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Arginine-Rich Motifs Are Not Required for Hepatitis Delta Virus RNA Binding Activity of the Hepatitis Delta Antigen

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Hepatitis delta virus (HDV) replication and packaging require interactions between the unbranched rodlike structure of HDV RNA and hepatitis delta antigen (HDAg), a basic, disordered, oligomeric protein. The tendency of the protein to bind nonspecifically to nucleic acids has impeded analysis of HDV RNA protein complexes and conclusive determination of the regions of HDAg involved in RNA binding. The most widely cited model suggests that RNA binding involves two proposed arginine-rich motifs (ARMs I and II) in the middle of HDAg. However, other studies have questioned the roles of the ARMs. Here, binding activity was analyzed *in vitro* using HDAg-160, a C-terminal truncation that binds with high affinity and specificity to HDV RNA segments *in vitro*. Mutation of the core arginines of ARM I or ARM II in HDAg-160 did not diminish binding to HDV unbranched rodlike RNA. These same mutations did not abolish the ability of full-length HDAg to inhibit HDV RNA editing in cells, an activity that involves RNA binding. Moreover, only the N-terminal region of the protein, which does not contain the ARMs, was cross-linked to a bound HDV RNA segment *in vitro*. These results indicate that the amino-terminal region of HDAg is in close contact with the RNA and that the proposed ARMs are not required for binding HDV RNA. Binding was not reduced by mutation of additional clusters of basic amino acids. This result is consistent with an RNA-protein complex that is formed via numerous contacts between the RNA and each HDAg monomer.

Hepatitis delta virus (HDV) is a unique human pathogen that increases the severity of liver disease in those infected with its helper virus, hepatitis B virus (HBV). The ~1,680-nucleotide (nt) single-stranded circular RNA genome assumes a characteristic unbranched rodlike structure in which ~70% of the nucleotides form Watson-Crick base pairs (1, 2). Genome replication occurs via a double-rolling-circle mechanism in which host RNA polymerase is redirected to synthesize genomic and antigenomic HDV RNAs, as well as the mRNA for hepatitis delta antigen (HDAg), the sole viral protein (reviewed in references 3 and 4). HDAg forms RNA-protein complexes (RNPs) with both genomic and antigenomic HDV RNAs, and these complexes play essential roles in this unique replication process (5–11). HDAg has been shown to transport HDV RNA to the nucleus, where replication occurs (12). In addition, HDAg interacts with RNA polymerase II (Pol II) (13, 14); this interaction may recruit the polymerase to bound HDV RNA and has been proposed to effect changes in the polymerase that permit RNA-directed transcription (14–17). RNPs formed by HDAg and HDV RNA are also packaged into virions (18, 19); these complexes include a modified form of HDAg that interferes with RNA synthesis (20–24). Understanding how HDV RNPs function depends on knowing their structural features, which remains a critical goal.

Several studies have attempted to identify regions of HDAg that directly contribute to binding HDV RNA and forming HDV RNPs, but a consistent picture has not yet emerged. In a widely cited model, two arginine-rich motifs (ARM I and ARM II) in the middle region of the protein are thought to form a bipartite RNA binding domain (8). This model is based largely on two *in vitro* binding studies using bacterially expressed HDAg fusion proteins. One of these studies showed that only fusion proteins containing the middle region of the protein, which includes the ARMs, could bind HDV RNA (25); no RNA binding activity was associated with the N-terminal 78 amino acids (aa). The other study showed loss of *in vitro* RNA binding activity when either of the ARMs, particularly ARM I, was disrupted by replacement of two basic residues with other amino acids (8). Both reports relied heavily on RNA-protein blots (Northwestern blots), which depend on the ability to remove bound detergent and properly refold proteins following electrophoresis and blotting. In contrast, other *in vitro* studies implicated the amino-terminal domain of HDAg in binding HDV RNA (26–28). However, these analyses were limited by the use of small segments of the protein; Poisson et al. used small peptide fragments (28), and Huang and Wu and Wang et al. employed an N-terminal 88-aa region that bound equally well to HDV and non-HDV RNAs (26, 27).

