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Progress toward the Development of a Genetically Engineered Attenuated Hepatitis A Virus Vaccine

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Mutations which positively affect growth of hepatitis A virus in cell culture may negatively affect growth in vivo. Therefore, development of an attenuated vaccine for hepatitis A may require a careful balancing of mutations to produce a virus that will grow efficiently in cells suitable for vaccine production and still maintain a satisfactory level of attenuation in vivo. Since such a balance could be achieved most directly by genetic engineering, we are analyzing mutations that accumulated during serial passage of the HM-175 strain of hepatitis A virus in MRC-5 cell cultures in order to determine the relative importance of the mutations for growth in MRC-5 cells and for attenuation in susceptible primates. Chimeric viral genomes of the HM-175 strain were constructed from cDNA clones derived from a virulent virus and from two attenuated viruses adapted to growth in African green monkey kidney (AGMK) and MRC-5 cells, respectively. Viruses encoded by these chimeric genomes were recovered by in vitro or in vivo transfection and assessed for their ability to grow in cultured MRC-5 cells or to cause hepatitis in primates (tamarins). The only MRC-5-specific mutations that substantially increased the efficiency of growth in MRC-5 cells were a group of four mutations in the 5′ noncoding (NC) region. These 5′ NC mutations and a separate group of 5′ NC mutations that accumulated during earlier passages of the HM-175 virus in primary AGMK cells appeared, independently and additively, to result in decreased biochemical evidence of hepatitis in tamarins. However, neither group of 5′ NC mutations had a demonstrable effect on the extent of virus excretion or liver pathology in these animals.

Hepatitis A virus (HAV) is endemic in many developing countries and continues to be a problem in some developed countries. In recent years in the United States, hepatitis A has accounted for as many as 50% of the cases of viral hepatitis reported to the Centers for Disease Control and Prevention (4), and the seroprevalence of antibody to hepatitis A was recently reported to be as high as 38.2% in the general population (36). Since infection with HAV results in considerably more morbidity in adults than in children, countries in which the socioeconomic standards (and therefore the average age of exposure to HAV) have risen are now observing more clinical hepatitis A. Therefore, the need for a safe and effective hepatitis A vaccine is evident. Although a formalin-inactivated hepatitis A vaccine has been licensed in the United States and is widely used in Europe, at least two doses of inactivated vaccine are required for long-lasting protection (29, 39). A live attenuated vaccine could potentially require only one dose, thereby decreasing the cost of administration and increasing long-term vaccine efficacy. Such a vaccine would be particularly important for regions in which the cost and delivery of vaccines pose impediments to their use.

Wild-type HAV grows very inefficiently in cell culture, and virus adapted to growth in one type of cultured cells may not grow efficiently in another type. Serial passage of HAV in cell culture has selected for variants that grow reasonably efficiently in cell lines suitable for vaccine production (1, 30). However, these variants were inadequately immunogenic in tamarins, chimpanzees, and/or humans (28, 30, 37), suggesting that some of the same mutations that promote growth in these cells may also attenuate the virus. Our goal is to use the information obtained from a systematic analysis of the HM175 strain of virus to engineer genetically a virus that is appropriately attenuated for vaccine use and that grows efficiently in MRC-5 cells, a licensed substrate for vaccine production.

In order to produce an HAV which grows efficiently in cell culture (Fig. 1), the wild-type HM175 virus was serially passaged in primary African green monkey kidney (AGMK) cells (10) (Fig. 1). The virus isolated after 32 passages is immunogenic without causing significant liver enzyme elevations in tamarins and chimpanzees (25), and it grows efficiently in AGMK cells (6). A full-length cDNA clone, pHAV/7 (7), was derived from passage 35 AGMK-adapted virus (Fig. 1). The virus encoded by this cDNA has 21 nucleotide changes compared with the wild-type HM175 virus (8) (Fig. 2) and in cell culture (18) and in animals (5) has characteristics similar to those of the passage 32 virus (Fig. 1). Neither the plasmid-derived virus nor the passage 32 virus grew efficiently in MRC-5 cells (17, 18). Therefore, the AGMK-adapted virus was serially passaged in MRC-5 cells to adapt the virus to growth in these cells. After nine passages, the MRC-5/9 virus, which grows efficiently in MRC-5 cells, was isolated (12).

During passage in MRC-5 cells, the virus acquired 13 new mutations and retained 20 AGMK-specific mutations (Fig. 2). In the presence of the AGMK-specific mutations, a cluster of four mutations in the 5′ noncoding (NC) region (bases 591, 646, 669, and 687) (Fig. 2) was shown to increase greatly the efficiency of growth in the MRC-5 cells (18). In addition, new MRC-5-specific mutations in the 2B-2C region and a cluster of mutations acquired in the 5′ NC region during passage in AGMK cells (bases 124, 131 to 134, 152, and 203) (Fig. 2) were found to increase the efficiency of growth in MRC-5 cells, but only when combined with the MRC-5-specific cluster of 5′ NC mutations (18). Studies of HAV growth in fetal rhesus kidney

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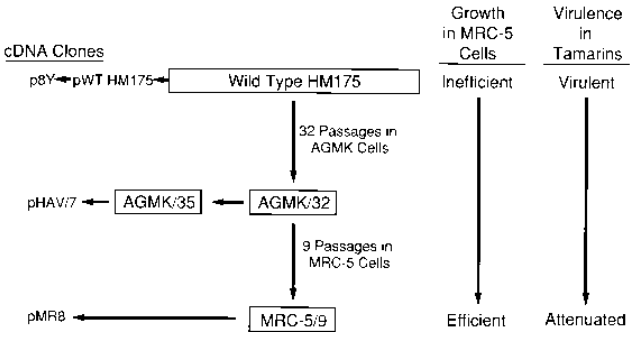

FIG. 1. Viruses and cDNA clones used in this study: virus passage history and phenotypes. Wild-type HM175 virus was previously cloned as cDNA (8), and a derivative infectious clone, p8Y (15), is used in the current study as a virulent control. Wild-type HM175 virus passaged 32 times in AGMK cells (25) was further passaged to provide the derivative cDNA clone from the AGMK passage 35 virus, pHAV/7 (7), or the MRC-5 cell passage 9 virus, MRC-5/9 (18), and its derivative cDNA clone, pMR8.

4 (FRhK-4) cells previously demonstrated that a few mutations which had a major impact on growth could be functionally replaced by a large number of mutations which individually had only a small impact (14). Therefore, although the MRC-5-specific mutations in the 5′ NC region are important, it is unclear whether MRC-5-specific mutations elsewhere in the genome can substitute for the 5′ NC mutations or whether these 5′ NC mutations are essential for growth in MRC-5 cells.

