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Mutations within the 5′ Nontranslated RNA of Cell Culture-Adapted Hepatitis A Virus Which Enhance Cap-Independent Translation in Cultured African Green Monkey Kidney Cells

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Mutations in the 5′ nontranslated RNA (5′NTR) of an attenuated, cell culture-adapted hepatitis A virus (HAV), HM175/P16, enhance growth in cultured African green monkey kidney (BS-C-1) cells but not in fetal rhesus monkey kidney (FRhK-4) cells (S. P. Day, P. Murphy, E. A. Brown, and S. M. Lemon, J. Virol. 66: 6533–6540, 1992). To determine whether these mutations enhance cap-independent translation directed by the HAV internal ribosomal entry site (IRES), we compared the translational activities of the 5′NTRs of wild-type and HM175/P16 viruses in two stably transformed cell lines (BT7-H and FRhK-T7) which constitutively express cytoplasmic bacteriophage T7 RNA polymerase and which are derived from BS-C-1 and FRhK-4 cells, respectively. Translational activity was assessed by monitoring expression of a reporter protein, chloramphenicol acetyltransferase (CAT), following transfection with plasmid DNAs containing bicistronic T7 transcriptional units of the form luciferase-5′NTR-CAT. In both cell types, transcripts containing the 5′NTR of HM175/P16 expressed CAT at levels that were 50- to 100-fold lower than transcripts containing the IRES elements of Sabin type 1 poliovirus or encephalomyocarditis virus, confirming the low activity of the HAV IRES. However, in BT7-H cells, transcripts containing the 5′NTR of HM175/P16 expressed CAT with four- to fivefold greater efficiency than transcripts containing the 5′NTR of wild-type virus. This translational enhancement was due to additive effects of a UU deletion at nucleotides 203 and 204 and a U-to-G substitution at nucleotide 687 of HM175/P16. These mutations did not enhance translation in FRhK-T7 or Huh-T7 cells (a T7 polymerase-expressing cell line derived from human hepatoblastoma cells) or in vitro in rabbit reticulocyte lysates. These results demonstrate that mutations in the 5′NTR of a cell culture-adapted HAV enhance viral replication by facilitating cap-independent translation in a cell-type-specific fashion and support the concept that picornaviral host range is determined in part by differences in cellular translation initiation factors.

Hepatitis A virus (HAV), a picornavirus classified within the genus *Hepatovirus*, is a hepatotropic virus which is a common cause of acute viral hepatitis in humans (28). Since the first demonstration that HAV may be propagated in cell culture (35), there have been many attempts to develop HAV vaccines. Formalin-inactivated HAV vaccines that are both efficacious and safe have been developed (22, 42). However, progress in the development of attenuated vaccine candidates has been considerably slower (39). In theory, an attenuated HAV vaccine could have an advantage over inactivated vaccines in terms of cost, the potential for oral administration, and the likelihood of providing long-term immunity following a single dose. While adaptation of wild-type HAV to efficient growth in African green monkey kidney cells has resulted in virus variants that are highly attenuated with respect to the ability to cause acute hepatocellular injury in susceptible primates, these cell culture-adapted HAVs replicate poorly in vivo and generally produce only low-level protective antibody responses (26, 31, 41). Furthermore, attenuated vaccine candidates which have been selected in this manner appear to be incapable of initiating infection by the oral route (40). Thus, recombinant DNA technology offers an attractive alternative for the rational design of improved attenuated vaccine candidates with more desirable replication properties in vivo. Definition of the molecular basis of attenuation associated with cell culture adaptation of HAV represents an important step in this direction.

Complete cDNA copies of the genomes of two independently isolated, attenuated cell culture-adapted variants of the HM175 strain of HAV have been molecularly cloned, and their sequences have been compared with that of their common wild-type parent (HM175/wt) (9, 25). Both of these attenuated viruses were adapted to grow in African green monkey kidney cells. One virus, HM175/P16, which replicates very poorly in primates compared with HM175/wt, contains 19 mutations from the wild-type sequence which are scattered throughout the 7.5-kb genome (25, 41). Seven of these mutations are found as similar or identical mutations in a second cell culture-adapted and attenuated HM175 strain variant, HM175/P35 (9). Although mutations in the P2 region (proteins 2B and 2C) have been shown to be of primary importance in adaptation of the virus to growth in cultured cells (14, 15), the presence of shared mutations within the 5′ nontranslated RNAs (5′NTRs) of these two independently isolated HM175 variants suggests that these mutations may also be important for cell culture adaptation (25). Previous studies with chimeric viruses rescued from infectious cDNAs containing the 5′NTR sequence of the HM175/wt, HM175/P16, or HM175/P35 genome indicated that several mutations in the 5′NTR of HM175/P16 virus (at nucleotides [nt] 152 and/or 203 and 204 and at nt 687) significantly enhance growth of the virus in cell culture (10, 11). Interestingly, the enhancement in replication conferred by these mu-

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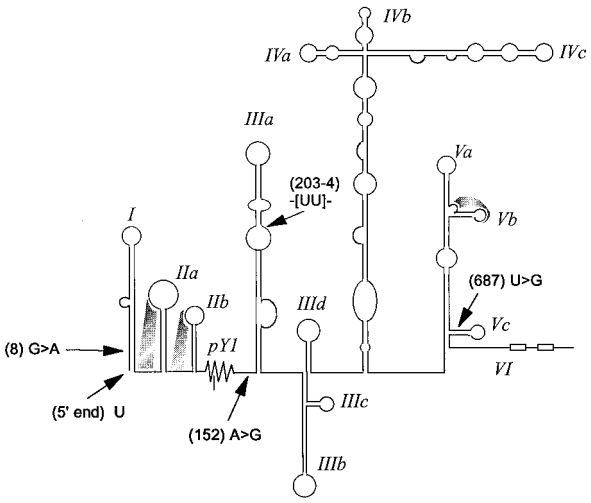


FIG. 1. Proposed secondary structure of the HM175/wt 5′NTR (5, 37). Major structural domains are labeled I through VI; the small open boxes indicate the locations of the two initiator AUG codons, Met-1 and Met-3. Arrows indicate the positions of the 5′NTR mutations present in the cell culture-adapted HM175/P16 virus.

tations was present in cultured African green monkey kidney (BS-C-1) cells but not in fetal rhesus kidney (FRhK-4) cells, which are also permissive for HAV (11).

