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A Hepatitis A Virus Deletion Mutant Which Lacks the First Pyrimidine-Rich Tract of the 5′ Nontranslated RNA Remains Virulent in Primates after Direct Intrahepatic Nucleic Acid Transfection

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Cell culture-adapted variants of hepatitis A virus (HAV) in which the first pyrimidine-rich tract (pY1; nucleotides 99 to 138) of the 5′ nontranslated region has been deleted (Δ96-137 or Δ96-139) replicate as well as parental virus in cultured cells (D. R. Shaffer, E. A. Brown, and S. M. Lemon, J. Virol. 68:5568–5578, 1994). To determine whether viruses with such large deletion mutations are able to replicate and to produce acute hepatitis in primates, we reconstructed the Δ96-137 deletion in the genetic background of a virulent virus which differs from the wild type by only one mutation in the 2B-coding region (HM175/8Y). Full-length synthetic Δ96-137/8Y RNA was injected into the livers of two HAV-seronegative marmosets (*Saguinus mystax*). Both animals developed serum liver enzyme elevations and inflammatory changes in serial liver biopsies within 3 to 4 weeks of inoculation which were comparable in magnitude to those observed previously following intrahepatic inoculation of marmosets with HM175/8Y RNA. Sequencing of RNA from virus shed in feces demonstrated the presence of the Δ96-137 deletion. These results indicate that the pY1 sequence of HAV is not required for efficient viral replication in hepatocytes in situ or for production of acute hepatic injury following intrahepatic RNA transfection in primates.

Hepatitis A virus (HAV) is a positive-strand RNA virus of the picornavirus family (genus *Hepatovirus*) which causes acute hepatitis in certain primate hosts (16). As with other picornaviruses, the genomic RNA of HAV contains a lengthy 5′ nontranslated region (5′NTR) with a complex secondary and tertiary structure (1) and sequence elements which are essential for cap-independent viral translation (1, 2, 13, 22) and viral RNA replication (20). Recent work has shown that a 40-nucleotide (nt) pyrimidine-rich sequence near the 5′ end of the 5′NTR, designated the first pyrimidine-rich tract [pY1; nt 99 to 138, numbered according to the sequence of the wild-type HM175 strain of HAV (HM175/wt) (3)], is not required for efficient replication of virus in cultured African green monkey or fetal rhesus kidney cells (19). The pY1 tract is located upstream of the HAV internal ribosomal entry site (IRES) (2, 20, 22) and contains a unique repetitive (U)UUCC(C) motif (Fig. 1). This region of the 5′NTR is sensitive to cobra venom nuclease V1 digestion centered on the cytidines within the repetitive motifs (19), suggesting that the pY1 domain is folded into an ordered structure, perhaps by virtue of noncanonical base pairing or via single-stranded helical stacking. The function of the pY1 domain remains unclear.

Studies undertaken with a murine picornavirus, mengovirus, have shown that deletion of a poly(C) tract which is similarly placed within the 5′NTR of that virus results in variants which are markedly attenuated in the natural host but which replicate well in cultured cells (5). Since the general features of the pY1 domain are conserved among all human hepatovirus strains studied to date, we have wondered whether an HAV mutant lacking the pY1 domain might be similarly attenuated during infection of otherwise susceptible primates. If this were the case, such a virus might have potential as a candidate live attenuated vaccine against hepatitis A.

The HAV mutants with large deletions involving the pY1 domain which we had studied previously (19) were constructed in the genetic background of a virus which is highly adapted to growth in cultured cells, HM175/P2.18f (23), and which is likely to be highly attenuated in primates (8, 21). Thus, to obtain a pY1 deletion mutant which could be tested for virulence in primates, the Δ96-137 deletion was reconstructed in the genetic background of a virulent virus (HM175/8Y). The nucleotide sequence of HM175/8Y is identical to that of HM175/wt except for a single C-to-U mutation at nt 3889 in the 2B-coding region (7–9, 11). The 3889 mutation substantially enhances the growth of wild-type virus in cultured cells, without significantly impairing the ability of the virus to replicate or cause disease in primates (8).