Analyses of HDAg-HDV RNA interactions in cells have also yielded conflicting interpretations. Wang et al. (11) found that mutation of ARM I severely diminished the ability of HDAg to package the RNA into secreted particles, a result that could suggest a role for ARM I in RNA binding. Consistent with that result, Chang et al. (18) observed that a large deletion (aa 89 to 163) from the middle region of HDAg eliminated packaging and RNP formation in cells. On the other hand, in an analysis of the ability of truncated and mutated forms of HDAg to transport HDV RNA to the nucleus of permeabilized cells, Chou et al. (12) showed that both ARMs are not required for nuclear import, which likely requires RNA binding. Moreover, these authors also observed that deletion of the N-terminal 27 amino acids in a truncated HDAg fusion protein eliminated nuclear import of the RNA and attrib-

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uted to this region a "cryptic" binding activity because it was revealed only when the C-terminal half of the protein was removed (12). Thus, although numerous reviews (see, e.g., references 29, 30, 31, and 32) suggest that ARM I and ARM II form the primary RNA binding motif of HDAg, the characterization of the HDAg regions in contact with the RNA remains incomplete.

Dissection of the structural features of HDV RNPs has been limited by the lack of robust experimental systems in which the full-length native protein binds specifically to HDV unbranched rodlike RNA to form discrete complexes. All of the *in vitro* experiments described above used HDAg preparations that were either severely truncated, had large fusion partners, or required refolding of the protein following denaturing procedures. Binding was found to be nonspecific, or specificity was not determined. More-recent analyses of full-length soluble HDAg binding to nucleic acids by electrophoretic mobility shift assay (EMSA) found that the 195-amino-acid protein did not bind specifically to HDV RNA (33, 34). Defenbaugh et al. (33) found that bacterially expressed HDAg, which was full length and contained a six-histidine N-terminal tag, bound both HDV and an unrelated RNA with similar low nanomolar affinities; Alves et al. (34) observed that a similar HDAg preparation, but without the His tag, bound not only non-HDV RNAs but DNA as well. In both studies, very large complexes, perhaps aggregates, formed as the protein concentration was increased.

The difficulties described above in analyzing the binding of this highly basic protein to RNA may have contributed to the lack of a clear consensus regarding the protein sequences involved in binding HDV RNA. Several factors likely contribute to nonspecific nucleic acid binding by HDAg. The protein includes 26 arginines and 22 lysines and has a predicted net charge of about +13 (http://pepcalc.com/protein-calculator.php), which could make large electrostatic contributions to binding nucleic acids. The effects of this high positive charge on binding could be augmented by the ability of HDAg to form stable oligomeric complexes comprising about 8 HDAg monomers (34–37). Finally, HDAg can be highly disordered (34); perhaps, in some protein conformations, binding is dominated by electrostatic interactions that are nonspecific.

We recently developed an *in vitro* binding assay using a C-terminally truncated 160-aa form of HDAg, termed HDAg-160, that is expressed in bacteria and purified via an N-terminal histidine tag (33, 37). HDAg-160 binds specifically to HDV unbranched rodlike RNA segments of at least about 300 nt to form discrete, nuclease-resistant complexes (33). Unlike full-length 195-aa HDAg, it forms no complexes with unrelated RNA or with fully double-stranded RNA (dsRNA) assembled from HDV RNA segments (33, 37). It is not yet clear why removal of the C-terminal 35 aa improves binding specificity of the protein. This region has been implicated in binding Pol II (14); it includes just 2 conserved basic amino acids, has a predicted net charge of zero, and is not thought to be directly involved in RNA binding. Perhaps disorder of aa 161 to 195, which has been predicted (34), affects RNA binding indirectly.

RNPs formed by the HDAg-160 oligomer with ~400-nt segments of the HDV unbranched rodlike RNA serve as a useful, simplified model for the analysis of the basic element of the HDV RNP *in vitro*. Not only is binding specific for the HDV unbranched rodlike RNA, it is remarkably length specific as well: RNAs shorter than about 300 nt are not bound (33). This length requirement, which is related to the ability of the protein to form oligomers prior to binding (37), recapitulates the length requirement observed for RNA packaging (18) and RNA stabilization by HDAg in transfected cells (33). Whereas a 395-nt HDV RNA segment is bound by a single HDAg oligomer, RNAs longer than about 700 nt are bound by two or more oligomers (37). Assuming that each oligomer consists of 8 HDAg monomers (34–36), these results suggest that 40 HDAg monomers or more are associated with the full-length RNA, which is in the lower range of estimates obtained from biophysical (38) and comparative quantitation of HDV RNA and HDAg in virions (39).

