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Mutations within the 5′ Nontranslated Region of Hepatitis A Virus RNA Which Enhance Replication in BS-C-1 Cells

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Passage of human hepatitis A virus (HAV) in cell culture results in attenuation of the virus as well as progressive increases in the efficiency of virus replication in cell culture. Because the presence of identical mutations within the 5′ nontranslated regions (5′NTRs) of several independently isolated cell culture-adapted HAV variants suggests that the 5′NTR may play a role in determining this change in virus host range, we constructed chimeric infectious cDNA clones in which portions of the 5′NTR of cell culture-adapted HM175/p35 virus were replaced with cDNA from either wild-type virus (HM175/wt) or a second independently isolated, but closely related cell culture-adapted virus (HM175/p16). Substitution of the complete 5′NTR of HM175/p35 with the 5′NTR of HM175/wt resulted in virus with very small replication foci in continuous African green monkey kidney (BS-C-1) cells, indicating that 5′NTR mutations in HM175/p35 virus are required for optimal growth in these cells. A chimera with the 5′NTR sequence of HM175/p16 retained the large foci of HM175/p35 virus, while the growth properties of other viruses having chimeric 5′NTR sequences indicated that mutations at bases 152 and/or 203 to 207 enhance replication in BS-C-1 cells. These findings were confirmed in one-step growth experiments, which also indicated that radioimmunofocus size is a valid measure of virus replication competence in cell culture. An additional mutation at base 687 of HM175/p16 had only a minor role in enhancing growth. In contrast to their effect in BS-C-1 cells, these 5′NTR mutations did not enhance replication in continuous fetal rhesus monkey kidney (FRhK-4) cells. Thus, mutations at bases 152 and/or 203 to 207 enhance the replication of HAV in a highly host cell-specific fashion.

Hepatitis A virus (HAV), now classified within the genus *Hepatovirus* of the family *Picornaviridae*, is a hepatotropic virus which commonly causes acute viral hepatitis in humans (13). Considerable efforts have been expended on the development of HAV vaccines since the first demonstration that the virus may be propagated in cultures of primate cells. Good progress has been made in the development of formalin-inactivated whole-virus vaccines, but progress has been much slower with attenuated-vaccine candidates, which offer substantial theoretical advantages over inactivated products in terms of cost, ease of administration, and perhaps duration of immunity (21). It has been shown that the adaptation of wild-type HAV to growth in cell culture and subsequent successive passaging of virus result in a marked reduction in the ability of the virus to cause acute hepatocellular disease in experimentally challenged primates (18). However, this attenuation of the hepatovirulence of HAV is accompanied by an apparent reduction in the replication competence of the virus in vivo, with the result that infection with cell culture-adapted, attenuated variants leads to only low-level antibody responses (17).

Because new approaches may be required for the development of better attenuated-vaccine candidates, we have been interested in the molecular mechanisms underlying the phenotypic changes which accompany the adaptation and passage of HAV in cell culture. In a previous study, we found that the particle/infectivity ratio of a cell culture-adapted HAV variant (HM175/p16 virus) was approximately 4,000-fold lower than that of its wild-type parent (HM175/wt virus), when the abilities of both viruses to form visible replication foci were studied over a period of 14 days in continuous cultures of African green monkey kidney (BS-C-1) cells (12). HM175/p16 virus contains only 19 mutations from the HM175/wt sequence throughout its 7.5-kb genome. Seven of these mutations are either identical with or very similar to mutations present in another independently isolated cell culture-adapted variant of the HM175 strain of HAV (HM175/p35 virus), suggesting that these mutations might be particularly important in adaptation (12). An analysis of chimeric infectious cDNAs with contributions from HM175/p35 virus and its wild-type parent (HM175/wt) has indicated that mutations within the P2 region of the genome (putatively proteins 2B and 2C) are required for efficient replication of HM175/p35 in cell culture (9). However, the occurrence of identical mutations within the 5′ nontranslated regions (5′NTRs) of the HM175/p16 and HM175/p35 viruses, as well as in a third cell culture isolate of HM175 virus (19), suggests that the 5′NTR may also play an important role in determining the growth characteristics of HAV in cell culture (12). This hypothesis was recently confirmed in studies examining the growth of HAV variants with chimeric genomes in cultured African green monkey kidney cells (6, 9).

To better define 5′NTR mutations which positively influence HAV replication in cell culture, we constructed a series of 5′NTR chimeras within the background genome of the infectious HM175/p35 clone. These chimeras contain cDNA representing segments of the 5′NTRs from HM175/wt and HM175/p16 viruses. Analysis of the growth properties of viruses rescued from these chimeras indicates that mutations at bases 152 and/or 203 to 207 of the 5′NTR strongly enhance replication competence in continuous cultures of African green monkey kidney cells.

