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An Infectious cDNA Clone of a Cytopathic Hepatitis A Virus: Genomic Regions Associated with Rapid Replication and Cytopathic Effect

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Rapidly replicating, cytopathic (*rr/cpe*$^+$) variants of hepatitis A virus (HAV) isolated from persistently infected BS-C-1 cells have numerous mutations from cell culture-adapted *rr/cpe*$^-$ HAV. To determine which mutations in one *rr/cpe*$^+$ virus, HM175/18f, determine enhanced replication in BS-C-1 cells, a series of chimeric viruses was rescued from infectious cDNAs in which HM175/18f genomic segments were placed within the background of a related *rr/cpe*$^-$ virus, HAV/7. Chimeric viruses containing the P2 region of HM175/18f produced replication foci in BS-C-1 cells that were larger than HAV/7, but not as large as HM175/18f virus. Enhanced viral replication required mutations in both 2B and 2C proteins, suggesting that these proteins remain closely associated during replication. Mutations in 5' nontranslated RNA (5'NTR) or P3 proteins had no independent effect, but acted cooperatively with mutations in P2 proteins to enhance replication and render the virus capable of conventional plaque formation. Cytopathic effects correlated with viral replication capacity and were not the result of any single mutation. Full expression of the *rr/cpe*$^+$ phenotype required mutations within the 5'NTR, P2, and P3 segments. These results suggest novel interactions between the 5'NTR and P2 proteins during HAV replication and provide useful new infectious cDNA clones. © 1995 Academic Press, Inc.

INTRODUCTION

Hepatitis A virus (HAV), a unique human picornavirus now classified within the genus *Hepatovirus*, remains a common cause of cases of acute viral hepatitis reported to public health authorities within the United States (Francki et al., 1991; Lemon and Shapiro, 1994). Although wild-type HAV has a characteristically slow and noncytopathic replication cycle in cultured cells, with serial passage the virus becomes progressively adapted to growth in cell culture (Provost and Hilleman, 1979; Binn *et al.*, 1984; Jansen *et al.*, 1988; Cohen *et al.*, 1987a). Cell culture-adapted variants of HAV have a lower particle-to-infectivity ratio in cultured cells and produce large replication foci after 14 days growth in radioimmunofocus assays, but continue to initiate persistent, generally noncytopathic infections (Jansen *et al.*, 1988). Under one-step growth conditions, these cell culture-adapted viruses may still require several days or longer to reach maximum virus yields. Because cell culture-adapted variants of HAV are often attenuated with respect to their ability to induce acute hepatocellular injury in susceptible primates (Provost *et al.*, 1982; Karron *et al.*, 1988; Taylor *et al.*, 1993), an understanding of the molecular mechanism(s) underlying cell culture adaptation could be useful in the rational design of novel attenuated HAV vaccine candidates.

Toward this end, the genomes of several independently isolated cell culture-adapted variants of the HM175 strain of HAV have been completely sequenced and compared with the sequence of wild-type (3rd marmoset passage) virus. From the wild-type sequence (Cohen *et al.*, 1987c), these isolates have been shown to contain a total of 19 mutations (HM175/P16, an isolate studied at the 16th cell culture passage level) (Jansen *et al.*, 1988), 25 mutations (HM175/P35) (Cohen *et al.*, 1987a), or 42 mutations (HM175/P59) (Ross *et al.*, 1989). Each of these three independent isolates shares common mutations in the 5' nontranslated region (5'NTR) and in the P2 and P3 regions of the genome. Experiments with chimeric infectious cDNA clones indicate that mutations in both the 5'NTR and P2 region (2B and 2C proteins) contribute to the ability of the virus to grow in cells of African green monkey kidney lineage (Day *et al.*, 1992; Emerson *et al.*, 1992, 1993).

Anderson (1987) and Cromeans *et al.* (1987, 1989) demonstrated a unique replication phenotype for HM175 variants that were rescued from cell cultures which had been persistently infected with cell culture-adapted HAV. This phenotype is characterized by a relatively rapid replication cycle, including the production of macroscopic foci of viral replication demonstrable in radioimmunofocus assays carried out only 6–7 days after infection of

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cell cultures and with intervals of less than 24 hr to maximal virus yields under one-step growth conditions (Cromeans *et al.*, 1989; Lemon *et al.*, 1991). In addition, these HM175 variants form visible plaques when infected cells maintained under agarose overlays are stained with vital dyes such as neutral red, despite the absence of host cell metabolic shutoff. We have characterized these viruses as *rr/cpe*$^{+}$ (positive for rapid replication and cytopathic effect) and recently reported the nearly complete nucleotide sequences of three clonally distinct *rr/cpe*$^{+}$ HM175 variants recovered from a single persistently infected cell culture (Lemon *et al.*, 1991). We found that the genomic sequences of each of these virus clones contain 31 to 42 mutations from that of HM175/P16 (Jansen *et al.*, 1988), a virus which is closely related by passage history to the virus which served to initiate the original persistent infection (Fig. 1). While the P1 (capsid protein-coding) segments (Rueckert and Wimmer, 1984) of the genomes of these three clonal *rr/cpe*$^{+}$ isolates contain distinctly different mutations, segments within the 5′ nontranslated RNA (5′NTR), P2, P3, and 3′NTR regions of each of these viruses share in common a number of identical mutations, including many within the polyprotein coding region which are silent (Lemon *et al.*, 1991). These findings have suggested that these virus genomes might be the products of recombination events during persistent infection and that the shared mutations were positively selected for their ability to contribute to enhanced fitness of the viral RNA in persistently infected BS-C-1 cells.

Here, we describe the construction of a series of infectious chimeric virus cDNAs containing segments of an *rr/cpe*$^{+}$ variant (HM175/18f virus) (Lemon *et al.*, 1991) within the background of a cell culture-adapted but *rr/cpe*$^{-}$ HM175 variant (HAV/7, representing the sequence of HM175/P35 virus) (Cohen *et al.*, 1987b) (Fig. 1). These experiments demonstrate the involvement of multiple genomic regions in determining the *rr/cpe*$^{+}$ phenotype and have resulted in the construction of several new infectious cDNA clones which generate virus with significantly enhanced capacity for growth in cell culture.

