Specific Interaction of Glyceraldehyde 3-Phosphate Dehydrogenase with the 5'-Nontranslated RNA of Hepatitis A Virus*

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Initiation of translation of hepatitis A virus (HAV) RNA occurs by internal entry and is likely to involve the interaction of trans-acting cellular protein factors with cis-acting structural elements of an internal ribosomal entry segment (IRES) within the 5'-nontranslated RNA. To characterize interactions between African green monkey kidney (BS-C-1) cell proteins and the predicted stem-loop IIIa (nucleotides 155–235) located at the 5' border of the HAV IRES, we utilized an electrophoresis mobility shift assay (EMSA) to identify a 39-kDa RNA-binding protein (p39). Amino-terminal amino acid sequencing of highly purified p39 revealed absolute identity with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The identity of p39 as simian GAPDH was further confirmed by antigenic and biochemical similarities between p39 and human GAPDH. Analysis of the RNA binding properties of simian GAPDH revealed that this cellular protein interacts with two additional sites in the HAV 5'-nontranslated RNA, one located between nucleotides 1–148 and the other between nucleotides 597–746. Competitive EMSAs also demonstrated that GAPDH and human polyphemus tract-binding protein, a putative picornavirus translation initiation factor, compete with each other for binding to stem-loop IIIa, suggesting that the relative cytoplasmic abundance of GAPDH and polyphemus tract-binding protein in individual cell-types may be an important determinant of viral translation activity. Human GAPDH was found to destabilize the folded structure of the stem-loop IIIa RNA based upon observed decreases in the circular dichroism spectra of this RNA following binding of the protein. This RNA helix-desstabilizing activity of GAPDH could directly influence IRES-dependent translation and/or replication of picornavirus RNA.

Human hepatitis A virus (HAV),1 an hepatovirus of the Picornaviridae family, is a positive-strand RNA virus with a genome length of approximately 7,480 nucleotides (1–3). The RNA genome is organized as a relatively long 734-nucleotide region (5'-NTR), a single large open reading frame encoding the viral polyprotein, and a short 3'-nontranslated region. Among HAV strains, the 5'-NTR is the most conserved region within the genome and contains extensive secondary and tertiary RNA structure (4). The highly ordered 5'-NTR contains an internal ribosomal entry segment (IRES), located between nt 152 and the first initiator AUG at nt 735 in the HAV genome, which regulates initiation of translation of the viral polyprotein by a cap-independent mechanism (5, 6). Compared with other picornaviruses, HAV IRES-directed translation is extremely inefficient, a feature that may contribute to the generally slow and noncytopathic replication of the virus in cell culture (5–7). In addition to its role in translation, it is likely that the 5'-NTR also contains regulatory elements for initiation of positive-strand RNA synthesis (8) and possibly encapsidation of genomic RNA.

The cap-independent initiation of translation is an important aspect of HAV replication, strongly influencing the growth of the virus in cell culture and probably contributing to its pathogenicity in primates. Attenuated, cell culture-adapted strains of HAV have mutations from the wild-type sequence throughout the RNA genome, but these are concentrated in the 5'-NTR as well as the P2 and P3 regions of the viral RNA, which encode nonstructural proteins involved in RNA replication (1, 9). Compared with wild-type viruses, these attenuated viruses demonstrate enhanced growth in cultured cells but reduced growth in primary chimpanzee hepatocytes.2 Although mutations in the P2 region are particularly important for cell culture-adaptation, studies of chimeric viruses containing 5'-NTR sequences from wild-type or cell culture-adapted HAVs indicate that several mutations in the 5'-NTR (at nt 152 and/or 203–204 and at nt 687) enhance viral growth in African green monkey kidney (BS-C-1) cells but not fetal rhesus monkey kidney (FRHK4)-cells (10–12). Furthermore, recent studies have shown that two of these mutations, a UU deletion at nt 203–204 of stem-loop IIIa of the 5'-NTR and a U-to-G substitution in stem-loop V, act to enhance IRES-directed translation with a cell-type specificity consistent with their effects on viral replication (17).