It is important to know which mutations are absolutely required for growth in MRC-5 cells because when the MRC-5/9 virus was tested as a live vaccine, seroconversion was not observed in some of the tamarins, chimpanzees (31), and humans (37) studied. Since the AGMK-adapted progenitor virus was sufficiently immunogenic as a live vaccine, some of the mutations acquired during passage in MRC-5 cells must have contributed to the low immunogenicity of the MRC-5/9 live virus. If the same mutations were responsible for growth in MRC-5 cells and for diminished immunogenicity, it would not be possible to produce an attenuated vaccine with this system. Therefore, it is also necessary to determine whether mutations which are required for propagation in cell culture affect virulence. The tamarin *Saguinus mystax* has been used in many animal studies (15, 25) because of previous work demonstrating the virulence of wild-type HAV in these animals (26) and because of their availability. Because wild-type HAV grows extremely slowly in cell culture (16), may mutate in the process, and sometimes cannot be recovered, it has been difficult to test in animals the virulence phenotype of virus amplified in tissue culture. Therefore, an important advancement for studying the virulence of mutant viruses was achieved with the successful infection of tamarins by direct intrahepatic inoculation of cDNA and transcribed RNA genomes of p8Y, which encoded a virus that differed from the wild type by a single mutation that permitted consistent recovery of the virus in cell culture but had no discernible effect on the virulence phenotype (15).

In the current study, chimeric viruses were constructed to evaluate the extent to which various mutations were important for growth in MRC-5 cells. Mutations determined to be critical for growth were then substituted into a cDNA clone (p8Y) encoding a virulent virus, and their effects on virulence in tamarins were tested.

MATERIALS AND METHODS

Cells. Since the continuous cell line of FRhK-4 cells was permissive for all viruses studied, these cells were used in most experiments as a positive control (18). A particularly robust subclone (11-1) of the FRhK-4 cell line was used in radioimmunofocus assays (RIFA). MRC-5 cells (ATCC CCL 171) were obtained from the American Type Culture Collection (Rockville, Md.). All cells were maintained in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, nonessential amino acids, glutamine, 50 μg of gentamicin sulfate per ml, and 2.5 μg of amphotericin B (Fungizone) per ml (10% Dulbecco modified Eagle's medium). After treatment with trypsin, MRC-5 cells were split 1:2 or 1:3 and FRhK cells were split 1:2 to 1:10.

cDNA clones and in vitro transcription. Three infectious cDNA clones in pGEM1 (Promega Corporation, Madison, Wis.) were used as the basis for new chimeric viruses: an infectious cDNA clone which encodes an attenuated virus adapted to efficient growth in AGMK cells (pHAV/7) (7), one which encodes a virulent virus which can be consistently recovered in cell culture (p8Y) (15), and one which represents most of the genome of the MRC-5-adapted virus (pMR8), constructed by sequential replacement of pHAV/7 restriction fragments with those generated by reverse transcription-PCR (RT-PCR) amplification of the MRC-5/9 virus genome (Fig. 1). Figure 2 identifies the 15 nucleotide changes that differentiate pHAV/7 from pMR8. The pHAV/7, p8Y, and pMR8 clones were used to construct additional chimeric infectious full-length cDNA clones (Fig. 3). After transformation of *Escherichia coli* with full-length cDNA clones, the plasmid cDNA was purified either by alkaline lysis and CsCl density gradients or with the Magic Maxipreps DNA purification system kit (Promega Corporation). The nucleotide sequences of all chimeric clones were determined at restriction enzyme sites used in cloning and at selected sites to confirm that the MRC-5/9-derived insert contained the expected MRC-5-specific mutation(s). The cDNA clones and PCR products were sequenced with the Applied Biosystems 373A automated DNA sequencer and a modified Sanger method. All mutations newly identified in this study were confirmed with the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corporation, Cleveland, Ohio) in accordance with the manufacturer's instructions. In vitro transcription from the Sp6 promoter of pGEM1 was performed to produce RNA for transfection (18).

In vitro and in vivo transfection. FRhK-4 cells were transfected by the DEAE-dextran method as previously described (18). Transfection of at least two sister clones was performed to confirm the phenotype. In vivo transfection of tamarins with a mixture of cDNA and transcribed RNA was performed by laparotomy with direct injection of the liver as previously described (15). One clone of each mutant cDNA was tested by inoculation of two animals with equal aliquots of the same transfection mixture. Full-length clones were transcribed in vitro by using 10 μg of DNA in each of two identical 100-μl transcription reaction mixtures, as previously described (15). These two reaction mixtures were combined and diluted in phosphate-buffered saline without calcium or magnesium. The final mixture was then divided into two equal aliquots, frozen on dry ice, and stored overnight at −80°C.

Growth curves. Growth curves of virus in MRC-5 and FRhK-4 cells were performed as previously described (18), except that cells were grown in 96-well plates and were inoculated with virus at a multiplicity of infection of 6 radioimmunofocus-forming units per cell. Since previous experience growing HAV in FRhK-4 cells suggested that the vast majority of the virus was cell associated (17), in initial experiments the medium was discarded. However, subsequent experiments showed that although the proportion of cell-associated virus did not vary significantly from one mutant virus to another, up to 30 to 50% of infectious virus was released from the MRC-5 cells. Thereafter, cell-associated and free virus were combined to estimate total virus. In order to keep the number of samples at a manageable level, duplicate determinations were not performed within experiments, but rather complete experiments were repeated at least twice with at least two sister clones to confirm the growth phenotype.

Virus quantification. Slot blot hybridization was used to quantify viral RNA. Cells and/or media were extracted either with Trizol reagent (Gibco BRL, Gaithersburg, Md.) as instructed by the manufacturer or with phenol after proteinase K digestion (5). Viral RNA blotted onto a nitrocellulose membrane was probed with a ^{32}P-labeled negative-strand RNA probe as described previously (18). When nylon membranes were used, the blot was probed with negative-strand RNA labeled with digitoxin by using the Genius System, version 2.0 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and signal was detected by chemiluminescence as instructed by the manufacturer. Autoradiographs were scanned with a model 620 densitometer equipped with the 1D Analyst software package (Bio-Rad Laboratories, Richmond, Calif.). Since slot blot hybridization measures the number of RNA genomes rather than the number of infectious particles, a RIFA was also performed to confirm the relative growth characteristics of every pair of viruses compared by slot blot hybridization. The RIFA was additionally able to quantify virus at early time points, when the virus concentration was below the level of detection of the slot blot. Harvested virus stocks were quantified by RIFA performed in FRhK-4 (subclone 11-1) cells as previously described (18). A RIFA to estimate focus size in MRC-5 cells was performed in the same manner as that in subclone 11-1 cells, except that an additional 5 ml of agarose overlay was added after 5 days of incubation.

Housing and maintenance of tamarins. Primates were individually housed under biosafety level biohazard containment. The housing, maintenance, and care of the animals met or exceeded all requirements for primate husbandry.

