JOURNAL OF VIROLOGY, Oct. 1987, p. 3035–3039
0022-538X/87/103035-05$02.00/0

Vol. 61, No. 10

Hepatitis A Virus cDNA and Its RNA Transcripts Are Infectious in Cell Culture

JEFFREY I. COHEN,* JOHN R. TICEHURST, STEPHEN M. FEINSTONE, BETSY ROSENBLUM, AND ROBERT H. PURCELL

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 16 April 1987/Accepted 23 June 1987

A full-length cDNA copy of an attenuated, cell culture-adapted hepatitis A virus (HAV HM-175/7 MK-5) genome was constructed in the *Pst*I site of plasmid vector pBR322. Transfection of monkey kidney cells with this plasmid failed to induce the production of hepatitis A virus (HAV). The HAV cDNA was excised from pBR322 and inserted, without the oligo(dG) · oligo(dC) tails, into an RNA transcription vector to yield plasmid pHAV/7. Transfection of monkey kidney cells with pHAV/7 DNA induced HAV infection. Transfection with RNA transcripts produced in vitro from pHAV/7 yielded about 10-fold more HAV than did transfection with pHAV/7 DNA. Marmosets inoculated with transfection-derived virus developed anti-HAV antibodies and had liver enzyme patterns that closely resembled the liver enzyme patterns seen in animals inoculated with virus from a comparable level of cell culture passage. Infectious RNA transcripts from HAV cDNA should be useful for studying the molecular basis of cell culture adaptation and attenuation as well as for studying specific viral functions.

Hepatitis A virus (HAV) is a plus-strand RNA virus which is a member of the picornavirus family. The genome of wild-type HAV strain HM-175 is 7,478 nucleotides long, is followed by a poly(A) tail, and encodes a polyprotein of 2,227 amino acids (4). Wild-type HAV grows poorly in cell cultures, is not cytopathic, and produces low yields of virus. Although HAV RNA (extracted from virions) is infectious in cell cultures (10, 18), direct manipulation of the viral genome (e.g., analysis of specific mutants and recombinants) is difficult because of its RNA composition. Nonetheless, direct identification of areas of the genome responsible for cell culture adaptation, viral attenuation, and specific viral functions requires this general strategy.

An alternative approach that does not use virion RNA has been used for genetic studies of other RNA viruses. Infectious cDNAs have been constructed for poliovirus types 1 to 3 (1, 14, 15) coxsackievirus B3 (8), bacteriophage Qβ (20), retroviruses (including human immunodeficiency virus [7]), and viroids (23). Infectious RNA transcripts have also been synthesized in vitro from cDNA clones of poliovirus (22), rhinovirus (12), plant viruses (6), viroids (23), and satellite viruses of plants (19).

We recently determined the nucleotide sequence of cDNA from an attenuated, cell culture-adapted HAV (3a). This virus is attenuated for chimpanzees and partially attenuated for marmosets but has not yet been tested in humans (R. Karron, R. Daemer, J. Ticehurst, E. D'Hondt, H. Popper, K. Mihalik, J. Phillips, S. Feinstone, and R. H. Purcell, submitted for publication). In this study we describe the assembly of cDNA clones from this virus to form a full-length cDNA copy of the genome. In addition, RNA transcripts were produced in vitro from the cDNA. Both the HAV cDNA and RNA transcripts were shown to be infectious when transfected into mammalian cells.

* Corresponding author.

