{32}P-labeled synthetic 40-mer oligonucleotide probe internal to PCR primers. The design of the primers was based on published nucleotide sequences of HCV cDNA (18, 19). Two sets of nested primers were used in the cDNA/PCR for the detection of HCV sequences; one was designed to detect the 5′ noncoding region of the genome and the other to detect the putative NS5 region. Sequences, polarity and locations, relative to the HCV genome, of 20-mer oligonucleotide primers are shown in Fig. 1. In the reverse transcription step, sense primer 1 or 5 was used to prime cDNA synthesis from minus-strand HCV RNA, and antisense primer 4 or 8 was used to prime cDNA synthesis from plus-strand RNA. For PCR, external primer pairs 1/4 and 5/8 were used, followed by internal primer pairs 2/3 and 6/7, respectively.

Control Viruses for cDNA/PCR. Rotavirus (YO strain) and poliovirus (Sabin 1 strain) were used as double- and single-stranded RNA controls, respectively. Ten-fold dilutions of ≈0.9 μg of RNA extracted from purified rotavirus were tested for the presence of each strand by cDNA/PCR with primers that begin at map position 689 of segment 9 of the virus genome and at position 1036 of the complementary strand. Ten-fold dilutions of nucleic acids extracted from 2 μl of purified poliovirus (1.55 ng/μl) were also tested by using primers beginning at map position 2506 (sense) and 2584 (antisense) of the virus genome. In addition, 10-fold dilutions of total RNA (2 μg/μl) extracted from NIH 3T3 cells infected with the Moloney mouse leukemia virus were tested for each viral RNA strand by cDNA/PCR with primers that begin at map positions 6281 and 7111 of the virus genome.

***In Situ* Hybridization.** The detection of intracellular HCV RNA by *in situ* hybridization was done according to the method reported by Negro *et al.* (20), with modifications. Cells were hybridized overnight at 55°C in a mixture containing ^{35}S-labeled HCV genomic-strand RNA probe (20,000 cpm/μl) for detecting HCV negative-strand RNA. The HCV cDNA fragment [533 base pairs (bp)] encoding the putative core region was excised from plasmid C740 (given by M. Hijikata at the Cancer Center, Japan) and ligated into Bluescript II (Stratagene). The ^{35}S-labeled genomic strand RNA transcript was produced with the TransProbe T kit (Pharmacia), purified chromatographically, and used in *in situ* hybridization reactions.

Immunofluorescence. Immunofluorescence staining of viral antigens was performed by an indirect method as described (21). Monoclonal antibodies were supplied by L. Mimms of Abbott Laboratories and C. Y. Wang of United Biomedical. The antibodies were produced by immunizing mice with synthetic peptides based on the published sequences of the core and NS4 regions of the HCV genome and were shown by immunofluorescence to be reactive with HCV-infected, but not with normal, chimpanzee livers.

RESULTS

Detection of HCV RNA. Ten-fold serial dilutions of a plasma containing HCV, strain H (12) ($10^{6.5}CID_{50}$ per ml of HCV) were tested for HCV genome by cDNA/PCR. HCV sequences were detected at a dilution of 10^{-7} but not at 10^{-8}, indicating that the cDNA/PCR assay yielded a similar or slightly higher titer relative to the *in vivo* infectivity titer in chimpanzees. In contrast, HCV sequences were not detected in a normal HCV-negative serum.

Detection of Strand-Specific Viral RNA. Specificity of PCR tests for strand-specific HCV RNA was determined with various RNA samples. RNA extracted from purified rotavirus was used as a representative double-stranded RNA and RNA from purified poliovirus provided a representative single-stranded RNA. The RNA sample from rotavirus yielded titers of 10^4 genomes per μl for both plus- and minus-strands by PCR. In contrast, the sample from poliovirus yielded titers of 10^4 genomes per μl for plus-strand but $<10^0$ genomes per μl for minus-strand. The total RNA extracted from NIH 3T3 cells infected with Moloney murine leukemia virus, used as a representative intracellular exclusively plus-strand viral RNA, titered 10^6 plus-strand Moloney viral genomes per μl and 10^1 minus-strand genomes per μl. Thus, if a minus strand were "detected" solely by an inadvertent amplification from a plus-strand, the plus-strand would have had to be detected at least in 10^4- to 10^5-fold excess. The reverse transcriptase activity of *Taq* DNA polymerase reported (22) was not detected in our assays because there was no measurable amplification when the reverse transcriptase was omitted.

