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cDNA Clone of Hepatitis A Virus Encoding a Virulent Virus: Induction of Viral Hepatitis by Direct Nucleic Acid Transfection of Marmosets

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Direct inoculation of marmoset livers with an in vitro transcription mixture containing cDNA and full-length genomic RNA transcripts of hepatitis A virus resulted in acute viral hepatitis. Elevations in serum levels of liver enzymes were correlated with appearance of antibody to hepatitis A virus. Genomes of infectious hepatitis A virus isolated from the feces of transfected marmosets contained the same mutation as the cDNA template used for transfection. Liver biopsies confirmed that the virus encoded by the cDNA clone induced histopathological changes equivalent to those caused by virulent wild-type virus.

Hepatitis A virus (HAV) is a major cause of acute viral hepatitis. HAV infection of humans and certain primates results in elevation of serum levels of liver enzymes and cytopathological changes in the target organ, the liver. Since HAV is generally not cytopathic in cultured cells, it is not clear why liver damage occurs. Although a killed vaccine for hepatitis A has recently been licensed in Europe, economic and administrative considerations provide reasons for developing an attenuated vaccine. Knowledge of which gene(s) of HAV is responsible for virulence of the virus and which mutation or constellation of mutations best attenuates the virus would greatly aid development of an efficacious and stably attenuated vaccine.

The availability of two infectious cDNA clones that appear to represent the genomes of virulent, wild-type HAV and a derivative, attenuated mutant provide an opportunity to determine the molecular basis for attenuation (4–6, 10). In theory, phenotypic analysis of chimeric genomes constructed from the two cDNA clones could be used to map determinants of virulence as has been done for other picornaviruses such as poliovirus and Theiler's murine encephalomyelitis virus (1, 11, 17). However, for this approach to work, it is crucial that each parent genome have a well-defined phenotype. Although the cDNA representing the attenuated virus has been shown to encode a virus with the same growth and attenuation phenotypes as the original attenuated virus (3), the cDNA representing the putative wild-type virus has not been shown to encode a virulent virus. Viruses derived by transfection of primary African green monkey kidney cells with full-length RNA transcripts of the putative wild-type clone or chimeric clones containing portions of the wild-type clone did not cause hepatitis when inoculated into marmosets (3, 8). The attenuation phenotype of these viruses could mean that a naturally occurring attenuated variant rather than the authentic wild-type virus had originally been cloned. In this case, construction of chimeras would not be useful for analysis of virulence. Alternatively, the genotype of the wild-type cDNA clone may not have been preserved during the required period of replication in cell culture. The transcripts from the putative wild-type clone were minimally infectious for cultured cells, and the resultant virus replicated to detectable levels only after an extensive incubation period in cell culture (10). Therefore, any mutant virus which replicated more efficiently than wild-type virus in cell culture would have a selective advantage. Since the original attenuated mutant was coincidentally isolated in cell culture by selection for a more rapidly replicating mutant (7), it may be that virus replication in cell culture usually selects for mutations which diminish or eliminate the virulence of the virus for primates.

Direct injection of liver with cloned DNA of the ground squirrel analog of hepatitis B virus or with RNA genomes purified from rabbit hemorrhagic disease virus virions resulted in production of the respective viruses, indicating that direct transfection of an animal could be achieved (14, 19). Therefore, to determine whether the putative wild-type cDNA represented virulent HAV, we attempted to bypass the cell culture step and transfect the animals directly. In this report, we demonstrate that in vivo transfection of marmosets with synthetic genomes of HAV, a positive-strand RNA virus, produced infectious virus and acute hepatitis.

MATERIALS AND METHODS

cDNA and in vitro transcription. During construction of the original plasmid, full-length HAV cDNA prepared from the liver of a marmoset experimentally infected with wild-type HAV, strain HM-175, was inserted downstream of the Sp6 promoter in a pGEM-1 (Promega Biotec, Madison, Wis.) expression vector. RNA from this cDNA was not infectious until the cDNA was modified to correct a single base deletion at position 4405. For the present study, we also introduced a C → T mutation at position 3889 (10). Plasmid DNA was partially purified by LiCl precipitation or more stringently purified by isopycnic banding in CsCl (9). Approximately 10 μg of linearized plasmid per 100-μl reaction volume was transcribed for 1 h at 37°C by Sp6 polymerase as described previously (9). The transcription reaction was terminated by adding 4 volumes of phosphate-buffered saline

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without calcium or magnesium (Whittaker Bioproducts, Walkersville, Md.). Aliquots (0.5 ml) were frozen on dry ice, stored at −80°C, and used within 24 h.