The cap-independent translation of the HAV polyprotein, as well as that of other picornaviruses, is likely to be dependent upon the interaction of trans-acting host cell proteins with the cis-acting IRES located within the 5'-NTR. This hypothesis is supported by several observations in addition to the cell-type specific action of the HAV mutations described above. First, efficient poliovirus and rhinovirus translation in rabbit reticulocytes lysate is dependent upon supplementation of the rabbit reticulocyte lysate with ribosomal salt wash (RSW) fractions

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1 The abbreviations used are: HAV, hepatitis A virus; nt, nucleotide(s); IRES, internal ribosomal entry segment; RSW, ribosomal salt wash; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mAb, monoclonal antibody; EMSA, electrophoresis mobility shift assay; PAGE, polyacrylamide gel electrophoresis; PTB, polyphemus tract-binding protein; NTR, nontranslated region.

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* S. F. Chao, R. E. Lanford, and S. M. Lemon, unpublished results.
from HeLa cells (13). Similarly, it has been suggested that HAV IRES-directed translation in rabbit reticulocytes lysate is enhanced following supplementation with cytoplasmic extracts from mouse liver (14). Finally, no picornavirus IRES has been reported to function in a wheat germ translation system, suggesting the absence of necessary trans-acting factors. Multiple cellular proteins, mostly derived from HeLa cells, have been shown to interact with picornavirus 5′NTRs. A 52-kDa nuclear autoantigen, La, binds with specificity to the poliovirus 5′NTR and appears to be the La cell factor responsible for correcting aberrant translation of poliovirus RNA in rabbit reticulocyte lysate (15, 16). The 57-kDa polypyrimidine tract-binding protein (PTB), which may have a role in nuclear pre-mRNA splicing, also binds to several picornavirus 5′NTRs, including that of HAV (17–22). Recent studies, in which PTB was depleted from translationally active HeLa and Krebs-2 cell extracts, suggest that PTB may also have a role in picornavirus translation in these cell types (18, 23). Other identified cellular proteins that bind to the 5′NTRs of picornviruses include the eucaryotic translation initiation factor, eIF-2A, and the elongation factor EF-1a (24, 25). Indeed, it is likely that all of the canonical translation initiation factors, with the possible exception of eIF-4f, are required for IRES-directed initiation of picornavirus translation, in addition to the novel putative translation factors mentioned above.

In previous studies, we utilized a UV cross-linking/label transfer assay to identify proteins of 30, 39, and 110 kDa (p30, p39, and p110), which are present in RSW prepared from BS-C-1 cells (10). Various cytoplasmic extracts from the plasma membranes of HAV RNAs were sequenced on a Model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy® Terminator Cycle Sequencing Kit (Applied Biosystems).

**MATERIALS AND METHODS**

**Reagents**—Monoclonal antibody (mAb) 40.10.09 to human uracil-DNA glycosylase/GAPDH was kindly provided by Michael Sirover (Temple University School of Medicine, Philadelphia, PA). Goat anti-mouse IgG + IgA + IgM and alkaline phosphatase-conjugated goat anti-mouse IgG + IgA + IgM were purchased from BRL. Anti-HAV mAb 1.93 has been described previously (28). Human erythrocyte GAPDH, ribo-homopolymers, and NAD⁺ were purchased from Sigma. Recombinant human PTB was a gift from Mariano Garcia-Blanco (Duke University, Durham, NC). Sephacryl S-300 HR, DEAE Sephacel, and heparin-Sepharose were purchased from Pharmacia Biotech Inc., and [α-32P]CTP (800 Ci/mmol) was obtained from Dupont NEN.

**Plasmids**—All HAV plasmid constructs contained sequences of the HM175/P16 strain of HAV (9) placed downstream of the promoter for a 12% SDS-polyacrylamide gel, and electrotransferred to a polyvinylidene difluoride membrane in 25 mM Tris, pH 8.3, 192 mM glycerol, 10% methanol. Protein bands were visualized by Coomassie Brilliant Blue staining, and the primary band corresponding to p39 was excised. For

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**Size Exclusion Chromatography of the BS-C-1 S100 Extract**—BS-C-1 S100 fraction (0.2 ml) was applied to a Sephacryl S-300 HR column (9 × 1.5 cm) equilibrated in buffer A with 25 mM KCl. Fractions (0.4 ml) were collected at a flow rate of 5 ml/h, and 5-μl aliquots of fractions were analyzed by EMSA as described above.