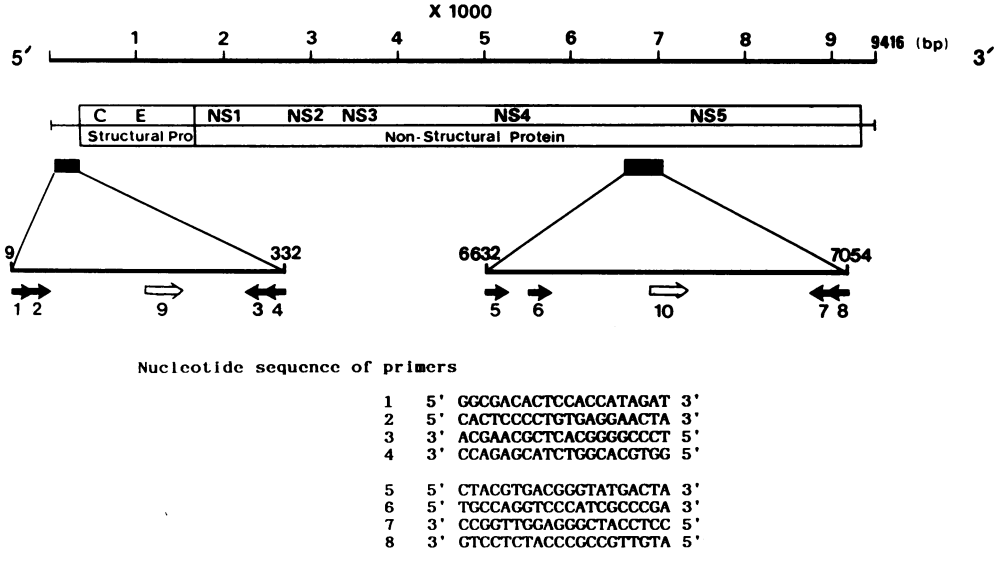

FIG. 1. Locations on the HCV genome (18), polarity, and nucleotide sequence of the primers for cDNA/PCR. The 5′ ends of primers 1, 2, 5, and 6 are at map positions 9, 29, 6632, and 6672, respectively. The 5′ ends of primers 3, 4, 7, and 8, from the complementary strand of the genome, are at 312, 332, 7034, and 7054, respectively. Solid boxes define the regions amplified by PCR, and open arrows indicate the locations of synthetic 40-mer oligonucleotide probes, the 5′ ends of which are at map position 161 or 6822, for Southern blot hybridization. Closed arrows, locations of primers for cDNA/PCR.

Detection of HCV Genomes in Chimpanzee Livers. As shown in Fig. 2*A*, both plus- and minus-strand RNAs were detected by the cDNA/PCR assay by using the primer pairs 5/8 and 6/7, respectively (refer to Fig. 1) in liver tissues from chimpanzees 177, 85, and 55 infected with HCV but were not detected in liver from uninfected chimpanzee 56. Specificity of the bands was confirmed by Southern blot hybridization using a ^{32}P-labeled 40-mer oligonucleotide (probe 10) (Fig. 2*B*). Comparable results were obtained with primer pairs 1/4 and 2/3. To determine the relative titers of plus- and minus-strand viral RNA in HCV-infected livers, 10-fold dilutions of ≈0.5 μg of cytoplasmic total RNA extracted from livers of infected chimpanzees 177, 85, and 55 were examined for each strand. Fig. 2*C* shows that the ratio of plus- and minus-strand HCV RNA appeared to be 1:1 for 177 and 10:1 for 85 and 55. These results generally agreed with the less quantitative results depicted in Fig. 2 *A* and *B*, except for chimpanzee 55.