PCR was used to amplify the HAV sequence between nt 18 and 170 of the genome-length clone, pΔ96-137 (19). The positive-strand PCR primer (wt +18; 5′-TTCAAGAGGG GTCTCCGG, nt 18 to 35) contained a *Bsp*EI site at nt 25, and the negative-strand PCR primer (wt −170; 5′-CCCTGAAC CTGCAGGAATTAATATTT, nt 170 to 145) contained a *Pst*I site at nt 162. With the exception of the Δ96-137 deletion, the sequence of the template cDNA between nt 18 and 170 differed from the HM175/8Y sequence only with respect to an A-to-G mutation at nt 152. Therefore, the negative-strand PCR primer was designed to change the G at nt 152 to A. The 0.15-kb PCR product was gel purified, digested with *Bsp*EI and

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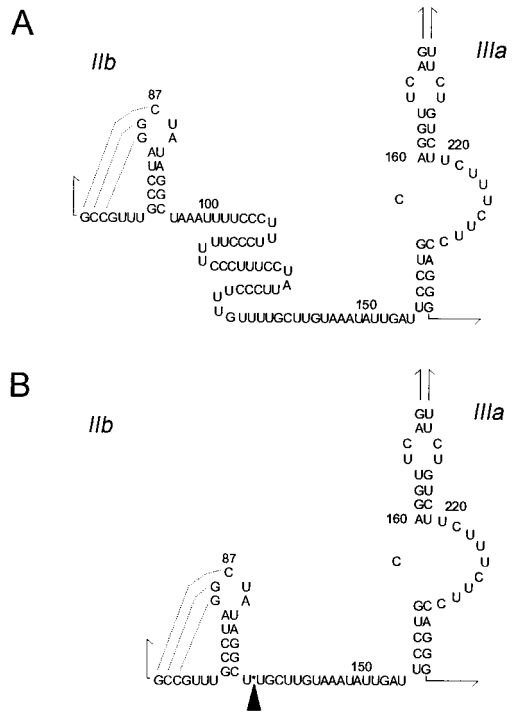

FIG. 1. Sequence and proposed secondary structure in the region of the pY1 domain of HM175/8Y virus and the Δ96-137/8Y pY1 deletion mutant.

*Pst*I, and cloned into the unique *Bsp*EI-*Pst*I site of pG3Zf8Y/0-632. pG3Zf8Y/0-632 is a subclone containing the 0.6-kb *Hin*dIII-*Bam*HI fragment of HM175/8Y cDNA (nt 0 to 632). The resulting plasmid was digested with *Bsp*EI and *Bam*HI, and the 0.6-kb fragment was gel purified and ligated with the 9.7-kb DNA fragment resulting from *Bsp*EI and partial *Bam*HI digestion of pHM175/8Y (11). The sequence of the resulting full-length cDNA clone, pΔ96-137/8Y, is identical to that of HM175/wt except for the deletion between nt 96 and 137 and the C-to-U mutation at nt 3889. A similar strategy was used to construct pΔ96-137/wt, which contains the deletion between nt 96 and 137 in the background of the fully wild-type HM175 sequence. The identity of cDNA clones was confirmed by restriction endonuclease mapping and by double-stranded DNA sequencing of the PCR-amplified sequence between nt 25 and 162 (Sequenase reagent kit; United States Biochemical Corp.).