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MATERIALS AND METHODS

Cell culture propagation of HAV. HM175 virus variants were grown in continuous cultures of African green monkey kidney (BS-C-1) or fetal rhesus monkey kidney (FRhK-4) cells maintained in Eagle's minimal essential medium with Earle's salts, 100 mM glutamine, and penicillin and streptomycin (MEM) supplemented with 2% fetal bovine serum. BS-C-1 cells were obtained from L. Binn of the Walter Reed Army Institute of Research, Washington, D.C., and used at passages 70 to 101. FRhK-4 cells were obtained from M. Sobsey, The University of North Carolina at Chapel Hill, and were utilized at passage 92.

HM175/p16 virus is a plaque-purified virus which was isolated from the liver of an experimentally infected marmoset (passage 6 of the wild-type human virus in marmosets and which has been passaged 16 times in low-passage or continuous cultures of African green monkey kidney cells (12). This virus has recently been shown to be substantially attenuated in primates (15). HM175/p35 virus was independently isolated from human feces and subsequently passaged 35 times in low-passage African green monkey kidney cells; this virus has been clonally isolated by limiting dilution passage, and it is attenuated in primates (4). The HM175/p35 virus employed in these studies was rescued from the infectious molecular clone, pG3HAV/7 (see below). Although independently adapted to cell culture, HM175/p16 and HM175/p35 viruses contain a number of identical mutations from the presumptive wild-type virus sequence, including mutations at bases 152 and 203 to 207 within the 5'NTR (Table 1) (12).

HAV cDNA clones. Plasmid stocks were purified from *Escherichia coli* TG1 transformed with cDNA constructs. The infectious genome-length cDNA of pHAV/7 (representing the genome of HM175/p35 virus) (5) was removed from the plasmid by partial *Hin*dIII and *Xba*I digestion and inserted into the multiple cloning site of transcriptional vector pGEM3 (Promega) downstream of the SP6 promoter to create pG3HAV/7. Chimeric genome-length cDNA clones were constructed from pG3HAV/7 by replacing restriction fragments representing segments of the 5'NTR of HM175/p35 virus with cDNA segments derived from HM175/wt (23) or HM175/p16 virus (12) (Fig. 1). cDNA representing the 5'NTR sequence of HM175/p16 virus was derived from the p5'p16 plasmid. This plasmid was constructed by amplification of an 895-bp fragment from the $pHAV_{CH}119$ plasmid (12) by polymerase chain reaction using primers A+x0 (5'-tcactcaagcTTTCAAGAAGGGTCTCCGG-3'), which corresponds to the 5' 19 bases of HM175/p16 virus preceded by an upstream *Hin*dIII site, and A-870 (5'-ATTGATCCA CAGAACT), complementary to bases 870 to 885. The polymerase chain reaction product was restricted with *Hin*dIII and *Xba*I and inserted into the multiple cloning site of pGEM3 downstream of the SP6 RNA polymerase promoter to create p5'p16. Chimeric genome-length cDNAs were constructed by ligation of appropriate restriction digestion fragments from pG3HAV/7 and p5'p16 as depicted in Fig. 1. An additional chimera was constructed in which the 5'NTR of HM175/p35 virus was replaced with cDNA from $pHAV_{LB}113$, which was derived from HM175/wt virus (23). The 5'NTR chimeras thus contained HM175/p16 cDNA from base positions 0 to 744 (pA/7-5'p16), 0 to 532 (pA/7-5'p16[0-532]), and 25 to 532 (pA/7-5'p16[25-532]) or contained wild-type HM175 cDNA from bases 25 to 532 (pA/7-5'wt) (Fig. 1). (Base 0 in HM175/p16 virus is an additional U residue at the 5' terminus of the genome [12].) Each construct retained the HAV polyprotein-coding region and 3' nontranslated region of pG3HAV/7 (HM175/p35 virus), including those mutations within the P2 region which Emerson et al. (9) have shown to be important for growth in cell culture. To confirm the correctness of the constructions, the chimeric cDNAs were sequenced through ligation sites by the dideoxynucleotide sequencing method of Sanger et al. (20), modified for double-stranded DNA.

In vitro transcription. Plasmid DNA (5 μg) was linearized with *Hae*II and the 3' overhang was filled in with Klenow fragment (5). The cDNA was extracted with phenol-chloroform, precipitated in ethanol, resuspended in nuclease-free water, and transcribed with SP6 RNA polymerase (Promega) according to the manufacturer's instructions. The cDNA template was removed by digestion of the transcription reaction mixture with RNase-free DNase (Promega), followed by phenol-chloroform extraction and ethanol precipitation. The RNA pellet was resuspended in 60 μl of nuclease-free water, and the quality of the transcription products was determined by electrophoresis in formaldehyde-agarose gels.