The 5′NTR of HAV, like that of other picornaviruses, forms a highly ordered secondary structure (Fig. 1) and contains elements which are necessary for both viral translation and RNA replication (1, 5, 6, 33, 37, 38). Picornavirus translation is initiated in a cap-independent fashion by a mechanism that involves the binding of the 40S ribosomal subunit at a site located hundreds of bases downstream of the 5′ end of the RNA which has been termed a ribosomal landing pad or an internal ribosomal entry site (IRES) (23, 34). Although the details of translation initiation by internal entry are unknown, it likely involves the interaction of a set of *trans*-acting cellular translation initiation factors with the *cis*-acting IRES, resulting in the binding of the 40S ribosomal subunit to the RNA. The existence of an HAV IRES has been demonstrated by experiments in which the 5′NTR sequence was placed within the intercistronic space of bicistronic RNAs, with the result that the second cistron was translated independently of the first cistron and in a manner dependent on the integrity of the 5′NTR sequence both in vitro and in vivo (6, 20, 43). Mutational analysis of the HAV 5′NTR has shown that the IRES is located downstream of nt 161 and extends to within 40 nt of the first initiator AUG, which is located at nt 734 (6).

While analysis of HAV translation in vitro in rabbit reticulocyte lysates has been useful in defining the 5′ and 3′ limits of the HAV IRES (6), the studies examining the impact of 5′NTR mutations on viral replication which are described above suggest that there may be cell-type-specific aspects to viral translation that could be appreciated only in an in vivo system (11). For this reason, we previously constructed an HAV-permissive cell line (BT7-H), derived from cultured African green monkey kidney (BS-C-1) cells, that constitutively expresses cytoplasmic bacteriophage T7 RNA polymerase (43). Transfection of these cells with plasmid DNA containing a T7 transcriptional unit results in efficient transcription of uncapped, cytoplasmic RNA transcripts which are very poorly translated if they do not contain an IRES (13, 43). We have shown previously that the translation of uncapped monocistronic and bicistronic T7 transcripts containing the reporter gene chloramphenicol acetyltransferase (CAT) fused in frame with the HAV 5′NTR is very inefficient in the BT7-H cells (43). It is likely that this low translational efficiency of the HAV IRES contributes to the normally slow growth of HAV in cultured cells.

Here, we describe experiments in which we examined the effects of mutations which are present in the 5′NTR of the cell culture-adapted and attenuated HM175/P16 virus on cap-independent translation in cultured monkey kidney cells. We established a second T7 RNA polymerase-expressing cell line (FRhK-T7), derived from FRhK-4 cells, so that we could carry out experiments in parallel in cells of adult African green monkey kidney and fetal rhesus kidney lineage. We found that the 5′NTR mutations present in HM175/P16 virus enhanced IRES-directed translation in BT7-H cells but not in FRhK-T7 cells, paralleling the cell-type-specific impact of these mutations on viral replication (11). The mutations responsible for this translational enhancement were the UU deletion at bases 203 and 204 and the U-to-G substitution at base 687. These results thus demonstrate that mutations in the 5′NTR of a cell culture-adapted HAV enhance viral replication by facilitating cap-independent translation in a cell-type-specific fashion and add to our current understanding of the determinants of the host range of HAV and other picornaviruses.

MATERIALS AND METHODS

Plasmids. Construction of the plasmids pHAV-CAT1, pEMCV-CAT, pLUC-P16-CAT (formerly pLUC-HAV-CAT), and pLUC-Δ355-532-CAT has been described previously (43). pLUC-EMCV-CAT and pLUC-SAB-CAT contain bicistronic T7 transcriptional units in which an upstream firefly luciferase coding region and a downstream CAT coding region are separated by either the encephalomyocarditis virus (EMCV) IRES (nt 243 to 836) or the entire Sabin type 1 poliovirus 5′NTR (nt 1 to 742) placed within the intercistronic space. pLUC-EMCV-CAT was constructed by subcloning the 1.7-kb *Eco*RI-*Pst*I fragment of pCITE-1 (Novagen), containing the EMCV IRES, into the *Acc*I-*Pst*I sites of pLUC-HAV-CAT, to create pCITE-LUC. To create pLUC-EMCV-CAT, the 4.3-kb *Apa*I-*Not*I fragment of pCITE-LUC was replaced with the 1.6-kb fragment from pEMCV-CAT. pLUC-SAB-CAT was constructed by PCR amplification of a 0.8-kb fragment containing the Sabin type 1 poliovirus 5′NTR with flanking *Hin*dIII and *Xba*I sites from pCAS7 (a generous gift from J. Almond, University of Reading) with the sense primer 5′-AGTGACCTAAGCTTAAAA CAGCTCTGGGGTTCC-3′ and the antisense primer 5′-AAACCTGTCTAGA CATTATGATACAATTGTCTG-3′. The 0.75-kb *Hin*dIII-*Xba*I fragment of pHAV-CAT1 containing the HAV 5′NTR was then replaced with the 0.75-kb *Hin*dIII-*Xba*I-digested PCR fragment containing the Sabin type 1 5′NTR. The resulting plasmid, pSAB-CAT, was digested with *Hin*dIII and *Not*I, and the 1.8-kb fragment was subcloned into the *Acc*I-*Not*I sites of pLUC-P16-CAT to create pLUC-SAB-CAT.

pLUC-WT-CAT contains a similar bicistronic T7 transcriptional unit containing the HM175/wt virus 5′NTR in the intercistronic space. It was constructed by replacing the 0.75-kb *Hin*dIII-*Xba*I fragment of pHAV-CAT1, containing the cell culture-adapted HM175/P16 virus 5′NTR, with the *Hin*dIII-*Xba*I fragment from pA/7-5′WT (11), containing the HM175/wt 5′NTR, to create the monocistronic plasmid pWT-CAT1. To create the bicistronic plasmid pLUC-WT-CAT, the 1.8-kb *Hin*dIII-*Not*I fragment from pLUC-P16-CAT was replaced with the corresponding *Hin*dIII-*Not*I fragment from pWT-CAT.

Plasmids p152, p203, p687, and p152.203.687 are identical to pLUC-WT-CAT except that they contain individual mutations (A-to-G substitution at nt 152; TT deletion at nt 203 and 204; T-to-G substitution at nt 687) which are present in the cell culture-adapted HM175/P16 virus at the positions indicated. For the construction of p152 and p203, mutations were introduced at nt 152 and nt 203 and 204 of the HM175/wt sequence by PCR mutagenesis. First, 0.17- and 0.22-kb fragments containing the 3′ end of the luciferase gene and part of the HAV 5′NTR were PCR amplified from pLUC-WT-CAT with the sense primer 5′-TA AGTGTATTCAGCGATGAC-3′ and either the 152 mutagenic primer 5′-CTT GTAAATATTGATTCCTGCAC-3′ or the 203-204 mutagenic primer 5′-AAGA ACACTCATTTCACGCTTTC-3′. An aliquot of these PCR products was then used as a primer with the antisense primer 5′-GTCAATCCACTCAATGCA TC-3′ to PCR amplify 0.6-kb fragments containing nt 1 to 545 of the HAV HM175/wt 5′NTR with either an A-to-G substitution at nt 152 or a TT deletion at nt 203 and 204. These fragments were then digested with *Hin*dIII and *Hpa*I, and the resulting 0.35-kb fragments were subcloned into the *Hin*dIII-*Hpa*I sites of pLUC-WT-CAT to create p152 and p203. p152.203 was constructed by PCR