In vivo transfection. Laparotomy was performed, and three aliquots (0.15 ml each) of diluted transcription mixture were injected directly into the liver tissue. Blood samples were drawn weekly and tested by standard methods (Metpath, Rockville, Md.) for the serum levels of alanine aminotransferase, isocitrate dehydrogenase, and gamma glutamyltransferase and with commercial assays (Abbott Laboratories, North Chicago, Ill.) for seroconversion to anti-HAV.

Fecal samples. Feces were collected thrice weekly and stored at −80°C. Fecal suspensions containing 10% (wt/vol) feces in 10 mM Tris (pH 7.2)–150 mM NaCl were prepared in a stomacher and clarified by low-speed centrifugation for use in hybridization or polymerase chain reaction (PCR) assays.

Slot-blot hybridization. The procedure for slot-blot hybridization was basically as described previously (10) except that the volume of each constituent was increased proportionally so that 100 μl of 10% fecal suspension was extracted and 150 μl of the final preparation was blotted onto nitrocellulose. The [^{32}P]CTP-labeled riboprobe was synthesized as described previously except the template was an 8-kb gel-purified *Hin*dIII fragment rather than the entire plasmid (9). Hybridization was performed for 16 h at 42°C and was followed by four washes at 54°C.

Cell culture. A 10% fecal suspension (0.5 ml) was centrifuged for 2 min in an Eppendorf centrifuge and then filtered through a 0.45-μm-pore-size filter. The filtrate was added to a drained monolayer of fetal rhesus kidney cells (FRhK-4) in a T-25 flask and incubated for 2 h at 34°C. The monolayer was washed once and overlaid with 5 ml of Dulbecco modified Eagle medium containing 10% fetal calf serum, nonessential amino acids, glutamine, 50 μg of gentamicin sulfate per ml, 2.5 μg of amphotericin B per ml, and 80 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (Sigma Chemical Co., St. Louis, Mo.). Five days later, cells were trypsinized, and half were transferred to coverslips. Immunofluorescence microscopy was performed as described previously (10). Cell lysates were prepared by adding 1 ml of lysing solution (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 8.6], 1% Triton N-101) to the drained monolayer in the T25 flask and removing the nuclei by centrifugation of the lysate for 1 min in an Eppendorf centrifuge. The lysate was stored at −80°C.

PCR. Fecal suspension or cell lysate (200 μl) was precipitated at 4°C overnight with 2 volumes of 15% polyethylene glycol 8000 in 1.5 M NaCl. Pellets were extracted by the guanidinium thiocyanate method (2), and the viral RNA was reverse transcribed at 42°C by using avian myeloblastosis virus reverse transcriptase (Promega Biotec) and a primer specific for the 2C gene. A two-step PCR was performed with nested primers and 35 cycles for each step. The PCR product was purified from 1% agarose gels by using Geneclean (Bio 101, Inc., La Jolla, Calif.). Sequencing was performed with Sequenase (United States Biochemical Corp., Cleveland, Ohio) in the presence of dimethyl sulfoxide (22).

Histopathology. Needle biopsy specimens of the liver were obtained before transfection and weekly thereafter, beginning 3 to 4 weeks after laparotomy. These specimens were fixed in formalin, embedded, sectioned, stained with hematoxylin and eosin, and mounted by standard procedures (American Histolabs, Inc., Gaithersburg, Md.).