Transfection assays. Transfections were performed in 60-mm-diameter cell culture dishes containing BS-C-1 cell monolayers that were approximately 85% confluent. Monolayers were washed three times with OPTI-MEM (GIBCO BRL) and then 3 ml of OPTI-MEM was added. A total of 50 μl of Lipofectin (GIBCO BRL) was added to 50 μl of RNA (approximately 5 μg of RNA), and this mixture was slowly added to the medium covering the cell monolayers. The cells were incubated overnight at 35.5°C in a 5% CO_2 atmosphere. The following day, 3 ml of MEM supplemented with 20% fetal bovine serum was added to each dish and the cells were incubated for an additional 24 h. The medium was then removed and replaced with MEM containing 2% fetal bovine serum. The cultures were incubated for 2 weeks at 35.5°C in a 5% CO_2 atmosphere, at which time supernatant fluids were removed and stored at −70°C for subsequent studies. To assess virus growth, the cells were fixed with acetone and stained with radiolabelled anti-HAV immunoglobulin G (IgG) as described previously (14).

Analysis of virus replication efficiency. The efficiency with which rescued viruses replicated in BS-C-1 or FRhK-4 cells was assessed in radioimmunofocus assays (14). Both the size of replication foci and the intensity of staining of individual radioimmunofoci with ^{125}I-labelled anti-HAV antibodies were evaluated as measures of virus replication. Briefly, rescued viruses were inoculated onto nearly confluent BS-C-1 cell monolayers in 60-mm-diameter cell culture dishes. After virus adsorption for 1 h, cell cultures were overlaid with agarose and incubated for 2 weeks, as described previously (14). Foci of virus replication were visualized by autoradiography after acetone fixation and staining of the cell sheets with radioiodinated anti-HAV IgG. The diameters of 30 representative replication foci were measured for each virus studied, and the mean areas were determined. Plastic discs representing the same foci were cut from the culture dish bottoms with a cork borer, and the quantity of radiolabelled anti-HAV IgG bound by each viral replication focus was determined by counting in a gamma counter. As none of the viruses studied were cytopathic, the relationship between focus size (specifically, area, which is proportionate to the number of cells within a focus) and the individual focus counts per minute provided an estimate of the average amount of viral antigen produced by individual cells within the replication focus. As an alternative measure of replication efficiency, the quantity of ^{125}I labelled anti-HAV anti-

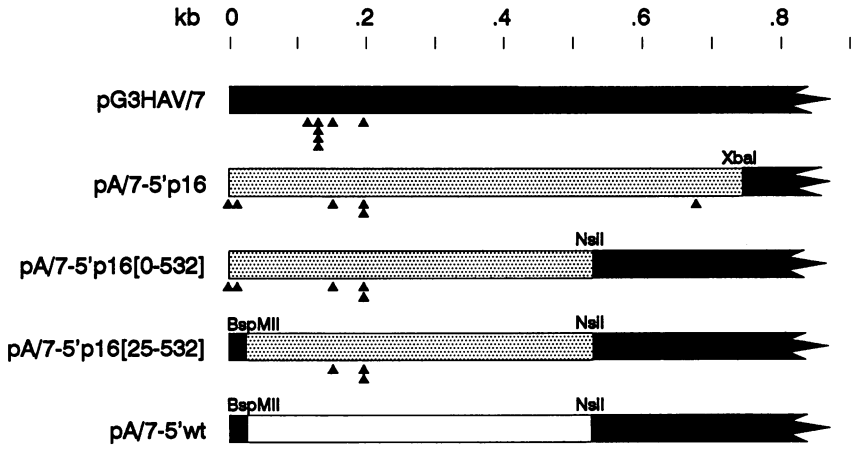

FIG. 1. 5'NTRs of plasmids containing infectious genome-length HAV cDNAs utilized for transfection of BS-C-1 cells. The NTR comprises the first 735 nucleotides of the genome. Black genome segments represent sequence derived from pG3HAV/7 (HM175/p35 virus), while stippled segments are derived from p5'/p16 (HM175/p16 virus) and the white segment is derived from $pHAV_{LB}113$ (HM175/wt virus). Arrowheads indicate mutations from the sequence of HM175/wt. Restriction sites used for construction of these plasmids are indicated.

bodies bound by entire cell culture dishes was determined by direct counting and related to the input viral titer measured in radioimmunofocus-forming units (14).

One-step HAV growth curves. The replication efficiency of selected viruses was assessed under approximate one-step growth conditions. BS-C-1 cells were grown until nearly confluent in 24-well plates and inoculated with 0.1 ml of virus representing a multiplicity of infection of approximately 2.0. Following a 1-h period of adsorption, cells were washed twice and 2.0 ml of maintenance medium was added to each well. The cells from individual wells were harvested and assayed for intracellular virus at 8, 24, 48, 72, and 144 h as follows. The medium was aspirated from each well, and the cell sheets were washed twice with Hanks' balanced salt solution. Cells were lysed by the addition of 1 ml of MEM containing 0.1% sodium dodecyl sulfate, and virus present in the lysate was quantified by radioimmunofocus assay (14 days of virus growth in BS-C-1 cells) (14).

