Replication of Hepatitis A Viruses with Chimeric 5' Nontranslated Regions

XI-YU JIA,¹ MICHAEL TESAR,² DONALD F. SUMMERS,^{1,3} AND ELLIE EHRENFELD^{2*}

Departments of Microbiology and Molecular Genetics,¹ Medicine,³ and Molecular Biology and Biochemistry,² University of California, Irvine, Irvine, California 92717

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The role of the 5' nontranslated region in the replication of hepatitis A virus (HAV) was studied by analyzing the translation and replication of chimeric RNAs containing the encephalomyocarditis virus (EMCV) internal ribosome entry segment (IRES) and various lengths (237, 151, or 98 nucleotides [nt]) of the 5'-terminal HAV sequence. Translation of all chimeric RNAs, truncated to encode only capsid protein sequences, occurred with equal efficiency in rabbit reticulocyte lysates and was much enhanced over that exhibited by the HAV IRES. Transfection of FRhK-4 cells with the parental HAV RNA and with chimeric RNA generated a viable virus which was stable over continuous passage; however, more than 151 nt from the 5' terminus of HAV were required to support virus replication. Single-step growth curves of the recovered viruses from the parental RNA transfection and from transfection of RNA containing the EMCV IRES downstream of the first 237 nt of HAV demonstrated replication with similar kinetics and similar yields. When FRhK-4 cells infected with recombinant vaccinia virus producing SP6 RNA polymerase to amplify HAV RNA were transfected with plasmids coding for these viral RNAs or with subclones containing only HAV capsid coding sequences downstream of the parental or chimeric 5' nontranslated region, viral capsid antigens were synthesized from the HAV IRES with an efficiency equal to or greater than that achieved with the EMCV IRES. These data suggest that the inherent translation efficiency of the HAV IRES may not be the major limiting determinant of the slow-growth phenotype of HAV.

The picornavirus family of single-stranded RNA animal viruses is classified into five genera of human and veterinary pathogens. These are the enteroviruses, including polioviruses (PV), coxsackieviruses, and echoviruses; the rhinoviruses; the cardioviruses; the aphthoviruses, or foot-and-mouth disease virus; and the hepatoviruses, including the etiologic agent of hepatitis A. Segregation of a sixth genus that includes echoviruses 22 and 23, which were formerly included among the enterovirus group, has been proposed recently (17, 37) on the basis of the physicochemical properties of the virus and molecular sequence comparisons of the genomes. All of these viruses contain long and highly structured RNA domains at the 5' termini of their genomes, which consist of 600 to 1,200 nucleotides (nt) and constitute approximately 10% of the viral genome. These complex 5' nontranslated regions (NTRs) are highly conserved within the virus groups, but there is little or no sequence homology among the groups. Computer-assisted RNA folding predictions indicate that enterovirus and rhinovirus 5' NTRs form similar structures (type 1), whereas cardiovirus and aphthovirus 5' NTRs present a different pattern of motifs (type 2) (reviewed in reference 42). It is not clear whether the hepatoviruses contain an independent type of 5' NTR structure or one that is related to the type 2 structure (8, 24).

The complex structural features of these 5' NTRs provide the regulatory determinants for several essential viral functions. Recognition signals for viral or cellular factors that mediate viral RNA replication are thought to reside in the most terminal domains. Removal of more than 100 5'-terminal nt from PV RNA eliminated viral RNA replication (29), and a

variety of more subtle changes within this region affected RNA synthesis (5, 23, 30, 39). A cloverleaf structure for the 5'-terminal 90 nt of PV RNA was suggested by computer modeling (31, 35) and verified by chemical and enzymatic modifications (5). Although the 5'-terminal nucleotide sequences of the RNAs of other picornaviruses vary, the potential to form a stable higher-order structure at the terminus is predicted in all cases (1). Sequences that direct cap-independent translation initiation comprise an internal ribosome entry site (IRES) (18), beginning some distance downstream of the 5' terminus and extending for several hundred nucleotides (42).