Here, we reinvestigate the regions of HDAg necessary for binding HDV RNA and forming RNP complexes using wild-type (wt) and site-directed mutant forms of HDAg-160 *in vitro* and site-directed mutant forms of full-length HDAg in cells. Our results question the role of the previously proposed bipartite RNA binding element containing the two ARMs in forming complexes with HDV RNA and suggest a dominant role for the N-terminal region of HDAg in contacting HDV RNA. That binding was not reduced by mutation of clusters of basic amino acids is consistent with an RNA-protein complex that is formed via numerous contacts between the RNA and each HDAg monomer.

MATERIALS AND METHODS

Plasmids. Mutations were generated in the plasmid pET-H_6HDAg-160 (33) by site-directed mutagenesis (QuikChange II; Agilent Technologies, Santa Clara, CA). HDAgN78 and HDAgM79-160, containing HDAg amino acids 1 to 78 and 79 to 160, respectively, with an N-terminal 6×His tag, were generated by site-directed mutagenesis of pET-H_6HDAg-160. All mutations were verified by DNA sequencing. The nonreplicating antigenomic HDV RNA expression plasmid pHDVΔx1-NR, the HDAg expression plasmid pCMV-AgS, and the HDAg mRNA frameshift expression plasmid pCMV-AgS(fs) were previously described (40). Plasmids containing coding sequences for HDAg mutARM I (pCMV-AgS-mutARM I, mutARM I) and mutARM II (pCMV-AgS-mutARM II, mutARM II) were created via site-directed mutagenesis of the pCMV-AgS construct. Mutations were confirmed by DNA sequence analysis.

Cell transfection. Human Huh7 hepatoma cells were cultured as described previously (41) and transiently transfected with pHDVΔx1-NR and either pCMV-AgS(fs) or mutant or wild-type HDAg-S cDNA vectors using FuGENE 6 (Roche). Transfections were performed by following the manufacturer's recommendations. Briefly, 3.5 μl FuGENE was mixed with a total of 1.6 μg plasmid DNA in 50 μl serum-free Dulbecco's modified Eagle's medium prior to addition to Huh-7 cells at 80% confluence in 12-well plates. Amounts of pHDVΔx1-NR were approximately 10-fold higher than amounts of HDAg expression constructs, which were varied to obtain similar levels of protein expression as determined by immunoblotting. RNA and protein were harvested using the RNA/Protein kit (Macherey-Nagel), sodium dodecyl sulfate (SDS) cell lysis buffer (2% SDS, 0.2 M Tris-HCl [pH 9.5], 1 mM EDTA) or the Nucleospin RNA II kit (Macherey-Nagel).

Analysis of HDV RNA editing. RNA editing assays were performed as described previously (40), with minor modifications. Briefly, samples were treated with TURBO DNase (Ambion) to digest residual plasmid DNA and subjected to reverse transcription-PCR (RT-PCR) with primers 7646 and 7647 (40); these primers are specific for the nonreplicating RNA derived from pHDVΔx1-NR and do not amplify cDNA products derived from the HDAg expression plasmids (40). Successful DNase treatment and lack of contaminants were confirmed by the absence of PCR products in control samples treated with DNase but no reverse transcriptase prior to PCR. PCR products were analyzed for RNA editing via ^{32}P labeling and restriction digestion with StyI (40, –44), which digests only PCR products derived from edited RNAs. Digested amplification products were electrophoresed on 6% polyacrylamide gels in 0.5× Tris-borate-EDTA and de-

tected using a Molecular Dynamics Storm 840 PhosphorImager. Percent editing was determined on the basis of the fraction of StyI digestion products in each lane. Percent inhibition was determined by using the following formula: 100 × (1 − percent edited with HDAg/percent edited without HDAg).