MATERIALS AND METHODS

Viruses

HAV/7 (HM175/P35) virus is a cell culture-adapted, *rr/cpe*$^{-}$ variant of the HM175 strain of HAV which was rescued from cells transfected with full-length RNA derived from the infectious clone pG3/7 derived from pHAV/7 (Cohen *et al.*, 1987b; Day *et al.*, 1992) (see below). HM175/18f virus ("clone B") is a *rr/cpe*$^{+}$ variant that was clonally isolated from agarose overlying replication foci of virus recovered from a persistently infected cell culture (Lemon *et al.*, 1991). These viruses represent independent isolates of the HM175 strain of HAV (Fig. 1). The genome of the HM175/18f virus contains a total of 31

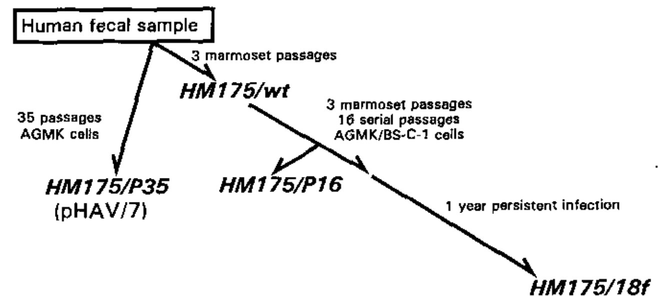

FIG. 1. Passage history and replication phenotypes of variants of the HM175 strain of HAV. HM175/wt (Cohen *et al.*, 1987c) was sequenced following 3 passages in marmosets. The cell culture-adapted but *rr/cpe*$^{-}$ variant HAV/7 (pHAV/7, HM175/P35) was isolated directly from human feces and sequenced after 35 passages in African green monkey kidney (AGMK) cells (Cohen *et al.*, 1987a). The *rr/cpe*$^{+}$ variant HM175/18f was rescued from persistently infected BS-C-1 cells (Lemon *et al.*, 1991). The virus initiating this persistent infection was closely related to the cell culture-adapted but *rr/cpe*$^{-}$ HM175/P16 virus (Jansen *et al.*, 1988), which was independently isolated from virus passaged 6 times in marmosets and sequenced following 10 passages in AGMK and 6 serial passages in BS-C-1 cells. All three cell culture-adapted viruses are clonal isolates.

mutations from that of HM175/P16, which is closely related to the virus which initiated the persistent infection from which HM175/18f was derived (Jansen *et al.*, 1988; Lemon *et al.*, 1991) (Fig. 1, Table 1). HM175/18f has 44 mutations from the wild-type (third marmoset passage) HM175 virus and 46 mutations from the sequence of HAV/7 virus (Table 1) (Cohen *et al.*, 1987a, c; Lemon *et al.*, 1991).

Cells

Hepatitis A viruses were propagated in continuously cultured African green monkey kidney (BS-C-1) cells (passage 90–110), as previously described (Binn *et al.*, 1984). RNA transfections were carried out in a fetal Rhesus kidney cell line (FRhK-4).

Chimeric infectious cDNA constructs

cDNA segments representing the 5′NTR, P2, P3, and 3′NTR regions of the HM175/18f virus genome were amplified from viral RNA by a combination of reverse transcription and polymerase chain reaction (RT/PCR). The oligonucleotide primers used in these reactions are shown in Table 2a. Each contained a unique restriction site near the 5′ terminus which facilitated the insertion of the amplified cDNA segment into plasmid vectors. As the sequence at the extreme 5′ terminus (nt 1–29) of the HM175/18f virus is not known (Lemon *et al.*, 1991), the sequence of the 5′NTR upstream primer was based on that of the related HM175/P16 virus (which contains two mutations from wild type in this region) (Table 1, and Jansen *et al.*, 1988). The amplified segments were digested at the terminal restriction endonuclease cleavage

TABLE 1

Mutations Present in the Genomes of the HM175/P16, HAV/7, and HM175/18f Genomes

Base position (protein–a.a.)	Wild type	HM175/P16	HAV/7	HM175/18f
5′NTR				
5′end	—	U	—	?[a]
8	G	A	—	?[a]
124	U	—	C	—
131–4	UUUG	—	dddd	—
152	A	G	G	G
154/155	—	—	—	UUGUAAAUAUUGAU
203–204	UU	dd	dU	dd
551	A	—	—	G
591	A	—	—	G
647	A	—	—	C
687	U	G	—	G
P1				
964 (1B–54)	A (Lys)	G (Arg)	G (Arg)	G (Arg)
1741 (1B–91)	C (Thr)	—	—	A (Lys)
1742	G	A*	A*	A*
2684	C	—	—	U*
3018 (1D–271)	U (Ser)	—	—	C (Pro)
3025 (1D–273)	A (Glu)	—	U (Val)	—
P2				
3196 (2A–??)	A (Asn)	—	G (Ser)	—
3248 (2B–2)	A (Lys)	—	—	U (Asn)
3281 (2B–13)	A (Ile)	G (Met)	—	G (Met)
3711 (2B–157)	G (Asp)	A (Asn)	—	C (His)
3867	U	C*	—	C*
3889 (2B–216)	C (Ala)	U (Val)	U (Val)	U (Val)
3919 (2B–226)	G (Gly)	—	C (Ala)	—
4043	U	—	C*	—
4049	C	U*	—	—
4060 (2C–22)	C (Ala)	—	—	G (Gly)
4066 (2C–24)	A (Tyr)	—	—	G (Cys)
4087 (2C–31)	A (Lys)	—	U (Met)	—
4185 (2C–64)	G (Glu)	A (Lys)	A (Lys)	A (Lys)
4222 (2C–76)	U (Phe)	—	C (Ser)	C (Ser)
4272 (2C–93)	C (His)	—	—	U (Tyr)
4369 (2C–125)	C (Ser)	—	—	U (Phe)
4419 (2C–142)	U (Tyr)	—	—	C (His)
4426 (2C–144)	A (Lys)	C (Thr)	—	—
4563 (2C–190)	G (Val)	—	A (Ile)	—
4607	U	—	—	C*
4955 (2C–320)	A (Glu)	—	—	U (Asp)
P3				
5010 (3A–4)	GAU (Asp)	—	—	ddd (d)
5172 (3A–58)	U (Phe)	—	—	G (Val)
5194 (3A–65)	G (Arg)	—	—	A (His)
5204	G	A*	A*	A*
5232 (3B–4)	C (His)	—	U (Tyr)	—
5255 (3B–11)	A (Gln)	U (His)	—	U (His)
5592 (3C–101)	C (Gln)	—	—	G (Glu)
6147-8 (3D–67)	GA (Asp)	GG (Gly)	AA (Asn)	GG (Gly)
6216	U	C*	—	C*
6461	A	—	—	G*
6522 (3D–192)	U (Ser)	A (Thr)	A (Thr)	A (Thr)
6619 (3D–224)	G (Arg)	—	—	A (Lys)
6633	U	—	—	C*
6920	C	—	—	U*
7247	U	—	—	C*
7304	U	—	—	C*
3′NTR				
7429	C	—	—	U
7430	A	G	—	G
7433	U	—	—	C

Note. d, deletion; *, silent mutation.

[a] Sequence at these positions is not known for HM175/18f virus; 5′NTR clones contain the sequence of HM175/P16 at these positions.

sites and inserted into the vectors pGEM3 (Promega) or M13mp18 using standard techniques.