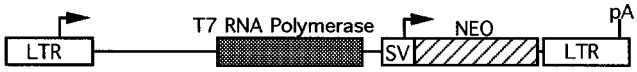


FIG. 2. Schematic diagram of the murine retrovirus vector pLXSN-T7. LTR, long terminal repeat; SV, SV40 early promoter, NEO, neomycin phosphotransferase gene (selectable marker); pA, polyadenylation site. Arrows depict transcriptional start sites.

amplifying a similar 0.58-kb fragment from pLUC-P16-CAT with the mutagenic sense primer 5′-CAGCCCAAGCTTCAAGAGGGGTCTCCGGGA-3′, which lacks the HM175/P16 mutations at nt 0 and 8, and the antisense primer SLA-545. The 0.35-kb *Hin*dIII-*Hpa*I fragment of pLUC-WT-CAT was replaced with the 0.35-kb *Hin*dIII-*Hpa*I fragment excised from the PCR-amplified product, which contained HM175/P16 mutations at nt 152 and nt 203 and 204. p687 was created by replacing the 1.3-kb *Nsi*I-*Not*I fragment of pLUC-WT-CAT with the 1.3-kb *Nsi*I-*Not*I fragment from pLUC-P16-CAT, which contains only the nt 687 T-to-G substitution. Finally, p152.203.687 was created by subcloning the 0.54-kb *Hin*dIII-*Nsi*I fragment from p687 into the corresponding *Hin*dIII-*Nsi*I sites of p152.203.

To confirm the sequences of the manipulated regions within the bicistronic plasmids listed above, plasmid DNAs were sequenced on a model 373A DNA Sequencer (Applied Biosystems), using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

Recombinant retrovirus vector expressing T7 polymerase. To produce a T7 RNA polymerase retrovirus expression vector, a 2.7-kb fragment representing the T7 RNA polymerase gene flanked by *Eco*RI and *Bam*HI sites was amplified by PCR from pOS2 (43) with the sense primer 5′-TGAAGAGGGAATTCAT GAACACGATTAACATCGCT-3′ and the antisense primer 5′-TTAGTGCA GGATCCTTACGCGAACGCGAAGTCCGAC-5′. The product was digested with *Eco*RI and *Bam*HI, and the resulting fragment was inserted into the multiple cloning site of the Moloney murine leukemia virus plasmid pLXSN (32) to create pLXSN-T7 (Fig. 2). To obtain packaged virus, pLXSN-T7 was transfected into the PA317 packaging cell line as described by Miller and Rosman (32). Replication-incompetent LXSN-T7 virus was recovered in the supernatant 3 days posttransfection.

Cells. To produce the T7 RNA polymerase-expressing cell lines FRhK-T7 and Huh-T7, FRhK-4 cells and Huh7 cells were infected with the LXSN-T7 virus (multiplicity of infection of ~0.001) in the presence of 8 μg of Polybrene per ml. After 2 h, virus-containing medium was replaced by growth medium (modified Eagle's medium supplemented with 10% fetal bovine serum, essential and nonessential amino acids, and penicillin-streptomycin). Following an additional 48-h incubation, cells were split 1:10 and 1:30 and fed with growth medium supplemented with 400 μg of Geneticin (active compound) per ml. After 7 days, Geneticin-resistant colonies were clonally isolated and screened for the ability to express CAT following transient transfection with pEMCV-CAT DNA (43).

DNA transfections and reporter gene assays. DNA transfections were carried out in six-well culture plates as described previously (43), but with 1 μg of plasmid DNA and 15 μl of Lipofectin (Gibco BRL) per well. For transfections of Huh-T7 cells, the cells were infected with recombinant vaccinia virus, vTF7-3 (18), expressing T7 RNA polymerase (multiplicity of infection of 10) 1 h prior to DNA transfections (see Results). At 20 to 24 h following transfection, cells were washed with phosphate-buffered saline, and 1 ml of TEN buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl) was added to each well. After 10 min, cells were mechanically removed from the plastic surface, and the resulting cell suspension was divided into 750- and 250-μl aliquots for CAT and luciferase assays. For the CAT assays, cells were pelleted, resuspended in 75 μl of 0.25 M Tris-HCl (pH 8.0), and lysed by freeze-thawing. CAT activity in the cell lysates was determined by a phase extraction assay which quantitates butyrylated [^{14}C]chloramphenicol products by liquid scintillation counting following xylene extraction (pCAT reporter gene systems; Promega) (43). To quantitate luciferase activity, cells were lysed in 1× cell culture lysis buffer (luciferase assay system E1500; Promega). Lysates (20 μl) were incubated with 100 μl of luciferin substrate (Promega), and luminescence was measured with a BioOrbit 1250 luminometer (LKB-Wallac, Turku, Finland) (43). For most experiments, CAT expression is reported as the activity present in 50 μl of extract, and luciferase expression is reported as the activity in 20 μl of extract (both levels corrected for background values in mock-transfected cells). However, where noted, both enzyme levels were normalized to protein content of these cell lysates, determined by a Coomassie blue assay (Bio-Rad).

RNase protection assays. An RNase protection assay for quantitation of RNA transcripts containing the CAT coding sequence was developed by using reagents and protocols from a lysate RNase protection assay kit (United States Biochemical Company). RNA probes included a 0.22-kb ^{32}P-labeled antisense CAT probe transcribed from *Ssp*I-linearized pHAV-CAT DNA with SP6 polymerase and a 0.14-kb ^{32}P-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antisense probe transcribed from a GAPDH control template (United States Biochemical) with T7 RNA polymerase. This latter probe was included to monitor the amount of total cellular RNA in each lysate. In the RNase protection assay, the antisense probes were designed to produce protected fragments of 0.21 and 0.10 kb, respectively. Transfected cells were washed and lysed with 1 ml of 4 M guanidine thiocyanate–25 mM sodium citrate–0.5% sarcosyl. The resulting cell lysates were hybridized with a mixture of the ^{32}P-labeled antisense RNA probes for 12 h at 37°C and then diluted and extensively digested with RNases and proteinase K. Protected RNA fragments were precipitated with isopropanol and electrophoresed on a 6% denaturing polyacrylamide gel. Bands representing the protected fragments of the CAT and GAPDH probes were detected by autoradiography, and their relative intensities were quantitated on a Hoefer Scientific GS 300 densitometer.

