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Mutations in both the 2B and 2C Genes of Hepatitis A Virus Are Involved in Adaptation to Growth in Cell Culture

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Oligonucleotide-directed mutagenesis of an infectious cDNA clone of wild-type hepatitis A virus was performed to determine which mutations acquired in the nonstructural 2B and 2C genes during adaptation to growth in cell culture were effective in enhancing virus growth in vitro. Results of transfection assays demonstrated that one mutation in the 2B gene and two mutations in the 2C gene were responsible for an increased efficiency in growth, but growth enhancement required the participation of at least two of the three mutations.

Hepatitis A virus (HAV) is a picornavirus that displays a limited capacity for growth in cultured cells. Whereas poliovirus, the prototype picornavirus, requires less than 8 h to complete its replication cycle and can produce 500 to 5,000 particles per infected cell, successful infection of cultured cells by HAV is asynchronous and produces approximately 20 particles per infected cell (1, 10). Although wild-type HAV normally grows extremely slowly in cultured cells, faster-growing variants have been selected by prolonged passage in culture. We are interested in determining the genetic basis for this adaptation to growth in cell culture, since such information would be important for optimizing virus propagation in vitro and should prove useful in defining the molecular pathway of viral replication.

The HM-175 strain of HAV, the prototype hepatitis A virus, was isolated from a human fecal sample and required months to grow to significant levels in cultures of primary African green monkey kidney cells (AGMK) (6). A cell culture-adapted (CC) variant derived from this wild-type HM-175 isolate by serial passage in AGMK cells grew much faster and differed from the parent virus in only 23 bases within the 7.5-kb genome (4). Chimeric viruses constructed from infectious cDNA clones of these two viruses displayed the CC phenotype when the 2B/2C region of the genome was derived from the fast-growing variant, demonstrating that mutations within this region were critical for adaptation (8). This region of the genome is believed to encode two nonstructural proteins and contains 6 of the 23 mutations distinguishing the two viruses.

In this study, we performed oligonucleotide-directed mutagenesis to introduce defined combinations of the 2B and 2C mutations into the wild-type cDNA clone. These altered genomes were then transfected into cultured cells in order to determine the effect of each mutation on growth in vitro. We show that one mutation in the 2B gene and two mutations in the 2C gene are responsible for the dramatic enhancement of virus growth in the cultured cells.

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MATERIALS AND METHODS

cDNA clones. Oligonucleotide-directed mutagenesis was performed by using the Amersham mutagenesis system as instructed by the manufacturer (Amersham Corp., Arlington Height, Ill.). Fragments of the cDNA clones containing the desired mutations were excised by digestion with the appropriate restriction enzyme (*Sac*I at base 2989, *Eco*RI at base 4977, and *Pfl*M1 at base 4196) and used to replace the homologous region in an infectious CC cDNA clone or wild-type cDNA clone that had the *Sac*I and *Eco*RI sites deleted from the polylinker. The *Avr-Eco* and *Sac-Eco* plasmids containing all six mutations found in the 2B/2C region of the CC variant have been described (8). Plasmid stocks were purified from transformed *Escherichia coli* HB101 by centrifugation through CsCl or by LiCl precipitation. Genotypes were verified by restriction digestion and sequencing of selected regions.

In vitro transcription. The preparation of RNA for transfection using SP6 polymerase and a *Hae*II-linearized plasmid has been described (8). In most experiments, three cDNA clones of each mutagenized construct were analyzed; one clone was purified by CsCl density banding, while the other two cDNA clones were purified less stringently by using a LiCl maxiprep procedure and omitting RNase digestion. The method of plasmid preparation had no discernible effect on the transfection results.

Synthesis of [^{32}P]CTP-labeled negative-strand RNA and slot blot analysis were performed exactly as described previously (8) except that unincorporated nucleotides were not removed and the 100-μl reaction mixture was diluted with 100 μl of 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.1% sodium dodecyl sulfate prior to freezing.

Transfection assays. The procedure has been described elsewhere (8). Briefly, the entire in vitro transcription mixture was mixed with DEAE-dextran and transfected into FRhK-4 cells or AGMK cells in T25 flasks. Cells were passed to coverslips, stained by hyperimmune chimpanzee antibodies to HAV as reported, and then stained with fluorescein-labeled goat anti-human immunoglobulin G antibodies (14). Stained coverslips were examined by fluorescence microscopy, and the number of cells containing viral antigen was estimated.