**Purification of BS-C-1 Cytoplasmic Extract**—BS-C-1 S100 extract (10 ml) was applied to a Sephacryl S-300 HR column (9 × 1.5 cm) that had been equilibrated in buffer A with 25 mM KCl. The flow-through fraction from the DEAE-Sepacel column was applied to a heparin-Sepacel column (2.5 × 1 cm) that had been equilibrated in buffer A containing 10 mM KCl. The fraction was collected at a flow rate of 20 ml/h, and the column was eluted with 30 ml of buffer A containing 0.5 mM KCl. The flow-through fraction from the DEAE-Sepacel was applied to a heparin-Sepacel column (2.5 × 1 cm) that had been equilibrated in buffer A containing 10 mM KCl. The column was eluted with 35 ml of a linear gradient of KCl (10–400 mM) in buffer A. Fractions (1 ml) were collected at a flow rate of 10 ml/h. Fractions 12–15, which contained the majority of the HAV stem-loop IIa binding activity (see “Results”) were pooled and dialyzed against buffer A containing 25 mM KCl.

**Amino Acid Sequencing**—Protein from the pooled heparin-Sepacel chromatography fractions was aceton precipitated, electrophoresed on a 12% SDS-polyacrylamide gel, and electrotransferred to a polyvinylidene difluoride membrane in 25 mM Tris, pH 8.3, 192 mM glycerol, 10% methanol. Protein bands were visualized by Coomassie Brilliant Blue staining, and the primary band corresponding to p39 was excised. For
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KCl. Following a 10-min incubation at 4°C.

glycine, 10% methanol.

Proposed secondary structure of stem-loop IIIa within Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet-40) and divided into three 100-μl aliquots.

Results were incubated with the uniformly labeled HAV stem-loop IIIa probe (Fig. 1) and electrophoresed on a 4% nondenaturing gel as described above in Materials and Methods. Molecular standards peaked at the following fractions: blue dextran, 8; aldolase (158 kDa), 11; bovine serum albumin (66.2 kDa), 15; and ovalbumin (42.7 kDa), 17. Lane A, stem-loop IIIa probe; lane B, probe plus S100 fraction; lanes C–K, probe plus S-300 HR column fractions.

**RESULTS**

Identification of a BS-C-1 Cytoplasmic Factor That Interacts with Stem-loop IIIa of the HAV 5' NTR—Potential interactions between proteins present in RSW or cytoplasmic extract prepared from HAV-permissive BS-C-1 cells and an RNA probe representing stem-loop IIIa of the 5' NTR of HM175/P16 virus (Fig. 1) were examined in an EMSA. This RNA segment represents the most 5' stem-loop within the IRES of HAV, as indicated by the fact that deletion of this stem-loop but not upstream RNA sequence significantly reduces the efficiency of internal initiation from bicistronic RNA transcripts (5, 8).

Furthermore, deletion of a UU dinucleotide at nt 206–207, located within a bulge loop of this RNA (Fig. 1), has recently been shown to enhance translation of the viral RNA in BS-C-1 cells (7). For these experiments, synthetic stem-loop IIIa RNA was uniformly labeled with [32P]CTP and incubated with either the RSW fraction or the cytoplasmic extract in the presence of yeast tRNA (200 μg/ml) as a nonspecific competitor. Electrophoresis of the reaction mixtures on a 4% nondenaturing polyacrylamide gel revealed retarded RNA-protein complexes with similar mass, suggesting that the RNA binding factor(s) present in these two cell fractions may be identical (Fig. 2, lane B, and data not shown). Binding of this factor(s) was inhibited in the presence of 0.1% SDS, suggesting that the factor is a protein and not nucleic acid (data not shown).

To determine the approximate molecular mass(es) of the protein(s) that binds to the stem-loop IIIa RNA, the BS-C-1 cytoplasmic extract was fractionated on a Sephacryl S-300 HR gel filtration column and fractions were assayed by EMSA. Under these conditions, the binding factor producing the re-
**FIG. 3. Isolation of a 39-kDa protein that binds to HAV stem-loop IIIa RNA.** A, BS-C-1 S100 fraction was applied to a DEAE-cellulose anion exchange column and the flow-through fraction was further fractionated by heparin-Sepharose chromatography as described under “Materials and Methods.” Fractions were assayed by EMSA for their stem-loop IIIa binding activity. Lane A, labeled stem-loop IIIa RNA probe. Lanes B-O contain RNA incubated with BS-C-1 S100 fraction (lane B), DEAE flow-through (FT) fraction (lane C); heparin-Sepharose flow-through fraction (lane D); and heparin-Sepharose elution fractions 3–33 (lanes E–O). B, heparin-Sepharose fractions 12–15 were pooled and further analyzed by 12% SDS-PAGE followed by silver staining. C, transfer of label from stem-loop IIIa RNA to a 39-kDa protein in the pooled heparin-Sepharose fractions following UV cross-linking and extensive RNase digestion.