RESULTS

Monkey kidney cell lines which stably express cytoplasmic bacteriophage T7 RNA polymerase. Mutations which occurred within the 5′NTR of HM175/P16 virus during its passage in cultured African green monkey kidney cells promote the growth of this virus in these (i.e., BS-C-1) cells but not in cultured fetal rhesus kidney (FRhK-4) cells, which are also permissive for HAV (11). Two of three implicated mutations map within the IRES of HAV (at nt 203 and 687), while the third mutation which might be involved in growth enhancement (at nt 152) is located upstream of this translational control element (6). To determine whether these mutations act to enhance replication by facilitating IRES-directed translation in a cell-type-specific fashion, we transformed and selected stable lines of cells which constitutively express bacteriophage T7 RNA polymerase from both the BS-C-1 and FRhK-4 cell lineages. The T7 polymerase expressed in the cytoplasm of these new cell lines, BT7-H and FRhK-T7, directs the transcription of RNA following the transfection of these cells with plasmid DNA containing T7 transcriptional units. Because these cytoplasmic RNA transcripts lack a 5′ m^{7}G cap structure, their efficient translation is dependent on the inclusion of an IRES element within the transcript (13, 43).

The BT7-H cell line has been described previously (43). It has provided a useful system for evaluating the relatively weak translational activity of the HAV IRES when this RNA structure is placed within the context of either monocistronic or bicistronic transcripts (43). To develop a companion T7-expressing cell line derived from FRhK-4 cells, we used a murine Moloney leukemia virus-based vector (32). FRhK-4 cells were infected with the recombinant replication-defective retrovirus LXSN-T7, which contains the coding sequence for bacteriophage T7 RNA polymerase under control of the retroviral long terminal repeat in addition to a selectable marker gene (*neo*) (Fig. 2). Stably transformed cells were clonally selected in the presence of Geneticin. Each of the Geneticin-resistant clones that were tested supported transcription from the T7 promoter, as demonstrated by the expression of CAT following transfection with pEMCV-CAT, which contains a T7 transcriptional unit with the EMCV IRES fused to the CAT coding sequence (data not shown). The clone producing the greatest CAT expression was designated FRhK-T7 and used for all subsequent experiments.

Analysis of IRES-directed translation in BT7-H and FRhK-T7 cell lines. To compare the abilities of BT7-H and FRhK-T7 cells to support IRES-dependent translation, we constructed several plasmids containing bicistronic T7 transcriptional units of the form luciferase-picornavirus 5′NTR-CAT (Fig. 3A). The upstream cistron (luciferase) within the uncapped bicistronic RNAs produced by these plasmids in cells expressing T7 polymerase is translated with very low efficiency by a 5′-end-dependent scanning mechanism (27, 43). Translation of the downstream cistron (CAT) is dependent on the presence of an IRES in the intercistronic space. Thus, while luciferase expression reflects both the efficiency of transfection and T7 transcription, the level of CAT activity relative to luciferase activity expressed in transfected cells reflects the

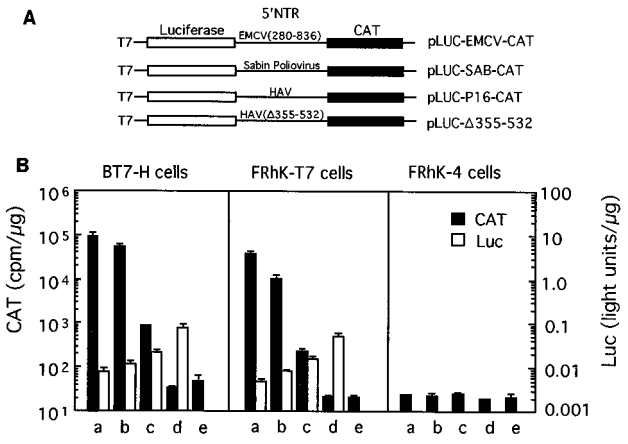


FIG. 3. Translation of bicistronic transcripts in BT7-H, FRhK-T7, and normal FRhK-4 cells. (A) Organization of T7 transcripts of the form luciferase-5′NTR-CAT which are expressed in the T7 cell lines following transfection with plasmid DNA. (B) CAT and luciferase (Luc) activities, each normalized to protein content, present in lysates of monkey kidney cells prepared 20 h after transfection with pLUC-EMCV-CAT (bar a), pLUC-SAB-CAT (bar b), pLUC-P16-CAT (bar c), pLUC-Δ355-532-CAT (bar d), or no plasmid (mock transfection; bar e). Results shown are means of duplicate transfections ± range.

translation-initiating activity of the IRES (43). Using a liposome-mediated transfection protocol, BT7-H, FRhK-T7, and normal FRhK-4 cells were transfected with several plasmid DNAs: pLUC-EMCV-CAT (which contains nt 280 to 836 of the 5′NTR of EMCV in the intercistronic space), pLUC-SAB-CAT (containing the entire 5′NTR of Sabin poliovirus type 1), pLUC-P16-CAT (containing the 5′NTR of the cell culture-adapted HAV, HM175/P16), and pLUC-Δ355-532-CAT (which contains this HAV 5′NTR with a large internal deletion which abolishes IRES-directed translation in BT7-H cells and rabbit reticulocyte lysates) (43) (Fig. 3A). CAT and luciferase activities were measured in cell lysates prepared 20 h following transfection (Fig. 3B). As expected, significant reporter gene expression from these plasmids was found only in transfected BT7-H and FRhK-T7 cells, indicating that expression was dependent on T7 RNA polymerase, as demonstrated previously for BT7-H cells (43).

Significant differences were noted in the translational activities of the three picornaviral IRES elements included in the experiment shown in Fig. 3B. CAT expression from pLUC-EMCV-CAT transcripts was high in both BT7-H (mean = 91,700 cpm/μg of protein) and FRhK-T7 (38,500 cpm/μg of protein) cell lysates, as expected from previous experiments which demonstrated high translational activity of the monocistronic plasmid, pEMCV-CAT, in BT7-H cells (43). CAT expression from pLUC-SAB-CAT was considerably lower in either BT7-H (55,200 cpm/μg of protein) or FRhK-T7 (10,100 cpm/μg of protein) cells, suggesting that the IRES of Sabin type 1 poliovirus is about one-half to one-fourth as active as that of EMCV in these cell lines. In contrast, CAT was expressed with much lower efficiency from pLUC-P16-CAT in both BT7-H (822 cpm/μg of protein) and FRhK-T7 (237 cpm/μg of protein) cells. Nonetheless, HAV-directed CAT expression was clearly measurable in comparison with CAT activity present following mock (48.2 cpm/μg of protein) or pLUC-Δ355-532-CAT (35.4 cpm/μg of protein) transfection of these cells (results are for BT7-H cells) (Fig. 3B). These results are consistent with the very low activity of the HAV IRES which we have previously noted (43) but provide for the first time a direct comparison of HAV and poliovirus IRES activities in vivo. With each plasmid tested, CAT expression was approximately two- to threefold higher in BT7-H than in FRhK-T7 cells, possibly reflecting differences in the efficiency of DNA transfection or the level of endogenous T7 polymerase activity in the two cell lines.