TABLE 1. Effect of wild-type substitutions on growth of the CC clone in FRhK-4 cells[a]

Construct	Substitution(s)						Days post-transfection[b] until 80% of cells were infected
	2B		2C				
CC	—	—	—	—	—	—	11
2B	3889	3919	—	—	—	—	24–33
$2C_1$	—	—	—	4087	—	4563	24
$2C_2$	—	—	—	—	—	4563	11
$2C_3$	—	—	—	—	4222	—	24–33

[a] The wild-type base was substituted for that of the CC clone at the base positions indicated. Virus was detected only after 117 days posttransfection of FRhK-4 cells with a fully wild-type clone (8).
[b] Infected cells were detected by immunofluorescence microscopy.

RESULTS

Choice of FRhK-4 cells for transfection. Although both the wild-type HM-175 virus and its CC variant were originally isolated from cultures of AGMK cells (5, 6), for two reasons we chose to perform the transfections with FRhK-4 cells. First, since we had been unable to demonstrate transfection of AGMK cells with genomes transcribed from wild-type cDNA clones but were able to transfect FRhK-4 cells with these genomic transcripts, FRhK-4 cells are more permissive than AGMK cells for transfection with the wild-type genome (8). Second, the immunofluorescent staining of HAV-infected FRhK-4 cells was easier to quantitate than that of AGMK cells (7).

Back-mutation of the 2B and 2C genes to wild-type sequence. To determine whether a single mutation in the 2B/2C region was responsible for adaptation of HAV strain HM-175 to growth in cell culture, oligonucleotide-directed mutagenesis was performed on the CC clone to reintroduce wild-type sequence at selected positions within these two genes. The effect of these back-mutations was determined by measurement of virus production after transfection of the altered genomes into FRhK-4 cells (Table 1). Wild-type sequence at position 4563 had no obvious effect on growth, since virus from either the CC parent clone or a cDNA clone modified at position 4563 had infected most cells by day 11 posttransfection. In contrast, it took at least twice as long to infect the majority of cells when the viral genomes contained wild-type sequence at positions 3889 and 3919 in 2B or in position 4087 or 4222 in 2C. These results suggested that mutations in both the 2B and 2C genes contributed to adaptation to cell culture. By comparison, only 5% of FRhK-4 cells were infected by day 132 after transfection with RNA transcribed from a cDNA clone of wild-type HM-175 (8), and less than 1% of FRhK-4 cells were infected by day 69 after infection with a human stool suspension containing HM-175 virions (7). Since the mutated viruses produced in each case still grew relatively rapidly and displayed growth rates much closer to that of the CC variant than of the wild-type virus, reversion of a single base from that of the CC variant to that of wild-type virus was not sufficient to restore the wild-type phenotype of severely limited growth in vitro.

Mutation of three positions in the 2B/2C region of the wild-type genome enhances virus growth in vitro. Because transfections with RNA transcribed from the wild-type cDNA clone either did not produce virus or produced detectable virus only after extended periods of cell culture (8), it seemed possible to use the transfection assay to identify mutations that increased even slightly the in vitro growth rate of wild-type virus. To this end, we constructed mutants that contained wild-type sequence everywhere except for a variable number of CC mutations in the 2B/2C region; these modified genomes were then monitored for growth after transfection of FRhK-4 cells (Table 2). Three constructs (6Y, 8Y, and 9Y) retained the wild-type growth phenotype and did not produce significant amounts of virus after transfection (Fig. 1, bottom). In three separate experiments utilizing three cDNA clones of each construct, virus was not detected after transfection with the 6Y or 9Y genome. In two experiments, single infected cells were observed after transfection with the 8Y clone, but the infection did not spread and the cultures were scored as negative for virus production.

The remaining seven constructs all produced virus that grew significantly better than did the wild-type virus, although viruses from the 5Y and 12Y constructs consistently appeared sooner after transfection than did those from the other five constructs. Although there was generally excellent agreement when two identical clones were compared within the same experiment, the actual length of time from transfection to appearance of virus varied from experiment to experiment. This variation presumably reflected subtle differences in cell culture or transfection conditions. However, a hierarchy of growth capability was suggested wherein viruses from the 5Y and 12Y and perhaps the 13Y constructs

TABLE 2. Genotype and in vitro growth phenotype of wild-type clones containing CC mutations