RESULTS

Viruses with different 5'NTR sequences generate radioimmunofoci with different sizes in BS-C-1 cell cultures. Four chimeric genome-length cDNA constructs were created by replacing regions of the 5'NTR of HM175/p35 virus with cDNA derived from either HM175/wt or HM175/p16 virus, as shown in Fig. 1. In each case, the remainder of the genome downstream of the 5'NTR was derived from HM175/p35 virus (pG3HAV/7). pA/7-5'p16 contained the entire 5'NTR sequence of HM175/p16, while pA/7-5'p16[0-532] and pA/7-5'p16[25-532] contained HM175/p16 sequence between bases 0 to 532 and 25 to 532, respectively. Within the 5'NTR, HM175/p35 virus contains mutations from the wild-type sequence only within the sequence flanked by bases 123 and 208 (Table 1). Thus, pA/7-5'wt, created by splicing cDNA from $pHAV_{LB}113$ and pG3HAV/7 at *Bsp*MII (base 24) and *Nsi*I (base 532) sites contained the entire 5'NTR sequence of HM175/wt. The pA/7-5'p16[25-532], pA/7-5'p16[0-532], pA/7-5'p16, and pG3HAV/7 constructs contained 3, 5, 6, and 7 single base changes from the wild-type 5'NTR sequence of pA/7-5'wt, in each case including common mutations at base 152 (A-to-G substitution) and at least one U deletion within a short oligo(U) tract at bases 203 to 207 (Table 1).

Runoff RNAs transcribed under direction of SP6 polymerase from plasmids linearized with *Hae*II were predominantly genome length (approximately 7.5 kb) (data not shown). However, we also observed two minor subgenomic species of approximately 4.6 and 1.6 kb, confirming previous observations (5). Virus was rescued by transfection of BS-C-1 cells with the RNA transcripts, using a liposome-mediated transfection procedure. The replication foci generated by these viruses in BS-C-1 cells ranged from large (similar to that obtained with HM175/p16 virus [12]) to very small (Fig. 2). Replacement of the 5'NTR sequence of HM175/p35 virus (pG3HAV/7) with the corresponding sequence of wild-type HM175 (pA/7-5'wt) resulted in virus with very small foci (diameter of ≤1 mm) resembling radioimmunofoci obtained after 2 weeks of growth of HM175/wt in BS-C-1 cells (12). Similar replication foci were obtained with viruses rescued from multiple identical cDNA constructs, arguing against the chance acquisition of a mutation that was detrimental to replication elsewhere in the genome. This result indicates that mutations within the 5'NTR of HM175/p35 virus are necessary for optimal growth in BS-C-1 cells.

Substitution of the complete 5'NTR of HM175/p35 with the 5'NTR of HM175/p16 (pA/7-5'p16 construct) resulted in virus with a focus size comparable to that of HM175/p35 (diameter, >3 mm) (Fig. 2) or HM175/p16 virus (data not shown). The fact that the 5'NTRs of HM175/p35 and HM175/p16 could substitute for each other without signifi-

TABLE 1. Phenotypes and 5'NTR mutations of HM175 virus variants

Virus	Cell culture growth[a]	Hepatitis in primates[b]	5'NTR mutation at base(s):						
			0	8	124	131–134	152	203–207	687
HM175/wt	+	++++		G	U	UUUG	A	UU	U
HM175/p16	+++	+	U	A	U	UUUG	G	dd[c]	G
HM175/p35	+++	+		G	C	dddd	G	dU	U

[a] +, very slow growth and low yields; +++, moderately rapid growth and yields.

[b] +, inconsistent, low-level serum enzyme elevations; ++++, consistent, marked serum enzyme elevations.

[c] d, deletion.

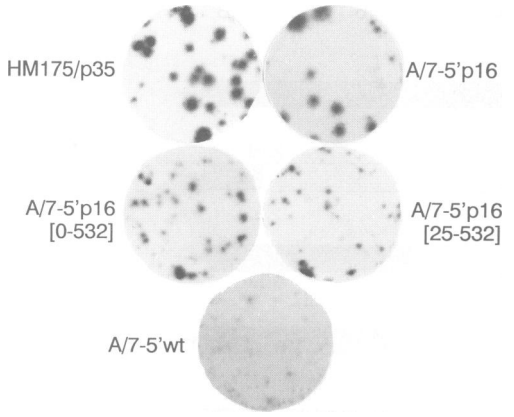

FIG. 2. Autoradiograms of radioimmunofocus assays (BS-C-1 cells) of viruses rescued from plasmids depicted in Fig. 1. The assay was completed 2 weeks after inoculation of the cells and represents cell culture passage 2 of the rescued virus.