Immunoblot analysis. Immunoblot detection of HDAg was as described previously (40, 44). Briefly, protein samples were separated via SDS-PAGE in NuPAGE 12% Bis-Tris gels. Proteins were transferred to nitrocellulose membranes, incubated 1 h in blocking milk, and then incubated overnight in a solution of human anti-HDAg T1/39 monoclonal primary antibody (1:1,000) (45). After several washes, the membrane was incubated in a solution of horseradish peroxidase (HRP)-conjugated anti-human IgG secondary antibody (KPL; 1:5,000) for 1 h. The membrane was washed and incubated either with ECL Plus, followed by detection on a Molecular Dynamics Storm 840 PhosphorImager or with fluorescent IRDye 800CW rabbit HRP-conjugated tertiary antibody (Li-Cor Biosciences) for 1 h, followed by detection using the Odyssey infrared imaging system (Li-Cor Biosciences).

Bacterial expression and purification of HDAg. His_6-tagged HDAg-160 and HDAg-145 were described previously (33, 37). The histidine tag is predicted to have a charge of less than +0.5 under the RNA binding conditions used (pH 7.5) and is not required for RNA binding because FLAG-tagged HDAg-160 exhibits identical binding behavior (B. L. Griffin and J. L. Casey, unpublished data). Full-length HDAg with an N-terminal histidine tag fully supports HDV RNA replication (13). Expression and purification of proteins were as described previously (33). Briefly, expression was performed in Rosetta(DE3) pLysS cells (Novagen) induced with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) and incubated overnight with shaking at 20°C. Cells were lysed on ice by sonication, and proteins were purified using the AKTApurifier system. Binding conditions on HisTrap FF Ni^{2+} columns were 0.5 M NaCl, 20 mM Tris (pH 7.5), 20 mM imidazole, and 25% glycerol. Bound HDAg was eluted in 0.5 M NaCl, 20 mM Tris (pH 7.5), 0.25 M imidazole, and 25% glycerol. Peak elution fractions were dialyzed in buffer containing 25% glycerol, 100 mM NaCl, 10 mM Tris (pH 7.0), 1 mM EDTA, and 0.1% Brij. Proteins were analyzed by SDS-PAGE and quantified by measuring UV absorbance at 205, 260, 280, and 320 nm in comparison to a bovine serum albumin (BSA) standard. Mutant proteins were prepared at least twice from different plasmid preparations.

RNA preparation. 395L RNA was transcribed and prepared as described previously (33). dsRNA was prepared by *in vitro* transcription and annealing of two complementary 154-nt segments of HDV RNA derived from one-half of the minimal-length 311-nt RNA that binds HDAg-160 (33) and is approximately the same length as this RNA. PCR templates for the dsRNA segments were amplified to transcribe nt 1484 to 1638 of the genome and the same segment from the antigenome. These two segments were annealed by heating to 90°C followed by slow cooling to generate a 154-bp dsRNA, which was then purified by nondenaturing polyacrylamide gel electrophoresis.

Electrophoretic mobility shift assays (EMSAs). Radiolabeled RNA (10 pM) was incubated with protein in a 20-μl mixture, including 10 mM Tris-HCl (pH 7.5), 80 mM NaCl, 5% glycerol, and 0.5 U/μl RNase inhibitor (Applied Biosystems). Protein concentrations were as indicated in the figure legends. Reaction mixtures were assembled on ice, incubated at 37°C for 30 m, and then electrophoresed on 6% native polyacrylamide gels in 0.5× Tris-borate-EDTA at 250 V for 2 h at room temperature. Results were visualized using a Molecular Dynamics Storm 840 PhosphorImager. Binding was calculated as the intensity of unbound RNA relative to the intensity of the entire lane minus the background. Dissociation constants (K_d) were determined by nonlinear regression analysis (GraphPad Prism).

UV cross-linking and CNBr cleavage. Cross-linking to RNA containing 4-thiouridine and CNBr digestion were based on the procedure of Amir-Ahmady et al. (46). Briefly, HDAg-160 and HDAg-145 proteins were incubated with 395L RNA, as above, except that the RNA was synthesized with a 1:10 ratio of 4-thiouridine triphosphate (TriLink, San Diego, CA) to UTP and the binding reaction mixture contained no RNase inhibitor. After binding at 37°C, samples were placed on ice and illuminated with 360-nm light for 15 min. Samples were digested with RNase A at 37°C, then electrophoresed on SDS-NuPage gels. Bands were localized by radioanalytic imaging and then excised. After elution overnight, protein samples were incubated overnight with CNBr in 70% formic acid and dried under vacuum. Proteins were separated on an SDS-NuPage gel, and transferred to nitrocellulose prior to imaging and immunoblotting.