Infection of MOLT-4 Cells with HCV. To examine the susceptibility of MOLT-4 and MOLT-4 Ma cells to HCV infection, cells were inoculated with HCV and cultured without medium changes for 7 days. The inoculum contained 10^3 genomes per 100 μl of plus-strand viral RNA by PCR, but minus-strand was not detected (Fig. 2). Cell suspensions harvested on days 1, 3, 5, and 7 were examined for intracellular minus-strand viral RNA by cDNA/PCR by using sense primer 5 for reverse transcription and primer pairs 5/8 and 6/7 for PCR. In the infected MOLT-4 Ma cells, minus-strand RNA was first detected on the third day of culture and increased to the maximum level on day 7. In similarly infected MOLT-4 cells, minus-strand viral RNA was first detected 7 days after inoculation (Fig. 3).

Because MOLT-4 Ma cells yielded stronger signals for minus-strand viral RNA, we chose them for additional experiments in which inoculated cells were maintained for ≈4 weeks by subculture at 3-day to 4-day intervals. The results are summarized in Table 1. Intracellular minus-strand viral RNA could not be detected during the first 4 days, but it was detected 6–9 days after inoculation. Both plus- and minus-strand HCV sequences were detected in the cells sporadically

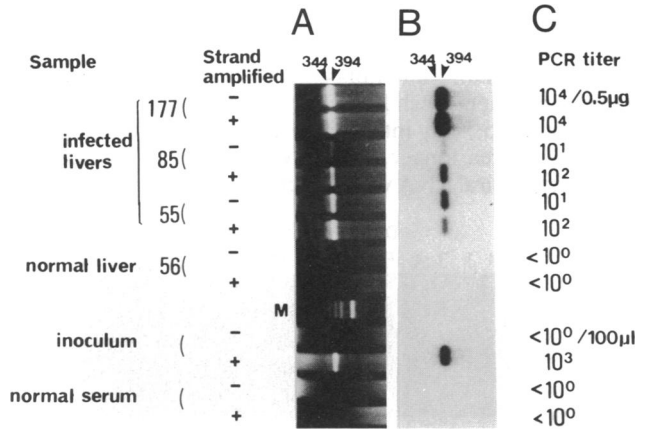

FIG. 2. Detection of minus- and plus-strand HCV RNA sequences in livers and in sera by cDNA/PCR. (*A*) Approximately 0.5 μg of cytoplasmic total RNA from the liver of HCV-infected chimpanzee 177, 85, or 55 or uninfected chimpanzee 56 was converted into cDNA using primer 5 for the detection of minus-strand or using primer 8 for plus-strand. cDNA was also synthesized from nucleic acids extracted from 100 μl of the serum used as the inoculum or from a normal serum (primer 5 or 8 was used). Each cDNA was amplified by PCR with primer pairs 5/8 and 6/7. PCR products were analyzed by gel electrophoresis and ethidium bromide staining. Lane M, 1-kilobase (kb) DNA ladder (BRL). (*B*) Southern blot hybridization with ^{32}P-labeled probe 10. Arrowheads indicate the positions of DNA fragments of 394 and 344 bp in the size marker. (*C*) PCR titer of minus- and plus-strand HCV RNAs in the livers and sera.

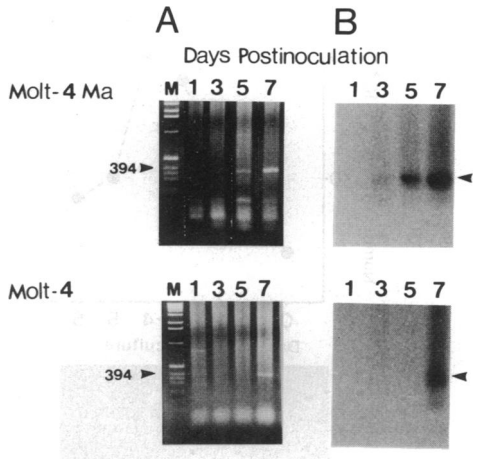

FIG. 3. Detection of intracellular minus-strand HCV RNA by cDNA/PCR in samples from MOLT-4 and MOLT-4 Ma cultures. cDNA/PCR was done with primer pairs 5/8 and 6/7. (*A*) Ethidium bromide staining. Lane M, 1-kb DNA ladder. (*B*) Southern blot hybridization with probe 10. Arrowheads indicate positions of 394-bp fragments.

thereafter for 2–3 weeks. Viral sequences were also detected sporadically in the supernatants of the cultures from day-8 to day-14 postinoculation.