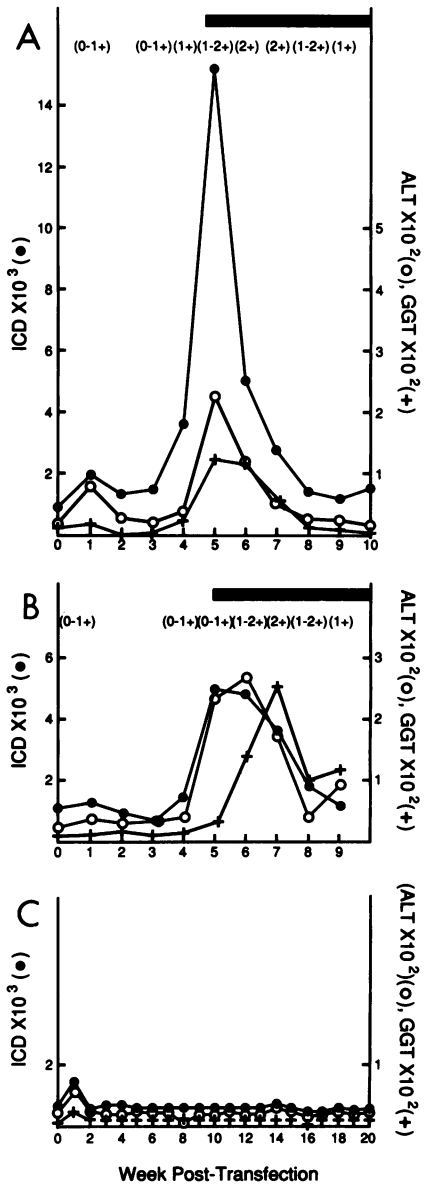

FIG. 1. Serum and biopsy results. Serum levels of isocitrate dehydrogenase (ICD), alanine aminotransferase (ALT), and gamma glutamyltransferase (GGT) are plotted for marmosets 682 (A), 641 (B), and 690 (C). Presence of antibody to HAV is denoted by the black bar. The degrees of necroinflammatory changes observed in the liver biopsy specimens are scored as 0 to 2+.

RESULTS

Induction of hepatitis. The cDNA clone used in these experiments encoded the putative wild-type HAV genome modified by a single mutation at nucleotide position 3889 in the 2B gene. Even though it was possible that this mutation attenuated the virulence of the virus, this cDNA clone was chosen for the initial experiments because it offered several advantages over that of the fully wild-type virus. First, since this mutation greatly increased the probability of recovering virus after transfection of cultured cells, its inclusion meant we could verify the intrinsic infectivity of the nucleic acid by in vitro transfection (9). Second, because this mutation is involved in adaptation to growth in cell culture, we felt it

TABLE 1. Comparison of severity of hepatitis

Virus source	Marmoset	Enzyme peak (wk)			Pathology peak (wk)[a]	Week seroconverted
		ALT[b]	ICD[c]	GGT[d]		
DNA	690	72 (1)	1,135 (1)	22 (1)	ND[e]	—[f]
DNA + RNA	682	226 (5)	15,159 (5)	124 (5)	2+ (6, 7)	5
DNA + RNA	641	272 (6)	5,041 (5)	256 (7)	2+ (7)	5
Stool	578[g]	1,099 (4)	39,777 (4)	90 (4)	2+ (4)	3
Stool	579[g]	274 (4)	8,449 (4)	39 (5)	1+ (6)	4

[a] Scored in arbitrary units.
[b] ALT, alanine aminotransferase; international units per liter.
[c] ICD, isocitrate dehydrogenase; units per milliliter.
[d] GGT, gamma glutamyltransferase; units per liter.
[e] ND, not determined.
[f] —, still negative at week 20 when experiment terminated.
[g] Data from Wong et al. (23).

would increase the probability of recovering virus from transfected animals (9). Finally, the mutation served as a marker to identify the source of any recovered virus and distinguish it from circulating wild-type HM-175 or cell culture-adapted mutants which lack this mutation (6, 20). The linearized cDNA, purified by LiCl precipitation, was transcribed in vitro by Sp6 polymerase. The entire transcription mixture containing the full-length RNA transcripts as well as the template cDNA was diluted with phosphate-buffered saline, and two marmosets were inoculated by the intrahepatic route.

One marmoset (682) subsequently developed acute viral hepatitis as evidenced by a dramatic rise in liver enzyme levels at the approximate time that antibodies to the capsid proteins of HAV were first detected (Fig. 1A). The pattern and magnitude of enzyme elevations were comparable to those observed in another study (23) in which marmosets were inoculated intravenously with the virulent, wild-type HM-175 virus (Table 1). Unfortunately, the second marmoset (681) inoculated in this study died 4 weeks postinjection before a significant rise in enzyme levels or HAV-specific antibodies was observed.