Monitoring of tamarins. Weekly serum samples were tested for alanine aminotransferase (ALT) and isocitrate dehydrogenase (ICD) levels by standard

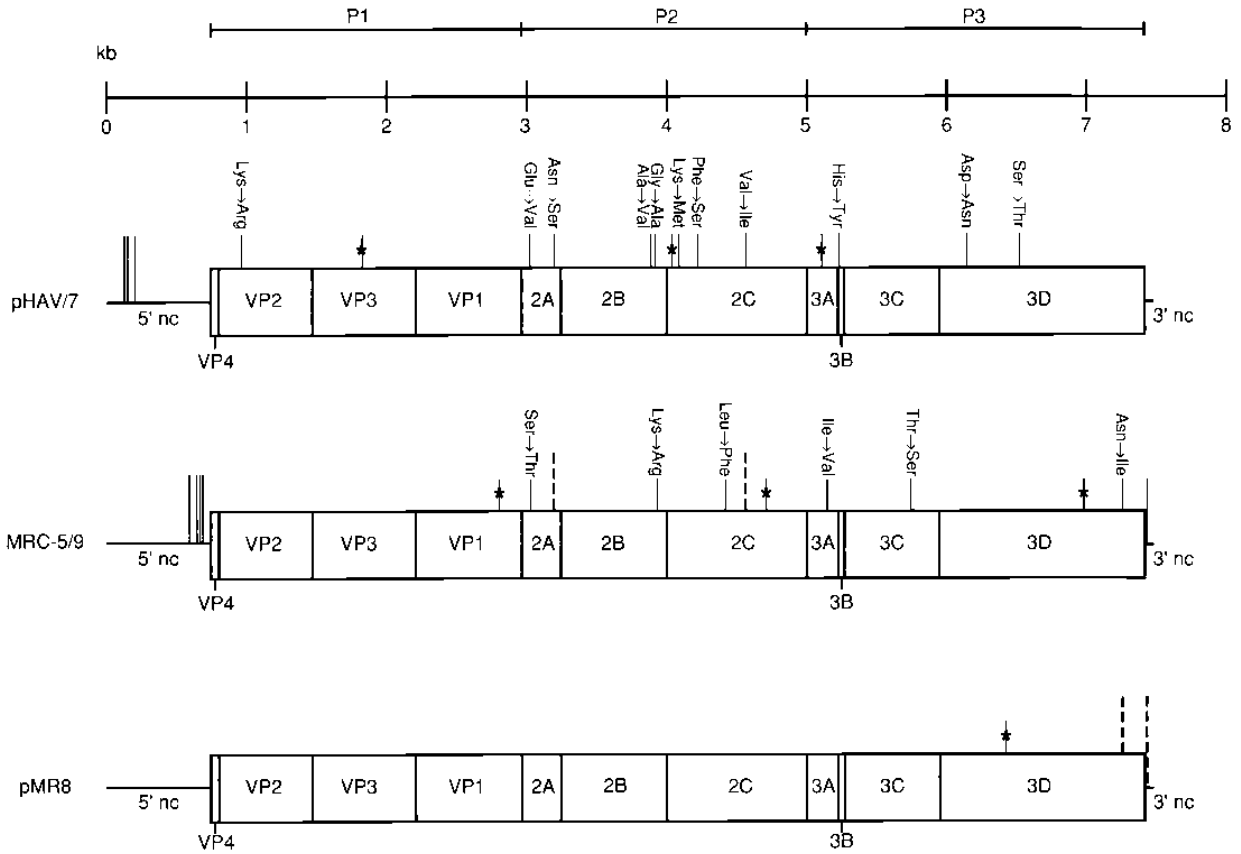

FIG. 2. Map of mutations which distinguish pHAV/7 from wild-type HM175 virus, MRC-5/9 virus from pHAV/7, and pMR8 from MRC-5/9 virus. The boundaries between genes VP1, 2A, and 2B are derived from recently available data on functional cleavage sites (33). A vertical line signifies a mutation which is not in the comparison virus, while a broken line signifies a mutation which is present in the comparison virus but is absent from the virus indicated. A silent mutation in the coding region of the genome is indicated by a line with an asterisk. A missense mutation is indicated by a line with the amino acid change written above it. A silent mutation at base 6383 in the 3D gene of pMR8 is not present in the MRC-5/9 virus and is presumed to be an artifact of PCR. Mutations at bases 7255 and 7430 are present in MRC-5/9 but not in pMR8. Two of the mutations present in the passage 35 AGMK-adapted virus (at bases 7032 and 7430) are not present in pHAV/7. The absence of mutations 3196 and 4563 in the 2A and 2C genes, respectively, of the MRC-5/9 virus most likely represents back mutations in the MRC-5/9 virus that occurred during passage in MRC-5 cells, since these mutations are present in the RT-PCR-amplified product of the progenitor passage 32 AGMK-adapted virus (17). The mutation at base 3934 resulting in a lysine-to-arginine amino acid change in the 2B region of the MRC-5/9 virus was previously reported incorrectly (18) to be a silent mutation. Mutations at bases 2864 in VP1 and 6216 in 3D, which were previously reported (18) to have occurred during passage in MRC-5 cells, were present in some of the wild-type HM175 clones (6, 8) and are both present in the RT-PCR-amplified product of the progenitor passage 32 AGMK-adapted virus (17).

methods (Metpath, Rockville, Md.) and for seroconversion to anti-HAV positivity with the HAVAB commercial assay kit (Abbott Laboratories, North Chicago, Ill.). Needle liver biopsies were performed prior to inoculation and on a weekly basis except for the first week. After standard processing and staining with hematoxylin and eosin, histopathology was evaluated under code by one of the authors (S.G.). Specimens were graded on an arbitrary scale of 0 to 4+, with 0 representing normal liver histology and 4+ representing severe hepatitis. Less severe histologic changes included evidence of periportal inflammation with lymphocytosis and/or monocytosis. Moderate to severe changes included extension of inflammation into the parenchyma with or without hepatocytolysis. Severe changes included extension of inflammation from one portal tract to the next (26).

Feces were collected weekly and stored at −20°C. Ten percent (wt/vol) fecal suspensions were prepared as previously described (15), except that phosphate-buffered saline without magnesium or calcium was used to prepare the suspension. Virus in feces was quantified by RIFA with subclone 11-1 cells as previously described (18), with the following modifications: the coverslips were not harvested until day 21 after inoculation, 0.1 mg of neomycin (G418; Gibco BRL) per ml was added to the agar overlay, and an extra 2.5 ml of agar overlay was added on days 7 and 14 after inoculation. Since the MR8 virus grew faster in cell culture than did all the other molecularly cloned viruses, RIFA cultures inoculated with stools of animals that received this viral construct were harvested on day 10. The peak titer of excreted virus was determined in duplicate, and the results were averaged. In several samples, RIFA was unsuccessful because bacteria in the feces were not inhibited by the antibiotics and destroyed the cells. In those cases, slot blot hybridization of a fecal extract was used to estimate the number of viral genomes. For this purpose, fecal extractions were performed by the proteinase K method (see above), and 150 μl of extract was blotted onto the nitrocellulose membrane. A preinoculation sample from the same animal served as a negative control. Samples were collected and tested weekly until the virus was no longer detected in the stool. Stools from the animals that were infected with the MR8 virus were also assayed for virus by RT-PCR. RT-PCR was performed on stools obtained at or near the peak of virus excretion as determined by RIFA. Primers for PCR amplified the 5′ NC region or the VP1 region.