To determine whether the occasional disappearance of HCV sequences in the cells during subcultures was related to the state of cell growth, MOLT-4 Ma cells were harvested 14 days after inoculation, washed once, diluted to 2 × 10^5 cells per ml with fresh medium, distributed into culture bottles, and subcultured without changing the medium for 7 days. The cells were examined daily for cell growth, and a pellet of ≈1

Table 1. Detection of HCV RNA in MOLT-4 Ma cultures

Days after inoculation	Detection of viral sequences by cDNA/PCR		
	Cells		Supernatant
	Minus-strand	Plus-strand	Plus-strand
0			
1	−		−
2	−		−
3	−	−	−
4	−		−
5			
6	+	−	
7	+	−	−
8	+	+	+
9	+	+	+
10			
11*	−	+	+
12			
13*	−	−	−
14	+	+	+
15*	−	+	−
16	+	+	−
17	−	−	−
18*	+	−	
19	−	−	
20		−	
21	−	+	−
22			
23	−	−	−
24*	−	+	−
25			
26	−	−	−
27	−		

*Days subcultures were performed.

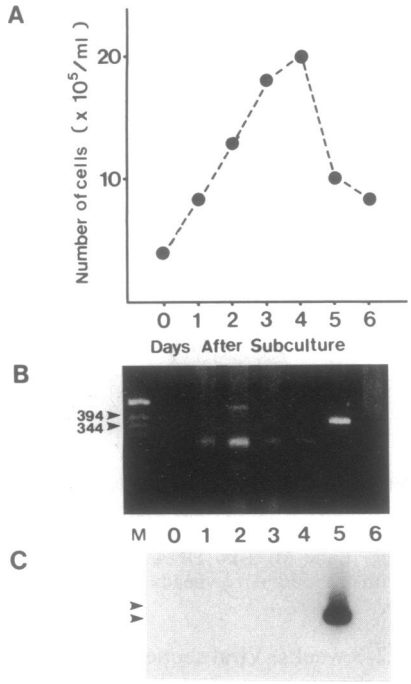

FIG. 4. Kinetics of cell growth (*A*) and detection of minus-strand viral RNA in MOLT-4 Ma cells by cDNA/PCR (*B*). For detection of HCV RNA, whole nucleic acids extracted from a pellet of 1×10^6 cells were subjected to cDNA/PCR as described. Primer 5 was used for cDNA synthesis, and primer pairs 5/8 and 6/7 were used for PCR. (*B*) Ethidium bromide staining. Lane M, 1-kb DNA ladder. (*C*) Southern blot hybridization with probe 10. Arrowheads indicate positions of DNA fragments of 394 and 344 bp.

$\times 10^6$ cells was tested for HCV sequences. Fig. 4 demonstrates the kinetics of cell growth and the detection of intracellular HCV minus-strand RNA by cDNA/PCR 5 days after subculture. Both plus- and minus-strand HCV RNAs had titers of 10^0 genomes per cell pellet by PCR at this time.

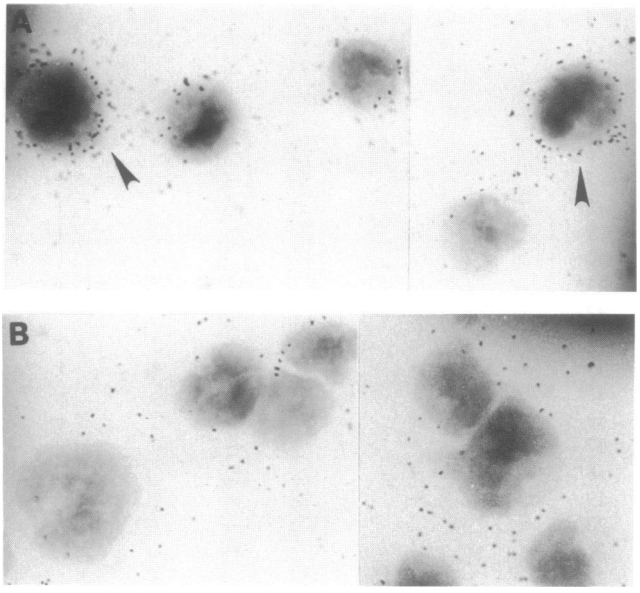

FIG. 5. Detection of HCV minus-strand RNA in MOLT-4 Ma cells by *in situ* hybridization. (*A*) MOLT-4 Ma cells harvested 7 days after inoculation with HCV and hybridized with a ^{35}S-labeled HCV genomic strand RNA probe; arrowheads indicate cells containing intracytoplasmic minus-strand viral RNA. (*B*) Lack of hybridization with the probe in uninoculated MOLT-4 Ma cells.