Interestingly, luciferase expression varied in an inverse fashion with CAT expression when plasmid DNAs containing IRES elements of widely varying activities were transfected into these cells (Fig. 3B). We have shown previously that expression of the upstream cistron, luciferase, in transfected BT7-H cells is due to a cap-independent mechanism and not related to cryptic nuclear synthesis of capped RNA transcripts (43). The ability to detect this very inefficient translation of uncapped transcripts which lack an IRES reflects the abundance of the T7 transcripts and the use of very sensitive reporter systems. In the experiment shown in Fig. 3B, luciferase expression was greatest from pLUC-Δ355-532-CAT, which lacks a functional IRES, followed by pLUC-P16-CAT and pLUC-SAB-CAT. The lowest level of expression of luciferase was with pLUC-EMCV-CAT. These results suggest that there is competition between the two cistrons for necessary cellular translation factors and that competition from the IRES element is related to its activity in promoting internal initiation of translation. We have noted similar effects in rabbit reticulocyte lysates programmed with bicistronic RNA transcripts (6).

To confirm that the differences in the levels of CAT and luciferase expression observed following transfection of BT7-H and FRhK-T7 cells were not due to differences in transfection or T7 transcriptional efficiency with individual plasmids, or in the stability of the transcripts, we examined the relative abundance of CAT transcripts in transfected cells by an RNase protection assay (Fig. 4). This assay used a mixture of an anti-sense RNA probe which hybridizes to CAT sequence within the RNA transcripts and a probe which hybridizes to cellular GAPDH mRNA. The second probe was included to monitor total cellular RNA levels in each lysate. A protected RNA fragment representing the expected size of the protected antisense CAT probe was present in each transfected cell lysate (Fig. 4). Quantitation of CAT and GAPDH protected fragments demonstrated that CAT/GAPDH ratios varied minimally in the plasmid transfections of both the BT7-H (0.149 to 0.197) and the FRhK-T7 (0.143 to 0.171) cells (data not shown). Thus, differences in the levels of CAT and luciferase activities in BT7-H and FRhK-T7 cells transfected with different bicistronic plasmids (Fig. 3B) reflect differences in the efficiency of IRES-directed translation and not differences in the abundance of RNA transcripts.

Comparison of IRES-directed translation from HM175/wt and HM175/P16 5′NTRs in monkey kidney cells. Since HAV translation can be monitored readily in both BT7-H and FRhK-T7 cells, it was possible to compare translation initiated by internal entry from the 5′NTR of the attenuated cell culture-adapted variant HM175/P16 with translation initiated from the wild-type 5′NTR in these cell types. For this analysis, we constructed an additional bicistronic plasmid, pLUC-WT-CAT, which contains the 5′NTR of HM175/wt virus within its intercistronic space (Fig. 5A). The expression of CAT and luciferase in a typical experiment in which BT7-H and FRhK-T7 cells were transfected with pLUC-P16-CAT or pLUC-WT-CAT is shown in Fig. 5B. Transfections were carried out in triplicate. In BT7-H cells, the ratio of CAT activity to luciferase activity was 5.1-fold greater from pLUC-P16-CAT (10,700 cpm/light unit, ± SEM [standard error of the mean] 245) than from pLUC-WT-CAT (2,110 cpm/light unit, ± SEM 288). In contrast, CAT/luciferase ratios were similar in FRhK-T7 cells transfected with either pLUC-P16-CAT or pLUC-WT-

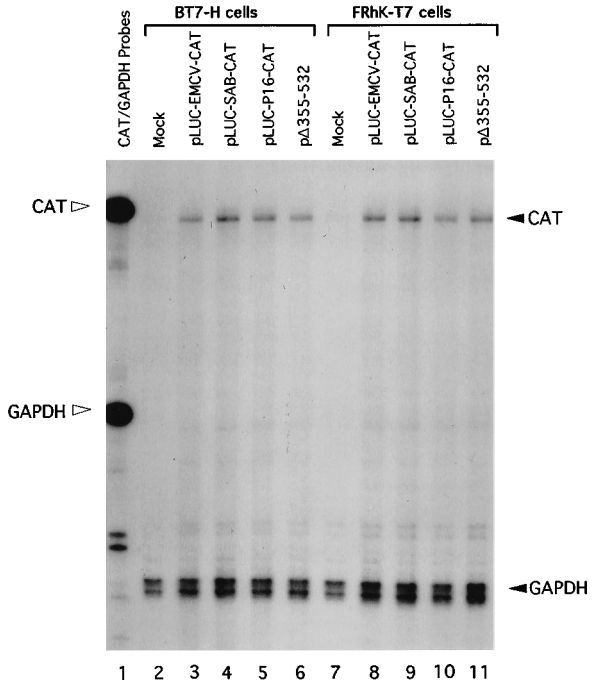


FIG. 4. Quantitation of RNA transcripts in transfected BT7-H and FRhK-T7 cells by RNase protection assay. Cells were harvested 20 h posttransfection, and lysates were hybridized overnight with ^{32}P-labeled antisense RNA probes complementary to CAT and GAPDH coding sequences. Following digestion with RNases and proteinase K, samples were electrophoresed on a 6% denaturing polyacrylamide gel. Lane 1, antisense RNA probes; lanes 2 to 11, protected RNA from BT7-H (lanes 2 to 6) and FRhK-T7 (lanes 7 to 11) cells. Cells were transfected with no DNA (lanes 2 and 7), pLUC-EMCV-CAT (lanes 3 and 8), pLUC-SAB-CAT (lanes 4 and 9), pLUC-P16-CAT (lanes 5 to 10), or pLUC-Δ355-532-CAT (lanes 6 to 11). Open arrowheads indicate CAT and GAPDH probes; solid arrowheads indicate protected CAT and GAPDH RNA fragments.

CAT (3,540 cpm/light unit or 3,910 cpm/light unit). The CAT/luciferase ratio should be a good measure of IRES activity over a wide range of transcript levels. However, to confirm that the expression of CAT and luciferase was not influenced by differences in T7 transcript levels, RNase protection assays were carried out as described above and demonstrated that transcript levels did not vary significantly following transfections in either the BT7-H or FRhK-T7 cells (data not shown). Although the magnitude of differences in the CAT/luciferase ratios might be exaggerated by intercistronic competition in comparisons of IRES elements which are widely different in their activities (such as the HAV and EMCV IRES elements; Fig. 3B), the modest difference observed in HM175/wt and HM175/P16 IRES activities did not appear to significantly affect translation of the upstream luciferase cistron (Fig. 5B). The data shown in Fig. 5 thus indicate that the translational effects of the 5′NTR mutations in HM175/P16 virus mimic their cell-type-specific effects on viral replication (11).