RESULTS

Mutation of ARM I or ARM II does not decrease RNA binding by HDAg-160 *in vitro*. Lee et al. (8) assessed the roles of the ARMs in binding HDV RNA by creating three mutated forms of HDAg that were expressed as fusion proteins in bacteria. Each of the mutated proteins consisted of substitutions of two arginines in the cores of either ARM I (RRRK) or ARM II (RRERR). HDV RNA binding activity was eliminated by mutation of two of the three arginines in the core of ARM I (RQGK; mutations underlined). Mutation of the first two arginines in ARM II (NGERR) disrupted *in vitro* binding activity more than mutation of the second two arginines (RRESG), which produced about a 2- to 3-fold decrease in binding; neither of the ARM II mutations eliminated binding (8). In order to more substantially abolish potential ARM function and minimize potential effects on the overall secondary structure of the protein, we created mutated forms of HDAg-160 in which alanines were substituted for either all three arginines in the core of ARM I (mutARM I, AAAK) or all four arginines in ARM II (mutARM II, AAEAA) (Fig. 1A). RNA binding activities of wild-type and mutated forms of HDAg-160 were assessed by electrophoresis in native polyacrylamide gels following incubation at 37°C. For specific binding, we used 395L, a 395-nt RNA derived from the HDV antigenome (33). This RNA binds a single HDAg oligomer (33, 37) and provides a means to analyze RNA-protein interactions in the absence of additional possible interactions between HDAg oligomers that might occur on longer HDV RNAs. HDAg-160 binds this RNA with the same initial K_d as that of full-length HDV RNA, although the longer RNA binds more oligomers (not shown).

Both mutARM I and mutARM II HDAg-160 bound 395L RNA with affinities indistinguishable from that for wild-type HDAg-160 (Fig. 1). The RNA-protein complexes formed by these mutants are likely similar to that formed by the wild-type protein, because the discrete complexes formed by both mutants migrated only about 10% slower in the gel than that formed by the wild-type protein (Fig. 1B, top). At protein concentrations above ~0.8 nM, both mutants formed additional complexes with even lower mobilities, whereas wild-type HDAg-160 did not. This difference could indicate that additional protein-protein or RNA-protein interactions occurred at higher concentrations of the mutant proteins. Binding curves generated by plotting the fraction of total RNA bound against HDAg concentration were indistinguishable for wt, mutARM I, and mutARM II HDAg-160 proteins, indicating nearly identical RNA-binding affinities (Fig. 1C). Similarly, the dissociation constants (K_d) for these three proteins were also indistinguishable (0.6 ± 0.1 nM, 0.6 ± 0.1 nM, and 0.5 ± 0.1 nM for wt, mutARM I, and mutARM II, respectively). We conclude from these results that ARM I and ARM II are not required for HDAg-160 binding of HDV unbranched rodlike RNA.

As mentioned above, HDAg is highly basic. Although the full-length 195-aa protein binds RNA with little specificity, the RNA