The parent infectious cDNA clone for these studies was pG3/7, which contains the HAV sequence of pHAV/7 in the vector pGEM3 (Cohen *et al.*, 1987b; Day *et al.*, 1992). This plasmid was modified by removing the resid-

TABLE 2

Oligonucleotide Primer Pairs Used for PCR Amplification of HAV cDNA

a	SLA+(0−10)	5′-tcactcaagcTTTCAAGAAGGGTCTCCGG-3′[a]
	SLA−0765	5′-TCAAGACCACTCCCAACAGT-3′
	SLA+2948	5′-TTATTTGTCTGTCACAGAACAAT-3′
	SLA−5282	5′-TATTTCCAAAGTTGACTGAGATTCTA-3′
	SLA+4887	5′-GATTTGATAATGGATGGACATAATG-3′
	SLA−7064	5′-GTAGCTGTCATGCCAAGTTTC-3′
	KGA+6963	5′-gtcgacAAGATTCTCTGTTATGGAGA-3′
	KGA-pU	5′-gaattcTTTTTTTTTTTTTTTTTTTT-3′
b	SLA+2891	5′-GGTTTCTATTCAGATTGCAAATTA-3′
	SLA-3998X	5′-accggaattccgGATATCTCTTAACCAGTTGG-3′
	SLA+4012X	5′-tcgcggatccgGATATCTGTTCTGGGATCACC-3′
	SLA-4987	5′-ACTCCATGAATTCAGTCAT-3′

[a] Lowercase letters reflect non-HAV sequence.

ual *Sac*I and *Eco*RI sites in the multiple cloning site of the pGEM3 vector sequence, yielding the plasmid pG3/7d. Using unique restriction sites within pG3/7 or pG3/7d, a series of chimeric plasmids (p5′.18f, pP2.18f, pP3.18f, p3′.18f) was constructed in which cDNA representing the 5′NTR, P2, P3, or 3′NTR segments of the HM175/18f virus genome replaced the corresponding segments in the full-length infectious clone, as shown Fig. 2. In these clones, the isolated 5′NTR segment contained the sequence from nt 0 (*Hin*dIII) to 744 (*Xba*I) positions in HM175 virus (for consistency, all of the map positions are those of the wild-type virus) (Cohen *et al.*, 1987c), the P2 segment contained sequence from nt 2993 (*Sac*I) to 4977 (*Eco*RI) and probably thus included the extreme carboxyl terminus of VP1 (see Results), the P3 segment contained sequence from nt 4978 to 7002, and the 3′NTR segment contained the sequence from nt 7003 (*Xho*I) to the end of the poly(A) tract (*Hae*II or *Sph*I). The clone containing the 3′NTR of HM175/18f had a 3′ poly(A) tract which was 20 nt in length, followed by an *Sph*I site. Additional chimeric plasmids, p5′P2.18f, p5′P2-3′.18f, p5′P3-3′.18f, pP2P3.18f, p5′P2P3.18f, and p5′P2P3-3′.18f, were constructed by ligation at the endonuclease cleavage sites noted in Fig. 2 using standard techniques.

Additional P2 region chimeras contained only the 2AB or 2C protein coding regions of the HM175/18f virus. cDNA segments representing the 2AB and 2C regions were PCR amplified from the DNA of pP2.18f, using the oligonucleotide primers shown in Table 2b. Because no suitable restriction sites exist between the 2AB and 2C coding regions of HAV, primers SLA−3998X and SLA+4012X were designed to place a unique *Eco*RV restriction site near the 2AB/2C junction (at the eighth codon of the 2C protein) by creation of a single silent mutation at position 4022 (T to C). The amplified 2AB and 2C cDNA segments were subcloned in shuttle vectors, fused at the newly created *Eco*RV site, and subsequently used to replace the P2 region of pP2.18f to create pP2.18fm. The sequence of pP2.18fm is identical to pP2.18f except for the silent mutation created for the *Eco*RV site at position 4022 and a second silent mutation occurring adventitiously at position 3899 (T to C). A similarly engineered *Eco*RV site was placed within the background of the full-length HAV/7 virus sequence, yielding pG3/7m. Finally, p2AB.18f and p2C.18f were constructed by replacing the 2AB (2993 to 4019) or 2C (4020 to 4977) regions of pG3/7m with the amplified 2AB or 2C segments of HM175/18f virus. p2ABC.18f, which contains

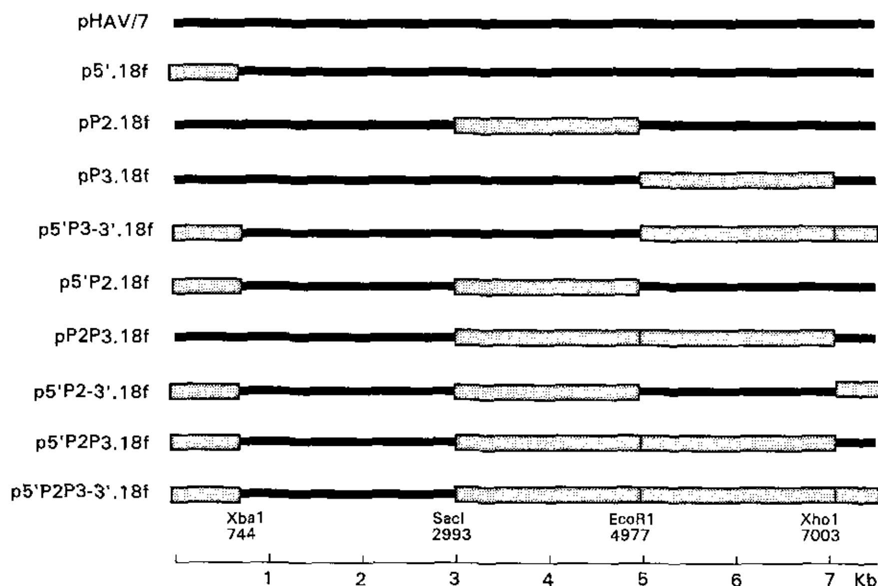

FIG. 2. Organization of chimeric infectious cDNAs containing sequences derived from the *rr/cpe*⁺ HM175/18f virus (stippled boxes) within the background of the *rr/cpe*⁻ HAV/7 virus (pG3/7, derived from pHAV/7) (Cohen *et al.*, 1987b) (solid bars). Nucleotide numbering is for the HM175/wt virus (Cohen *et al.*, 1987c).

the entire P2 region of HM175/18f, was reconstructed from these two plasmids as described under Results.

All of the PCR-amplified cDNA segments were sequenced as DNA to determine whether any additional mutations were introduced during the amplification process. In addition, the construction of chimeric plasmids was validated by DNA sequencing at selected sites of replacements.

RNA transcription and transfection reactions

Plasmid DNAs were linearized by digestion with *SphI* (plasmids containing the HM175/18f 3′NTR segment) or *HaeII* (plasmids containing the HAV/7 3′NTR segment). RNA transcription and liposome-mediated transfection of FRhK-4 cells were carried out as described previously (Shaffer *et al.*, 1994). The quality of the transcription products was assessed by electrophoresis of sample product in 0.1% sodium dodecyl sulfate agarose gels. The products of transcription reactions with different chimeric plasmids contained approximately the same quantity of full-length 7.5-kb RNA. The transfections were carried out in 60-mm-diameter cell culture dishes containing FRhK-4 cell monolayers which were approximately 80% confluent.

Virus stocks were harvested 14 days after transfection by mechanically removing cells into 3 ml medium. The cell suspension was subjected to three freeze–thaw cycles, a brief sonication, and clarification by low-speed centrifugation followed by an equal-volume chloroform extraction. Aliquots of virus were stored at −70°.