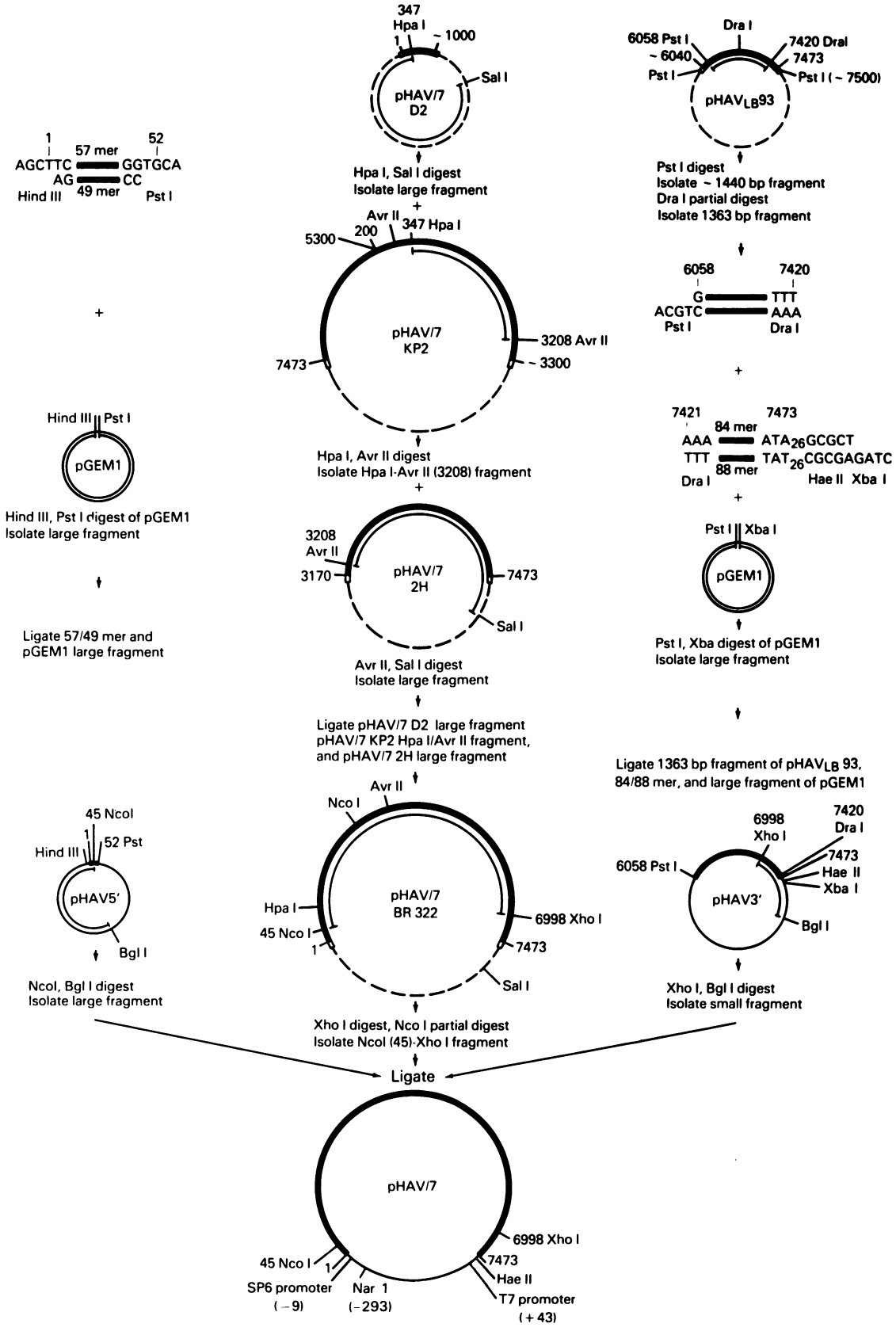
MATERIALS AND METHODS

Construction of full-length HAV cDNA. Molecular cloning of HAV HM-175/7 MK-5 was described previously (3a). Three cDNA clones, pHAV/7 D2, pHAV/7 KP2, and pHAV/7 2H, spanned the entire genome of the virus. Restriction endonuclease fragments from these three clones were ligated together (Fig. 1, column 2), and the resulting plasmid, pHAV/7 BR322, contained a full-length cDNA copy of HAV HM-175/7 MK-5 in plasmid vector pBR322. The HAV cDNA insert in this plasmid was flanked by oligo(dG) · oligo(dC) tails remaining from the cloning procedure.

To construct full-length HAV cDNA without oligo(dG) · oligo(dC) tails, we synthesized double-stranded oligonucleotides representing the 5′ and 3′ termini of the viral genome. Two oligonucleotides (57-mer and 49-mer) corresponding to the 5′ end of wild-type HAV HM-175 virion RNA (and its complementary strand) with *Hin*dIII and *Pst*I restriction sites at the 5′ and 3′ ends, respectively, were synthesized (model 380A DNA synthesizer; Applied Biosystems, Foster City, Calif.) (Fig. 1, column 1). After gel purification and annealing of the complementary oligonucleotides, the resulting double-stranded DNA was ligated to plasmid vector pGEM1 (Promega Biotec, Madison, Wis.) to yield pHAV5′.