Construct	Mutation(s)[a]				Growth[b]
	VP1	2A	2B	2C	
Sac-Eco	3025	3196	3889 3919	4043 4087 4222 4563	Yes
2Y	—	—	— —	4043 4087 4222 4563	Yes
3Y	—	—	3889 —	— 4087 — —	Yes
5Y	—	—	3889 —	— 4087 4222 4563	Yes
6Y	—	—	— —	— — 4222 4563	0
7Y	3025	3196	3889 3919	4043 4087 — —	Yes
8Y	—	—	3889 —	— — — —	0
9Y	—	—	— —	— 4087 — —	0
11Y	—	—	3889 —	— 4087 — 4563	Yes
12Y	—	—	3889 —	— 4087 4222 —	Yes
13Y	—	—	3889 —	— — 4222 —	Yes
Avr-Eco	—	—	3889 3919	4043 4087 4222 4563	Yes

[a] CC mutations included in the cDNA clone are identified by nucleotide position.
[b] Yes indicates a growth rate greater than that of the wild-type virus, as shown in Fig. 1.

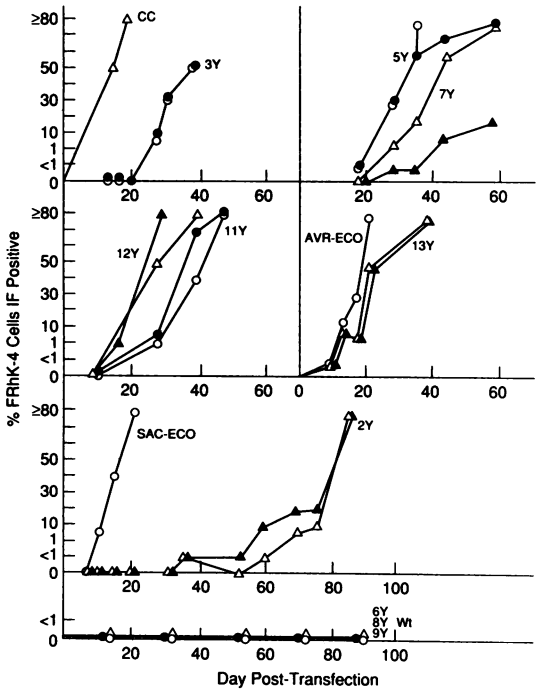

FIG. 1. Transfection of FRhK-4 cells by wild-type genomes containing CC mutations. Each panel represents data from one experiment. Results from two clones of each genotype are shown for clarity, although a third was usually included and gave similar results. Identical open (clone 1) and closed (clone 2) symbols in a single panel represent data points for different clones of a single genotype. The fully adapted cDNA clone (CC) and two clones (*Avr-Eco* and *Sac-Eco*) containing all six mutations in the 2B/2C region are included for comparison. The number of infected cells was estimated by immunofluorescence microscopy. The genotype of each mutant is summarized in Table 2.

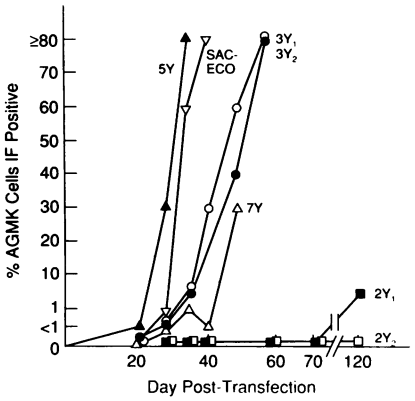

FIG. 2. Transfection of AGMK cells by wild-type genomes containing CC mutations. The number of infected cells was estimated by immunofluorescence microscopy. The genotype of each mutant is summarized in Table 2. One clone of 5Y (▲), *Sac-Eco* (▽), and 7Y (△) and two clones of 3Y (○, ●) and 2Y (□, ■) were tested.

were apparently produced sooner after transfection than were those from the other four constructs. With one exception, all constructs that yielded virus with enhanced growth capacity in vitro contained the 3889 mutation in the 2B gene and either the 4087 or 4222 mutation in the 2C gene (Table 2). The exceptional construct, 2Y, lacked the mutation in the 2B gene but contained both the 4087 and 4222 mutations in the 2C gene. Virus from this construct was detected only after periods of culture longer than were required for the other genomes but significantly shorter than were required for the wild-type genome. Therefore, mutations in both 2B and 2C regions are involved in the adaptation of HM-175 virus to growth in FRhK-4 cells.