Although HAV RNA was at least 10 times more efficient than cDNA in transfection of cells in culture (5), it could be argued that, in vivo, the DNA might resist nucleases better and so be more effective than RNA after intrahepatic transfection. To determine whether the cDNA itself was infectious, we prepared identical transcription reaction mixtures containing CsCl-purified DNA, but added Sp6 polymerase to only one reaction mixture. After incubation, the mock transcription reaction mixture containing only cDNA and the complete reaction mixture containing both cDNA and the transcribed RNA were injected into the livers of marmosets 690 and 641, respectively, as described previously. The mixture containing the RNA was again infectious and induced significant elevations of liver enzymes and seroconversion to HAV by 5 weeks posttransfection (Fig. 1B and Table 1). Although the peak enzyme elevations were lower than those in the previous experiment, they remained elevated for a longer time, thus confirming the original results. However, the marmoset inoculated with only the cDNA preparation did not become infected or develop hepatitis since enzyme levels remained normal and antibodies to HAV were not detected during a 20-week interval postinjection (Fig. 1C and Table 1).

Identification of HAV in the feces. To confirm that the injected viral nucleic acids had actually transfected the liver cells and produced infectious virus, we analyzed feces from the two marmosets in the first experiment. Slot-blot hybridization assays detected HAV nucleic acid in feces collected just before seroconversion of marmoset 682 (Table 2). The feces that were collected just before marmoset 681 died were also positive for HAV RNA by hybridization analysis (Table 2). Comparisons of the intensity of the hybridization signals with that from a titered HAV standard indicated that at the peak of shedding, the feces from marmosets 681 and 682 (as well as marmoset 641) contained greater than $10^{8.8}$ 50% infectious doses per g of stool. Therefore, it appeared that HAV was being shed in the feces of both animals in amounts equivalent to the mean of $10^{7.9}$ 50% infectious doses reported after infection with the wild-type virus (12).

We attempted to recover infectious HAV by inoculating FRhK-4 cell cultures with aqueous suspensions of feces that were positive by slot-blot hybridization analysis. To further increase the possibility of isolating HAV, we treated the cell cultures with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, an adenosine analog which enhances the growth of wild-type HAV in cell culture (8, 21). Cell cultures inoculated with the fecal suspensions became infected with HAV since the cells were subsequently stained with antibodies to HAV in an immunofluorescence microscopy assay (Table 2). Therefore, infectious HAV was present in the feces of both transfected marmosets.

To confirm that the virus recovered from the marmosets had indeed originated from the transfecting nucleic acid, we

TABLE 2. Identification of HAV in posttransfection samples

Marmoset	Day[a]	Feces		Cell culture[b]	
		Hybridization[c]	3889 mutation[d]	% IF[e]	3889 mutation
681	18	++	Present	5	ND[f]
	20	+++	Present	10	Present
	22	++++	ND	5	Present
682	18	±	ND	1	ND
	25	+	Present	5	Present
	32	+	ND	30	Present
	41	−	ND	ND	ND

[a] Day posttransfection feces were collected.
[b] Inoculated with feces and examined 6 days later.
[c] Signal on slot-blot probed with ^{32}P-labeled riboprobe.
[d] PCR consensus sequence.
[e] Percent cells positive by immunofluorescence microscopy.
[f] ND, not determined.