To determine which individual mutation(s) are responsible for this translational enhancement in BT7-H cells, we constructed a series of bicistronic plasmids containing the wild-type 5′NTR with various combinations of the HM175/P16 mutations at positions 152, 203 and 204, and 687 (Fig. 6A). These are the mutations which previous results indicate are responsible for enhanced replication in BS-C-1 cells (11). The bicistronic plasmids were transfected into BT7-H cells, and CAT and luciferase activities were measured as before (Fig. 6B). Consistent with the results shown in Fig. 5B, the CAT/luciferase ratios were 2,100 cpm/light unit, ± SEM 300, for pLUC-WT-CAT and 10,100 cpm/light unit, ± SEM 320, for pLUC-P16-CAT. The CAT/luciferase ratio following transfection of plasmid p152 was similar to that for pLUC-WT-CAT, indicating that the A-to-G substitution at nt 152 does not have an enhancing effect on translation. In contrast, p203, containing a UU deletion at nt 203 and 204, and p687, containing a U-to-G substitution at nt 687, both produced approximately 3.5-fold increases in the CAT/luciferase ratio (6,930 cpm/light unit, ± SEM 610, and 6,710 cpm/light unit, ± SEM 720), compared with that observed with pLUC-WT-CAT (Fig. 6B). In cells transfected with p152.203.687, which contains both the nt 203-204 and 687 mutations as well as the substitution at nt 152, the CAT/luciferase ratio (10,900 cpm/light unit, ± SEM 460) was nearly identical to that for pLUC-P16-CAT, which contains all five of the HM175/P16 5′NTR mutations (Fig. 6A).

To confirm that the nt 203-204 and 687 mutations had no effect on IRES-dependent translation in fetal rhesus monkey kidney cells, we transfected the same series of bicistronic plasmids into FRhK-T7 cells (Fig. 7). As expected from the results shown in Fig. 5B, CAT/luciferase ratios did not vary significantly with the bicistronic plasmids containing different combinations of the HM175/P16 mutations. These results further demonstrate that the HM175/P16 mutations do not enhance IRES-directed translation in fetal rhesus kidney cells.

Comparison of HM175/wt and HM175/P16 IRES-directed translation in a human liver cell line. The Huh7 cell line is derived from a human hepatocellular carcinoma and is permissive for HAV replication (data not shown). To assess the effects of the 5′NTR mutations on viral translation in these cells, we established a stably transformed subline, Huh-T7, which constitutively expresses T7 RNA polymerase. However, the levels of T7 transcription and/or the efficiency of translation of bicistronic transcripts containing the EMCV or HAV 5′NTR were low in these cells, resulting in only very low levels of CAT and luciferase expressed following transfection with either pLUC-EMCV-CAT or pLUC-P16-CAT (data not shown). To enhance this level of expression, we infected the Huh-T7 cells with recombinant vaccinia virus vTF7-3 immediately prior to DNA transfections. While this is likely to result in the presence of a 5′ cap structure in a small proportion of the T7 transcripts (17), it should not affect internally initiated translation from bicistronic transcripts. CAT and luciferase expression following transfection with pLUC-WT-CAT or pLUC-P16-CAT in vTF7-3 infected Huh-T7 cells is shown in Table 1. Although the absolute level of expression was much higher than in the other T7-expressing cell lines which were not infected with the recombinant vaccinia virus (Fig. 3), the CAT/luciferase ratio was similar. Importantly, the CAT/luciferase ratio following transfection with pLUC-WT-CAT (1,230 cpm/light unit, ± SEM 63) was similar to that observed with pLUC-P16-CAT (1,320 cpm/light unit, ± SEM 62). These values were much less than the CAT/luciferase ratio following transfection of the positive control pLUC-EMCV-CAT (35,100 cpm/light unit, ± SEM 3,400). These results strongly suggest that the HM175/P16 mutations do not enhance or reduce IRES-directed translation in this cell type.

DISCUSSION

Mutations responsible for the adaptation of HAV to efficient growth in cultured cells have been mapped to the 5′NTR as well as the P2 region of the genome, within the sequence encoding the 2B and 2C proteins (11, 14). A mutation at nt 3889, which results in an alanine-to-valine substitution within the 2B protein, appears to be particularly important in deter-

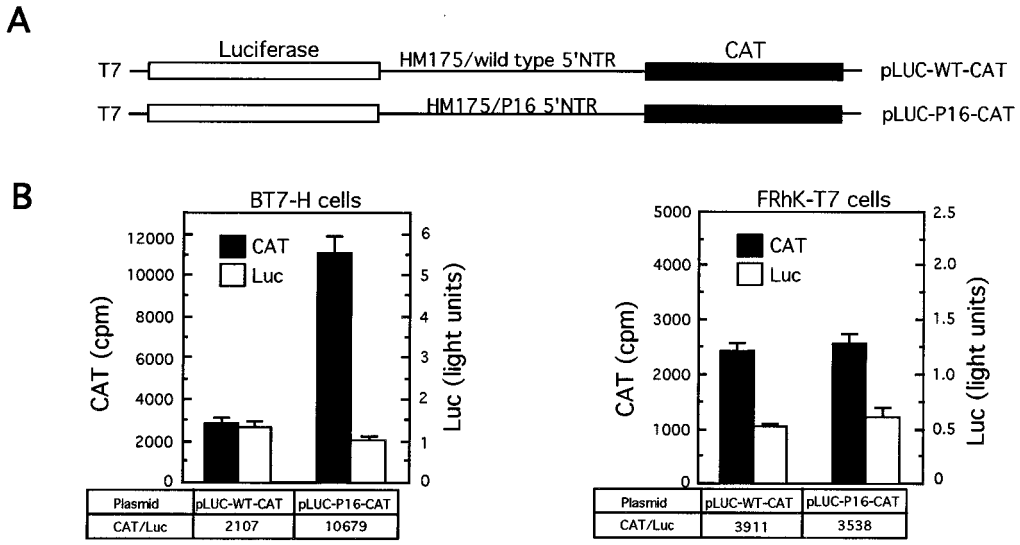


FIG. 5. Translation directed by bicistronic constructs containing either the HM175/wt or HM175/P16 5′NTR in two T7 RNA polymerase-expressing monkey kidney cell lines. (A) Schematic of pLUC-WT-CAT and pLUC-P16-CAT transcripts. (B) CAT and luciferase (Luc) activities present in BT7-H or FRhK-T7 lysates prepared 20 h after transfection with pLUC-WT-CAT or pLUC-P16-CAT. Results of triplicate transfections are presented as means ± SEM. Calculated CAT/luciferase ratios are shown at the bottom.