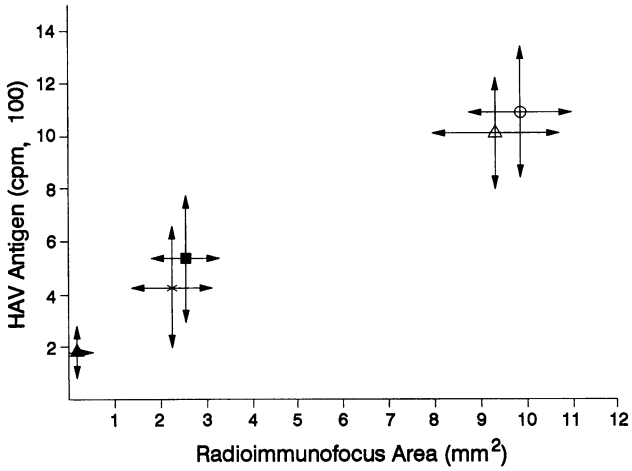

FIG. 3. Viral antigen content versus size (area) of individual radioimmunofoci produced by 5'NTR chimeric viruses in BS-C-1 cells. Symbols: △, HM175/p35; ○, A/7-5'p16; *, A/7-5'p16[0-532]; ■, A/7-5'p16[25-532]; ▲, A/7-5'wt. Each point represents the mean values of 30 representative replication foci, while lines with arrowheads in both dimensions indicate standard deviations.

cant reduction in the growth characteristics of the virus suggested that the mutations which these viruses share in common within this region (at bases 152 and 203 to 207 [Table 1]) may be particularly important to the ability of HAV to replicate efficiently in BS-C-1 cells. This hypothesis was tested by the construction of additional plasmids in which HM175/p16 mutations at base 687 (pA/7-5'p16[0-532]) or bases 0, 8, and 687 (pA/7-5'p16[25-532]) were excluded (Fig. 1). In multiple radioimmunofocus assays in BS-C-1 cells, viruses rescued from these constructs generated substantially larger replication foci than virus with the 5'NTR sequence of HM175/wt virus (Fig. 2). These results confirm that it is the 5'NTR mutations at bases 152 and/or 203 to 207 that are necessary for efficient replication. Although we noted variability in different radioimmunofocus assays, the replication foci produced by these viruses were often smaller (diameter of 1 to 3 mm) than those produced by HM175/p35 or A/7-5'p16 virus (Fig. 2). These results suggest that the mutation at 687 has a minor role in further enhancing virus replication in BS-C-1 cells (6).

Expression of HAV antigen in vivo by viruses with different 5'NTR sequences. Considerable attention has been focused recently on the role of the 5'NTR in controlling translation of the proteins of picornaviruses, including HAV (1, 10). In an attempt to examine the influence of 5'NTR mutations on viral translation in vivo, we measured the quantity of radiolabelled anti-HAV IgG bound to individual replication foci produced in BS-C-1 cells by viruses rescued from the constructs shown in Fig. 1. This measure provides an estimate of the amount of HAV antigen produced within each individual focus, while the size (area) of the focus is proportionate to the number of cells infected within the focus. As shown in Fig. 3, when we analyzed 30 representative radioimmunofoci produced by each virus, we found a roughly linear correlation between the mean area of replication foci and the mean quantity of ^{125}I-labelled anti-HAV antibodies bound by each focus. Thus, the amount of viral antigen produced within individual cells was not measurably different with any of the viruses studied, while the viruses with greater replication efficiency spread to a larger number of cells within individual replication foci over the 2-week period of incubation. However, it is important to point out that we do not know the linearity of the relationship between counts per minute bound in radioimmunofocus assays and the intracellular viral antigen content. It may be that ^{125}I-labelled anti-HAV antibody staining saturates at relatively low intracellular antigen concentrations.

One-step growth analysis of 5'NTR mutant viruses. Because the radioimmunofocus assay measures the spread of HAV antigen from an infectious focus within a cell sheet, determination of focus size in this assay should be a good measure of the adaptation of virus to growth in cell culture. To validate this hypothesis, we assessed the replication of three of the rescued viruses in BS-C-1 cells under one-step growth conditions (Fig. 4). To generate sufficient virus for these assays, it was necessary to pass each virus once in cell culture after its rescue from cDNA. Thus, these one-step growth experiments represented passage 3 of these viruses in cell culture after rescue, while the radioimmunofocus assays for quantitation of the one-step growth harvests represented passage 4 in cell culture after virus rescue.

These one-step growth experiments confirmed the earlier results and also demonstrated that radioimmunofocus size is a valid measure of cell culture adaptation. The replication of A/7-5'wt virus (Fig. 4B) was significantly slower than that of A/7-5'p16[25-532] virus (Fig. 4A). Although the infectious titers of A/7-5'p16[25-532] and A/7-5'wt were nearly identical at 8 h, the A/7-5'wt yields were significantly lower at 24 and 48 h and approximately 10-fold less at 72 h. Intracellular A/7-5'p16[25-532] virus plateaued at 72 h, so that the titers of these viruses were approximately the same at 144 h. While the infectious titers of HM175/p35 virus (Fig. 4C) were less at 8 and 24 h, the replication kinetics of this virus indicated somewhat more rapid growth than for the A/7-5'p16[25-532] virus. The differences in the replication rates of these viruses thus reflect the different radioimmunofocus sizes generated by these viruses (Fig. 2). The results of the one-step growth analysis, therefore, confirm that the mutations at bases 152 and/or 203 to 207 enhance the growth of HAV in BS-C-1 cells.