Analysis of viral replication efficiency

The efficiency of virus replication in BS-C-1 cells was assessed by radioimmunofocus assay carried out in BS-C-1 cells (Lemon *et al.*, 1983). Briefly, virus stocks were inoculated onto 60-mm-diameter cell culture dishes containing nearly confluent cell monolayers. After 2 hr of viral adsorption, cells were overlaid with 5 ml medium containing 0.5% agarose and placed in a 5% CO_2 environment at 35.5° for 1 to 2 weeks (depending on viral growth properties). The overlay was gently removed and the cells were fixed with 80% acetone and stained with radioiodinated human polyclonal anti-HAV IgG. Foci of viral replication were visualized by autoradiography.

HAV plaque assay

The cytopathic effect of selected chimeric viruses was demonstrated by conventional plaque assay (Lemon *et al.*, 1991). The conditions of infection were the same as for the radioimmunofocus assay except that the agarose overlay contained 25 m*M* $MgCl_2$. Following incubation at 35.5° for 6–8 days, a second 0.5% agarose overlay containing 25 m*M* $MgCl_2$ and 0.013% neutral red was added. Visible plaques were counted 6 to 24 hr later.

Analysis of virion RNA sequence

To confirm the identity of selected *rr/cpe*$^+$ viruses, virion RNA was sequenced in the P1 region using an antigen-capture RT/PCR method, followed by direct sequencing of PCR products (Jansen *et al.*, 1990). The absence of HM175/18f virus mutations within the sequenced region confirmed that these viruses were derived from infectious cDNA constructs. The negative-sense primer used for sequencing was SLA-1771 (5′-ATCTGACAAATAGAAGCCA-3′).

RESULTS

Mutations in the P2 region contribute to the rapid replication of HM175/18f virus

HM175/18f virus is one of three *rr/cpe*$^+$ viruses which were clonally selected from a virus stock harvested from BS-C-1 cells which had been persistently infected with a cell culture-adapted HAV for a period of approximately 1 year (Lemon *et al.*, 1991). These three viruses contain numerous mutations throughout the genome from the virus which initiated the persistent infection. Although each virus has unique mutations within the P1 and the P3 ($3C^{pro}$) genomic regions, they each share common mutations in the 5′NTR, 3′NTR, P2, and P3 (3A and $3D^{pol}$) regions. The presence of identical mutations in these segments of all three *rr/cpe*$^+$ genomes suggests that these mutations may be particularly important for the rapid replication phenotype (Lemon *et al.*, 1991). To test this hypothesis and to determine which genomic region(s) contribute to the replication phenotype of *rr/cpe*$^+$ virus, three chimeric genome-length cDNA constructs were created by replacing the 5′NTR, P2, or P3 regions of HAV/7 virus with cDNA derived from HM175/18f virus, as shown in Fig. 2 and described under Materials and Methods. Virus stocks were prepared 14 days after transfection of FRhK-4 cells with RNA transcribed from these constructs. The replication capacity of these viruses was subsequently examined in radioimmunofocus assays carried out in BS-C-1 cells. Previous studies have shown that the size of HAV replication foci in radioimmunofocus assays correlates well with viral replication under one-step growth conditions (Day *et al.*, 1992; Shaffer *et al.*, 1994).

Only very small or inapparent replication foci were present in radioimmunofocus assays harvested 1 week following infection of BS-C-1 cells with HAV/7, 5′.18f, and P3.18f viruses (5′.18f represents the virus rescued from the plasmid p5′.18f) (Fig. 3A). The growth characteristics of these viruses were consistent with the previously observed replication properties of HAV/7 virus in BS-C-1 cells. In contrast, replication foci generated by P2.18f virus were considerably larger, but only intermediate in size between HAV/7 and HM175/18f viruses (Fig. 3A). In radioimmunofocus assays harvested at 2 weeks postin-

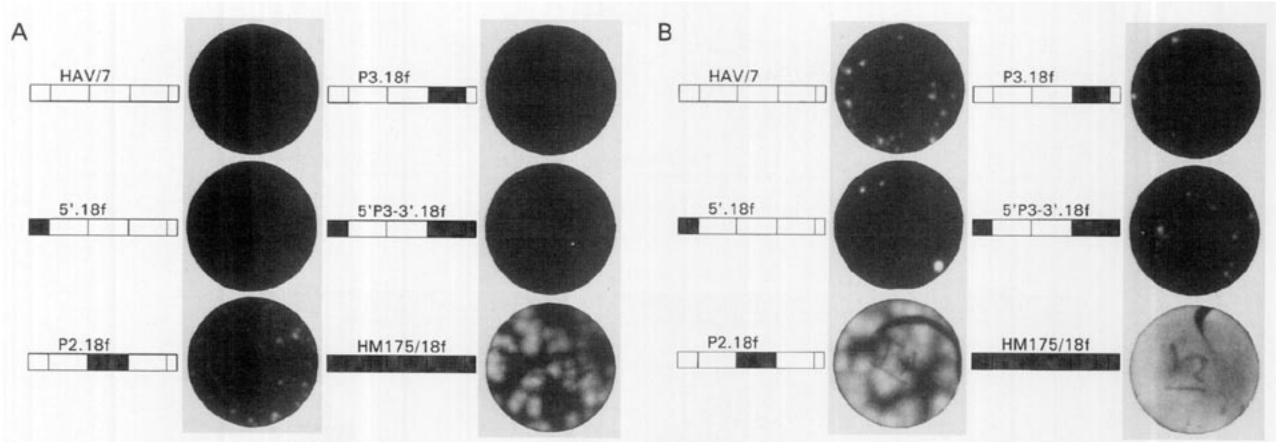

FIG. 3. Negative-image autoradiograms of radioimmunofocus assays carried out with BS-C-1 cells inoculated with viruses rescued in FRhK-4 cells from infectious RNAs which had been transcribed *in vitro* from chimeric cDNAs (HAV/7; 5'.18f; P2.18f; P3.18f; and 5'P3-3'.18f) or with the *rr/cpe*$^+$ HM175/18f virus. For each virus, genomic segments (5'NTR, P1, P2, P3, and 3'NTR) which are shaded indicate HM175/18f sequence. Cells were processed for detection of replication foci after (A) 6 days infection or (B) 14 days infection.

fection, all of the rescued viruses generated readily visible replication foci (Fig. 3B). P2.18f virus foci were much larger than those of HAV/7 virus, but smaller than those of HM175/18f virus. 5'.18f and P3.18f foci were similar to those of HAV/7 virus. Thus, the HM175/18f virus mutations present in the P2 region contribute to the enhanced replication of this *rr/cpe*$^+$ virus in BS-C-1 cells, but are not sufficient for complete expression of this phenotype. Mutations in the 5' or P3 segments have no independent effect on viral replication.