Similarly, two oligonucleotides (84-mer and 88-mer) corresponding to the 3′ end of wild-type HAV HM-175 virion RNA (and its complementary strand) with a 26-base oligo(dA) · oligo(dT) tail followed by *Hae*II and *Xba*I restriction sites were synthesized (Fig. 1, column 3). After gel purification and annealing, the resulting DNA was ligated to a restriction fragment from $pHAV_{LB}93$ (a cDNA clone from wild-type HAV HM-175 [21]) and pGEM1 to yield pHAV3′.

The appropriate restriction fragments from pHAV5′, pHAV/7 BR322, and pHAV3′ were ligated together to yield plasmid pHAV/7. This plasmid contains a full-length cDNA copy of HAV HM-175/7 MK-5 in pGEM1, except for two nucleotide changes. Nucleotides at positions 7027 and 7425


1
57 mer
52
AGCTTC
GGTGCA
AG
CC
Hind III
49 mer
Pst I
+
Hind III Pst I
pGEM1
Hind III, Pst I digest of pGEM1
Isolate large fragment
Ligate 57/49 mer and
pGEM1 large fragment
347
Hpa I
1
~ 1000
Sal I
pHAV/7
D2
Hpa I, Sal I digest
Isolate large fragment
+
Avr II
347 Hpa I
5300
200
pHAV/7
KP2
3208 Avr II
7473
~ 3300
Hpa I, Avr II digest
Isolate Hpa I-Avr II (3208) fragment
+
3208
Avr II
3170
pHAV/7
2H
7473
Sal I
Avr II, Sal I digest
Isolate large fragment
Ligate pHAV/7 D2 large fragment
pHAV/7 KP2 Hpa I/Avr II fragment,
and pHAV/7 2H large fragment
Dra I
6058 Pst I
~ 6040
Pst I
7420 DraI
7473
Pst I (~ 7500)
pHAV$_{LB}$93
Pst I digest
Isolate ~ 1440 bp fragment
Dra I partial digest
Isolate 1363 bp fragment
6058
7420
G
TTT
ACGTC
AAA
Pst I
Dra I
+
7421
7473
84 mer
AAA
ATA$_{26}$GCGCT
TTT
TAT$_{26}$CGCGAGATC
88 mer
Dra I
Hae II Xba I
+
Pst I Xba I
pGEM1
Pst I, Xba digest of pGEM1
Isolate large fragment
Ligate 1363 bp fragment of pHAV$_{LB}$ 93,
84/88 mer, and large fragment of pGEM1
45 NcoI
1
52 Pst
Hind III
pHAV5'
Bgl I
NcoI, Bgl I digest
Isolate large fragment
Avr II
Nco I
Hpa I
45 Nco I
1
pHAV/7
BR 322
6998 Xho I
7473
Sal I
Xho I digest, Nco I partial digest
Isolate NcoI (45)-Xho I fragment
6998
Xho I
7420
Dra I
7473
Hae II
Xba I
6058 Pst I
pHAV3'
Bgl I
Xho I, Bgl I digest
Isolate small fragment
Ligate
pHAV/7
6998 Xho I
45 Nco I
1
7473
Hae II
SP6 promoter
(-9)
Nar 1
(-293)
T7 promoter
(+43)

correspond to those of wild-type HAV HM-175 (4). The nucleotide sequence at the ligation junctions was determined directly from plasmid DNA by using the method of Zagursky et al. (24). All constructs had the expected junction sites.

In vitro transcription. Plasmid pHAV/7 was digested with *Hae*II [cuts immediately after the poly(A) tail], and the resulting linear DNA was extracted sequentially with phenol-chloroform and chloroform and then precipitated with ethanol. RNA transcription was performed with SP6 polymerase (Promega Biotec) in a reaction mixture containing 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine hydrochloride, 10 mM NaCl, 10 mM dithiothreitol, RNasin (Promega Biotec), 2 μg of linearized pHAV/7, and 0.5 mM each ATP, CTP, UTP, and GTP. The reaction mixture was incubated at 37°C for 1 h, DNase I (20 U/ml; RQ1; Promega Biotec) was added, and incubation was continued at 37°C for 15 min. The reaction mixture was extracted sequentially with phenol-chloroform and chloroform and then precipitated twice with ethanol.