Purification and Amino-terminal Sequencing of the Cytoplasmic Factor That Binds to Stem-loop IIIa RNA—The ability to detect stem-loop IIIa RNA binding activity by EMSA following gel filtration chromatography suggested that EMSA could be utilized to monitor RNA binding activity following other chromatographic separation techniques. Thus, in an effort to purify the relevant RNA-binding factor, BS-C-1 cell cytoplasmic extract was applied to a DEAE-Sephacel column, and RNA binding activity was recovered in the flow-through fraction (Fig. 2A, lane C). This was followed by heparin-Sepharose chromatography; which resulted in a peak of RNA binding activity eluting at approximately 150 mM KC1 (Fig. 2A, lanes H and I). Analysis of the pooled fractions (12–15) containing this peak binding activity by 12% SDS-PAGE followed by silver staining revealed a major protein band of 39 kDa that was approximately 85% pure (Fig. 3B). The UV cross-linking/label transfer assay using the pooled fractions also revealed a 39-kDa protein as the primary species to which 32P label was transferred from the stem-loop IIIa RNA (Fig. 3C). Together, these data provided strong evidence that the 39-kDa protein was responsible for producing the RNA-protein complex in the fractions analyzed by EMSA. These data also suggest that p39 exists as a high molecular weight complex in BS-C-1 cells. Alternatively, these data could be interpreted as showing a unique HAV RNA-binding protein that was not identified by the earlier UV cross-linking/label transfer assay employed by Chang et al. (19).

Confirmation That the 39-kDa BS-C-1 RNA Binding Factor Is Simian GAPDH—To confirm that the RNA binding factor p39 is in fact GAPDH, a series of experiments were carried out comparing p39 and human erythrocyte GAPDH (Sigma). First, purified p39 and human GAPDH were probed in immunoblots with the anti-human uracil DNA glycosylase/GAPDH mAb 40.10.09 (Fig. 4A). As expected, the 40.10.09 mAb detected a single band of 37 kDa representing human GAPDH (Fig. 4A, lane B). In addition, the 40.10.09 mAb also recognized the BS-C-1 p39 protein, which migrated with a slightly higher apparent molecular mass than human GAPDH (Fig. 4A, lane B). Comparison of these two proteins by SDS-PAGE and silver staining also revealed this slight difference in apparent molecular mass (data not shown).

As with BS-C-1 p39, human GAPDH was found to be labeled after UV cross-linking with 32P-labeled stem-loop IIIa RNA followed by extensive RNase digestion (Fig. 4B, lanes A and F). As additional evidence that p39 was in fact GAPDH, the resulting radiolabeled RNA-protein complexes could be immunoprecipitated with the anti-GAPDH 40.10.09 mAb but not with a control mAb directed against HAV capsid, 1.191 (Fig. 4F). Finally, purified GAPDH formed a primary complex with stem-loop IIIa RNA in EMSA, with a shift in mobility similar to the p39-RNA complex (Fig. 4C, lanes B and E). When the ratio of protein to stem-loop IIIa RNA was increased, a second minor RNA-protein complex with more retarded mobility formed with both p39 and GAPDH (data not shown). A very small amount of this secondary complex is apparent in Fig. 4C (lanes B and G). The addition of the 40.10.09 anti-GAPDH mAb further re-

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**TABLE I**

| Protein          | Amino acid sequence* | Position |
|------------------|----------------------|----------|
| BS-C-1 p39       | GVVGSGFGCELGVLTRAAFGSG | 2–28     |
| Human GAPDH      | GVVGSGFGCELGVLTRAAFGSG |          |