Cooperative action of mutations in the 2B and 2C proteins

Although the VP1/2A cleavage site of HAV remains undefined, epitope mapping studies (S. M. Lemon *et al.*, unpublished data) suggest that RNA encoding the carboxyl terminus of VP1 is present within the *Sac*I/*Eco*RI fragment used to replace the P2 region of HAV/7 with that of the HM175/18f virus (Fig. 4). Both of these viruses contain a single mutation from the wild-type genome in this region: a Glu to Val substitution at VP1 residue 273 in HAV/7, and Ser to Pro at VP1 residue 271 in HM175/18f (Table 1). However, it is unlikely that this latter mutation contributes to the rapid replication phenotype, because it was not present in other *rr/cpe*$^+$ viruses recovered from the persistently infected BS-C-1 cells (Lemon *et al.*, 1991). The location of the 2A/2B cleavage shown in Fig. 4 differs from that proposed by Cohen *et al.* (1987c) and is based on recent identification of this cleavage site in HAV proteins expressed by recombinant vaccinia viruses in eucaryotic cells (A. Martin *et al.*, 1995). There are no mutations in the 2A protein of the *rr/cpe*$^+$ HM175/18f virus (Fig. 4). Thus, the growth enhancement noted with P2.18f virus is most likely due to mutations within the 2B and 2C proteins. To determine how these two proteins contribute to the enhanced replication phenotype of the P2.18f virus, additional constructs were created which contained only the 2AB or 2C coding regions of the HM175/18f virus within the HAV/7 background (plasmids p2AB.18f and p2C.18f) (Fig. 4). These constructions were facilitated by creation of a silent mutation at nt 4022, which established a unique *Eco*RV site close to the sequence at the 2B/2C cleavage site (see Materials and Methods).

In contrast to P2.18f virus, no radioimmunofoci were visible 1 week after infection of BS-C-1 cells with viruses rescued from these two cDNAs in FRhK-4 cells (data not shown). By 2 weeks after infection, small replication foci were apparent, as shown in Fig. 4. The size of the replication foci formed by 2AB.18f virus approximated that of the HAV/7 parent virus, while 2C.18f replication foci appeared to be somewhat smaller. To confirm that neither the silent mutation at nt 4022 which created the unique *Eco*RV site used for construction of these clones nor an adventitious silent mutation at 3899 in the amplified 2B sequence of HM175/18f (see Materials and Methods) was responsible for the small replication focus phenotypes of the 2AB.18f and 2C.18f viruses, the 5' and 3' proximal halves of the p2AB.18f and p2C.18f genomes were fused at the *Eco*RV site to create a new chimera, p2ABC.18f (Fig. 4). This construct contained the entire P2 region of HM175/18f virus and was identical to pP2.18f (Fig. 3) except for the two silent mutations. Virus rescued from p2ABC.18f demonstrated rapid growth in BS-C-1 cells with replication foci significantly larger than HAV/7 virus and similar to those observed at 2 weeks with the P2.18f virus (Fig. 4). This result suggests that mutations in the 2B and 2C proteins of the cytopathic HM175/18f virus act cooperatively to enhance viral replication in BS-C-1 cells, and that these proteins are thus likely to remain

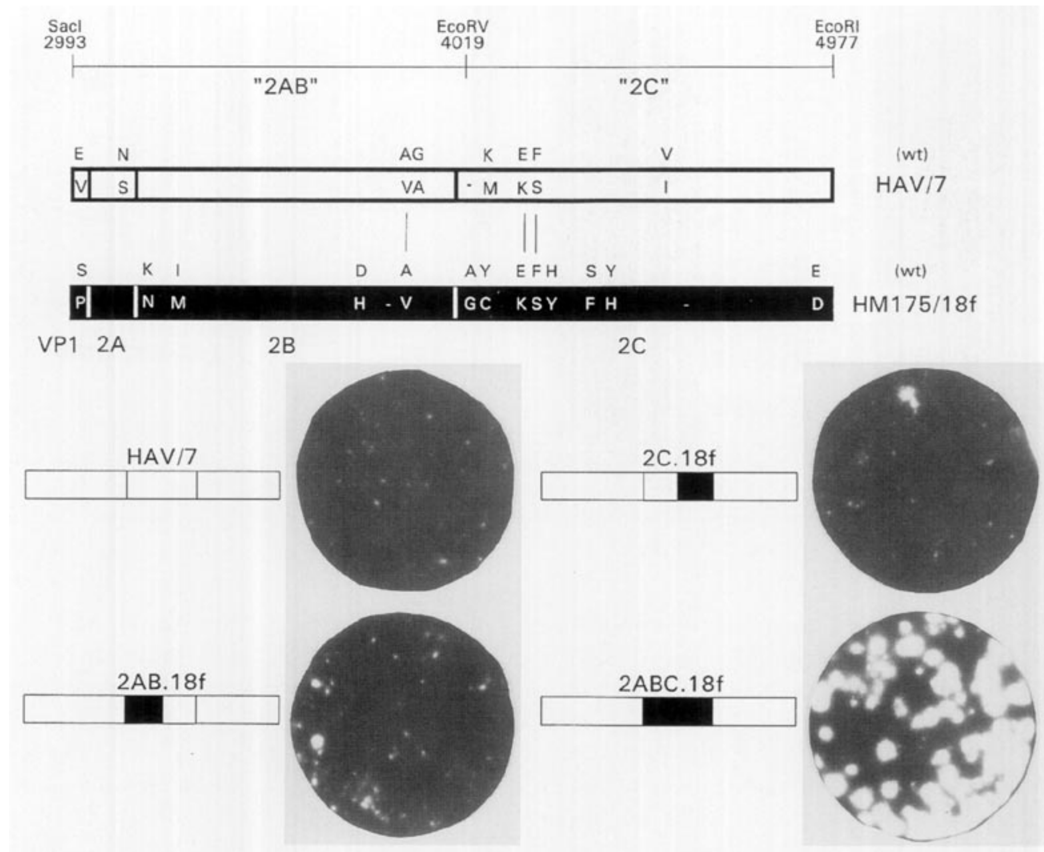

FIG. 4. Radioimmunofocus assays demonstrating cooperative action of mutations in the 2AB and 2C segments of the HM175/18f genome. Amino acid substitutions in the region spanning the *SacI/EcoRI* sites of the HAV/7 and HM175/18f cDNAs are shown at the top, in comparison with the HM175/wt sequence. Vertical lines indicate mutations from wild type which are present in both of the cell culture-passaged viruses. Each of the viruses shown here contains the silent mutation at nt 4022, which created a unique *EcoRV* site near the 2AB/2C junction. p2ABC.18f was reconstructed by fusion of the p2AB.18f and p2C.18f plasmids at this *EcoRV* site. Cells were processed for detection of replication foci 2 weeks after infection. See also Table 1 and legend to Fig. 3.

closely associated with each other during replication of the virus (see Discussion).

Mutations in the 5′NTR and P3 regions cooperate with P2 region mutations to facilitate replication

Since the sizes of the replication foci generated by P2.18f virus were smaller than those generated by HM175/18f virus (Fig. 3), it seemed likely that mutations in other regions of the genome must also contribute to the rapid replication phenotype. Although viruses with chimeric genomes containing only the 5′NTR or P3 segments of the *rr/cpe*$^+$ HM175/18f virus did not demonstrate enhanced replication (Fig. 3B), it remained possible that mutations in these regions could enhance viral replication by cooperating with mutations in the P2 or other region(s) of the genome. Thus, additional chimeric constructs were created which contain the P2 region mutations in combination with mutations present in the 5′NTR, P3, or 3′NTR segments of the HM175/18f virus genome (Fig. 2). As in the experiments described above, virus stocks were rescued following RNA transfection of FRhK-4 cells and the replication competence of each virus was evaluated by radioimmunofocus assay in BS-C-1 cells.