Replication competence of 5'NTR chimeras in FRhK-4 cells. To determine whether the differences in the replication competence of viruses which we observed in BS-C-1 cells were also present in a different cell type, we compared

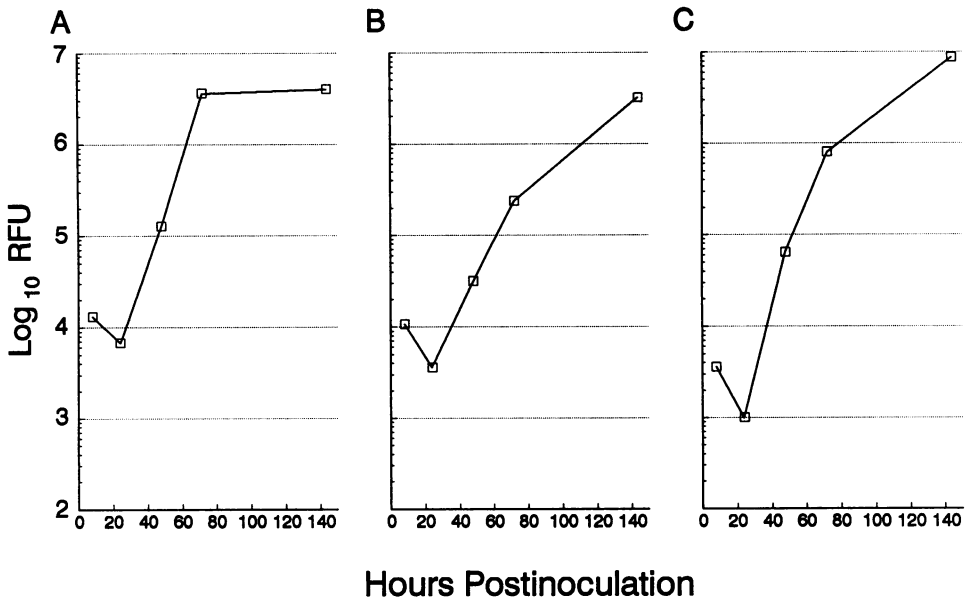

FIG. 4. Intracellular virus accumulation measured under approximate one-step growth conditions in BS-C-1 cells infected with virus rescued from pA/7-5'p16[25-532] (A), pA/7-5'wt (B), and pG3HAV/7 (C). RFU, radioimmunofocus-forming units.

radioimmunofocus size and viral antigen production in BS-C-1 cells and continuous cultures of fetal rhesus monkey kidney (FRhK-4) cells. Although both HM175 strain variants we studied were adapted to growth in African green monkey kidney cells, FRhK-4 cells have been used widely for propagation of HAV in cell culture and in a previous study of mutations responsible for the cell culture adaptation phenotype of HM175 virus (9). Parallel cultures of FRhK-4 and BS-C-1 cells were inoculated with increasing quantities of viruses rescued from the constructs shown in Fig. 1, as well as HM175/p16 virus. Cells were maintained under agarose overlays for 2 weeks, then fixed, and stained with radiolabelled anti-HAV antibodies. We found less than a 3.3-fold difference in the infectious titers of each of the rescued viruses when these were determined by independent radioimmunofocus assays carried out in BS-C-1 and FRhK-4 cells (data not shown). However, there were dramatic differences in the size of replication foci in these cell lines (Fig. 5). Differences in the sizes of replication foci of the HM175/p35, A/7-5'p16, and A/7-5'wt viruses were again noted in BS-C-1 cells. The replication foci obtained with each of the rescued viruses were smaller in FRhK-4 cells than in BS-C-1 cells. Moreover, there were no apparent differences in the size of replication foci obtained with viruses having wild-type or mutant 5'NTR sequences in FRhK-4 cells (Fig. 5), indicating that the 5'NTR mutations present in HM175/p35 and HM175/p16 viruses do not enhance growth in this cell line. In both BS-C-1 and FRhK-4 cells, individual replication foci were occasionally significantly larger than others, suggesting the possibility that new mutations were accumulating with continued passage of rescued viruses in cell cultures. This experiment represented passage 3 of the rescued viruses in cell culture. A similar phenomenon was noted in the one-step growth experiments described above (data not shown).