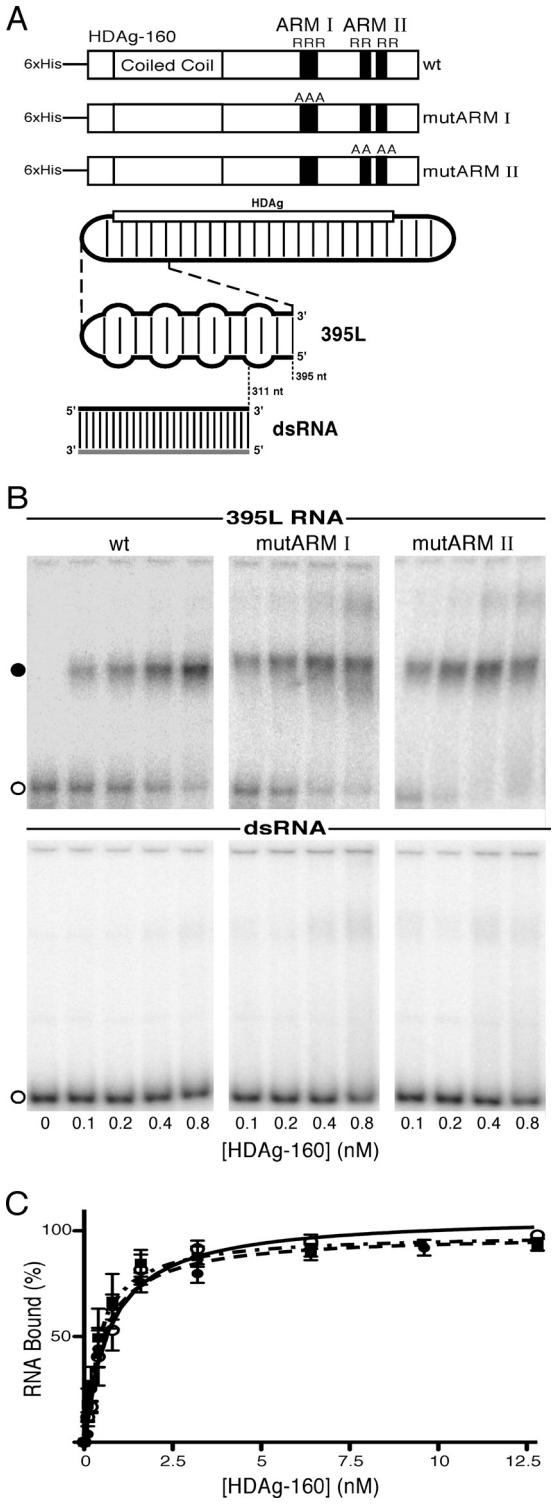

FIG 1 mutARM I HDAg-160 and mutARM II HDAg-160 bind HDV RNA with affinities indistinguishable from that for wt HDAg-160 *in vitro*. (A, top) Schematic of wild-type (wt) and mutant HDAg-160. The two previously proposed arginine-rich motifs (ARM I and ARM II) implicated in binding HDV RNA (8) are shaded black; the core arginines of these motifs are shown above. The coiled-coil domain involved in dimerization and oligomerization (20, 35) is also indicated. Site-directed mutations were made by substituting alanine for the 3 core arginines in ARM I or for the 4 core arginines in ARM II to generate mutARM I and mutARM II, respectively. (Bottom) The ~1,680-nt HDV RNA genome is shown as a rounded rectangle with the HDAg open reading frame indicated as an open rectangle. Vertical lines denote 70% base pairing of the unbranched rodlike structure. The 395-nt unbranched rodlike segment 395L is depicted at an expanded scale; bulges in the horizontal lines loosely represent internal loops and bulges. The 154-bp (308-nt) dsRNA used to assess nonspecific RNA binding is shown with more-closely spaced vertical lines to indicate 100% base pairing. This RNA is derived from a 311-nt RNA, 311L, which is the previously identified minimal-length RNA that binds HDAg-160 (33). (B) Electrophoresis of 395L RNA (top) and dsRNA (bottom) after incubation with different concentrations of wt, mutARM I, or mutARM II HDAg-160. RNAs (6 to 10 pM) were incubated with the indicated concentrations of HDAg proteins for 30 min at 37°C prior to electrophoresis in native 6% polyacrylamide gels for 2 h. Free (open circles) and bound (solid circle) RNAs are indicated. (C) Binding curves showing affinity of HDAg-160 proteins for HDV RNA. Solid line, open circles, wt; dashed line, filled circles, mutARM I HDAg-160; dot/dash line, squares, mutARM II HDAg-160. Lines are nonlinear regression fits for single-site binding (GraphPad Prism). Apparent dissociation constants from these fits are 0.6 ± 0.1 nM for wt HDAg-160, 0.6 ± 0.1 nM for mutARM I HDAg-160, and 0.5 ± 0.1 nM for mutARM II HDAg-160. Data are from five independent experiments for each HDAg-160 protein; error bars indicate standard deviations.