Constructs p5′P2.18f and p5′P2-3′.18f (Fig. 2) were created to determine whether mutations in the noncoding regions of the genome promote viral replication by acting in a cooperative fashion with mutations in the P2 region. One week after infection of BS-C-1 cells, 5′P2.18f and 5′P2-3′.18f replication foci were significantly larger than P2.18f virus foci, but still somewhat smaller than those generated by HM175/18f virus (Fig. 5). The 5′P2.18f and 5′P2-3′.18f replication foci were similar in size. Because the 5′NTR mutations did not enhance replication of the virus in the absence of the P2 region mutations (Fig. 3), these results indicate that mutations in the 5′NTR (but not the 3′NTR) of HM175/18f virus contribute to the rapid replication phenotype by acting in cooperation with mutations in the P2 region.

To similarly evaluate the role of the P3 region mutations, we constructed a chimera containing both the P2 and P3 segment mutations of HM175/18f virus (pP2P3.18f, Fig. 2). The resulting P2P3.18f virus generated replication foci which were substantially larger than

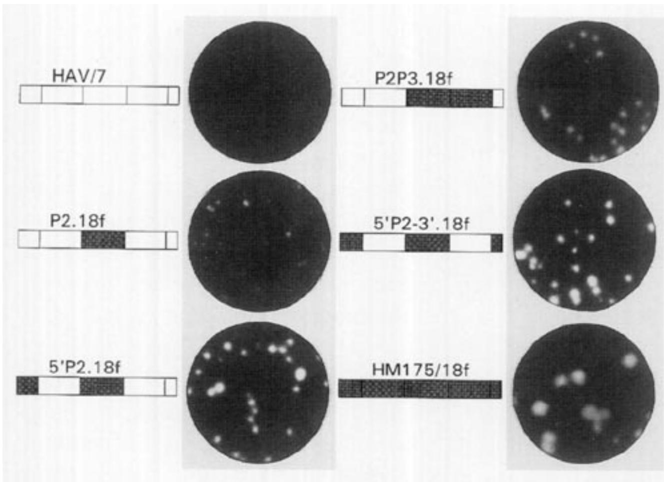

FIG. 5. Radioimmunofocus assays of chimeric viruses containing combinations of 5′NTR and P2, P2, and P3, or 5′NTR, P2, and 3′NTR segments of the HM175/18f virus genome within the background of HAV/7. Cells were processed for detection of replication foci after 6 days infection. See legend to Fig. 3.

P2.18f replication foci, but still smaller than those of HM175/18f virus 1 week following infection of BS-C-1 cells (Fig. 5). These data indicate that mutations in the P3 region also act to enhance viral replication. However, since the P3 mutations by themselves had no effect on viral replication (Fig. 3), they also facilitate replication only in cooperation with mutations in the P2 region.

In contrast to these results, no interaction was noted between the 5′NTR and P3 region mutations. Virus rescued from p5′P3-3′.18f did not generate visible replication foci by 1 week after infection (Fig. 3A), while at 2 weeks, 5′P3-3′.18f replication foci were similar in size to those of HAV/7 (Fig. 3B). Thus, the combination of the 5′NTR, 3′NTR, and P3 region mutations did not enhance the growth of HAV/7 virus. These data further support the notion that mutations in the P2 region are necessary for the rapid replication phenotype. In the radioimmunofocus assays shown in Fig. 5, there were no differences in the sizes of 5′P2.18f and 5′P2-3′.18f replication foci. Thus, the mutations in the 3′NTR of HM175/18f virus do not appear to enhance replication.

Mutations in the 5′NTR, P2, and P3 regions confer the rapid replication phenotype

The data presented above suggest that mutations in the 5′NTR, P2, and P3 regions of the HM175/18f virus act cooperatively to facilitate replication of the virus. Thus, in an effort to construct an infectious cDNA clone with optimal replication properties, and to further evaluate the potential role of the 3′NTR mutations, two additional chimeric plasmids were created. p5′P2P3.18f contains the 5′NTR, P2, and P3 regions of the HM175/18f virus sequence, leaving HAV/7 virus sequence only in the P1 and 3′NTR segments (Fig. 2). p5′P2P3-3′.18f represents the entire HM175/18f virus sequence except for the P1 region which corresponds to HAV/7. By 1 week after infection of BS-C-1 cells, viruses rescued from both of these constructs generated very large replication foci which approximated the size of the HM175/18f foci (Fig. 6). Viruses rescued from these two constructs thus appear to have the same growth characteristics as the HM175/18f virus. The size of the replication foci was not influenced by the presence or absence of the 3′NTR mutations found in HM175/18f virus, consistent with the results of earlier experiments which suggested that the mutations in the 3′NTR do not enhance viral replication (Fig. 5).

To confirm that these rapidly replicating viruses were in fact derived from infectious cDNA and not simply HM175/18f virus contaminants, partial P1 segment RNA sequences of the P2P3.18f, 5′P2P3.18f, and 5′P2P3-3′.18f viruses were determined using an antigen-capture RT/PCR method (Jansen *et al.*, 1990). Each of these three viruses contained unique HAV/7 sequence within the P1 region, indicating that these *rr/cpe*$^+$ viruses had been rescued from cDNA (data not shown).

Cytopathic effects of chimeric HM175 viruses

Unlike HAV/7 and most other cell culture-adapted HAV variants, the HM175/18f virus is cytopathic and capable of forming conventional plaques that can be visualized with vital staining of infected cells maintained under agarose overlays (Lemon *et al.*, 1991). To determine whether the rapidly replicating chimeric viruses described above were capable of forming conventional plaques, BS-C-1 cells were infected with selected virus stocks and overlaid with agarose containing 25 m*M* $MgCl_2$. One week after inoculation, the cultures received a second overlay containing neutral red (Fig. 7). Plaques were readily visualized in cells infected with HM175/18f virus, as expected. Similar plaques were also observed in cells infected with the 5′P2.18f, P2P3.18f, and 5′P2P3-3′.18f viruses (Fig. 7), as well as 5′P2P3.18f (data not shown). Plaque number was related to inoculum size,

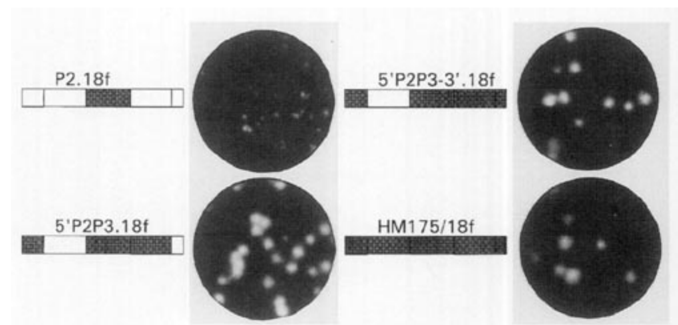

FIG. 6. Radioimmunofocus assays of chimeric viruses containing combinations of 5′NTR, P2, and P3, or 5′NTR, P2, P3, and 3′NTR segments of the HM175/18f virus genome within the background of HAV/7. Cells were processed for detection of replication foci after 6 days infection. See legend to Fig. 3.