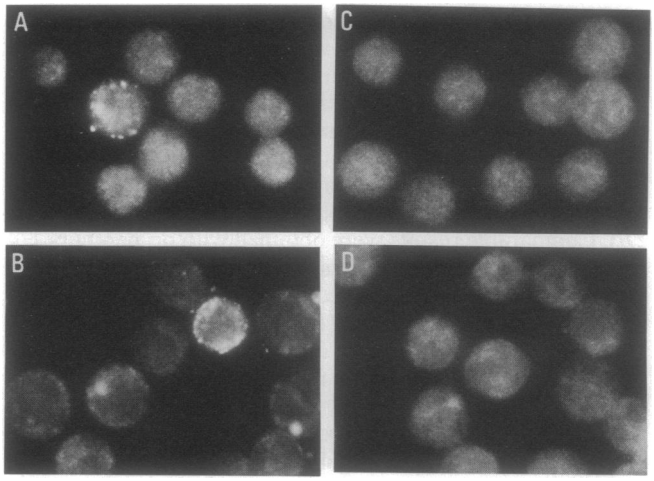

FIG. 6. Indirect immunofluorescent staining of MOLT-4 Ma cells with mouse monoclonal anti-HCV antibodies. Positive reactions with antibody to putative HCV core (*A*) or HCV nonstructural protein (NS4) (*B*) antigens in MOLT-4 Ma cells harvested 7 days after inoculation with HCV. Lack of staining with anti-HCV core (*C*) or anti-NS4 (*D*) in uninoculated MOLT-4 Ma cells.

To confirm these observations, *in situ* hybridization, using a ^{35}S-labeled HCV plus-strand RNA probe, was used on MOLT-4 Ma cells harvested 7 days after virus inoculation in another experiment. Fig. 5*A* shows that minus-strand HCV RNA was detected in the infected MOLT-4 Ma cells; about 1% of the cells were positive. Uninoculated MOLT-4 Ma cells were negative for HCV RNA when hybridized with the probe (Fig. 5*B*).

To test for expression of virus-encoded proteins, the cells were examined for putative HCV core and NS4 antigens by the indirect immunofluorescence method with mouse monoclonal antibodies. MOLT-4 Ma cells were found positive for both viral antigens 7 days after inoculation (Fig. 6 *A* and *B*); about 1% of the cells were positive for both antigens. Uninoculated cells were negative (Fig. 6 *C* and *D*).

Selected experiments were repeated with the well-characterized H-strain of HCV (12). A 2.25-ml sample of cell suspension containing 1.5×10^6 MOLT-4 Ma cells was inoculated with 0.25 ml of plasma containing HCV, strain H. In this experiment fresh MOLT-4 Ma cells were added to the cultures at 2- to 5-day intervals in an attempt to maintain the positive cultures longer. As shown in Fig. 7, intracellular minus-strand viral RNA was detected on day 1 and again after

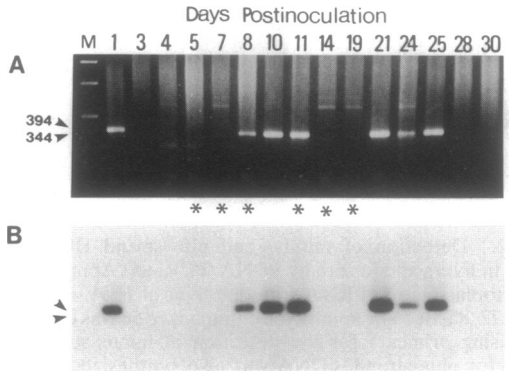

FIG. 7. Detection of intracellular minus-strand viral RNA in MOLT-4 Ma cultures after inoculation of HCV-containing plasma H. cDNA/PCR was done with primer pairs 5/8 and 6/7. Asterisks (*) indicate the days fresh MOLT-4 Ma cells were added to the cultures. (*A*) Ethidium bromide staining. (*B*) Southern blot hybridization with probe 10. Arrowheads indicate the positions of DNA fragments of 394 and 344 bp in the size marker.