The 5'-terminal cloverleaf and the downstream IRES structures appear to function as relatively independent nonoverlapping elements that control RNA replication and translation, respectively. Evidence for their function as independent modules comes from the viability of recombinant viruses in which individual segments of the 5' NTRs have been exchanged. Although replacement of the entire 5' NTR of PV type 3 with the 5' NTR from human rhinovirus 14 produced chimeric RNAs that failed to replicate, the IRES portion of the 5' NTR of human rhinovirus 14 substituted quite efficiently for the corresponding region of the PV type 3 5' NTR (32). In addition, the encephalomyocarditis virus (EMCV) IRES region of the 5' NTR can replace the PV IRES (2, 32), despite the absence of any primary sequence or predicted secondary-structure similarity between the type 1 and 2 IRESes (42). Thus, a compatible interaction appears to be required between the terminal cloverleaf region of the 5' NTR and the coding sequences and/or 3' NTR of the viral genome; however, the downstream IRES can be transferred en bloc between viruses and still support virus growth.

Recently, the independence of the RNA replication and translation signals has been questioned by the demonstration that mutations in the middle of the IRES region of the PV 5' NTR significantly reduced viral RNA replication, although

* Corresponding author. Mailing address: Dept. of Molecular Biology and Biochemistry, 3205 Bio Sci II, University of California, Irvine, Irvine, CA 92717. Phone: (714) 824-3264. Fax: (714) 824-8551. Electronic mail address: eehrenfe@uci.edu.

translation in a mixed rabbit reticulocyte lysate (RRL)-HeLa cell extract was only minimally affected (6). In addition, an earlier report showed that a lesion in the stem-loop formed by nt 10 to 34 in the 5'-terminal cloverleaf of PV caused a fivefold reduction in translation efficiency in infected cells (34). A functional coupling of RNA translation and replication is an attractive model for regulation of viral growth and could be an incentive for nonindependence of the two domains. This would require that the EMCV IRES in the chimeric virus constructs fortuitously be able to provide whatever replication signals are needed for interaction with the PV cloverleaf or replication machinery. A mechanism for this coupling has not been proposed.

The 5' NTR of hepatitis A virus (HAV) has been characterized less well than those of some of the other picornaviruses. The existence of an IRES between nt 161 and 734 of the 5' NTR of HAV has been demonstrated by analyzing translation in RRL programmed with monocistronic RNAs or bicistronic RNAs that contained sequences from the 5' NTR of HAV in the intercistronic space (8, 14). The HAV IRES has been shown to be relatively inefficient for translation in RRL *in vitro* (8, 19), and it also was demonstrated to be inefficient in BT7-H cells (a HAV-permissive cell line derived from BS-C-1 cells that constitutively expresses T7 RNA polymerase) when evaluated following transfection of plasmids containing a chloramphenicol acetyltransferase reporter gene fused to the HAV 5' NTR under control of the T7 promoter (41). It was suggested that the low translation efficiency of the HAV IRES contributes to the slow and poor-yielding replication cycle of HAV in cultured cells. The replication domain at the 5' terminus of the HAV 5' NTR has not been well characterized.

In this study, we constructed chimeric RNAs in which the HAV IRES is replaced by that from EMCV. As with other 5' NTR chimeras, viable virus was recovered from cells transfected with chimeric RNA, which stably retained the EMCV sequences for at least four passages. More than 151 nt from the 5' terminus of HAV were required to support virus replication. Translation of all of the chimeric RNAs in RRL *in vitro* exhibited improved translational efficiencies, characteristic of EMCV IRES-driven initiation. The data indicate either that the signals governing RNA replication are localized to the 5'-terminal portion of the HAV 5' NTR or that any replication signals that extend within the HAV IRES can be functionally provided by the EMCV IRES sequence-structure. Despite the improved translational efficiency conferred by the EMCV IRES in RRL *in vitro*, the chimeric viruses growth rates and yields similar to those of the complete HAV in FRhK-4 cells. When translation efficiencies of the HAV and chimeric 5' NTR constructs were tested by measuring HAV capsid protein synthesis in transfected cells infected with recombinant vaccinia virus to generate mRNAs, the HAV 5' NTR directed protein synthesis with an efficiency equal to or greater than that of chimeric mRNAs containing the EMCV IRES.