RESULTS

Growth characteristics of MR8 virus. The MR8 virus, which was encoded by a cDNA plasmid containing most of the MRC-5-specific mutations, was compared with the AGMK-cell-adapted virus (HAV/7) and to the MRC-5-cell-adapted virus (MRC-5/9) for growth in cell culture. Samples collected at various time points on 7-day growth curves of the three viruses in MRC-5 cells were assayed by RIFA. The MRC-5/9 and MR8 viruses both increased in titer by a factor of $10^{2.5}$ to $10^{3.0}$ in the MRC-5 cells, whereas growth of HAV/7 was not detected over the same time period (Fig. 4). Radioimmunofocus sizes of the MRC-5/9 and MR8 viruses were compared in MRC-5 and FRhK-4 subclone 11-1 cells. Both the MRC-5/9 virus and the MR8 virus produced a greater average radioimmunofocus size in MRC-5 cells than in 11-1 cells. However, in MRC-5 cells, the MR8 virus had a somewhat smaller focus size

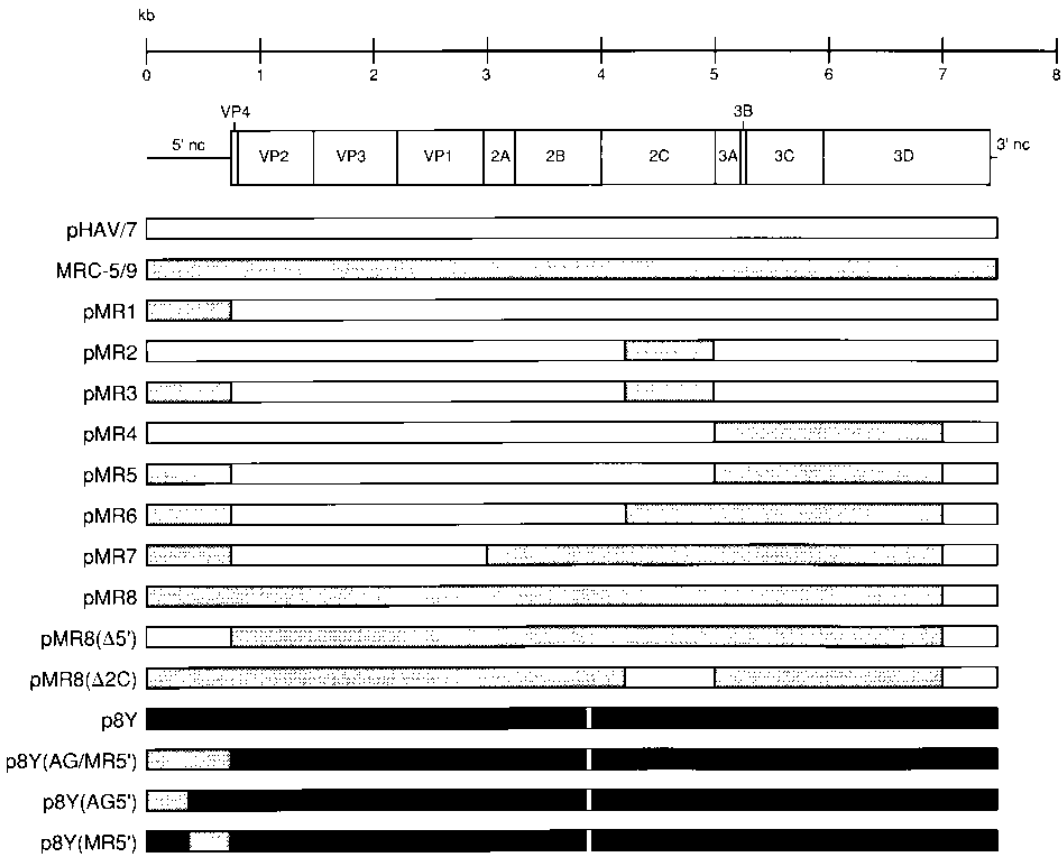

FIG. 3. Genomic diagrams of viruses studied. Regions of the genomes derived from the cDNA clones pHAV/7 (open bar) and pWT HM175 (solid bar) and from RT-PCR fragments amplified from the MRC-5/9 virus (shaded bar) are indicated.

than did the MRC-5/9 virus (Fig. 5). Therefore, the comparative RIFA assays detected subtle differences between the MR8 and MRC-5/9 viruses that were not obvious from the growth curves. The presence of the MRC-5-specific mutation at base 7255 in 3D and the AGMK-specific mutation at base 7430 in the 3′ NC region in the MRC-5/9 virus but not in the MR8 virus, the differences in the lengths of the termini (9, 22), or the presence of the silent mutation at base 6383 in the MR8 virus but not in the MRC-5/9 virus (see the legend to Fig. 2) most likely accounts for the small differences noted.

5′ NC mutations are more important than coding mutations for efficient growth in MRC-5 cells. Whereas the chimera containing MRC-5-specific mutations only in the 5′ and 2C regions (MR3) grows relatively efficiently in MRC-5 cells, the MRC-5/9 virus grows significantly better in these cells (18). The MR8 virus, which contained 12 of the 13 MRC-5-specific mutations (including mutations in 2A, 2BC, 3A, 3C, and 3D), appeared to grow much more like the MRC-5/9 virus (Fig. 4) than like the MR3 virus (data not shown), suggesting that MRC-5-specific mutations outside of the 5′ NC and 2C regions of the genome were contributing to the efficiency of growth. In order to test this hypothesis, chimeras identical at all nucleotide sites except the site(s) of interest (Fig. 3) were compared in multiple growth curve experiments. Virus replication was quantified at two to six time points per growth curve by slot blot hybridization. Tabulations from multiple experiments included assessment of the hierarchy of growth at each time point and the mean $\log_{10}$ increase in RNA accumulation over the entire time course of each experiment.

The effect of the MRC-5-specific mutations in the P3 region on growth was tested by comparing growth of the MRC-5/9 virus to that of chimeras which contained the P3 mutations in the absence (MR4) or presence (MR5) of the 5′ NC mutations. Viruses were inoculated onto MRC-5 cells, and growth was assayed by slot blot hybridization. Only the MRC-5/9 virus and the MR5 chimera, which had the MRC-5-specific 5′ NC mutations, were able to replicate sufficiently well to produce a signal (Fig. 6A), and the MR5 chimera with the 5′ NC and P3 mutations still grew less efficiently than the MRC-5/9 virus. Since analysis of multiple growth curves suggested that the MR5 virus grew with an efficiency similar to that of MR1 (with only the 5′ NC mutations), the P3 mutations assayed appeared to have little independent effect on growth and certainly could not substitute for the 5′ NC MRC-5-specific mutations. We then examined other MRC-5-specific mutations in the open reading frame for their effect on growth. The MRC-5-specific mutations in 2C [MR2 versus HAV/7, MR1 versus MR3, or MR8 versus MR8(Δ2C)], 2AB (MR6 versus MR7), or VP1 (MR8 versus MR7) had no significant incremental effect on growth efficiency in MRC-5 cells, since chimeras containing the region of interest grew no better than chimeras lacking that region.