Synthesis of minus-strand RNA was identical to that described above, except that pHAV/7 was linearized with *Nar*I (cuts 293 bases before the 5′ end of HAV) and transcription was performed with T7 polymerase.

Transfection of cells and detection of HAV. RNA transfections were performed in African green monkey kidney (AGMK) cells (second passage) or CV-1 cells by a modification of the procedure of Van der Werf et al. (22). About 4 μg of RNA (one-half of the transcription reaction product) was dissolved in 0.5 ml of HBSS buffer (21 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose [pH 7.05]), and 0.5 ml of DEAE-dextran (1 mg/ml in HBSS) was added. AGMK cells in 6-cm dishes (80% confluent) were washed once with Dulbecco modified Eagle medium (DMEM), 1 ml of RNA in DEAE-dextran was added, and the cells were placed at room temperature for 30 min. DMEM (2 ml) supplemented with 10% (vol/vol) fetal calf serum was added, and the cells were incubated at 35°C for 6 h with rocking every hour. After 6 h the medium was removed, the cells were washed twice with DMEM, DMEM supplemented with 10% (vol/vol) fetal calf serum was added, and the cells were incubated at 35°C. DNA transfections were performed as described by Racaniello and Baltimore (15), except that 5 μg of plasmid DNA was used for each 6-cm dish and the glycerol shock was performed for 1 min at room temperature.

Two weeks after transfection, the cells were trypsinized and plated onto glass cover slips. The cells were assayed with fluorescein-labeled antibody (11) for the production of hepatitis A antigen each week thereafter.

Animal studies. Five weeks after transfection, AGMK cells were pelleted and lysed, and 1 ml of DMEM supplemented with 10% fetal calf serum was added. A 25% suspension (vol/vol) of AGMK cell lysate in fetal calf serum was injected intravenously into marmosets (*Saguinus mystax*). Animals were monitored weekly for the determination of serum isocitrate dehydrogenase (ICD) and anti-HAV antibody (Abbott Laboratories, North Chicago, Ill.). Serum ICD levels that rose to twice the mean preinoculation value for each animal were considered elevated if they occurred within 4 weeks preceding or following seroconversion.

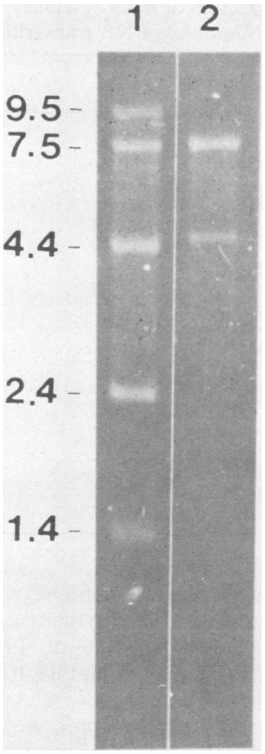


FIG. 2. RNA transcribed from pHAV/7 with SP6 polymerase. RNA was denatured with 50% formamide and 6.4% formaldehyde, electrophoresed on a 1% agarose gel containing 2% formaldehyde, and stained with ethidium bromide. Lane 1 indicates RNA size markers, and lane 2 indicates plus-strand RNA. Each marker band contains 0.17 μg of RNA; RNA in lane 2 represents 2% of the SP6 polymerase reaction. The sizes of the marker bands are in kilobases.

RESULTS

Analysis of RNA transcripts. RNA transcribed in vitro from pHAV/7 should correspond to virion RNA except for (i) the absence of a genome-linked viral protein (VPg) at the 5′ terminus, (ii) 10 additional nucleotides at the 5′ end (GAATACAAGC), (iii) two nucleotide changes corresponding to wild-type HAV HM-175 RNA, and (iv) a shortened (26-base) poly(A) tail. Virion RNA contains a 40- to 80-base poly(A) tail (18). RNA from the transcription reaction was denatured with formamide and formaldehyde (5) and analyzed on a 1% agarose gel (Fig. 2). The predominant RNA species was 7.5 kilobases in length, but two additional bands (4.6 and 1.6 kilobases) were noted. Two additional bands