day 7. Positive results were obtained sporadically for >3 weeks. The titer of HCV strain H in the inoculum was 10^6 genomes per 100 μl for plus-strand HCV RNA and 10^2 genomes per 100 μl for minus-strand RNA. Thus, it is not possible, based on the PCR data, to discriminate among (*i*) inadvertant amplification of plus-strand during PCR, (*ii*) carry-over of preexisting minus-strand from the inoculum, or (*iii*) viral replication as the source of the minus-strand signal in cells inoculated with the H strain of HCV. Minus-strand was also detected by Fong *et al.* (23) in this same HCV-containing plasma. However, in the present study, the positive signal on day 1 disappeared and did not reappear until after 7 days in culture, suggesting that actual HCV replication took place.

DISCUSSION

Shortly after infection of cells with positive-strand RNA viruses, minus-strand RNA is transcribed from the genomic RNA, and this minus-strand becomes the template for the synthesis of progeny plus-strand RNAs. Although the mode of HCV replication is not fully understood, minus-strand HCV RNA would be expected to be present in the cells when active viral replication occurs. Thus, in the present study, HCV replication in MOLT-4 and MOLT-4 Ma cells was evaluated primarily by detection of intracellular minus-strand replicative intermediates. The data suggest that the HCV genome replicated in both MOLT-4 and MOLT-4 Ma cells, as detected by demonstration of intracellular minus-strand viral RNA by cDNA/PCR. It was unlikely that HCV sequences detected in the infected cultures were derived from the inoculum for the following reasons: (*i*) intracellular minus-strand HCV RNA was not detected during the first 1–5 days of culture, and the intensity of the signals increased with time; if the signal had been due to carry-over, the signal should have been progressively lost. (*ii*) In the principal inoculum used in this study, minus-strand could not be detected. (*iii*) HCV RNA was detected in the cells for a period spanning 24 days after inoculation. In the culture supernatants, viral RNA remained detectable up to 14 days (Table 1). During the 24 days of culture the cells multiplied with a doubling of 24 hr to attain a 10^7-fold increase, and on day 14 the culture medium had been diluted $\approx 10^{10}$-fold by centrifugation/resuspension steps during subcultures. Thus, carry-over HCV RNA from the inoculum (10^3 genomes per 100 μl by PCR) should have been diluted 10^4-fold and 10^7-fold beyond the estimated titers of residual genomes in the cells (day 24) and supernatant (day 14), respectively. (*iv*) Had the detection of minus-strand in the infected cells been caused by the nonspecific amplification of plus-strand from the inoculum, we should have detected plus-strand in 10^4-fold excess, based on the experiments described above; this actually was not the case. In the experiment shown in Fig. 4, the PCR titers of plus- and minus-strands were both 10^0 genomes per cell pellet and, as shown in Table 1, the minus-strand could be detected even when the positive-strand was undetectable. (*v*) We demonstrated intracellular HCV minus-strand by *in situ* hybridization with a strand-specific probe, using a modification of a technique recently shown to be specific for HCV sequences in infected hepatocytes (24). (*vi*) We demonstrated viral core and NS4 antigens by immunofluorescence with monoclonal antibodies from two different sources. Taken together, these findings strongly suggest that the HCV genome replicates and expresses viral gene products in MOLT-4 Ma cells.

Although being essentially hepatotropic, a lymphotropic character of HCV has been suggested by the data of Hellings *et al.* (9, 10). They demonstrated the transmission of presumed hepatitis C to chimpanzees by leucocyte preparations from a human patient and from an infected chimpanzee. Our present results are consistent with these findings.

The present data suggest that HCV is able to replicate in a human T-cell line. Establishment of *in vitro* systems for propagation of HCV will be valuable for further characterization of this virus and for development of *in vitro* assays of virus infectivity, virus neutralization, etc.

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