MATERIALS AND METHODS

Plasmids and DNA manipulation. Plasmid pHAV/7 contains a complete copy of infectious HAV cDNA under control of the SP6 promoter (11). Plasmid pE5LVP0, originally obtained from A. Palmenberg, contains the IRES from EMCV (27). To construct pH237EH, 554 bp of the EMCV sequence, starting immediately downstream of the poly(C) tract and including the complete IRES from pE5LVP0 with an extra *SalI* site inserted at the 5' end of the EMCV sequence, was generated by PCR. The primers were 5'-CCTTGCTAGC CACTGGTCGACCTAACGTTACTGGCCGAA-3' for plus-strand synthesis and 5'-GGAACTTCTAGACATATTATCATCGTGTTC-3' for minus-strand synthesis. The fragment was digested with *SalI* and *XbaI* restriction enzymes. Another PCR fragment containing the first 237 nt of HAV with an extra *SalI* site at the 3' end and some of the vector sequence upstream of an SP6 promoter were also generated by PCR with primer 5'-GATCTGGCTAGCGAT

FIG. 1. Schematic diagram of the 5' NTR of HAV and chimeric constructs. Each hybrid plasmid name indicates the number of HAV nucleotides retained at the 5' end, the source of the IRES (E), and the identity of the coding sequence (H).

GACCCTG-3' for plus-strand synthesis and primer 5'-GCACCTGGTCGA CAGCCCTGGAAGAAAGAAGA-3' for minus-strand synthesis. The PCR fragment was digested with *SalI* and *NheI*. A third, large fragment, starting at nt 739 of HAV and extending through the rest of the HAV sequence, as well as some of the vector sequence, was generated by partial *XbaI* and *NheI* digestion of pHAV/7. These three fragments were ligated to generate plasmid pH237EH. The final construct contained nt 1 to 237 of HAV cDNA connected via a *SalI* site, GTCGAC, to 554 nt of the EMCV IRES fused to the entire HAV coding sequence and 3' NTR (see Fig. 1). Subclones were constructed from both pHAV/7 and chimeric plasmid pH237EH, which contained only the P1 capsid coding region of HAV cDNA, terminating at the *SacI* site at nt 2988. These were designated pHAV-P1 and pH237EH-P1. Six independent clones of pH237EH-P1, resulting from ligations of fragments generated by two independent PCRs, were isolated.

Two additional constructs, pH151EH and pH98EH, shown in Fig. 1, were made by replacing the HAV sequence in pH237EH upstream of the *SalI* site with fragments containing nt 1 to 151 or 1 to 98 of HAV cDNA generated by PCR. Each construct was named to indicate the number of 5'-terminal HAV-specific nt retained at the 5' end, the source of the IRES (E), and the identity of the coding sequence (H). The junctions of all of the ligated fragments were confirmed by sequence analysis.

RNA transcription and transfection. Plasmids (1 µg) were linearized with *SmaI* and transcribed with SP6 RNA polymerase (Promega) in a 30-µl reaction mixture, generally in accordance with the manufacturer's instructions. Nucleic acids were extracted with phenol and precipitated with ethanol, and a portion was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Approximately 4×10^4 FRhK-4 cells were seeded in each well of a six-well plate and grown to about 60% confluency. Cells were washed with Dulbecco's modified minimum essential medium (DMEM). Liposome-mediated transfection was accomplished by adding 7 µl of lipofectin reagent (Bethesda Research Laboratories) mixed with 10 µg of RNA in 0.5 ml of DMEM to each well. Cells were incubated for 4 h at 37°C in a CO₂ incubator, after which the cells were washed, fed with 1.5 ml of DMEM containing 10% fetal bovine serum, and further incubated at 35°C.

DNA transfections were performed simultaneously with infection by recombinant vaccinia virus, which expressed SP6 RNA polymerase (40). In this combined transfection-infection procedure, 8 µl of lipofectin was mixed with recombinant vaccinia virus (approximately 5 PFU per cell) and up to 2 µg of DNA in 0.5 ml of medium, and the mixture was added to each well of a six-well plate. Cells were incubated at 37°C for 4 h, washed, fed with 1.5 ml of DMEM containing 10% serum, and further incubated at 37°C.

Virus infection and titration. Fivefold serial dilutions of virus stocks were prepared in DMEM, and 0.5 ml was used to infect FRhK-4 cells in a 24-well plate. After 3 weeks of incubation at 35°C, cells were harvested and analyzed for the presence of HAV VP1 antigen by Western immunoblot after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Infectious-virus titers were determined by end-point titration of infectivity determined by detection of VP1 antigen.

In vitro translation of RNA transcripts. Translation reactions were performed in an RRL (Promega) at 30°C for 1 h as previously described (19). Reactions were terminated by treatment with RNase A (100 µg/ml) for 15 min. Finally, a 1.5-µl translation mixture was added to 40 µl of SDS-PAGE sample buffer and subjected to electrophoresis in 10% polyacrylamide gels. Gels were dried and exposed to X-ray film at -70°C for 2 to 3 days.