FIG. 1. Construction of full-length HAV HM-175/7 MK-5 cDNA in pGEM1. Single lines indicate pGEM1 DNA, dashed lines indicate pBR322 DNA, solid bars indicate HAV cDNA, open bars indicate oligo(dG) · oligo(dC) tails on the ends of the cDNA, and inner circles indicate restriction fragments used for ligations. Numbers refer to nucleotides of HAV HM-175/7 MK-5 RNA (3a), and numbers in parentheses refer to nucleotides of pGEM1. Column 1 shows construction of the 5′ end of HAV cDNA in pGEM1, column 2 shows construction of full-length HAV cDNA in pBR322, and column 3 shows construction of the 3′ end of HAV cDNA in pGEM1. Plasmid HAV/7 KP2 contains two discontinuous portions of the HAV genome arranged head-to-head. $pHAV_{LB}93$ is a cDNA clone from wild-type HAV HM-175 (21); however, nucleotide positions have been renumbered (5 bases deleted) to correspond to those of HAV HM-175/7 MK-5. bp, Base pairs.

TABLE 1. Transfection of monkey kidney cells with HAV cDNA and its RNA transcripts

Cells and nucleic acid	HAV produced
Secondary AGMK	
RNA DEAE-dextran	
pHAV/7 + DNase I	+
pHAV/7 + DNase I + RNase A	−
pHAV/7 minus-strand + DNase I	−
DNA calcium phosphate	
pHAV/7	+
pHAV/7 + RNase A	+
pHAV/7 + DNase I	−
pHAV/7 BR322	−
CV-1	
RNA DEAE-dextran	
pHAV/7 + DNase I	+
pHAV/7 + DNase I + RNase A	−

(5.5 and 3.0 kilobases) were also noted when minus-strand RNA was synthesized with T7 polymerase. When poliovirus cDNA was transcribed with SP6 or T7 polymerase, an additional band attributed to premature transcription termination was seen (9).

DNA and RNA transfections. Three weeks after transfection with plus-strand RNA transcripts from HAV/7, about 10% of AGMK cells contained hepatitis A antigen (Table 1). By 5 weeks after transfection, about 80% of the cells were producing antigen. RNA transcripts treated with RNase A (50 μg/ml) before transfection and minus-strand RNA transcripts did not induce the production of hepatitis A antigen.

Five weeks after transfection, AGMK cells that had been transfected with RNA transcripts from pHAV/7 were trypsinized, pelleted, and suspended in 1.0 ml of DMEM supplemented with 10% (vol/vol) fetal calf serum. The cells were then frozen, thawed, sonicated three times, and inoculated into uninfected BS-C-1 cells. One week later hepatitis A antigen was detected in BS-C-1 cells. The original AGMK cell lysate contained about $10^{7.8}$ 50% tissue culture infective doses per ml. Hepatitis A antigen was not detected in BS-C-1 cells inoculated with cell cultures from the control transfections.

Three weeks after transfection with pHAV/7 DNA, about 1% of AGMK cells contained hepatitis A antigen. Transfection with pHAV/7 DNA treated with RNase A also yielded antigen; however, transfection with pHAV/7 BR322 DNA or pHAV/7 DNA treated with DNase I (20 U/ml) failed to induce the production of antigen.

Marmosets inoculated intravenously with 0.125 ml of AGMK cell suspension ($10^{6.9}$ 50% tissue culture infective doses) developed anti-HAV antibodies within 6 weeks of inoculation (Table 2). The geometric mean peak ICD level was 2,252 sigma units/ml (2.99 times the mean preinoculation value). Marmosets inoculated with AGMK cell lysate obtained after transfection with an earlier HAV construct (which failed to yield detectable virus in vitro) did not develop anti-HAV antibodies (data not shown).

TABLE 2. Liver enzymes of marmosets receiving virus recovered from transfections

Marmoset no.	Mean pre-inoculation ICD value (sigma units/ml)	Peak ICD level (sigma units/ml)	Wk after inoculation until:	
			Peak ICD level	Positive anti-HAV antibody
457	1,047	1,903	8	4
458	668	4,038	8	4
475	766	1,167	3	5
476	621	2,798	5	6

DISCUSSION

Transfection of AGMK cells with HAV cDNA or plus-strand RNA transcripts from HAV cDNA induced HAV infection. Thus, transfections with HAV nucleic acid resemble those with poliovirus in which cDNA (15) or plus-strand RNA transcripts (22) are infectious. In contrast, RNA transcripts from rhinovirus cDNA were infectious, but the cDNA alone was not (12). Transfection with RNA transcripts of HAV cDNA was more efficient than that with HAV cDNA, as evidenced by the higher percentage of cells infected. Van der Werf et al. (22) observed that RNA transcripts from poliovirus cDNA were at least 100-fold more infectious than was the cDNA.