mining this host range change (15). Although not proven, it is likely that mutations in the P2 region act to enhance replication of the viral RNA in cultured cells. Within the 5′NTR, previous studies have shown that an A-to-G substitution at nt 152 and/or the deletion of two U residues at nt 203 and 204 substantially enhance viral replication in a cell-type-specific fashion (11). Compared with a chimeric virus containing the 5′NTR of HM175/wt, viruses containing these mutations replicate substantially more efficiently in BS-C-1 cells (which are derived from adult African green monkey kidney). In contrast, there is no difference in the replication of these viruses in FRhK-4 cells (which are of fetal rhesus kidney origin). In addition to the mutations at nt 152 and 203, a U-to-G substitution at nt 687 has also been shown to enhance viral replication in BS-C-1 cells (10, 11).

Both the nt 152 and the nt 203-204 mutations are found in the cell culture-adapted HM175/P16 and the HM175/P35 viruses, although the HM175/P35 virus contains only a single U deletion at base 203 (9, 25). The U deletion at base 203 is also found in a third cell culture-adapted variant of this strain of HAV, HM175/P59, that was independently isolated in African green monkey kidney cells (36). The nt 687 mutation is present in both the HM175/P16 and HM175/P59 viruses and in cell culture-adapted variants of HAV strain GBM (9, 21, 36). Although it is not present in HM175/P35 virus, subsequent adaptation of this virus to growth in human diploid (MRC-5) cells resulted in the appearance of the nt 687 mutation, in addition to others within the IRES (19). The presence of the mutations at nt 203 and 687 in each of three independently isolated cell culture-adapted HAV variants suggests that they play a role in allowing the virus to overcome a restriction to growth which is normally present in cultured African green monkey kidney cells. Here, we have shown that this restriction is related to viral translation.

We examined translation directed by the HAV IRES in two monkey kidney cell lines, BT7-H and FRhK-T7, both of which constitutively express T7 RNA polymerase. Following transfection of these cells with plasmid DNAs containing bicistronic T7 transcriptional units of the general form luciferase-5′NTR-CAT, the efficiency of internally initiated translation was estimated by comparing levels of CAT and luciferase activities present in cell lysates (43). Although in this study we examined only translation directed by bicistronic transcripts, the BT7-H and FRhK-4 cells offer a unique system for the evaluation of IRES activities within the context of monocistronic RNAs be-

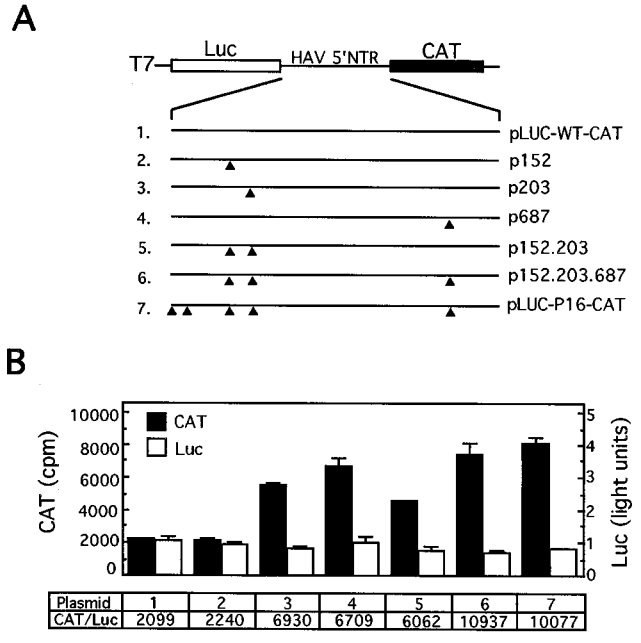
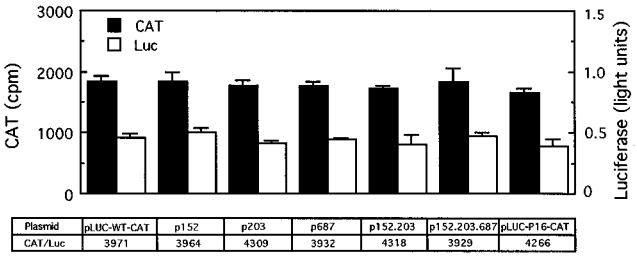


FIG. 6. (A) Schematic representation of T7 transcripts expressed from bicistronic plasmids. pLUC-WT-CAT contains the HM175/wt 5′NTR, and pLUC-P16-CAT contains the HM175/P16 5′NTR. Plasmids 2 to 6 contain the wild-type 5′NTR with HM175/P16 mutation(s) at the positions indicated by triangles (see Fig. 1). Luc, luciferase. (B) CAT and luciferase (Luc) activities 20 h after transfection of BT7-H cells. Results shown are means of triplicate transfections ± SEM. Calculated CAT/luciferase ratios are shown at the bottom.



Plasmid	pLUC-WT-CAT	p152	p203	p687	p152.203	p152.203.687	pLUC-P16-CAT
CAT/Luc	3971	3964	4309	3932	4318	3929	4266

FIG. 7. Translation of HAV bicistronic transcripts in FRhK-T7 cells. Results shown are means of triplicate transfections ± SEM. See the legend to Fig. 6 for details.

cause the T7 transcripts produced in these cells are not capped and thus closely resemble picornaviral RNAs (43). Both BT7-H and FRhK-T7 cells are derived from HAV-permissive cell lines which are often used for propagation of this virus in the laboratory.

We first compared the abilities of BT7-H and FRhK-T7 cells to support translation from bicistronic transcripts containing IRES elements derived from EMCV, the Sabin type 1 poliovirus, and HAV. Since the EMCV IRES efficiently directs cap-independent translation in a wide range of mammalian cell types (12, 24), it was not surprising that we found bicistronic RNAs containing the IRES of EMCV initiated translation in both BT7-H and FRhK-T7 cells more efficiently than bicistronic RNAs containing the poliovirus 5′NTR and much more efficiently than transcripts containing the HAV 5′NTR (Fig. 3B). The very low activity of the HAV IRES was consistent with our previous observation that the IRES in the cell culture-adapted HM175/P16 virus is approximately 200-fold less efficient than the EMCV IRES in directing translation from monocistronic transcripts (43). In bicistronic transcripts produced in both BT7-H and FRhK-4 cells, we found the HAV IRES in this study to be approximately 50-fold less efficient than the poliovirus IRES and 100-fold less efficient than the EMCV IRES (Fig. 3B).