To confirm that RNA derived from these mutant clones remained infectious, 20 μl of an SP6 in vitro RNA transcription mix containing 1 μg of plasmid DNA (and visually estimated to contain approximately 5 μg of full-length HAV RNA) was used to transfect fetal rhesus monkey kidney (FRhK-4) cells. Parallel transfections were carried out with pΔ96-137/8Y, pHM175/8Y, pΔ96-137/wt, and pHM175/wt RNAs. Transfection was accomplished by electroporation using 8×10^6 cells per 0.4-cm cuvette and a voltage of 1.50 kV/25 μF, applied twice. Following electroporation, cells were immediately diluted into 4 ml of growth medium in a 60-mm-diameter dish. Cells were maintained at either 31 or 37°C with weekly medium changes before harvest of cell lysates in either 0.05 or 0.1% sodium dodecyl sulfate at 4 or 7 weeks as previously described (4). Serial dilutions of transfected cell lysates were tested for viral antigen content by a solid-phase radioimmunoassay, carried out as previously described (17).

As shown in Table 1, viral replication was documented by the presence of detectable HAV antigen 4 weeks after transfection of either the HM175/8Y or Δ96-137/8Y RNA, with further increases in viral antigen by 7 weeks. Previous experiments have shown that cell culture-adapted virus containing the Δ96-137 deletion replicates with comparable if not slightly greater efficiency at 37 than at 31°C (19), as does the parental cell culture-adapted virus. In contrast, pY1 deletion mutants in which the deletion extended downstream of nt 141 are temperature sensitive as a result of a defect in RNA synthesis (19, 20). The results from the experiment shown in Table 1 are consistent with these observations, in that the Δ96-137/8Y RNA produced virus which was not temperature sensitive, with more viral antigen following transfection at 37 than at 31°C. Transfection of the Δ96-137/8Y RNA produced at least as much, if not more, viral antigen as the HM175/8Y RNA at both temperatures. These radioimmunoassay results were confirmed by radioimmunofocus infectivity assays of lysates of transfected cells which were carried out in BS-C-1 cells (data not shown). Thus, the Δ96-137 deletion does not impair the replication of the virulent HM175/8Y virus in cultured FRhK-4 or BS-C-1 cells. Cells transfected at either temperature with the HM175/wt or Δ96-137/wt RNA were negative for antigen at both 4 and 7 weeks, confirming the importance of the 3889 mutation (present in HM175/8Y) in facilitating replication in cultured cells (9, 11).

TABLE 1. Radioimmunoassay detection of HAV antigen in lysates of FRhK-4 cells transfected with synthetic viral RNAs

RNA	cpm			
	4 wk		7 wk	
	31°C	37°C	31°C	37°C
HM175/8Y	483	560	1,146[a]	2,560[a]
Δ96-137/8Y	569	1,313	1,982	5,165[a]
HM175/wt	46	46	ND[b]	32
Δ96-137/wt	40	39	46	47[a]
None (mock transfection)	ND	33	ND	87

[a] Mean value from two replicate transfections.
[b] ND, not done.

To assess the replication competence and pathogenicity of Δ96-137/8Y virus in primates, two marmosets (*Sanguinus mystax*) were infected by direct intrahepatic nucleic acid transfection (10). Scaled-up SP6 RNA transcription reactions were carried out to produce Δ96-137/8Y RNA for intrahepatic inoculation. Each marmoset (approximately 0.6 kg in size) received a total inoculum of 100 μl of transcription mix containing 10 μg of pΔ96-137/8Y cDNA as a template, given as three separate injections involving both lobes of the liver (10). The marmosets were bled weekly for 2 weeks prior to intrahepatic nucleic acid transfection and at weekly intervals thereafter for the next 15 weeks. Fecal samples and percutaneous needle biopsies of liver were also obtained at weekly intervals.

As shown in Fig. 2, both marmosets developed evidence of acute hepatitis within 3 to 4 weeks of intrahepatic transfection of the Δ96-137/8Y RNA. The severity of disease was similar to that seen in previous intrahepatic transfections with HM175/8Y RNA (10). Serum isocitrate dehydrogenase, alanine aminotransferase, and γ-glutamyl transpeptidase activities all became elevated to levels typical of acute hepatitis A in the marmoset. By 7 weeks posttransfection, the serum enzyme levels had returned to near normal in both animals.