Marmosets which were inoculated with virus produced following transfection had a mean peak ICD of 2,252 sigma units/ml. Two of the four animals had ICD levels more than twice their preinoculation value. Marmosets inoculated with HAV HM-175/7 MK-2 (three cell culture passages earlier than the virus used for cDNA cloning in this study) had a mean peak ICD of 1,686 sigma units/ml, and two of the five animals had ICD levels more than twice their preinoculation value. In contrast, four marmosets which received wild-type HAV HM-175 had a mean peak ICD of 6,076 sigma units/ml, and all four had ICD levels more than twice their preinoculation value (Karron et al., submitted). Thus, marmosets inoculated with transfection-derived virus developed liver enzyme levels that closely resembled the liver enzyme levels seen in animals inoculated with virus from a comparable level of cell culture passage. Hence, the phenotype for attenuation was retained by the molecularly cloned virus.

The RNA transcripts from HAV cDNA had 10 additional nucleotides at the 5′ end but no additional nucleotides after the poly(A) tail. While pHAV/7 DNA was infectious, we were unable to demonstrate infectivity with pHAV/7 BR322 DNA. This latter construct was the parent of pHAV/7 and differed from it only by (i) the plasmid vector used (pBR322 versus pGEM1), (ii) the presence of oligo(dG) · oligo(dC) tails, (iii) the length of the poly(A) tail (about 5 bases versus 26 bases in pHAV/7), and (iv) 2 bases in pHAV/7 that correspond to wild-type HAV HM-175 RNA (see Materials and Methods). We suspect that the oligo(dG) · oligo (dC) tails may be responsible for the lack of infectivity of pHAV/7 BR322.

Infectious RNA transcripts of HAV cDNA should facilitate further study of the biology of HAV. Chimeric HAVs derived from recombinants of wild-type and attenuated (cell culture-adapted) infectious cDNAs (or RNAs) would be useful for mapping areas of the genome responsible for attenuation and cell culture adaptation. Experiments with chimeric polioviruses have shown that attenuation of the live poliovirus type 1 vaccine strain is due to multiple nucleotide changes scattered over wide areas of the genome (13). Chimeric viruses might also be produced from recombinants of HAV and other picornaviruses. Semler et al. (17) produced a temperature-sensitive recombinant virus by inserting a portion of the 5′-noncoding region of the coxsackie-

virus B3 genome into an infectious poliovirus cDNA. Similar experiments might be done with HAV; however, the nucleotide and amino acid sequences of HAV are less homologous to those of any other picornavirus than the latter sequences are to each other (4). Thus, many chimeric DNAs might not yield infectious viruses. A more important concern is that chimeric DNAs that do prove to be infectious could yield viruses with potentially dangerous phenotypes.

In vitro mutagenesis of HAV cDNA might be used to produce viruses with new phenotypes. Transfection with poliovirus cDNA containing insertions in the 2A, 3A, or 3'-noncoding regions produced poliovirus which failed to inhibit host cell translation (3), was cold sensitive (2), or was temperature sensitive (16), respectively. When more is learned about the molecular basis for attenuation of HAV (and other picornaviruses), site-directed mutagenesis of HAV cDNA might be used to produce stable candidate viruses for live, attenuated hepatitis A vaccines.

ACKNOWLEDGMENTS

We thank Vincent Racaniello and Bert Semler for useful discussions, John Brady for advice on DNA transfections, Richard Kuhn for advice on RNA transfections, Max Shapiro and William London for assistance with animal studies, Robert Chanock for constant encouragement and support, and Linda Jordan and Debra Brunelle for editorial assistance.