* X denotes amino acid residues that could not be clearly identified in the Edman degradation.
Fig. 4. Antigenic relatedness of BS-C-1 p39 and human GAPDH. A, immunoblot of purified BS-C-1 p39 (lane A) and human GAPDH (lane B) probed with mouse monoclonal anti-human GAPDH antibody 40.10.09. B, immunoprecipitation of UV cross-linked BS-C-1 p39 and human GAPDH. Purified p39 (lanes A–E) or human GAPDH (lanes F–I) were UV cross-linked to 32P-labeled stem-loop IIIa RNA and extensively digested with RNases prior to SDS-PAGE. Labeled proteins (lanes A and F) were tested for their ability to be immunoprecipitated with anti-GAPDH monoclonal 40.10.09 (supernatant, lanes B and G; pellet, lanes C and H) or control mouse monoclonal antibody to HAV capsid 1.193 (supernatant, lanes D and E; pellet, lanes E and J). C, mobility supershift of BS-C-1 p39-RNA and human GAPDH-RNA complexes with anti-GAPDH antibody. Lane A, labeled HAV stem-loop IIIa RNA probe. EMSA reactions contained RNA incubated with BS-C-1 p39 (lanes B–D) or human GAPDH (lanes E–G) in the presence of no antibody (lanes B and E), anti-GAPDH monoclonal 40.10.09 (lanes C and F), or control antibody 1.193 (lanes D and G). Lanes H and I represent probe reacted with antibody 40.10.09 (lane H) or 1.193 (lane I) in the absence of p39 or GAPDH.

Fig. 5. EMSA demonstrating competition between the co-enzyme NAD⁺ and stem-loop IIIa RNA for binding to purified BS-C-1 p39 and human GAPDH. Lane A, labeled stem-loop IIIa RNA probe. EMSA reactions contained probe incubated with BS-C-1 p39 (lanes B–E) or human GAPDH (lanes F–I) in the absence of added NAD⁺ (lanes B and F) or in the presence of 0.01 (lanes C and G), 0.1 (lanes D and H), or 1 not NAD⁺ (lanes E and I).

The RNA binding properties of BS-C-1 p39 and human GAPDH were compared with each other to further confirm the identity of p39 as simian GAPDH. Nagy and Rigby (36) previously demonstrated that GAPDH binds RNA in its NAD⁺ co-enzyme binding site (otherwise known as the “Rossman fold”). In competitive binding experiments with p39 or human GAPDH, NAD⁺ inhibited binding of both proteins to stem-loop IIIa RNA at nearly identical concentrations (Fig. 5). In addition, various unlabeled ribohomopolymers competed with 32P-labeled stem-loop IIIa RNA for binding with p39 or human GAPDH with approximately equal efficiencies (Fig. 6). As demonstrated previously in competitive binding experiments with the 3’NTR of the interferon-γ 3’ mRNA (36), poly(U) was the strongest competitor of RNA binding to GAPDH as well as the BS-C-1 p39 protein (Fig. 6, lanes D and E). A lesser degree of binding competition was observed at the highest concentration of poly(A), whereas poly(C) had no apparent effect on stem-loop IIIa binding to GAPDH or p39.

Specificity of Simian GAPDH Binding to the HAV 5’NTR—To localize the binding sites of simian GAPDH (BS-C-1 p39) within the HAV 5’NTR and confirm previous observations of the specific binding of p39 to HAV RNA, we determined the ability of various unlabeled RNA probes representing different structural elements of the HAV 5’NTR to compete for binding to purified p39 in the UV cross-linking/label transfer assay. The unlabeled competitor probes are shown in Fig. 7A and included RNA transcripts corresponding to nt 1–148 (representing stem-loops I–IIb in addition to the most 5’ pyrimidine-rich tract, pY1), nt 155–235 (stem-loop IIIa), nt 249–632 (stem-loops IIIb-IV), and nt 596–744 (stem-loop V to the initiator AUG codon) (4, 37). In addition, a 269-nt transcript (GEM-t) derived from pGEM3ZR(−) was utilized as a nonspecific competitor RNA in these experiments. In the UV cross-linking/label transfer assay, the 1–148 and 155–235 nt probes were the strongest competitors, followed by the 596–744 nt probe (Fig. 7B). Both the 249–632 nt HAV probe and the control GEM-t were relatively weak competitors showing little if any effect at molar concentrations 200-fold in excess of the radiolabeled stem-loop IIIa RNA. These data thus support the specific nature of the interaction between GAPDH and stem-loop IIIa RNA but confirm earlier observations that p39 (GAPDH) binds to several sites within the 5’NTR (19). Importantly, the three sites that specifically bound purified simian GAPDH in the UV cross-linking/label transfer assay did not differ from those that were previously found to bind the 39-kDa protein identified in a crude BS-C-1 cell extract (19). This provides further evidence that these proteins are identical. Because the 249–632-nt and 596–744-nt HAV RNA probes overlap each other by 37 nt, the low affinity GAPDH binding activity of the latter probe is likely to be located between nt 633 and 744 or to be dependent upon the presence of an intact stem-loop Va (4).