Effect of mutations on growth in AGMK cells. To confirm the effects of these mutations on the generalized adaptation to growth in vitro, certain of the constructs were also transfected into AGMK cells (Fig. 2). The results were consistent with those obtained with the FRhK-4 cells. Virus was produced relatively rapidly after transfection with genomes (5Y, *Sac-Eco*) containing the three mutations deemed critical for growth in FRhK-4 cells. Virus production was less rapid when only two of the critical mutations were present (3Y and 7Y) and was very slow when only the mutations in 2C were included (2Y). One isolate of the 2Y clone required over 100 days to produce virus, while the other isolate had not produced detectable virus by the end of the experiment. The 6Y construct which contained the mutations at 4222 and 4563 and failed to grow in FRhK-4 cells also failed to grow in the AGMK cells.

DISCUSSION

Growth of HAV in cell culture is inefficient, and apparently the requirements for growth in vitro are complex. Certain regions of the genome appear to vary in importance for growth in vitro according to the cell type (8). However, in contrast to the host range effect of these mutations, mutations in the 2B/2C region are sufficient for enhanced growth of HM-175 in CV-1 cells, AGMK cells, and FRhK-4 cells, suggesting that mutations in these genes may be critical for growth in many, if not all, permissive cell types.

Of the six mutations in the 2B/2C region, three had no detectable effect on virus growth in FRhK-4 cells. Neither mutation at 3919 or 4043 (7Y virus) nor that at 4563 (11Y virus) was required for enhanced growth, since the 7Y and 11Y viruses grew no better than the 3Y viruses which lacked these mutations. However, since the 3919 mutation was always paired with the mutation at 3889, we cannot rule out the possibility that the 3919 mutation can substitute for the mutation at 3889. In contrast, the three remaining mutations at 3889 (Ala→Val), 4087 (Lys→Met), and 4222 (Phe→Ser) were all found to promote growth in cell culture, although no one of these three mutations was effective by itself. Thus, constructs containing the CC mutation only at 3889 (8Y genome), 4087 (9Y genome), or 4222 (6Y genome) were similar to the fully wild-type genome in that they had not produced significant amounts of virus by 88 days posttransfection (Fig. 1). In contrast, a combination of any two of these three mutations greatly increased the ability of the virus to grow in cell culture: constructs containing mutations at either 4087 plus 4222 (2Y virus), 3889 plus 4087 (3Y virus), or 3889 plus 4222 (13Y virus) all produced virus that grew in cell culture much better than did wild-type virus, although the 2Y virus did not grow as well as did the other two viruses. Without total sequence verification of each virus produced, we cannot rule out the possibility that additional potentiating mutations occurred during the course of these experiments. However, for a number of reasons, we believe that the occurrence of such additional mutations is unlikely. First, each infection was initiated de novo by transfection of RNA from a clone of cDNA so there was no prior selective

TABLE 3. Comparison of cell culture-grown HAV amino acid sequences at the critical sites in 2B/2C

Virus[a]	Sequence				Cells[b]
	3889	4087	4222	4066	
HM-175 m3	GCA (Ala)	AAG	TTT (Phe)	TAT (Tyr)	None
HM-175 p35	GTA (Val)	ATG (Met)	TCT (Ser)	—	AGMK
HM-175 m3 m3 p16	GTA (Val)	—	—	—	AGMC, BS-C-1
HM-175 m3 m3 p16 cyto[c]	GTA (Val)	—	TCT (Ser)	TGT (Cys)	AGMK, BS-C-1, FRkH-4
HM-175 m3 m3 p59	GTA (Val)	—	TCT (Ser)	TGT (Cys)	AGMK, BS-C-1
MBB	GTA (Val)	AAC (Asn)	—	—	Human hepatoma
LA	—	—	—	—	Not reported

[a] The HM-195 viruses were all derived from the same human stool and were passaged in vitro (p) or in marmosets (m) for the indicated number of times. HM-175 m3 and HM-175 p35 represent the wild-type and CC variant used in this study. The MBB isolate and LA isolates were derived from two different human stools.
[b] Cells used for in vitro culture of the virus.
[c] Cytopathic variant derived from additional passage of HM-175 m3 m3 p16.

pressure for new potentiating mutations. In each case, two or more individual clones of each genotype were tested, and they gave similar and reproducible results (Fig. 1). In addition, the combined data from all 10 genotypes tested were totally consistent in supporting the conclusion that the three mutations (3889, 4087, and 4222) were critical. Finally, the importance of these mutations was confirmed in experiments performed with CC clones which were back-mutated to wild-type sequence. Individual reversion of each of these three mutations measurably retarded the growth of the virus in vitro, although growth was still much better than that of fully wild-type virus (Table 1). This partially enhanced growth relative to wild-type virus presumably reflected the retention of two of the three critical mutations in 2B and 2C, although other regions of the genome may also have contributed. The transfection data suggest that certain combinations of the three mutations are more effective than others in promoting virus growth in vitro. However, the transfection assays contain more variables than do infection assays, and further studies comparing precise rates of growth after infection of cells with the mutated viruses are required to answer this question.