When bicistronic transcripts containing IRES elements of widely differing activities, such as the EMCV IRES and the HAV IRES, were expressed in these cells, the efficiency with which the upstream cistron, luciferase, was translated was inversely related to the efficiency of translation of the downstream cistron, CAT (Fig. 3B). Previous studies indicate that translation of the upstream cistron in these T7 transcripts is not due to the presence of a 5′ cap structure, as it is enhanced by coexpression of the 2A protease of poliovirus (43). The intercistronic competition which is evident from the data shown in Fig. 3 indicates that the translation of the upstream cistron, which probably occurs by a 5′-end-dependent scanning mechanism, and the IRES-dependent translation of the downstream cistron utilize common cellular translation factors. These data further suggest that T7 transcripts are present in these cells at or near saturating levels with respect to translation.

A comparison of translation initiated from bicistronic RNAs containing either the HM175/wt or the HM175/P16 5′NTR demonstrated that the mutations in HM175/P16 virus enhance translation by internal entry in BT7-H cells (Fig. 5B). These results support the hypothesis that these HM175/P16 mutations enhance viral replication in BS-C-1 cells (11) by facilitating cap-independent, internal initiation of viral translation. As with the effects of these mutations on viral growth, the enhancement of translation mediated by the HM175/P16 mutations was highly host cell specific. The mutations had no effect on viral translation in either FRhK-T7 cells (Fig. 5B and 7) or Huh-T7 cells (Table 1) or in vitro in rabbit reticulocyte lysates (11) (data not shown). The UU deletion at nt 203 and 204 and the U-to-G substitution at nt 687 contributed additively to this translational enhancement (Fig. 6B). These results are thus in close agreement with previous studies which examined the effect of these 5′NTR mutations on viral replication (11).

The impact of these mutations on IRES-directed translation provides additional support for the location of the HAV IRES as determined in earlier deletion mutagenesis studies (6). We previously mapped the 5′ limits of the HAV IRES to stem-loop IIIa (Fig. 1) (6), which includes the site of the UU deletion at nt 203 and 204. The U-to-G substitution at nt 687 lies approximately 50 nt upstream of the initiator AUGs and is also within the mapped limits of the IRES. However, although enhancement of viral replication via a secondary effect of the nt 203-204 and 687 mutations on RNA replication would seem to be a very remote possibility, it is worth noting that Borman et al. (3) have suggested that the poliovirus IRES may contain signals necessary for RNA replication as well as translation. The other mutations within the 5′NTR of HM175/P16, which include the addition of a U at the extreme 5′ end of the genome and substitutions at nt 8 and 152 (Fig. 1), are located upstream of the HAV IRES (6) and had no apparent effects on viral translation in either of the monkey kidney cell types (Fig. 5B). Nonetheless, the A-to-G substitution at nt 152 is present in at least two independently isolated, cell culture-adapted strains, suggesting that it may also play a role in cell culture adaptation. If so, it is likely that this mutation has a primary effect on RNA replication, since it is part of a single-stranded domain in which deletion mutations (at nt 140 to 144) have been shown to result in a temperature-sensitive RNA replication phenotype (38). Studies examining this possibility are in progress.

The mechanism by which the UU deletion at nt 203 and 204 and the U-to-G substitution at nt 687 of the HAV 5′NTR enhance viral translation in a cell-type-specific fashion is unknown. Both mutations likely alter the secondary structure of the 5′NTR only minimally. The UU deletion at nt 203 and 204 would shorten a UUUUU tract normally present in HM175/wt virus, resulting in a reduction in an unpaired bulge loop near the top of stem-loop IIIa (5, 37) (Fig. 1). The substitution at nt 687 would minimally destabilize a putative stem-loop structure (stem-loop Vc) located just upstream of the initiator AUGs, resulting in a change in free energy from −12.9 to −8.7 kcal (1 cal = 4.184 J)/mol, according to energy values of Freier et al. (16). Although minimal, our data indicate that these structural changes affect the affinity of the IRES for essential cellular translation initiation factors which thus far remain poorly defined (24, 29, 30).

Using a UV cross-linking/label transfer assay, Chang et al. (7) previously identified three ribosome-associated proteins (p30, p39, and p110) that are present in both BS-C-1 and

TABLE 1. Translation mediated by the 5′NTRs of HM175/wt and HM175/P16 viruses in vTF7-3-infected Huh-T7 cells[a]

Plasmid	CAT (10^6 cpm ± SEM)	Luciferase (light units ± SEM)	CAT/luciferase (cpm/light unit ± SEM)
pLUC-WT-CAT	5.38 ± 0.51	4,370 ± 280	1,230 ± 63
pLUC-P16-CAT	5.88 ± 0.44	4,460 ± 160	1,320 ± 62
pLUC-EMCV-CAT	84.6 ± 4.1	2,410 ± 300	35,100 ± 3,400

[a] Huh7-T7 cells were infected with vTF7-3 (multiplicity of infection of 10) prior to transfection with plasmid DNA (see Materials and Methods). Results are means of duplicate transfections ± SEM.

FRhK-4 cells and which bind to specific domains within the 5′NTR of HAV. While the binding of these proteins was not specifically localized to the sites of the nt 203-204 and 687 mutations, a role for these nucleotides in determining the binding affinity of these proteins cannot be excluded on the basis of available data. However, it remains unknown whether any of these proteins have a function related to HAV IRES-mediated translation. Their role in the cell culture adaptation of HM175/P16 virus is equally difficult to assess, since similar proteins were identified by UV cross-linking/label transfer experiments with extracts prepared from both BS-C-1 and FRhK-4 cells despite the functional differences that we have shown exist in HAV translation in these two cell types (7). It is interesting, however, that UV cross-linking/label transfer studies carried out with extracts of a human cellular carcinoma cell line, HepG2, identified an alternate set of ribosome-associated proteins which resemble those previously identified in HeLa cells (7, 8). These include the 57-kDa pyrimidine tract-binding protein, which has been suggested to be an important cellular factor involved in picornaviral translation initiation (2, 4, 21a, 24).

While we do not know which HAV RNA-binding proteins are present in human liver, the results shown in Fig. 3 and 5 suggest that the cellular factors involved in IRES-directed translation within the liver differ significantly from those present in cultured African green monkey kidney cells. This conclusion is consistent with the data shown in Table 1, which indicate that the HM175/P16 mutations do not have a positive impact on IRES-mediated translation in Huh-T7 cells, which are derived from human hepatocytes. Viruses which contain these 5′NTR mutations are highly attenuated in otherwise susceptible primates (26, 41). Although changes elsewhere in the genome are likely to play an important role in attenuation (15), it would be interesting to know the effects of these mutations on translation within the liver.

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