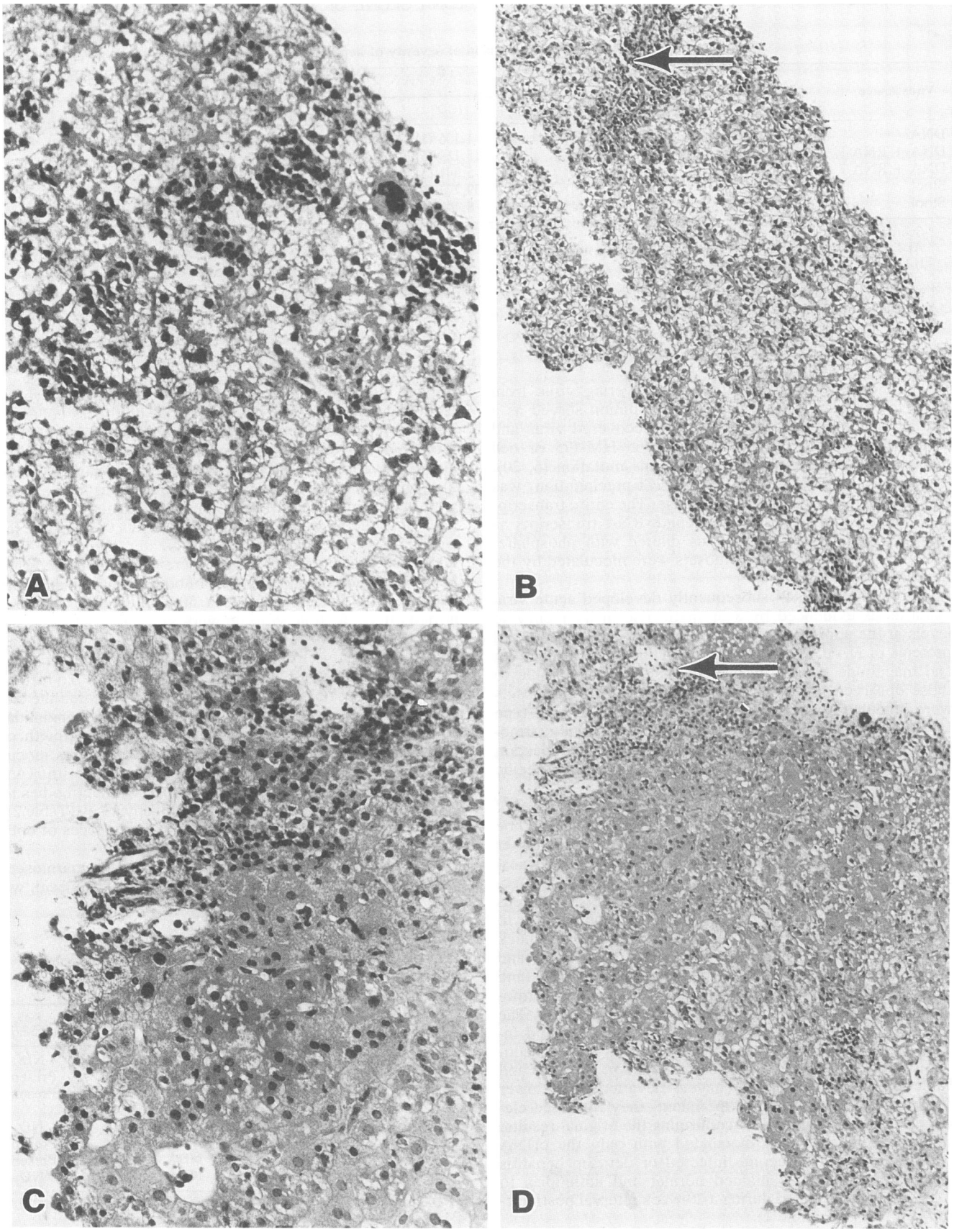

FIG. 2. Photographs of biopsy samples from marmosets 682 (A and B) and 641 (C and D). (A) Parenchymal necroinflammatory changes (×120); (B) parenchymal necroinflammatory changes (×60) and portal inflammatory expansion (arrow); (C) portal mononuclear inflammatory infiltration (×120); and (D) portal area with inflammatory infiltration (arrow) and multifocal parenchymal necrosis (×60).

used the PCR to amplify the 2B region of the viral genome for direct sequencing. The PCR consensus sequence was obtained for the 2B region spanning nucleotides 3781 to 3995 of three fecal and four cell culture samples. For all seven samples, the C → T mutation at position 3889 of the template cDNA was found in the PCR product (Table 2). In six samples, the remainder of the PCR consensus sequence was identical to that of wild type. The base at position 3885 could not be determined for the seventh sample that was derived from cell culture, but the rest of the sequence was that of wild type. Therefore, the virus shed in the feces must have originated from the transfected genomes.