Mutations other than the three identified in this study may also potentiate growth in cell culture. Of five other isolates of human HAV sequenced in the 2B/2C region to date, none has all three adaptive mutations. The in vitro growth parameters of one isolate (LA) have not been described sufficiently to allow a meaningful comparison (15). However, it is striking that the remaining four isolates all contain the adaptive valine in 2B encoded by mutation 3889, suggesting that this mutation might be particularly critical (Table 3). The 8Y construct containing this mutation was the only one of the three single mutations which produced detectable virus, albeit infected cells were extremely rare. Three of the isolates share a common early history in that they are strain HM-175 viruses passaged three or more times in marmosets before adaptation to cell culture in different laboratories. An early-passage virus (m3 m3 p16) derived by passage in AGMK cells followed by passage in BS-C-1 cells contains neither the 4087 nor the 4222 mutation, but a cytopathic variant derived from it had acquired the mutation at 4222 (12). The m3 m3 p59 isolate passaged in AGMK cells and then in BS-C-1 cells also contains the mutation at 4222 (17). Although both the m3 m3 p16 and m3 m3 p59 isolates lack the mutation at 4087, they both contain an identical mutation at 4066 (Tyr→Cys) which is of interest because they both have acquired it and because of its proximity to 4087. The MBB strain, which is not directly related to the other isolates, lacks the mutation of lysine to methionine at 4087 (16) but has another amino acid at this position, suggesting that perhaps it is the removal of lysine rather than the inclusion of methionine that promotes in vitro growth of HM-175. It should be informative to test the ability of the mutation at 4066 or mutations that replace the critical lysine at 4087 with residues other than methione to substitute for the present mutation at 4087 in the CC variant.

It is unclear how these mutations are affecting growth because we know nothing about the function of these genes in HAV. The mutations that we identified as critical for in vitro growth map to the region of the genome that in other picornaviruses encodes two nonstructural proteins, 2B and 2C. In poliovirus, one or both of these proteins is involved in viral RNA synthesis (2, 3, 11, 13), while the 2C protein of rhinoviruses has been implicated as a determinant of host range (18). Although the exact boundary between 2B and 2C of HAV has not been defined, published computer-based predictions place it between bases 3889 and 4087 (5, 15, 16). Therefore, both the 2B and 2C genes must be able to affect the ability of the virus to grow in vitro. Despite the identification of an important mutation in 2B, enhanced growth also occurred when mutations were limited to 2C (2Y viruses), demonstrating that mutations in both genes are not simultaneously required. However, growth appeared to be better when the 2B mutation was included. The data suggest that enhanced growth may reflect an altered interaction between the 2B and 2C regions which is affected by a combination of any two of the three mutations. Such interaction might simply be a requirement for a specific conformation of the 2BC precursor prior to proteolytic processing. In this case, the mutations may alter processing and have no direct effect on the function of the individual proteins. If this is so, the experiments reported here cannot resolve whether enhanced growth requires 2B, 2C, or both proteins.

Alternatively, the 2B and 2C proteins might interact as a functional complex so that each protein has an intimate role in replication either as individual polypeptides or as the 2B/2C precursor protein. Although the functions of these proteins are not defined for the other picornaviruses, a current model for poliovirus replication suggests that organization of vesicles into replication complexes involves the 2B/2C region and that one of these two proteins holds the viral RNA in the complex (3). Although there is no evidence that the 2B and 2C genes of HAV and poliovirus are functionally equivalent, there are some notable similarities. Computer plots of these proteins reveal that the putative 2B protein of HAV and the 2B protein of poliovirus have a similar hydropathic profile which is dominated by two major hydrophobic domains bracketing a hydrophilic domain (7);

this structural similarity is consistent with their sharing a similar function. Since the 2C protein is so much larger, comparisons between these proteins from HAV and poliovirus are more difficult. However, a motif identified as the purine nucleoside triphosphate-binding sequence pattern is present in the 2C proteins of both of these viruses (9), suggesting a functional similarity. These data demonstrate that one or both of the 2B and 2C proteins are critical for viral replication, but they do not provide insight as to why they are important. It now appears that in order to understand HAV replication, it will be necessary to define the precise functions and interactions of these two nonstructural proteins which play such a basic role in virus propagation.

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