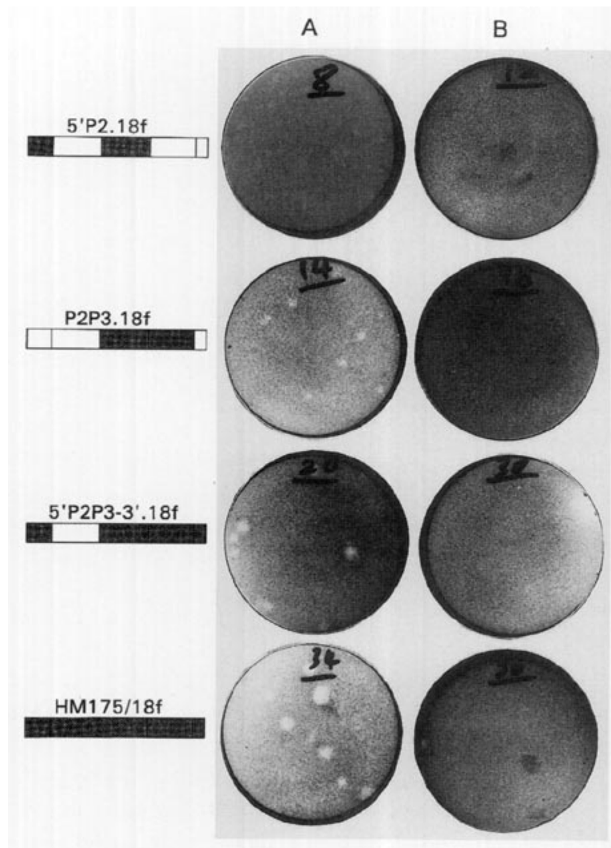

FIG. 7. Plaque assay demonstrating cytopathic effects of selected chimeric viruses. BS-C-1 cells were inoculated with indicated viruses and overlaid with agarose as described under Materials and Methods. Cells were inoculated with virus (A) before or (B) after neutralization with anti-HAV antibody. Following incubation for 1 week, cultures received a second overlay which contained neutral red.

and plaque formation was inhibited by incubation of the virus with neutralizing anti-HAV antibodies prior to inoculation of cells (Fig. 7). Thus, the rapidly replicating chimeric viruses were also cytopathic. Very small plaques may also have been formed by the P2.18f virus, but there was no suggestion of plaque formation by HAV/7 virus (data not shown).

DISCUSSION

The HM175/18f virus is one of several cell culture-adapted HM175 variants which were rescued from persistently infected BS-C-1 cells and which have a rapid replication phenotype associated with cytopathic effects (*rr/cpe*$^+$) in cultured monkey kidney cells (Lemon *et al.*, 1991). The nucleotide sequences of these viruses demonstrate the presence of numerous mutations from the sequence of the more slowly replicating, noncytopathic (*rr/cpe*$^-$) parent virus. These mutations are scattered throughout the genome. However, there is conservation of the mutations within the 5′NTR, P2, and P3 regions of all three *rr/cpe*$^+$ viruses, which contrasts with substantial differences in the mutations present in intervening segments of the genome. These findings led us to suggest previously that mutations in each of these regions may contribute to the enhanced replication capacities of these viruses in cultured cells (Lemon *et al.*, 1991). We have now confirmed this hypothesis by evaluating the replication properties of a large series of chimeric viruses in which segments of the *rr/cpe*$^+$ HM175/18f genome were placed in the background of the *rr/cpe*$^-$ HAV/7 virus.

In these experiments, the primary measure of the replication capacity of chimeric viruses was the size of replication foci observed in radioimmunofocus assays carried out in infected BS-C-1 cells (Lemon *et al.*, 1983). While the size of conventional viral plaques is not necessarily a good measure of viral replication capacity, radioimmunofocus size correlates well with the replication capacity of HAV as assessed by much more labor-intensive one-step replication studies in cultured cells (Day *et al.*, 1992; Shaffer *et al.*, 1994). This is not surprising, as the diameter of a replication focus visualized in a radioimmunofocus assay is exponentially related to the number of infected cells which express viral antigen in quantities sufficient for the binding of detectable amounts of radiolabeled anti-HAV antibody. The size of the replication focus is also proportionate to the duration of the infection. The *rr/cpe*$^+$ HM175/18f virus forms large foci within 6–8 days of inoculation, while 14 days or longer may be required to form similarly sized foci of the *rr/cpe*$^-$ HAV/7 virus (Fig. 3, compare A and B).

The results of these studies indicate that mutations in the P2 region (specifically proteins 2B and 2C) of HM175/18f virus are necessary but not sufficient for the full expression of the rapid replication properties of this virus (Figs. 3–5). This finding is consistent with earlier observations that mutations in the 2B and 2C proteins are particularly important in the early host range change that occurs during the adaptation of wild-type HAV to growth in cultured monkey kidney cells (Emerson *et al.*, 1992). While the 2A sequence of HM175/18f does not differ from the wild-type sequence, the 2B and 2C proteins contain a number of mutations from the wild-type as well as the *rr/cpe*$^-$ HM175/P16 and HAV/7 viruses (Table 1, Fig. 4). The inclusion of only the 2C protein coding sequence of HM175/18f failed to enhance the replication of HAV/7 virus (Fig. 4). Mutations in 2C were only beneficial to viral replication in the presence of the mutations in 2B, which by themselves also had no impact on viral replication (Fig. 4). A single Ser to Pro substitution in the putative carboxyl terminus of VP1 was included with the 2AB sequence of HM175/18f in the chimeric viruses shown in Fig. 4. However, it is unlikely that this mutation plays any role in determining the rapid replication phenotype as, unlike the 2B and 2C mutations, this mutation is not present in other *rr/cpe*$^+$ HM175 variants (Lemon *et al.*,

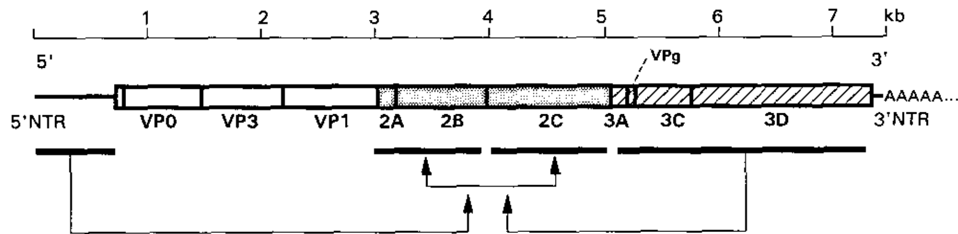

FIG. 8. Diagram indicating positive cooperative actions of mutations in different segments of the HM175/18f virus genome.