Since there were clearly mutations outside the 5′ NC region contributing to growth efficiency (Fig. 6A), but small sets of mutations in the open reading frame appeared to have little effect on growth, we hypothesized that mutations in multiple genes in the open reading frame must be combined in order to have a significant effect on growth efficiency. Therefore, to

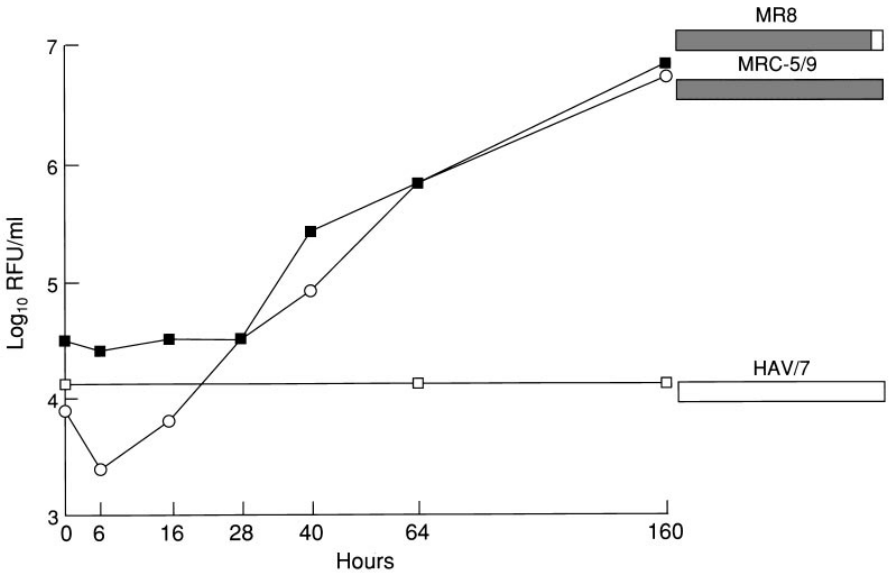

FIG. 4. Growth curves generated in MRC-5 cells. Values at each time point represent virus titer (log_{10} radioimmunofocus-forming units [RFU] per milliliter) as quantified by RIFA. Biologically cloned MRC-5/9 virus (○) was compared with cDNA-cloned, rescued viruses MR8 (■) and HAV/7 (□). Total virus was plotted as the sum of the titers of free and cell-associated virus.

determine whether MRC-5-specific mutations in the open reading frame could collectively replace the function of the mutations in the 5′ NC region, we removed the four 5′ NC mutations from the MR8 clone to produce MR8(Δ5′) (Fig. 3). Growth curves for this chimeric virus and MR8 virus, performed in MRC-5 cells and in FRhK-4 cells (a permissive control cell line), were compared, and samples obtained at various time points were assayed by slot blot hybridization (Fig. 6B). Although removal of the MRC-5-specific 5′ NC mutations led to a slight decrease in growth, both viruses still grew efficiently in the FRhK-4 cells. In contrast, whereas growth of the MR8 virus was comparable in FRhK-4 and MRC-5 cells, growth in MRC-5 cells was dramatically reduced by removal of the 5′ NC mutations. These data demonstrated that the sum contribution of mutations in the open reading frame to growth in MRC-5 cells was much less than that of the 5′ NC mutations. In combination with the above data on the lack of incremental effects of mutations in VP1, 2AB, 2C, and P3, these data furthermore suggested that the growth of the MR8(Δ5′) virus in MRC-5 cells (Fig. 6B) was most likely due to the cumulative action of multiple genes.

Effect of the 5′ NC mutations on virulence in tamarins. From the in vitro growth data, it was clear that an MRC-5-adapted live HAV vaccine would have to include some or all of the mutations in the 5′ NC region of the MRC-5/9 virus in order to make production practical. Since the MRC-5/9 virus was significantly less immunogenic than its progenitor (31), some or all of the mutations acquired during adaptation to growth in MRC-5 cells may have been responsible and might have to be eliminated to produce a virus with the proper balance between attenuation and virulence. The most critical question, then, was whether the 5′ mutations required for efficient growth in MRC-5 cells were also responsible for the increase in attenuation after passage in MRC-5 cells or whether the two phenotypes could be dissociated. Since both the AGMK-specific and MRC-5-specific mutations in the 5′ NC region enhance the efficiency of growth in MRC-5 cells (18), we analyzed the effect of the two groups of mutations, individually and together, on the virulence phenotype.

In order to weight the duration as well as the degree of elevation of biochemical parameters of hepatitis, virus excretion, and histopathology scores, each was summed to produce a comprehensive measure of biological response (Table 1). ALT and ICD enzymes displayed similar patterns of elevation (Fig. 7); therefore, for simplicity, only one biochemical parameter (ICD) is discussed here. For each animal, the geometric mean titer of five preinoculation ICD levels was compared with the peak postinoculation level (Table 1). The ICD levels during the period of viral excretion were also summed as a measure of the cumulative biochemical response during the presumed period of active hepatitis, and the geometric mean for each pair of animals was determined.

The substitution of the AGMK-specific, the MRC-5-specific, or the AGMK- and MRC-5-specific 5′ NC mutations into the background of the 8Y virulent genome decreased biochemical evidence of hepatitis in tamarins infected with the respective viruses compared with those infected with 8Y (Fig. 7; Table 1). The geometric means of the preinoculation ICD levels were

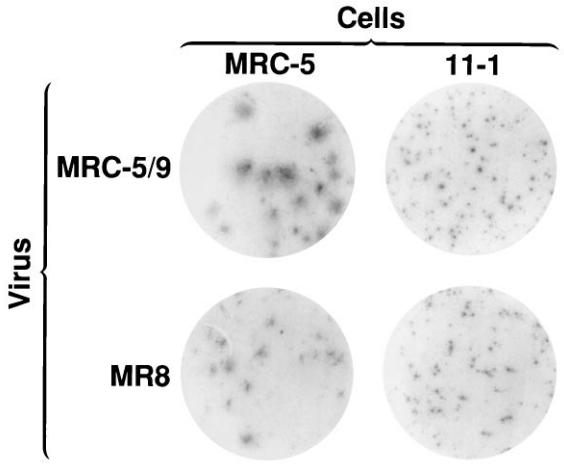

FIG. 5. Autoradiograms showing radioimmunofocus sizes of the MR8 and MRC-5/9 viruses in MRC-5 and 11-1 cells.