GAPDH and PTB Compete for Binding to HAV Stem-loop
Several lines of evidence suggest that GAPDH and PTB, a putative picornavirus translation initiation factor (18, 21), have similar RNA binding specificities. This includes the similar binding specificity of these proteins for ribohomopolymers (36, 38) and previous observations suggesting that BS-C-1 p39 and HeLa cell p57 (PTB) compete for similar binding sites on the HAV 5’ NTR (19). To determine whether GAPDH and PTB compete for binding to a functionally significant region of the HAV 5’ NTR, we carried out UV cross-linking/label transfer assays with uniformly labeled stem-loop IIIa RNA and reaction mixtures containing both human GAPDH and PTB in varied molar ratios (Fig. 8). As expected, in reactions with GAPDH or PTB alone, the label was transferred to proteins of 37 or 57 kDa, respectively, with label transfer to PTB being more efficient than to GAPDH (Fig. 8, lanes A and I). However, in reactions with equimolar quantities of the two RNA-binding proteins, label transfer to GAPDH was significantly reduced, whereas label transfer to PTB was not impaired (Fig. 8, lane E). When the molar concentration of PTB was 10-fold higher than GAPDH, label transfer to GAPDH was not detected, whereas a 10-fold molar excess of GAPDH resulted in only a modest reduction of label transfer to PTB. These results are consistent with competition between GAPDH and PTB for binding to similar or overlapping sites on the stem-loop IIIa RNA and indicate that the binding affinity of PTB is substantially higher than that of GAPDH.

Circular Dichroism Spectra of Stem-loop IIIa RNA Complexed with Human GAPDH—Previously, both yeast GAPDH and human GAPDH (P8 protein) were found to possess a helix destabilizing activity that could depress the melting temperature of poly(A-U) by as much as 28 and 24 °C, respectively (39). By definition, helix destabilizing proteins bind single-stranded polynucleotides with higher affinity than double-stranded helical polynucleotides, with the resulting equilibrium favoring the single-stranded conformation (40). A common experimental property of several helix destabilizing proteins, including yeast GAPDH and the prototype T4 bacteriophage gene 32 protein (39, 40), is that they affect a substantial decrease in the CD spectra of helical nucleic acids. This phenomenon has been interpreted as evidence for protein-induced melting of the polynucleotide helix accompanied by unstacking of nucleic acid bases.

To determine whether the reported helix-destabilizing activity of GAPDH extended to its interaction with stem-loop IIIa RNA, we examined the effect of GAPDH binding on the CD spectra of this RNA. Fig. 9A depicts the CD spectra of isolated stem-loop IIIa RNA in the absence of protein at temperatures
ranging from 10 to 80°C. Inspection of these spectra reveals a positive band centered at approximately 268 nm, with a progressive reduction in the molar ellipticity between 250 and 290 nm with increasing temperatures. Because the structure of stem-loop IIIa RNA has previously been shown to have significant double-stranded character (4, 37), the reduction in intensity of the stem-loop IIIa RNA spectra with increasing temperature is likely to reflect disruption of base pair interactions that are present in the folded conformation of this RNA. Fig. 9B shows the CD spectra of stem-loop IIIa RNA (250 nM) at 15°C in the presence of three different concentrations of human GAPDH (1, 2, and 4 μM), as well as the CD spectra of isolated human GAPDH at concentrations of 2 and 4 μM. The minimal ellipticity of isolated GAPDH at wavelengths greater than 250 nm (Fig. 9B) allowed a straightforward assessment of the effect of this protein on the CD spectra of the RNA between 250 and 290 nm. The addition of even low concentrations of GAPDH (1–2 μM) to stem-loop IIIa RNA resulted in a significant reduction in the intensity of the RNA spectra at wavelengths between 250 and 290 nm (Fig. 9B). This effect was dependent upon the concentration of GAPDH and resembled the reduction in ellipticity, which was observed upon thermal denaturation of the RNA (Fig. 9A). In contrast to GAPDH, the addition of a 10-fold molar excess of albumin to stem-loop IIIa RNA had no effect on the CD spectra of the RNA between 250 and 290 nm (data not shown). These data thus indicate that the binding of GAPDH to stem-loop IIIa RNA significantly destabilizes the secondary structure of this RNA, suggesting that GAPDH binds the single-stranded RNA with greater affinity than helical segments of this stem-loop or that it converts helical segments to unpaired strands.