RNA extraction and hybridization. RNA was extracted from FRhK-4 cells by the protocol of Chomczynski and Sacchi (10). Total RNA from approximately 2×10^5 cells was resuspended in 50 µl of water; 10 µl was added to 100 µl of 12× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-40% formaldehyde and applied to nitrocellulose membranes prewetted with 10× SSC. The membranes were subjected to UV cross-linking and hybridized as described previously (9) to a ³²P-labeled EMCV or HAV probe. The EMCV RNA probe was

the negative strand of the EMCV IRES region from pE5LVP0; the HAV RNA probe was complementary to nt 2069 to the 3' end of HAV RNA from pHAV/7, transcribed with T7 RNA polymerase.

Reverse transcription PCR and DNA sequence analysis. RNA samples extracted from FRhK-4 cells were used to synthesize cDNA. The cDNA synthesis and PCR were performed as described previously (38). The primers used for PCR were (i) HAV positive-strand sequence nt 143 to 164 and (ii) the EMCV negative-strand sequence complementary to nt 31 to 50 downstream of the poly(C) tract. The expected PCR product from vH237EH cDNA was 150 nt long. Amersham's PCR sequence kit was used for PCR sequence analysis.

Antibody and Western blot analysis. Anti-VP1 serum and Western blot analysis have been described before (22).

RESULTS

Construction of HAV-EMCV chimeric cDNAs. All of the picornaviral RNAs contain elements of several hundred nucleotides within defined boundaries that are able to direct internal cap-independent initiation of protein synthesis (1, 13, 36). To determine whether the HAV IRES could be replaced with the corresponding segment from a different, distantly related picornavirus, three chimeric cDNAs were constructed (Fig. 1). The parent plasmid was pHAV/7, which contains an infectious cDNA copy of the HAV genome (11). Variable lengths of the 5'-terminal HAV cDNA were retained in the different constructs, from 98 to 237 nt, fused to 554 nt of EMCV RNA containing the entire IRES and terminating in the first of two AUG codons that introduce the HAV coding sequence (38). Nucleotide sequences at both junctions of the HAV and EMCV cDNAs were confirmed by dideoxy sequencing. All viral sequences are located downstream of an SP6 promoter that generates transcripts with an extra 10 nt at the 5' terminus (11). Transcripts of this set of plasmids, linearized downstream of the insert with *Sma*I, migrated in agarose gels in accordance with their predicted sizes (data not shown).

Translation of chimeric RNAs in vitro. The ability of the EMCV IRES to direct translation in the context of the HAV sequences was tested in an RRL in vitro. Each plasmid shown in Fig. 1 was linearized with *Bgl*II at nt 1807 near the middle of the VP3 gene and transcribed in vitro with SP6 RNA polymerase. Transcripts were used to direct translation in an RRL. Figure 2A shows the translation products of decreasing amounts of RNA, from 100 to 1.2 μ g/ml, from pHAV/7 (lanes 1 to 5) and pH237EH (lanes 6 to 10). The predominant product is the predicted 39.5-kDa truncated HAV capsid protein. Other, more rapidly migrating bands represent smaller polypeptides initiated aberrantly from internal AUG codons, as described previously (20). RNAs containing the EMCV IRES were translated with greater efficiency than the transcripts containing the HAV IRES. Translation directed by all HAV-EMCV chimeric 5' NTRs occurred with equal efficiency, independent of the length of the 5'-terminal HAV sequences (lanes 6, 11, and 12). Figure 2B shows the relative incorporation of [35 S]methionine into acid-precipitable protein directed by HAV-EMCV chimeric RNA versus HAV RNA. The EMCV IRES conferred an approximately fivefold increase in translation efficiency in the RRL system.