1991). The strongly cooperative action of mutations within the 2B and 2C proteins of HAV (Fig. 8) indicates that these proteins are likely to remain closely associated during viral replication. This possibility was also suggested by Emerson *et al.* (1992), who observed that mutations in the 2B and 2C proteins of HAV/7 virus, while each having independent effects on viral replication, contributed to an ostensibly greater than additive enhancement of growth in FRhK-4 cells. Consistent with this interpretation, the 2B and 2C proteins of poliovirus are found in close physical proximity within the membrane-bound poliovirus replication complex (Bienz *et al.*, 1990, 1992).

Mutations in the 5′NTR and P3 segments of the cytopathic HM175/18f virus, either alone or in combination with each other, did not noticeably enhance the replication of the virus (Fig. 3). However, when these mutations were placed in the background of a virus containing the P2 mutations of HM175/18f virus, there were substantial increases in the size of viral replication foci (Fig. 5). These data provide support for several interactions between diverse segments of the picornaviral genome (Fig. 8).

Although we found that mutations in the 5′NTR of the cytopathic virus significantly enhanced replication only if placed in the background of virus containing additional mutations in 2B and 2C, there are few other lines of evidence supporting direct interactions between the 5′NTR and P2 proteins of picornaviruses. While potential interactions have been noted between the 5′NTR and 2A proteins of poliovirus (Hambidge and Sarnow, 1992; Macadam *et al.*, 1994), the 2A protein of HAV lacks the proteinase activity of the poliovirus 2A protein and shows little other evidence of relatedness. Moreover, there are no mutations in the 2A protein of HM175/18f, indicating that this protein plays no role in determining the *rr/cpe*$^+$ phenotype. Funkhouser *et al.* (1994) demonstrated that a combination of mutations in the 5′NTR and 2C protein of an MRC-5 cell-adapted HAV promoted the growth of the virus in MRC-5 cells. However, unlike the 5′NTR and 2C mutations of the *rr/cpe*$^+$ HM175/18f virus that we studied, both the 5′NTR and 2C mutations of the MRC-5 adapted virus appeared to have independent although lesser effects on viral replication in these cells.

It seems likely that interactions between the P2 (2B and 2C) proteins and the 5′NTR of HAV could occur within the context of a putative membrane-bound HAV replication complex. An essential step in the synthesis of poliovirus RNA involves the assembly of a ribonucleoprotein complex at the 5′ end of the viral RNA (Andino *et al.*, 1990a, 1993). RNA replication occurs in tight association with small membranous structures, the formation of which appears to involve both 2B and 2C proteins (Bienz *et al.*, 1990, 1992; Cho *et al.*, 1994). While 3AB and 3CD^{pro} are thought to interact directly with the 5′NTR during assembly of the picornaviral ribonucleoprotein replication complex (Andino *et al.*, 1990b; Allaire *et al.*, 1994; Harris *et al.*, 1994), the interactive nature of the 2BC and 5′NTR mutations observed in the present study suggests the possibility of a similar role for the 2B and 2C proteins of HAV.

In light of this interpretation of our results, it is interesting to note that the most striking change which is present in the 5′NTR of the *rr/cpe*$^+$ viruses is a 14-nt sequence reduplication involving nt 140–153 (Table 1) (Lemon *et al.*, 1991). This sequence comprises a single-stranded domain within the 5′NTR of HAV (Shaffer *et al.*, 1994). It is located immediately upstream of the IRES of HAV as mapped in our laboratory (Brown *et al.*, 1994), and small deletions within it (nt 140–144) result in a temperature-sensitive defect in viral RNA synthesis (Shaffer *et al.*, 1994, 1995). However, the 5′NTR of HM175/18f also contains several mutations between nt 551 and 687 which are clearly within the IRES (Table 1), including a U to G substitution at nt 687 which we have recently shown acts to facilitate HAV IRES-directed translation in BS-C-1 cells (Shultz *et al.*, manuscript submitted for publication). Thus, further studies will be required to determine whether the cooperative action of mutations in the 5′NTR and P2 proteins facilitates RNA replication or results in improved translation efficiency.

The additional cooperative action of the P2 and P3 segment mutations in facilitating the replication of HM175/18f virus (Figs. 5 and 8) is consistent with the notion that translation of the P2 and P3 proteins is *cis*-active with respect to replication of picornaviruses. The *cis*-dominant role of these proteins has been inferred from genetic complementation studies and analysis of naturally occurring poliovirus defective interfering particles (reviewed by Wimmer *et al.*, 1993), as well as the lack of viability of bicistronic poliovirus genomes in which P2 and P3 proteins are translated from different cistrons

(Schmid and Wimmer, 1994). The fact that the P3 mutations present in HM175/18f enhance viral replication only in the presence of the mutations in the P2 proteins (Figs. 3 and 5) provides evidence for P2–P3 protein interactions in the replication of HAV (Fig. 8). Although it is also possible that the cooperative action of these mutations could reflect altered processing of the polyprotein (Emerson *et al.*, 1992), this seems much less likely. The multiple interactions between mutations in various segments of the HAV genome which were observed in this study (Fig. 8) reflect both the compact organization of the picornaviral genome and the assignment of multiple functions to many of the viral proteins (Wimmer *et al.*, 1993). The fact that mutations in multiple regions of the genome of HM175/18f virus contribute to the *rr/cpe*$^+$ phenotype of this virus is consistent with previous observations which indicate that the adaptation of wild-type virus to growth in cultured cells involves mutations in several viral proteins as well as the 5′NTR (Emerson *et al.*, 1992, 1993; Day *et al.*, 1992). Thus, the *rr/cpe*$^+$ phenotype can be considered an extension of the adaptation of the virus to growth in cultured cells (Lemon *et al.*, 1991).

Morace *et al.* (1993) noted the deletion of aspartic acid residues near the amino terminus of the 3A proteins of unrelated cytopathic HAVs and suggested that this mutation (which is present in HM175/18f virus) alters an amphipathic helical structure putatively located in this region of the 3A protein. These investigators postulated a primary role for this mutation in causing the cytopathic effects observed with rapidly replicating HAV variants in eucaryotic cells. However, we found that P3 region mutations were not essential for the cytopathic effect of HM175/18f (Fig. 7). Rather, our results suggest that the HAV cytopathic effect is closely related to the overall replication capacity of the virus, as previously suggested (Lemon *et al.*, 1991), and not due to any single mutation. Significant shutdown of host cell protein synthesis, as observed with other cytopathic picornaviruses, does not appear to occur in cells infected with HM175/18f (data not shown) or other HAVs (Gauss-Muller and Deinhardt, 1984).

In addition to providing new information concerning the molecular basis of the enhanced replication capacity and cytopathic effects of *rr/cpe*$^+$ strains of HAV, the studies described here have resulted in new cDNA clones which should prove useful for future research. While 2 weeks or longer are generally required for rescue of HAV from the pHAV/7 infectious clone, this period of time is reduced to 6 days or less for the p5′P2P3-3′.18f clone. The pP2.18f construct, which contains only the P2 region of HM175/18f virus and has an intermediate growth phenotype (Fig. 3), has already proven very useful for the creation of temperature-sensitive 5′NTR deletion mutants (Shaffer *et al.*, 1994, 1995). It is likely that the availability of fully *rr/cpe*$^+$ infectious clones (p5′P2P3-3′.18f) will facilitate additional genetic studies of HAV.

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