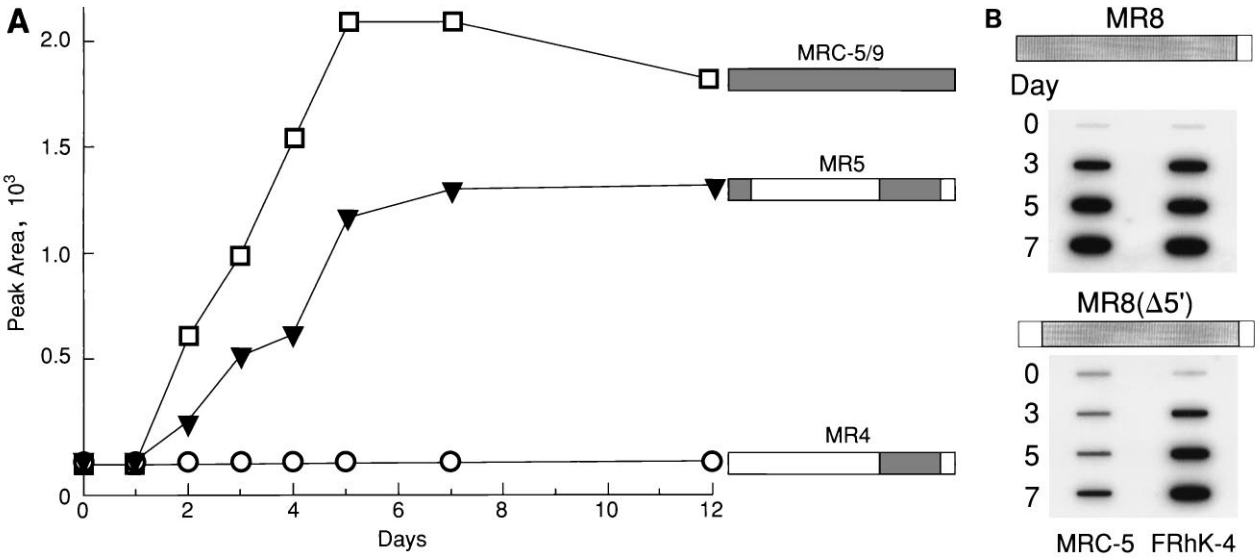

FIG. 6. (A) Growth curves generated in MRC-5 cells. Cell-associated virus was harvested, and total viral genomic RNA was quantified by slot blot. The *y* axis represents peak area in optical density in millimeters (see reference 18, Fig. 3), determined by densitometry of autoradiograph. (B) Autoradiograms of slot blot hybridization assays of growth curve samples of chimeric HAVs in MRC-5 cells and FRhK-4 cells. Cell-associated and free virus were harvested together at the indicated intervals, and virus RNA was quantified by slot blot hybridization with a ^{32}P-labeled negative-strand RNA probe. The bar diagram represents the viral genome, with the shaded bars representing MRC-5/9 sequences and the white bars representing HAV/7 sequences.

similar among all the animals (Table 1). The peak ICD level of one animal that received the 8Y genome was threefold higher than that of the second animal that received the same inoculation. However, their cumulative ICD levels differed by only 1.5-fold, reflecting the fact that whereas animal 682 had a very high peak elevation, animal 641 displayed a more moderate but prolonged peak elevation (15). The tamarins that received the 8Y genome modified to include one or the other set of 5′ NC mutations had cumulative ICD levels that were similar to each other but were 1.5 to 3 times lower than those in the animals receiving the 8Y genome itself. The two animals that received the 8Y genome modified to contain both sets of 5′ NC mutations displayed even lower cumulative ICD levels, but they were still higher than those of the animals that received the MR8 genome. This trend of decreasing cumulative ICDs was confirmed by comparing the geometric mean titers for each pair of animals in the study. The geometric mean cumulative ICD levels ($\times\!\div$ standard deviations of the means) of animals that received wild-type 8Y RNA (21,530 $\times\!\div$ 1.3 U/ml) were greater than those of animals that received RNA containing only one group [8Y(AG 5′)] or the other [8Y(MR 5′)] of mutations in the 5′ NC region (geometric mean cumulative ICD, 9,890 $\times\!\div$ 1.2 U/ml and 10,634 $\times\!\div$ 1.1 U/ml, respectively), which were greater than those of animals that received RNA

TABLE 1. Biochemical, immunologic, virologic, and histopathological responses of 10 tamarins to intrahepatic inoculation of mutant HAV genomes

Virus	Tamarin number	ICD concn (sigma U/ml)			Cumulative virus excreted in stool (RFU/g of stool)[c]	Cumulative liver histopathology score[d]	Week of seroconversion
		Preinoculation geometric mean[a]	Peak	Cumulative during virus excretion[b]			
8Y	641	1,034	5,041	17,477	1.4×10^8 (6)	11+	6
	682	820	15,159	26,523	6.6×10^7 (5)	7+	5
8Y(AG5′)	775	433	4,618	11,579	3.2×10^8 (6)	10.5+	3
	776	627	6,094	8,448	1.5×10^6 (3)	3.5+	4
8Y(MR5′)	780	1,012	6,860	11,551	3.4×10^7 (5)	4+	4
	781	534	4,329	9,790	7.2×10^7 (4)	6.5+	4
8Y(AG/MR5′)	778	554	3,164	5,143	1.7×10^8 (4)	20+	4
	779	630	2,897	6,039	3.8×10^7 (4)	8.5+	3
MR8	795	772	1,156	3,363	2.8×10^5 (4)	0	7
	796	666	1,107	1,699	8.0×10^5 (3)	0	23

[a] The values are geometric means of the data for five preinoculation serum samples.
[b] The values are the sums of ICD levels obtained during the period in which virus was recovered from the stools by RIFA.
[c] The values are the sums of the radioimmunofocus-forming units (RFU) per gram of feces isolated from weekly stool samples from the time of inoculation until the first negative virus culture was obtained, as detected by RIFA. The values in parentheses are the total numbers of weeks of virus excretion.
[d] The values given are the sums of all histopathology scores from weekly liver biopsies (rated on a scale of 0 to 4+) through week 12 postinoculation (with the exception of tamarin number 641, who died after 9 weeks). Since many animals had baseline scores of 1+, only scores over 1+ were included in the cumulative scores.