Hematoxylin-and-eosin-stained sections of percutaneous

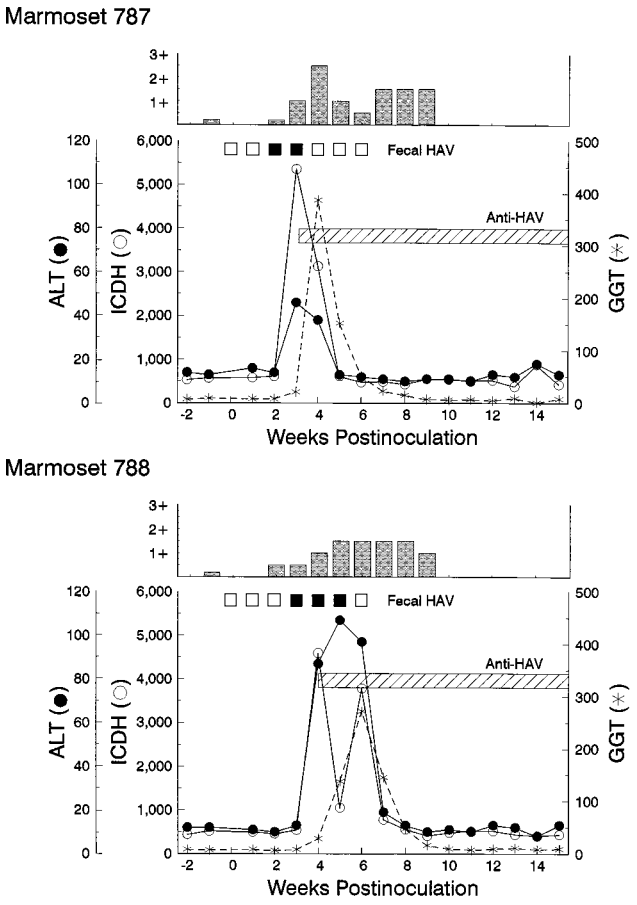

FIG. 2. HAV infection following intrahepatic transfection of two marmosets with Δ96-137/8Y RNA. The bar graph at the top of each panel represents histologic scores (0 to 3+) of percutaneous needle biopsy samples of liver (see text). Solid squares represent fecal samples strongly positive for HAV RNA by slot blot hybridization, while open squares represent fecal samples which were negative for viral RNA. Serum alanine aminotransferase (ALT; ●) and isocitrate dehydrogenase (ICDH; ○) activities are represented with the appropriate scales shown at the left, while γ-glutamyl transpeptidase (GTP; *) activity is shown with the scale at the right. The presence of serum anti-HAV antibody detectable by a competitive inhibition immunoassay is indicated by the striped horizontal box.

liver biopsy samples were examined without knowledge of the identity of samples. Sections were graded from 0 to 4+, based on the degree of necroinflammatory changes. Previous intrahepatic transfections of marmosets with HM175/wt or HM175/8Y RNA have typically resulted in histologic scores of 2+, rarely 3+, and never 4+ (6, 10). As shown in Fig. 2, both animals transfected with the Δ96-137/8Y RNA developed necroinflammatory changes in the liver which were similar in timing and severity to those observed previously in animals transfected with HM175/8Y RNA (10).

Viral RNA was detected in the feces of transfected animals by 2 to 3 weeks following transfection (Fig. 2). By semiquantitative slot blot hybridization (12), both animals transfected with the Δ96-137/8Y RNA were estimated to have shed virus equivalent to approximately $10^{7.0}$ to $10^{7.8}$ 50% tissue culture infective doses of cell culture-adapted virus per g of stool on days of maximal virus shedding. The timing of maximal virus excretion (2 to 3 weeks posttransfection) and the duration of shedding (approximately 2 to 3 weeks) were similar to those observed during previous transfections of marmosets with HM175/8Y RNA (10).