Transfection of FRhK-4 cells with HAV and chimeric RNAs. Full-length RNA transcripts from each plasmid were introduced into FRhK-4 cells by lipofectin-mediated transfection. After 3 weeks, the cultures were harvested and cell lysates were analyzed by SDS-PAGE and a Western immunoblot probed with anti-VP1 serum. Cells transfected with RNA from pHAV/7 or pH237EH contained immunoreactive proteins; those transfected with pH151EH or pH98EH transcripts showed no detectable VP1-containing proteins (data not shown). Putative low yields of infectious virus were amplified by passage in fresh monolayers of FRhK-4 cells, and after 2

FIG. 2. Translation of HAV and EMCV-HAV chimeric RNAs in RRL. Plasmids were digested with *Bgl*II and transcribed with SP6 RNA polymerase to generate transcripts containing HAV nt 1 to 1807, which were used to direct translation in RRL in the presence of [35 S]methionine. (A) Translation products were resolved by SDS-PAGE and subjected to autoradiography. Lanes 1, 6, 11, and 12 show the products of translation of 1 μ g of the indicated RNA per 10- μ l reaction mixture. Lanes 2 to 5 and 7 to 10 contained 0.33, 0.11, 0.037, and 0.012 μ g of the indicated RNAs, respectively. The numbers on the left are molecular weights in thousands. (B) Two microliters of each translation reaction directed by pH237EH transcripts was precipitated with 5% trichloroacetic acid, collected on glass fiber filters, and counted in a liquid scintillation spectrometer. Incorporation is shown as a function of RNA concentration in the translation reaction.

weeks, cells were collected for analysis of VP1-specific protein by Western immunoblot. Figure 3, lane 4, shows cells infected with virus generated after transfection with pHAV/7 transcripts. Lane 1 shows that pH237EH transcripts were infectious in FRhK-4 cells and that a progeny virus which could be subsequently passaged was formed. No detectable virus was recovered, however, following transfection with RNAs containing 151 or fewer HAV 5'-terminal nt (lanes 2 and 3). Lane 5 shows a mock-infected negative control.

To confirm that the virus generated after amplification con-

FIG. 3. Immunoblot of proteins from cells infected with HAV and chimeric HAV-EMCV. FRhK-4 cells were transfected with RNA transcripts of the wild-type and chimeric HAV cDNAs (shown in Fig. 1) indicated above the lanes. After 3 weeks, cell lysates were used to infect fresh cultures for 2 weeks. Cells were harvested, and proteins were resolved by SDS-10% PAGE prior to Western blot analysis with antibody against HAV VP1. Molecular weights are indicated on the left in thousands. The position of HAV VP1 is indicated on the right.

tained the original chimeric 5' NTR, a first-passaged virus stock was extracted with phenol and subjected to reverse transcription PCR with primers complementary to sequences in the EMCV IRES and the HAV P1 coding region. Figure 4 shows that only the chimeric viral RNA generated a PCR product of 150 bp, which corresponds to the size expected of the fragment that crosses the downstream EMCV-HAV junction between the primer sequences. This fragment was excised from the gel and sequenced, confirming that the original sequence introduced into pH237EH was retained in the progeny virus (data not shown).

Growth of chimeric virus. Since RNA from the chimeric cDNA pH237EH replicated in FRhK-4 cells and generated infectious progeny, we sought to determine whether the replication cycle was affected by the presence of the EMCV IRES. Virus produced from transfected cells was amplified by one passage, and the infectivity titer of the resulting stock was determined by RNA hybridization and end-point dilution. Du-

FIG. 4. Agarose gel analysis of reverse transcription PCR products. Two primers specific for the H237EH virus were used in the PCRs. Lanes 1, 2, and 3 contained the PCR products from HAV-infected, H237EH-infected, and mock-infected cells, respectively. Lane M contained DNA length markers, whose sizes are shown on the left in kilobases. The position of a 150-bp PCR product is shown on the right.

plicate fivefold serial dilutions were used to infect FRhK-4 cells in individual wells of multiwell plates, and after 2 weeks, the cells were analyzed for production of viral antigen by Western immunoblot. The titers of HAV and chimeric HAV(237)-EMCV in the first-passaged stocks were similar and yielded approximately 5×10^8 viral RNA molecules per ml and approximately 4×10^6 and 8×10^6 infectious doses per ml, respectively. These stocks were then used to infect FRhK-4 cells at a multiplicity of infection of 5 per cell. Figure 5 shows the kinetics of virus replication measured by slot blot hybridization with an RNA probe complementary to the 3' terminus of HAV RNA during 18 days of infection. Both HAV and the HAV containing the EMCV IRES downstream of the first 237 nt of HAV replicated with similar kinetics to produce similar yields. When probed with an RNA complementary to the EMCV IRES, only the cells infected with the chimeric virus gave a positive signal (data not shown).