Fig. 9C shows the molar ellipticity of RNA-GAPDH mixtures at the peak wavelength (268 nm) as a function of the molar ratio of monomeric GAPDH to stem-loop IIIa RNA (held constant at 250 nM) (molar ellipticity normalized for the spectral contribution of GAPDH). These results show that a molar ratio of monomeric GAPDH to RNA of approximately 4:1 is required for a half-maximal change in the RNA structural perturbation. If GAPDH is predominantly tetrameric under the conditions of this experiment, these results would suggest that a ratio of one GAPDH tetramer/RNA molecule is required for half-maximal destabilization of the RNA structure. However, although GAPDH is predominantly a tetrameric protein, further interpretation of these results is hindered by the fact that GAPDH...
can exist as an equilibrium mixture of tetramers, monomers, and possibly dimers under conditions where the concentration of protein is relatively low, such as those described in Fig. 9C (41–43). Although the monomeric form of GAPDH has been reported to bind poly(dU) and to possess an associated uracil DNA glycosylase activity (34), it is still not clear which form(s) of GAPDH bind to RNA. Furthermore, it is also unknown whether GAPDH binding to RNA affects the relative abundance of tetramers, dimers, and monomers.

**DISCUSSION**

Although the precise mechanism by which picornavirus translation is initiated remains unknown, it is likely to depend upon the interaction of trans-acting cellular factors with cis-acting elements of the 5′ NTR, which result in the binding of the 40 S ribosomal subunit at a position that is favorable for translation to be initiated at the proper AUG initiator codon. Studies that examined the translation of HAV RNA in rabbit reticulocyte lysates indicated that the HAV IRES is located between nt 151–735 of the 5′NTR (5). Interestingly, the 5′ border of the HAV IRES coincides with a stem-loop structure (domain IIIa), which suggests that base pairing in this region is important for IRES-directed translation (Figs. 1 and 7A) (4, 5, 37). Immediately 5′ of this stem-loop there is an extended single-stranded domain within which deletions do not significantly impair translation but do result in a temperature-sensitive replication phenotype due to a reduction in RNA synthesis (8, 37). Within stem-loop IIIa, a deletion of two U residues at nt 206–207, which normally contribute to a bulge-loop in this RNA, has been shown to enhance the growth of cell culture-adapted virus in continuously cultured African green monkey kidney (BS-C-1) cells due to a specific enhancement of IRES-dependent translation in these cells (7, 12). Thus, several independent lines of evidence indicate that stem-loop IIIa plays an important role in the initiation of HAV translation by internal entry.

Here, we show that a protein of approximately 39 kDa (p39), which is present in BS-C-1 cell cytoplasmic extracts and RSW fractions and which binds with relative specificity to stem-loop IIIa RNA, is simian GAPDH. The identification of this protein as GAPDH was based upon several comparisons of the purified p39 RNA-binding protein with human GAPDH, a tetrameric protein that is composed of four identical 37-kDa subunits (26, 27). First, amino-terminal amino acid sequencing of p39 revealed absolute identity between p39 and human GAPDH at the 25 amino acid residues that were identified in the first 27 cycles of Edman degradation (Table I). In addition, as with the BS-C-1 p39 protein, human GAPDH also was UV cross-linked in RNA binding reactions with radiolabeled stem-loop IIIa RNA and formed a complex with this RNA in EMSA that migrated in similar fashion to the p39-RNA complex in a non-denaturing acrylamide gel (Fig. 4). Furthermore, p39 and human GAPDH were found to be antigenically related based upon immunoblot analysis, immunoprecipitation of stem-loop IIIa RNA-protein complexes with anti-GAPDH monoclonal antibody, and a mobility supershift of this complex by anti-GAPDH antibody in EMSA (Fig. 4). Competition binding experiments with NAD+ and the ribohomopolymers poly(U), poly(C), and poly(A) demonstrated strong similarities between p39 and human GAPDH (Figs. 5 and 6). Finally, because GAPDH exists primarily as a tetrameric protein (26, 27), the identification of p39 as simian GAPDH is also consistent with our finding that the RNA binding activity of p39 elutes from a size exclusion column with an apparent molecular mass of approximately 150 kDa (Fig. 2).