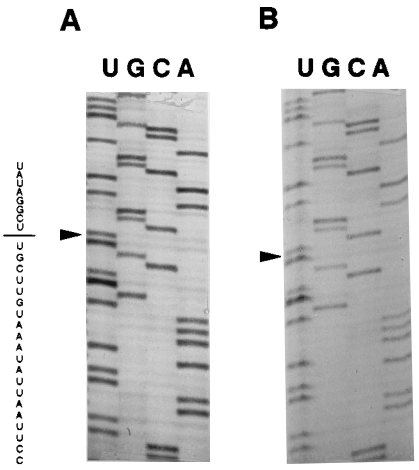

FIG. 3. RNA sequence of virus recovered following transfection of FRhK-4 cells (A) and from the stool of marmoset 788 (B), which was inoculated intrahepatically with Δ96-137/8Y RNA. The arrowhead at the left of each sequence indicates the position of the Δ96-137 deletion.

Weekly serum samples were tested for anti-HAV antibodies by a competitive inhibition immunoassay (HAVAB-EIA; Abbott Laboratories). Animal 787 seroconverted 3 weeks posttransfection, and animal 788 seroconverted 4 weeks posttransfection (Fig. 2). Although biological variability in these outbred, wild-caught marmosets may have some effect on the course of infection, animals that develop high serum liver enzyme elevations generally develop antibodies to HAV by 6 weeks posttransfection (6). In contrast, in infected animals with normal or only slightly elevated serum liver enzyme activities, seroconversion occurs later, most often between weeks 7 to 9 but occasionally as late as 22 weeks with some attenuated virus variants. Thus, the appearance of anti-HAV antibodies in marmosets 787 and 788 was consistent with the pattern observed in prior experiments involving intrahepatic transfection with virulent HAV RNA. In both animals, seroconversion coincided with peak serum liver enzyme activities, as is typical in acute hepatitis A (16).

Because the Δ96-137/8Y virus did not appear to be impaired with respect to either replication or the ability to induce acute hepatitis in marmosets, it was of interest to determine whether the Δ96-137 deletion was retained by virus shed in feces. Sequencing of viral RNA from lysates of Δ96-137/8Y RNA-transfected FRhK-4 cells and from marmoset fecal samples was carried out by using an antigen capture PCR method followed by cycle sequencing using ΔTaq DNA Polymerase (United States Biochemical Corp.) (15, 19). As shown in Fig. 3, the original Δ96-137 deletion was present in the virus rescued following RNA transfection of FRhK-4 cells as well as in the virus shed in stool by marmoset 788. Similar sequencing results were obtained with virus from the stool of marmoset 787 (data not shown). Thus, the Δ96-137/8Y virus is not impaired in replication following intrahepatic nucleic acid transfection of susceptible marmosets.

pY1 domains, containing at least three repeats of the (U)UUCC(C) sequence motif, are present in all human hepatovirus strains which have been sequenced to date (19). Although the pY1 domain occupies a position within the 5′NTR which is immediately upstream of the IRES (2, 20, 22), analogous to that occupied by poly(C) tracts in cardioviruses (5), this unique sequence element is not found in any other picornavirus. One would expect that the pY1 domain would be

TABLE 2. Influence of deletions in the upstream pyrimidine-rich tracts of the 5′NTRs of different picornaviruses on growth of virus in cultured cells and virulence in animals

Genus	Virus	Pyrimidine tract variant	Growth in cell culture[a]	Virulence
Cardiovirus[b]	Encephalomyocarditis virus	$C_{115}UCUC_3UC_{10}$[c]	+++++	$<10^{2}$[d]
		C_4	+++	3×10^{3}[d]
		C_9	+++	1[d]
	Mengovirus	$C_{50}UC_{10}$[c]	+++++	10[d]
		$C_{13}UC_{10}$	+++++	8×10^{6}[d]
Aphthovirus[e]	FMDV A_{12}	C_{185}[c]	+++++	3[f]
		C_{35}	+++++	1.5[f]
		C_2	+	1.5[f]
Hepatovirus	HM175/8Y	$U(U_3C_3)_2U_4C_3U_3C_2UAU_2C_3U_3GU_4$[c]	+++++	++[g]
		U_2[h]	+++++	++(+)[g]