Translation directed by the HAV or chimeric IRES in FRhK-4 cells. HAV RNAs containing the EMCV IRES were translated with significantly greater efficiency than those containing the HAV IRES in RRL *in vitro* (Fig. 2), yet virus containing these RNAs replicated equally well in FRhK-4 cells (Fig. 5). To determine whether viral protein synthesis from the chimeric HAV-EMCV RNA in FRhK-4 cells reflected the enhanced efficiency of the EMCV IRES manifested *in vitro*, cells were transfected with transcripts from subclones of pHAV/7 and pH237EH that included only the P1 capsid coding region of the HAV cDNA and analyzed 16 h later for the presence of HAV VP1-containing protein by immunoblot analysis of cytoplasmic extracts. Under these conditions, no viral antigen was detected in cells transfected with either RNA (data not shown). These data confirmed previous experiments that showed that detection of viral antigens in transfected cells

FIG. 5. Slot blot hybridization of RNA extracts from cells infected with HAV and a chimeric virus. HAV-specific ^{32}P -labeled probes were used to monitor viral RNA accumulation as a function of time after infection. RNA from approximately 10^4 cells was used for the slot blot. Harvest times are indicated on the left. RNA from mock-infected cells was blotted on the bottom left. Known amounts of RNA transcripts from pHAV/7 and pH237EH were blotted on the bottom right.

required replication and amplification of the input RNA (6, 16). To increase the sensitivity of this analysis, cells were transfected with supercoiled plasmid DNA in the presence of recombinant vaccinia virus carrying the gene for SP6 RNA polymerase. This simultaneous infection-transfection results in the production of relatively large amounts of viral RNA from efficient utilization of the SP6 promoter by the SP6 RNA polymerase, as well as increased expression of viral proteins in vaccinia virus-infected cells (26). Under these conditions, viral antigens were readily detected within 16 h after transfection (Fig. 6). Figure 6A shows the immunoblot analysis of cells transfected with twofold serial dilutions of full-length pHAV/7 (lanes 1 to 5) or pH237EH (lanes 7 to 11), from 0.5 to 0.03 μg of DNA per well (approximately 4×10^4 cells). The predominant VP1-containing protein in the cells under these conditions is a polypeptide with an apparent molecular mass of ~ 40 kDa, most likely pX, a VP1-2A fusion (3). Surprisingly, at all concentrations of DNA tested, synthesis of viral protein was approximately three- to fivefold more efficient when directed by the HAV IRES than when directed by the EMCV IRES inserted directly upstream of the HAV protein coding sequence. A similar preferential utilization of the HAV 5' NTR over the HAV-EMCV 5' NTR was observed at saturating DNA concentrations ($>0.5 \mu\text{g}$ per well) in HeLa cells (data not shown). This result contrasts with the relative translation efficiencies of these two RNAs in RRL *in vitro* (Fig. 2). Co-transfection of plasmid pH98EH (Fig. 1) with recombinant vaccinia virus yielded the same translation efficiencies as pH237EH. This suggests that the EMCV IRES in pH237EH RNA was not specifically constrained by the artificial junction between HAV and EMCV sequences.

FIG. 6. VP1 immunoblot analysis of cells transfected with HAV cDNAs containing HAV or a chimeric 5' NTR sequence. (A) FRhK-4 cells were transfected with twofold serial dilutions of pHAV/7 (lanes 1 to 5) or pH237EH (lanes 6 to 10), from 0.5 to 0.03 μg of DNA, in the presence of recombinant vaccinia virus encoding SP6 RNA polymerase. Cells were harvested 24 h posttransfection, and proteins were analyzed by Western immunoblot probed with anti-HAV VP1 serum. Prestained molecular weight markers were analyzed on the same gel. (B) FRhK-4 cells were transfected with fourfold serial dilutions of pHAV-P1 (lanes 1 to 5) and pH237EH-P1 (lanes 6 to 10), from 2.0 to 0.008 μg of DNA, in the presence of recombinant vaccinia virus encoding SP6 RNA polymerase. Cells were harvested and analyzed as for panel A. The arrow indicates the uncleaved capsid polypeptide with a molecular mass of ~ 84 kDa.