We also measured viral antigen accumulation in the infected BS-C-1 and FRhK-4 cell cultures maintained under agarose overlays (Fig. 6). These results, which were not obtained under one-step growth conditions, indicated an approximately 10-fold reduction in the expression of antigen by BS-C-1 cell cultures infected with virus containing the wild-type 5'NTR sequence compared with cell cultures infected with an equivalent quantity of virus containing the 5'NTR sequence of either HM175/p35 or HM175/p16 virus (Fig. 6A). In contrast, the antigen content of infected FRhK-4 cells was much less than that of BS-C-1 cells infected in parallel with the same inocula, and little difference was evident in antigen expression by FRhK-4 cells infected with viruses having different 5'NTR sequences (Fig. 6B).

DISCUSSION

Previous studies have indicated that mutations responsible for the adaptation of HAV to growth in cell culture are located within the region of the genome encoding the puta-

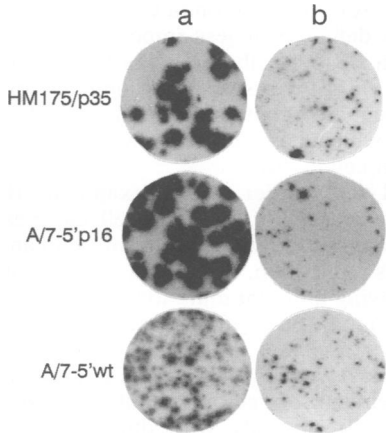

FIG. 5. Radioimmunofoci generated by virus rescued from pG3HAV/7 (HM175/p35), pA/7-5'p16, and pA/7-5'wt in continuous cultures of African green monkey (BS-C-1) (a) and fetal rhesus monkey kidney (FRhK-4) (b) cells.

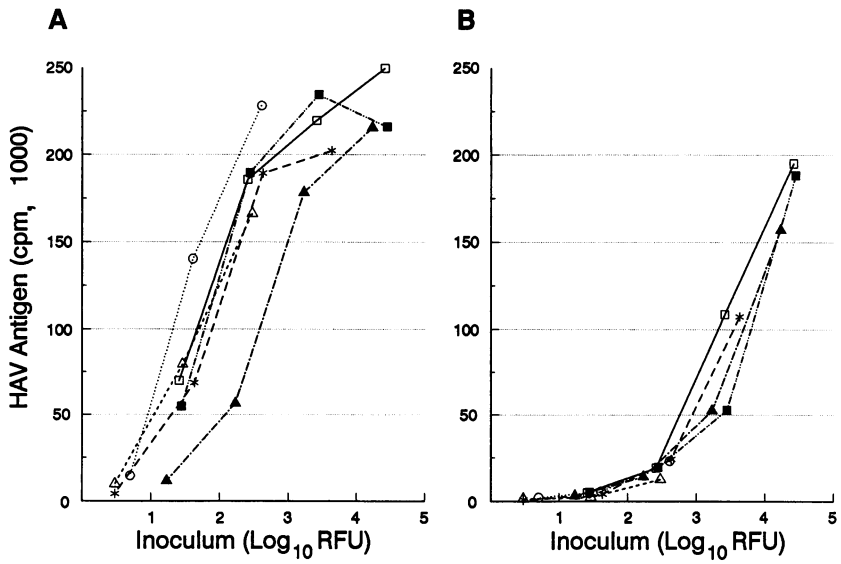

FIG. 6. Accumulation of HAV antigen in BS-C-1 (A) and FRhK-4 (B) cells infected with viruses rescued from cDNA constructs depicted in Fig. 1. Symbols are as described in the legend to Fig. 3, □ = HM175/p16 virus, which differs from the other viruses with respect to mutations outside of the 5'NTR (11). Cells were maintained beneath agarose overlays for 14 days after infection, then fixed, and stained with ^{125}I-labelled anti-HAV antibodies. The counts per minute (cpm) bound by each culture dish is shown as a function of inoculum size (radioimmunofocus-forming units [RFU] measured in BS-C-1 cells). Background binding of ^{125}I-labelled anti-HAV antibodies ranged from 1,939 to 3,376 cpm.

tive 2B and 2C proteins (8, 9). In this report, however, we present data which demonstrate that mutations within a small region of the 5'NTR of the HAV genome also substantially enhance the replication of HM175 variants in continuous cultures of African green monkey kidney (BS-C-1) cells. Mutations which enhance viral replication in BS-C-1 cells include an A-to-G substitution at base 152 and/or the deletion of one or two U residues within a short oligo(U) tract at bases 203 to 207. Both mutations are present in the cell culture-adapted HM175/p16 and HM175/p35 viruses. In addition, there is also a U deletion at bases 203 to 207 in HM175/p59 virus, a third HM175 variant which was independently adapted to growth and serially passaged in African green monkey kidney cells (19). Thus, it may be this mutation which is the most important in enhancing growth in BS-C-1 cells. A third mutation, a U-to-G substitution at base 687, plays a definite but less important role in enhancing replication in BS-C-1 cells (6) and is found in both HM175/p16 and HM175/p59 viruses. All of the infectious constructs which we examined contained the 2B and 2C mutations of HM175/p35 which have been shown to be important for replication in cell culture.