LITERATURE CITED

1. **Almond, J. W., G. D. Westrop, A. J. Cann, G. Stanway, D. M. A. Evans, P. D. Minor, and G. C. Schild.** 1985. Attenuation and reversion to neurovirulence of the Sabin poliovirus type-3 vaccine, p. 271–277. *In* R. A. Lerner, R. M. Chanock, and F. Brown (ed.), Vaccines 85: molecular and chemical basis of resistance to parasitic, bacterial, and viral diseases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bernstein, H. D., P. Sarnow, and D. Baltimore.** 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. J. Virol. **60**:1040–1049.
3. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.

3a. **Cohen, J. I., B. Rosenblum, J. R. Ticehurst, R. J. Daemer, S. M. Feinstone, and R. H. Purcell.** 1987. Complete nucleotide sequence of an attenuated hepatitis A virus: comparison with wild-type virus. Proc. Natl. Acad. Sci. USA **84**:2497–2501.

4. **Cohen, J. I., J. R. Ticehurst, R. H. Purcell, A. Buckler-White, and B. M. Baroudy.** 1987. Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses. J. Virol. **61**:50–59.
5. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology, p. 143–145. Elsevier Science Publishing, Inc., New York.
6. **Dawson, W. O., D. L. Beck, D. A. Knorr, and G. L. Grantham.** 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Natl. Acad. Sci. USA **83**:1832–1836.
7. **Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biological activity. Nature (London) **316**:262–265.
8. **Kandolf, R., and P. H. Hofschneider.** 1985. Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. Proc. Natl. Acad. Sci. USA **82**:4818–4822.
9. **Kaplan, G., J. Lubinski, A. Dasgupta, and V. R. Racaniello.** 1985. *In vitro* synthesis of infectious poliovirus RNA. Proc. Natl. Acad. Sci. USA **82**:8424–8428.
10. **Locarnini, S. A., A. G. Coulepis, E. G. Westaway, and I. D. Gust.** 1981. Restricted replication of human hepatitis A virus in cell culture: intracellular biochemical studies. J. Virol. **37**:216–225.
11. **Mathiesen, L. R., S. M. Feinstone, R. H. Purcell, and J. Wagner.** 1977. Detection of hepatitis A antigen by immunofluorescence. Infect. Immun. **18**:524–530.
12. **Mizutani, S., and R. J. Colonno.** 1985. In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. J. Virol. **56**:628–632.
13. **Omata, T., M. Kohara, S. Kuge, T. Komatsu, S. Abe, B. L. Semler, A. Kameda, H. Itoh, M. Arita, E. Wimmer, and A. Nomoto.** 1986. Genetic analysis of the attenuation phenotype of poliovirus type 1. J. Virol. **58**:348–358.
14. **Racaniello, V. R.** 1984. Poliovirus type II produced from cloned cDNA is infectious in mice. Virus Res. **1**:669–675.
15. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
16. **Sarnow, P., H. D. Bernstein, and D. Baltimore.** 1986. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. Proc. Natl. Acad. Sci. USA **83**:571–575.
17. **Semler, B. L., V. H. Johnson, and S. Tracy.** 1986. A chimeric plasmid from cDNA clones of poliovirus and coxsackievirus produces a recombinant virus that is temperature-sensitive. Proc. Natl. Acad. Sci. USA **83**:1777–1781.
18. **Siegl, G., G. G. Frosner, V. Gauss-Muller, J.-D. Tratschin, and F. Deinhardt.** 1981. The physicochemical properties of infectious hepatitis A virions. J. Gen. Virol. **57**:331–341.
19. **Simon, A. E., and S. H. Howell.** 1987. Synthesis *in vitro* of infectious RNA copies of the virulent satellite of turnip crinkle virus. Virology **156**:146–152.
20. **Taniguchi, T., M. Palmieri, and C. Weissmann.** 1978. Qβ DNA-containing hybrid plasmids giving rise to Qβ phage formation in the bacterial host. Nature (London) **274**:223–228.
21. **Ticehurst, J. R., V. R. Racaniello, B. M. Baroudy, D. Baltimore, R. H. Purcell, and S. M. Feinstone.** 1983. Molecular cloning and characterization of hepatitis A virus cDNA. Proc. Natl. Acad. Sci. USA **80**:5885–5889.
22. **Van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**: 2330–2334.
23. **Visvader, J. E., A. C. Forster, and R. H. Symons.** 1985. Infectivity and *in vitro* mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors. Nucleic Acids Res. **13**:5843–5855.
24. **Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman.** 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2**: 89–94.