Previous studies have shown that GAPDH is an abundant, multifunctional enzyme that is primarily cytoplasmic but also present in the nucleus (35). In addition to its role in glycolysis, several unrelated activities have been attributed to GAPDH including DNA repair (34), protein phosphorylation (44), and interaction with RNA (35, 36), DNA (34, 45), microtubules (46), and red cell membranes (47). Similar to our finding that GAPDH binds to several regions within the HAV 5′NTR, GAPDH has been shown recently to bind to certain tRNAs and eucaryotic mRNAs (35, 36). It has been reported that GAPDH binds yeast tRNA\textsuperscript{Sec} with an apparent dissociation constant (K\textsubscript{D}) of approximately 1.8 × 10\textsuperscript{−8} M and other tRNAs with high affinity and has been suggested to participate in the export of tRNAs from the nucleus (35). Although we have not measured the K\textsubscript{D} of GAPDH for different segments of the HAV 5′NTR, the effects of GAPDH on HAV IRES function are similar to those of GAPDH on the yeast tRNA\textsuperscript{Sec} tRNA. Our finding that GAPDH affects the binding of p39 to stem-loop IIIa RNA is unlikely to be due to a reduction in RNA synthesis (8, 12). Thus, several independent lines of evidence indicate that stem-loop IIIa plays an important role in the initiation of HAV translation by internal entry.

Competitive binding experiments confirmed that GAPDH (p39) binds to multiple sites within the HAV 5′NTR. GAPDH was found to bind RNA probes representing nt 1–148, 155–235, and 596–746 but with greatest affinity to the nt 1–148 (which contains the pY1 pyrimidine-rich tract) and nt 155–235 (stem-loop IIIa) RNAs (Fig. 7). In previous UV cross-linking/label transfer studies, the 39-kDa protein present in BS-C-1 cell extracts was found to have similar binding specificities, which strongly suggests that our purified p39 is identical to this previously identified protein (19). GAPDH is thus similar to PBV in its interaction with the 5′NTR, because PBV also binds to multiple sites within the 5′NTRs of HAV and other picornaviruses (18–22). We previously found that p39 binds specifically to RNA probes representing the IRES of encephalomyocarditis virus, a picornavirus, as well as the 5′NTR of hepatitis C virus, a member of the Flaviviridae that also translates its polyprotein by an IRES-directed mechanism (19, 48, 49). In addition, in preliminary studies we have found that both simian GAPDH and human GAPDH interact with the poliovirus 5′ cloverleaf in a UV cross-linking/label transfer assay.3 These data raise the possibility that GAPDH may be identical to a 36-kDa cellular protein of HeLa cells that has been implicated in promoting the binding of the poliovirus 3CD\textsuperscript{pro} protein to the 5′ end of the viral plus-strand RNA, in a step that is critically important for replication of the viral RNA (50). The affinity of GAPDH for the 5′NTRs of several different positive-strand RNA viruses suggests that it might contribute functionally to the replication of these viruses by one or more specific mechanisms.

Although we have not determined whether GAPDH plays a role in the replication of HAV, the ability of GAPDH to decrease the melting temperature of poly(A-U) (39) suggested that it might function to destabilize RNA structural elements that regulate translation initiation or RNA replication. In support of this hypothesis, we found that GAPDH specifically reduces the intensity of the circular dichroism spectra of stem-loop IIIa RNA (Fig. 9B), indicating that GAPDH binding favors the single-stranded form of this RNA over its predicted stem-loop conformation (Fig. 1). Because the functional activities of IRESs are strongly dependent upon their secondary RNA

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3 D. E. Schultz, and S. M. Lemon, unpublished results.
structure (51), it is tempting to speculate that the destabilization of stem-loop IIa structure by GAPDH would adversely influence internal initiation of translation directed by the HAV IRES. However, different mechanisms can be envisioned whereby GAPDH could have a positive or negative impact on translation, depending upon whether the initial contact site of the 40 S ribosome subunit translates RNA. Further experiments will be required to test these possibilities.

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