[a] Plaque or replication focus size relative to wild-type virus (+++++).
[b] From Hahn and Palmenberg (14).
[c] Wild-type virus.
[d] Fifty percent lethal dose following intracranial inoculation of 4- to 6-week-old mice with virus.
[e] From Rieder et al. (18).
[f] Fifty percent lethal dose following intraperitoneal inoculation of 7-day-old mice with virus.
[g] Hepatic necroinflammatory activity in marmosets following intrahepatic transfection (10).
[h] Δ96-137/8Y virus (also lacks upstream AAA; Fig. 1).

rapidly lost from all hepatovirus strains if it were not important for some phase of the viral life cycle. However, previous studies have shown that the pY1 domain is not required for efficient replication of cell culture-adapted HAV in cultured monkey kidney cells (19). These findings were confirmed here in transfections of cultured cells with viral RNA lacking the pY1 domain as well as all but one of the mutations shown previously to contribute to growth in cultured cells (4, 7–9, 11). More importantly, the experiments described here clearly demonstrate that the pY1 domain is not required for efficient replication of HAV in primate hepatocytes in situ or for production of acute hepatocellular injury.

These results suggest that the pY1 domain of HAV may have a function which is distinct from that of the large pyrimidine-rich tracts which are located upstream of the IRESs in the 5′NTRs of the aphthoviruses and cardioviruses (5, 18). The latter are substantially longer, nearly pure poly(C) tracts, while the pY1 domain of HAV contains numerous uridylic acids, as described above (Table 2). Genetically engineered short poly(C) tract variants of foot-and-mouth virus (FMDV) were unstable during passage in cell culture and rapidly reverted to variants with longer poly(C) tracts (C_{75} to C_{140}) (18). In addition, a short poly(C) (C_2) FMDV variant which was genetically stable but replicated poorly in cell culture was shown to be virulent in mice (18). These data suggest that the poly(C) tract of FMDV is required for efficient replication in cultured cells but not for virulence in mice. On the other hand, the poly(C) tract of mengovirus is not necessary for efficient replication in cultured cells but is required for virulence in mice following intracranial or intraperitoneal inoculation (5). Recently, Hahn and Palmenberg (14) reported that short poly(C) tract variants of encephalomyocarditis virus replicated nearly as well as parental virus in cultured cells but were only minimally attenuated in mice. In contrast to FMDV and mengovirus (and resembling the situation with encephalomyocarditis virus), the pyrimidine-rich tract of HAV is required neither for replication in either cultured cells or hepatocytes in situ nor for virulence in intrahepatically transfected primates (Table 2).

The role of this unique hepatovirus sequence element remains unclear. The single-stranded RNA segment immediately downstream of the pY1 domain appears to play a critical role in replication of the viral RNA (20), but thus far it has not been possible to assign a function to the oligopyrimidine tract. It is possible that the pY1 domain is required for infection of cells within the gastrointestinal tract or in circulating leukocytes, both of which could serve as sites of primary viral replication prior to infection of hepatocytes during natural HAV infections. However, it is important to note that the mechanisms by which the virus reaches the liver and the existence of any putative sites of primary virus replication are as yet undefined (16). Because the marmoset infections described here were carried out by direct intrahepatic nucleic acid transfection, impairment in earlier steps of the infectious cycle in the primate host would not have been observed. Further studies in which Δ96-137/8Y virus rescued by RNA transfection of cultured cells is inoculated into susceptible primate hosts perorally will be required to determine whether the pY1 domain in some way enables HAV to establish infection within the liver.

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