Compared with earlier studies examining the effects of mutations on growth of HAV in cell cultures (8, 9), our findings are enhanced by our use of more-quantitative measures of virus replication. We compared the replication rates of viruses having different mutations under one-step growth conditions (Fig. 4). Such an approach is difficult to apply to large numbers of viruses, however, because of the slow and generally noncytolytic growth of HAV. However, quantitative measurements of radioimmunofocus size and antigen content (Fig. 3) correlate with differences in replication kinetics under one-step conditions and provide a simple and direct measure of replication competence.

The exact mechanism by which these 5'NTR mutations affect viral replication in certain cell cultures and hence the host range of the virus is not understood. However, the 5'NTRs of other picornaviruses have been shown to initiate viral translation by an unusual process of internal ribosomal entry, an event occurring many hundreds of bases downstream of the uncapped 5' terminus of the RNA (10). Recent work has confirmed the existence of a similar internal ribosomal entry site (IRES) within the 5'NTR of HAV (2). Comparison of a model of the secondary structure of the HAV 5'NTR with the structure proposed for the 5'NTR of encephalomyocarditis virus and analysis of in vitro translation experiments using 5' truncated HAV RNAs have suggested that the 5' terminus of the HAV IRES is located upstream of base 355 (1). This has been confirmed in a more recent study examining translation in rabbit reticulocyte lysates programmed with bicistronic constructs in which the HAV 5'NTR controls translation of the downstream reporter gene (2). Thus, available data suggest that the locations of the 5'NTR mutations which we have shown to positively influence the replication of HAV in BS-C-1 cells lie within the HAV IRES element or very close to its 5' limit. Modeling studies suggest that nucleotides 203 to 207 are located near the base of a conserved stem-loop structure and that U deletions at bases 203 to 207 reduce the size of a small bubble within this structure (1) (Fig. 7). Base 152 is located near the 3' terminus of a putatively single-stranded pyrimidine-rich tract. Deletion of sequence downstream of base 151 significantly impairs HAV IRES-directed translation in vitro (2).

While a primary effect on RNA replication cannot be excluded, the locations of these mutations thus suggest the possibility that they act to enhance replication by positively influencing translation of the HAV polyprotein. We found no measurable increase in the binding of radiolabelled antibody to cells infected with chimeric viruses containing wild-type or cell culture-adapted 5'NTR sequences (Fig. 3), but the ability of this in vivo assay to distinguish different levels of intracellular antigen accumulation remains unproven. In

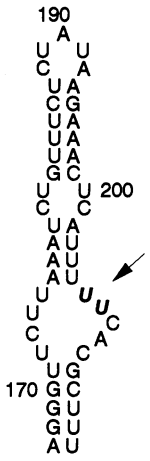

FIG. 7. Proposed secondary structure of part of domain III of the HM175/wt 5'NTR (1). The arrow indicates the location of mutations (U deletions) at base positions 203 to 207 which are present in HM175/p16 virus.

addition, it may be that the spread of virus to adjacent cells occurs only after a certain level of viral replication (intracellular antigen accumulation) is reached. In fact, data from the one-step growth experiments suggest that viruses with and without these mutations eventually reach the same intracellular titers (compare Fig. 4A and B) but that virus lacking the mutations takes longer to do so. Examination of the translational efficiencies of RNAs transcribed from the chimeric 5'NTR constructs in rabbit reticulocyte lysates has failed to reveal any enhancement that could be attributed to specific mutations (2). However, these data do not exclude a role for these mutations in enhancing translation of HAV RNA in vivo. We found the positive influence of these mutations on virus replication to be present in BS-C-1 cells, which are derived from African green monkeys, but not in FRhK-4 cells which are derived from the kidneys of fetal rhesus monkeys. The 5'NTR mutations thus act in a highly host cell-specific fashion, as suggested by Emerson and coworkers (9), and an effect on translation would not be expected in reticulocyte lysates.

As both HM175/p16 and HM175/p35 virus were adapted to growth in cultures of African green monkey kidney cells, it is not surprising that a very host cell-specific effect might be seen only in closely related cells. The 5'NTRs of the picornaviruses bind several host cell proteins which are likely to play roles in virus translation and/or viral RNA replication (7, 11, 16). Recent evidence from our laboratory suggests that the 5'NTR of HAV similarly binds several BS-C-1 cell proteins (3). The 5'NTR mutations which we have shown to positively influence the replication of the virus in these cells may thus act by enhancing or reducing the affinity of the 5'NTR for these cellular RNA-binding proteins. Further investigation of this hypothesis is likely to provide additional information on the mechanisms by which these mutations enhance replication of HAV in cell culture and in a larger sense may provide insights into the mechanisms of attenuation mediated by 5'NTR mutations in other picornaviruses such as poliovirus (22).

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