Comparison of the severity of hepatitis. Although the increases in serum levels of liver enzymes in the transfected animals were comparable to those induced by infection with virulent wild-type virus (Table 1), it was necessary to compare the degrees of liver pathology before a final assessment of virulence could be made. Sections of liver obtained by weekly needle biopsy were compared by one of us (S.G.), under code, with biopsy specimens obtained from marmosets infected with wild-type virus.

The liver biopsies of the transfected marmosets 641 and 682 revealed an identical, moderately severe (2+) degree of acute viral hepatitis (Fig. 1 and 2 and Table 1). The portal inflammation consisted of mononuclear cells; the parenchyma revealed multifocal hepatocytolysis distributed throughout the lobule. Both animals had evidence of extramedullary hematopoiesis. The biopsy specimens from both marmosets infected with wild-type virus (578 and 579) had prominent portal inflammation. However, the lobular necroinflammatory activity was much more prominent in animal 578 (2+) than in animal 579 (1+). The degree of necrosis in marmoset 578 was similar to that of marmosets 641 and 682, consistent with a diagnosis of acute viral hepatitis. The sections of liver from marmoset 579 revealed milder necroinflammatory changes in comparison with the others. Therefore, the hepatitis induced by the transfecting genomes was as severe as that caused by infection with authentic wild-type virus.

DISCUSSION

These experiments demonstrated that a full-length cDNA clone of HAV encoded a virulent HAV that could be recovered after direct transfection of a primate host. The virus encoded by the synthetic genome was as virulent as authentic wild-type HM-175 virus since both viruses caused similar pathological changes in the liver. In addition, the detection of elevated liver enzyme values in the serum (Table 1), as well as the detection of large amounts of virus in the feces, indicated that the transfection-derived virus had the virulence of wild type. A simple comparison of individual animals can be misleading since there is considerable biological variability in the serum enzyme responses of individual animals to infection by HAV. However, in another study from this laboratory in which four marmosets were infected with wild-type HM-175 virus, the mean maximum pathology score for the four animals was 1.75+, while the geometric mean maximum serum isocitrate dehydrogenase level was 6,076 U/ml with a relative standard error of 1.43 (12). Therefore, in the present study, the liver histopathology and isocitrate dehydrogenase elevations were as expected for infection by wild-type virus except that the serum enzyme response of marmoset 578 was exceptionally high. The slightly longer period required for the transfected animals to become ill most likely reflects a lower initial dose of virus since Purcell et al. (16) have reported that the viral incubation period is inversely proportional to virus dose. The cDNA clone we used differed from the putative wild-type cDNA clone by a single mutation that is important for adaptation to growth in cell culture (9). Since the viral genome with the single mutation was apparently fully virulent, it should now be possible to map the genetic determinants of HAV virulence by assaying chimeric genomes constructed from the infectious cDNA clones of this virulent virus and the HM-175 derivative, attenuated virus, HAV/7.

The mutation at 3889, in the 2B gene, was of interest because, when combined with mutations in the 2C gene, it enabled HAV to grow much more efficiently in cell culture (9). There appears to be very strong selective pressure for this mutation during replication in cell culture because four independently isolated, cell culture-adapted variants have acquired the identical mutation (4, 13, 15, 18). Interestingly, this mutation was not selected against in vivo but was retained in the consensus sequence of PCR products amplified directly from the feces of the transfected marmosets. The data clearly demonstrated that the single mutation at 3889 was not sufficient to attenuate the wild-type virus for marmosets. Since this mutation has a dramatic effect on growth in cell culture only when combined with other mutations (9), it will now be interesting to determine whether it contributes to attenuation when combined with additional mutations.

The in vivo transfection technique is unique in that it permits the recovery of a DNA virus (19) or an RNA virus (this study) from a cDNA clone in the total absence of passage in cell culture. Therefore, this technique may provide a means of recovering an infectious RNA virus encoded by a cDNA clone even when it has not been possible to grow that virus in cell culture. Additionally, this technique may be critical for recovery of virus mutants which might be selected against during growth in cell culture. For hepatitis viruses, the in vivo transfection procedure has obvious applications for hepatitis A, C, and E viruses. It remains to be seen whether nonhepatatropic viruses can be recovered by transfection of liver or whether other organs can be successfully transfected.

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