We considered the possibility that a translation product of the HAV genome specifically enhanced the utilization of the HAV IRES in cultured cells. To test this possibility, we utilized subclones which contained truncated HAV cDNAs (terminating at the *SacI* site at nt 2988), such that transcripts produced by SP6 RNA polymerase directed the synthesis of only capsid protein sequences. Figure 6B shows the results of transfection of FRhK-4 cells with serial fourfold dilutions, from 2.0 to 0.008 μg of DNA per well, of the truncated plasmids derived from pHAV/7 (pHAV-P1; lanes 1 to 5) and pH237EH (pH237EH-P1; lanes 7 to 11). The predominant translation product is the uncleaved polypeptide with a molecular mass of ~ 84 kDa. In

FIG. 7. Diagram of sequences in the HAV 5' NTR proposed for different functions. The numbers indicate nucleotide sequences of pHAV/7. REP, replication-related sequence.

this experiment, translation from the HAV IRES was about 10-fold more efficient than that from the EMCV IRES in the chimeric construct. A similar analysis of six independent clones isolated from two independent plasmid constructions of the same HAV(237)-EMCV chimeric 5' NTR yielded the same results.

The possibility that the chimeric RNA was translated more poorly than the HAV RNA because of increased degradation or an otherwise decreased RNA concentration was excluded by slot blot hybridization of the same samples used for the immunoblot analysis shown in Fig. 6B. Intracellular RNA levels were quite comparable in both sets of samples. When probed with EMCV sequences, only RNA from cells transfected with the chimeric RNA was reactive (data not shown).

DISCUSSION

The 5' NTR of picornaviral RNA contains sequences and structural motifs required for RNA replication and ribosome binding and possibly for other functions, such as RNA packaging and assembly of virus particles. Mutational mapping studies and structural analyses conducted predominantly by the Lemon laboratory (7, 8, 33) have generated a model which is schematically depicted in Fig. 7. At the extreme 5' terminus is a series of stable stem-loop structures with the potential to form two or more pseudoknots. This element includes the first 94 nt of the HAV sequence and appears to be analogous to the cloverleaf element formed by the first 90-nt sequence at the 5' terminus of PV RNA (5) that contains the recognition signals for protein interactions required for RNA replication (4). Similar pseudoknot structures have been predicted for members of the cardiovirus and aphthovirus groups (24). Immediately downstream, there is a pyrimidine-rich sequence, called pY1, which extends from nt 99 to 138 and contains a series of tandem repeats of a U_3C_3 sequence motif that are stacked into an ordered structure that is resistant to single-strand-specific RNases (33). Surprisingly, this region could be deleted from the viral RNA with no apparent effect on virus replication in BS-C-1 or FRhK-4 cells (33). Extension of the deletion downstream, past nt 139 into an apparently single-stranded region (nt 140 to 153), resulted in virus replication defects. The segment that directs ribosome binding for initiation of viral protein synthesis (IRES) appears to begin downstream of nt 152 and extends to a point very near the beginning of the coding region (8), although one earlier report showed an effect of further upstream sequences on translation (14).

The ability to substitute the IRES from another picornavirus into the 5' NTR of HAV allowed us to separate the translation function from the other replication functions of the 5' NTR. Insertion of the EMCV IRES downstream of the first 237 nt of HAV generated a viable, infectious virus that grew in FRhK-4 cells with rates and yields similar to those of the parent strain of HAV. The growth of this chimeric virus supports a model in which the upstream virus-specific replication element functions independently of the IRES, which can be functionally transferred to any RNA coding sequence. Retention of the 5'-terminal 98 nt of HAV upstream of the EMCV IRES was

expected to permit formation of the pseudoknot-containing replication element (Fig. 7) but removed the pY1 and adjacent single-stranded sequences upstream of the IRES. This construct was originally designed to test whether the pY1 and adjacent sequences participate in RNA replication or translation reactions. Since the pY1 element appears to play no essential role in virus growth in cultured cells, however, the H98EH RNA is likely noninfectious because of the absence of sequences between pY1 and the IRES (nt 140 to 153). It was previously shown that nucleotides at the 5' end of this region (nt 140 to 144) are essential for efficient replication in cultured cells (33), but it was uncertain whether they participate in RNA replication or possibly assembly and packaging or whether this deletion affects folding of downstream IRES structures and therefore affects translation. The approach described in this report, in which a functional IRES from EMCV was inserted, supports the former explanation.