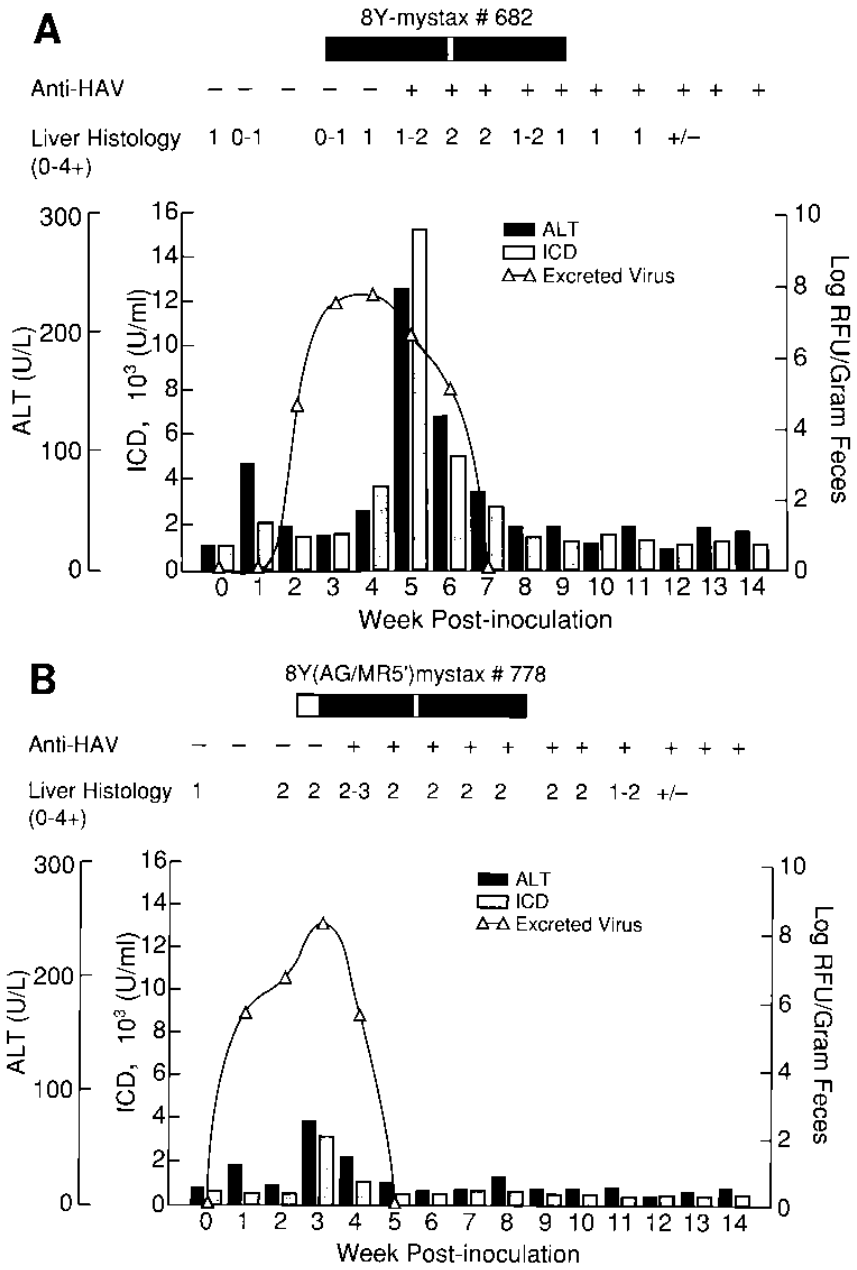

FIG. 7. Examples of the biological responses of individual tamarins to intrahepatic injection with RNA transcribed from the wild-type HM175 genome with a C-to-U mutation at base 3889 (p8Y) (A), from p8Y with MRC-5-specific and AGMK-specific 5′ NC mutations [p8Y (AG/MR 5′)] (B), and from a cDNA clone derived from the MRC-5-adapted virus (pMR8) (C). ICD and ALT levels in serum are shown, as are quantities of infectious virus excreted in the feces. Results of the liver histology and serology tests are noted at the top of each graph.

containing both groups [8Y(AG/MR 5′)] of mutations (5,573 $\times/\div$ 1.1 U/ml), which were greater than those of animals that received the attenuated MR8 genome (2,390 $\times/\div$ 1.6 U/ml).

Despite the apparent effect of the 5′ NC mutations on levels of liver enzymes in serum, other measures of hepatitis were not obviously affected by the presence of these mutations alone. A correlation was not found between cumulative virus excretion or the length, the peak quantity, or the timing of virus excretion with the presence or absence of 5′ NC mutations in the virulent background. All animals that were inoculated with RNA of a virulent background showed significant histopathological evidence of hepatitis (Fig. 7; Table 1), though the extent and duration varied. We did not detect a correlation between histopathology and the presence or absence of the 5′ NC mutations.

In contrast, the two animals that were inoculated with MR8 RNA had normal liver histology. Whereas animal number 795 seroconverted 7 weeks after transfection with MR8 RNA, animal number 796 seroconverted after 23 weeks, significantly later than all of the other animals (Table 1). The stools of both animals inoculated with MR8 RNA were investigated by RT-PCR and RIFA for evidence of virus excretion. Stools from both animals were positive for virus by RIFA for at least 3 consecutive weeks and by RT-PCR for at least 6 consecutive

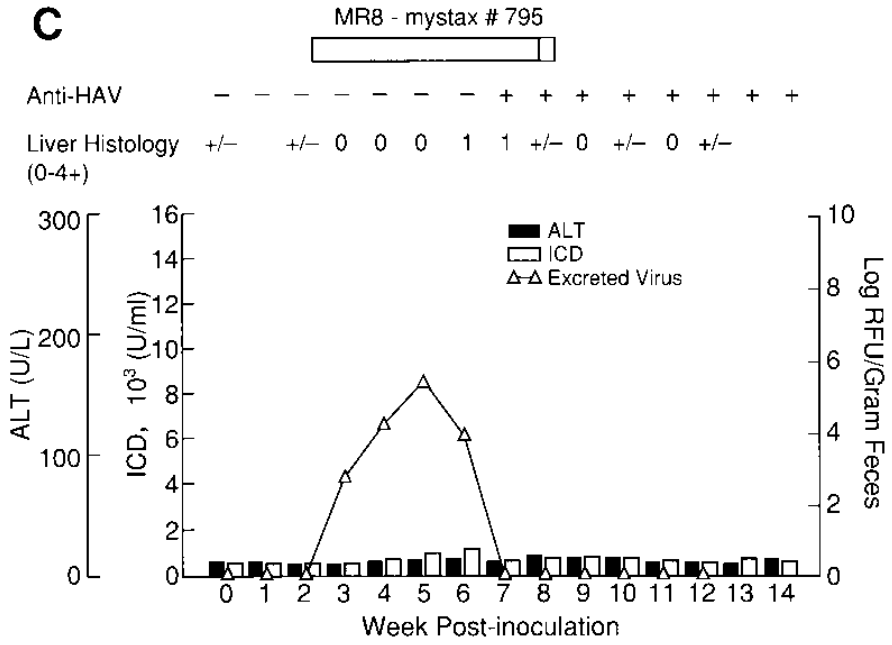

FIG. 7—*Continued.*

weeks. However, the quantity of virus excreted was considerably lower than that from animals receiving either the 8Y or the 8Y/5′ NC chimeric genomes (Table 1).

RT-PCR was performed on stools of every animal at or near the peak of virus excretion in order to confirm that identifying mutations were retained during the infection. In every case, the virus recovered from the stool had the same nucleotide sequence in the relevant region of the genome as had been present in the cDNA clone and RNA inoculated into that animal's liver.

DISCUSSION

The major challenge in development of a live vaccine has been to achieve a balance among attenuation, adaptation of the virus to efficient growth in cell culture, and retention of sufficient immunogenicity in the host to provide adequate protection without producing disease. Several groups have been actively pursuing the development of live attenuated HAV vaccines by serial passage of wild-type virus in cell culture to increase the efficiency of growth. The experiences with these vaccines in primates and human subjects that have been described in publications to date suggest that a high dose of attenuated vaccine given subcutaneously or intramuscularly is required for seroconversion (28, 37) and that seroconversion may be impossible to achieve by oral administration (37). Questions about safety remain for vaccines that are highly immunogenic (32). Therefore, the delicate balance of satisfactory attenuation and safety of a live virus that efficiently grows in a cell line suitable for vaccine production has not yet been achieved. By genetic mapping of the mutations responsible for the HAV phenotypes of attenuation in vivo and efficient growth in vitro, we are hoping to move closer to the construction of a virus with an appropriate level of attenuation and reasonably efficient growth in cell culture.

In the current study, we demonstrated that a set of four mutations in the 5′ NC region was absolutely required for efficient growth of the MRC-5/9 virus in MRC-5 cells. Although a chimera lacking the MRC-5-specific 5′ NC mutations did replicate to a very low level in MRC-5 cells (Fig. 6B), this replication required mutations in multiple genes and perhaps most, if not all, of the other mutations which accumulated during adaptation to growth in these cells. Individual or small groups of mutated genes were unable to promote growth in the MRC-5 cells (18) (Fig. 6A). The 5′ NC mutations had such a dramatic effect on growth that it proved extremely difficult to evaluate the contributions of individual genes since each mutated gene had to be assayed in the context of the dominant 5′ NC mutations and hence in the presence of significant baseline growth. We reported previously that the mutations in the 2C gene augmented growth when paired with the MRC-5-specific 5′ NC mutations. In the current study we found that this supplementation was not consistently different than that contributed by the P3, 2A-2B, or VP1 mutations. Therefore, although each of these mutated genes or regions might have an incremental role in increasing growth, the assay systems available are not sensitive enough to rank them for importance, and they probably have a serious impact only in aggregate. Thus, the MRC-5-specific 5′ NC mutations are the only ones that are required to be incorporated into a vaccine strain to allow propagation in MRC-5 cells; therefore, they were the only set of MRC-5-specific mutations we tested for their effect on virulence.