The failure to recover infectious virus from cells transfected with H151EH RNA is more surprising. This construct was predicted to have retained all of the HAV-specific replication-requiring elements, including the terminal pseudoknot-forming structure and the single-stranded sequence between pY1 and the IRES. It is possible that the fusion of HAV and EMCV sequences is just at the border of the essential region, and a small number of important nucleotides are missing or unable to function in the chimeric context. It may also be that important replication sequences extend downstream or are located within the HAV IRES element, between nt 151 and 237. The possibility that the H151EH RNA might have generated virus at reduced temperatures or under other restricted conditions was not examined.

The finding that the EMCV IRES directed translation in the chimeric RNAs more efficiently *in vitro* than the HAV IRES (Fig. 2) was not unexpected. The EMCV IRES is known to function with high efficiency in RRL (12), and previous experiments have shown by direct comparison that the EMCV IRES is more efficiently utilized than the HAV IRES *in vitro* (8, 20). The weak activity of the HAV IRES in RRL may be due to the absence in those lysates of cell- or tissue-specific translation factors utilized by HAV (14a) or to a relatively weak affinity for IRES-specific factors which may be rate limiting in RRL. Similar poor translation is directed by the PV IRES in RRL, although it translates well in extracts prepared from HeLa cells (28).

When viral cDNAs were introduced into FRhK-4 cells in the presence of recombinant vaccinia virus that produces SP6 RNA polymerase to generate viral RNA transcripts, the HAV sequences were translated more efficiently from the intact HAV 5' NTR than from a chimeric NTR containing the EMCV IRES. This occurred regardless of whether there were 98 or 237 nt of HAV upstream of the EMCV IRES. This result contrasts with those reported by Whetter et al. (41), in which both monocistronic and bicistronic RNAs which contained a chloramphenicol acetyltransferase reporter sequence fused to the HAV IRES were translated with much lower efficiency than the same reporter sequence fused to the EMCV IRES in cells that were engineered to constitutively express T7 RNA polymerase to generate the reporter gene transcripts. The explanation for this discrepancy is not apparent. A significant difference between the two experiments is that Whetter et al. used a reporter sequence to monitor the translation reaction, whereas we measured production of HAV capsid protein sequences by using expression of the uninterrupted viral RNA sequence as a reporter. If the HAV IRES element extended downstream of the initiating AUG, then fusion to a reporter gene sequence at that site might weaken its activity. However,

there is no reported evidence of IRES domains extending beyond the HAV 5' NTR, although substitution of the HAV 5' noncoding region by the EMCV 5' noncoding region on several different HAV coding sequences produced surprisingly variable effects on the translation of the resulting protein sequences (21). We considered the possibility that a HAV nonstructural protein might specifically enhance utilization of the HAV IRES, and thus we would have observed a stimulation of translation not occurring with the reporter gene fusion construct. This type of specific enhancement of translation driven by the PV IRES has been attributed to the PV 2A protein or activity (15). Expression of the HAV VP1 antigen from truncated transcripts from which all nonstructural protein coding sequences had been removed, however, eliminated this possibility. Another difference between the constructs used in the experiments reported here and those performed previously (41) is the presence of the HAV 5'-terminal end sequence rather than the entire EMCV 5' NTR. If a significant contribution to translational activity resided in the first 100 nt of the HAV sequence, then more efficient translation directed by the chimeric 5' NTR might be expected compared with the complete EMCV 5' NTR. Most of the data, however, suggest that sequences upstream of nt 152 have little or no effect on translation (8). Finally, the possibility of an unidentified debilitating mutation that occurred during the cloning of the EMCV sequence used in these experiments cannot be excluded, especially since a large amount of PCR-generated DNA was incorporated. To minimize this possibility, two independent PCRs using minimal amplification were performed, and six independent clones were isolated and tested for translation efficiency after transfection of FRhK-4 cells. All exhibited similar translation efficiencies.

Regardless of the relative translational efficiencies of the HAV and EMCV IRES elements in FRhK-4 cells, it appears that substitution of the HAV IRES with that from EMCV did not produce a virus with improved growth properties in FRhK-4 cells. The replication cycle of wild-type HAV in hepatocytes during a natural infection has not been characterized, although the lag of several weeks between infection and maximal virus shedding is consistent with a slow replication pattern in a human host. It has been suggested that slow, nonlytic replication is advantageous to a virus that requires a functioning liver for secretion in the bile and excretion in feces for optimal spread in the population (25). If this is the case, then it is likely that many viral processes have coevolved to generate a slow-growth phenotype, and the efficiency of IRES utilization may represent only one determinant of the growth rate phenotype of HAV.

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