The AGMK-specific and MRC-5-specific groups of 5′ NC mutations, together or independently, did appear to decrease one parameter of virulence, since serum levels of both ALT and ICD were lower in animals receiving genomes with these mutations than in animals receiving the unmodified 8Y genome. The biochemical data also suggested that the effects of the two groups of 5′ NC mutations were additive. These conclusions differ from a previous report suggesting that the AGMK-specific 5′ NC mutations had no clear effect on virulence (5). The most likely reason for this discrepancy in the two studies is that the 5′ NC mutations were assayed in different genetic backgrounds. In the current study, the 5′ NC mutations were assayed in a background which normally produced very

high enzyme levels, so moderate decreases were obvious, whereas previously the 5′ NC mutations were evaluated for their ability to further decrease enzyme levels that were already close to baseline because of mutations in the P2-P3 region. However, in the current study, even though the enzyme levels were decreased, neither the level of virus excretion nor histopathological evidence of hepatitis appeared to be affected by the presence or absence of 5′ NC mutations. These data suggested that whereas the 5′ NC mutations may have diminished one marker of hepatitis, they clearly were not sufficient to cause overt attenuation.

It is not clear how the 5′ NC mutations affect either virulence or growth in cell culture. The AGMK-specific mutations are located between bases 124 and 207 near a poly(C · U) tract (35) and may overlap the internal ribosome entry site (20, 23), the 5′ end of which may start somewhere between bases 151 and 251 (3, 19). Interestingly, deletion of the poly(C · U) tract from base 96 to base 137 had no apparent effect on viral replication in cell culture or in tamarins (35), suggesting that only very particular changes in the RNA secondary structure in this region of the genome have a significant effect on the phenotype. One or more of the AGMK-specific mutations have a cell-specific effect on growth efficiency; they are required for efficient growth in CV-1 cell culture but not in FRhK-4 cell culture (16), and the mutation at base 152 in this group also increases growth in BS-C-1 cells (11). Recently, Schultz et al. (34) reported that two mutations (the UU deletion at bases 203 and 204 and the U-to-G mutation at base 687) in the 5′ NC region of another cell culture-adapted HM175 variant (24) increase the efficiency of cap-independent translation in BS-C-1 cells but not in FRhK-4 or Huh7 cells. The identical mutation at base 687 of the MRC-5/9 virus might therefore be expected to increase translation in MRC-5 cells as well, and it is possible that the analogous deletion of one U between bases 203 and 207 in the MRC-5/9 virus also has a cell-specific effect on translation. Certainly, the cell specificity of the effects of the group of AGMK-specific mutations suggests an interaction with cellular factors, and the proximity of the internal ribosome entry site suggests that translation might be involved. However, a role for at least some of these mutations in RNA replication is also a possibility and would not be unprecedented among picornaviruses, since at least 126 nucleotides at the 5′ end of the rhinovirus-14 5′ NC region may be involved in the initiation of plus-strand RNA synthesis (27). The MRC-5-specific mutations, which also have a cell-specific effect on growth, occur between bases 591 and 687 in the putative stemloop V (2) within the internal ribosome entry site and just before the translation start codon, so they appear to be prime candidates for translation modulators. This hypothesis is supported by the data of Schultz et al. (34) mentioned above. Poliovirus provides a precedent for attenuation mediated by a mutation which affects translation in vitro (apparently to different degrees in the presence of different cellular factors) and which alters the ability of the virus to grow in different cells (21). Since both sets of 5′ NC mutations greatly affect the ability of HAV to grow in specific cells, it will be interesting to determine if the decreased liver enzyme levels in serum that are observed in the presence of histopathological changes and the high levels of viral excretion reflect a differential ability to replicate in some cell types in the liver.

Although a considerable body of data suggests that host response, particularly the cellular immune response (38), may play a significant role in the development of liver damage in response to HAV, none of the studies evaluating host response has addressed the effect of small differences in viral sequence on that response. In our study, differences in the level of attenuation as measured by three different parameters (biochemical evidence of hepatitis, quantity of virus shedding, and histopathology) were easily detected and consistent among the parameters when comparing viruses that were different at multiple loci (i.e., 8Y versus MR8). Karron et al. (25) also found a consistent correlation among these three parameters when studying three viruses that were quite different in passage history (the wild-type HM175 virus, the passage 21 AGMK-adapted HM175 virus, and the passage 32 AGMK-adapted HM175 virus). However, we found what appeared to be divergent results in biochemical evidence of hepatitis versus histopathology and viral excretion when we compared the animals that had received virulent virus (8Y) and viruses with 5′ NC mutations in the same virulent background [8Y(AG/MR 5′), 8Y(AG 5′), and 8Y(MR 5′)]. The consistency of the findings between each of the two animals that received a given construct applied to all three parameters, suggesting that the divergent results with some mutant viruses (Table 1) may be a reproducible biological phenomenon and not simply due to biological variation in the host response. Although it is thought that elevation in liver enzyme levels in serum results from hepatocyte lysis, data from tamarins (26) and chimpanzees (13) with disease caused by wild-type HAV show that marked histologic abnormality can persist long after liver enzymes normalize. Since the histopathological scoring in this study represents a summary of a number of histologic parameters, including the extent of both hepatocyte lysis and infiltration of inflammatory cells, it is not clear whether the abnormality in histology seen in the animals in this study with divergent histopathological and biochemical responses to hepatitis reflects cell lysis with little enzyme elevation or inflammatory infiltration with little enzyme elevation. This question is the subject of ongoing investigation.

Previous studies have clearly shown that tamarins, chimpanzees, and humans can have different biological responses to a given strain of live HAV vaccine (30). Neither animal model appears to predict consistently the human biological response to HAV, and to date there is no reliable in vitro predictor of the in vivo virulence phenotype. From a practical point of view, since neither liver pathology nor elevated liver enzymes in primate animal models are acceptable for a candidate vaccine, both pathological and biochemical evidence of hepatitis need to be followed closely, and several different animal models need to be tested in the preclinical study of candidate HAV vaccines.

The present study did show that the phenotypes of efficient growth in MRC-5 cells and attenuation for tamarins overlap and are coordinately affected by the MRC-5-specific 5′ NC mutations. However, whereas these mutations were absolutely required for efficient growth in MRC-5 cells, they had much less of an impact on virulence and did not significantly impair the ability of the virus to replicate in vivo. Therefore, the production of an attenuated HAV vaccine in MRC-5 cells remains feasible. The challenge now will be to incorporate into the 8Y genomes containing 5′ NC mutations a subset of mutations selected from the coding region of the MR8 virus genome to produce a combination of mutations that will permit efficient growth in cell culture and attenuate the virus sufficiently to reduce clinical hepatitis but will still permit enough virus replication to induce a strong antibody response. The ability of HAV to grow efficiently in cell culture is clearly determined by multiple mutations throughout the genome (5, 14, 16, 18). This pattern of interdependence of mutations may also hold true for the virulence phenotype. Therefore, many combinations of mutations will probably have to be tested before a promising vaccine candidate is identified.

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