TABLE 1 Binding of wild-type and mutated forms of HDAg-160 to 395L RNA and dsRNA

HDAg construct	Residues mutated[a]	K_d (nM)	
		395L RNA	dsRNA
wt		0.6 ± 0.1	NB>[b]
mutARM I	R103, R104, R105	0.6 ± 0.1	4.8 ± 0.6
mutARM II	R139, R140, R142, R143	0.5 ± 0.1	NB
I	R3, R7, R10, R13	0.9 ± 0.2	10.0 ± 2.5
II	K25, K26	0.1 ± 0.03	6.4 ± 0.6
III	R32, R35, K36	0.3 ± 0.1	NB
IV	K38, K39, K40	0.2 ± 0.1	19.6 ± 0.4
V	K42, K43	0.8 ± 0.2	21.6 ± 2.0
VI	K60, K61	0.4 ± 0.1	15.5 ± 0.9
VII	K72, R73	0.2 ± 0.05	15.4 ± 2.6

[a] All residues were changed to alanine.
[b] NB, no binding.

binding activity of wild-type HDAg-160 is specific for HDV unbranched rodlike RNAs (33, 37). In particular, HDAg-160 does not bind dsRNA, even at concentrations 1,000-fold higher than required for binding the unbranched rodlike RNA (37). At present, it is not clear what accounts for the different binding specificities of the 195-aa and 160-aa proteins, but it is possible that even a few amino acid changes in HDAg-160 could lead to increased nonspecific binding. In order to determine whether the binding of mutARM I HDAg-160 and mutARM II HDAg-160 is also specific for unbranched rodlike RNA, we examined the binding of these mutant proteins to a dsRNA that is derived from and approximately the same length as the minimal-length 311-nt RNA previously reported (33) (Fig. 1B, bottom). At protein concentrations below 1 nM, binding to dsRNA was very low to undetectable. Thus, it is clear that the abilities of mutARM I HDAg-160 and mutARM II HDAg-160 to bind HDV unbranched rodlike RNA are not due to nonspecific binding.

Binding of dsRNA to wt HDAg-160 and mutARM II HDAg-160 was barely detectable at protein concentrations up to 13 nM, and no K_d could be calculated (Table 1). However, at higher protein concentrations binding was observable for mutARM I HDAg-160 (Fig. 2). The binding of dsRNA to mutARM I HDAg-160 was clearly distinct from that of unbranched rodlike RNA 395L. First,

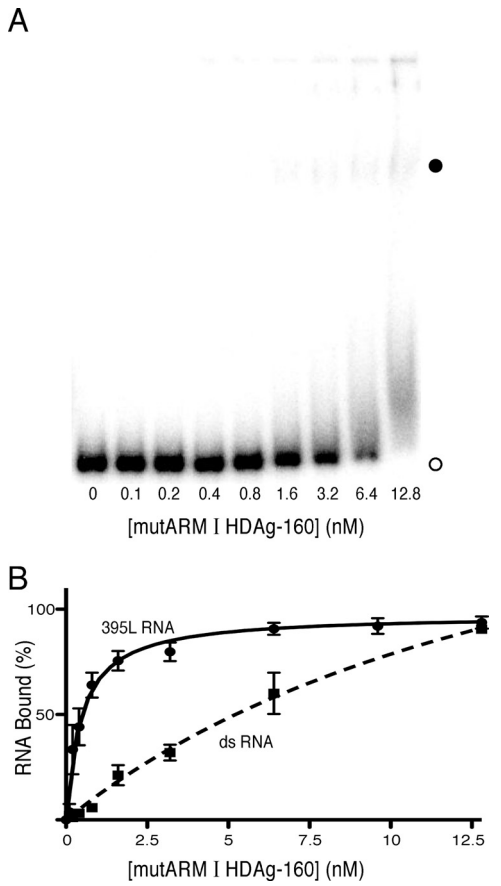

FIG 2 mutARM I HDAg-160 binds double-stranded RNA with low affinity. (A) Electrophoresis of dsRNA after incubation with increasing concentrations of mutARM I HDAg-160. The RNA (10 pM) was incubated with the indicated concentrations of HDAg proteins for 30 min at 37°C prior to electrophoresis in native 6% polyacrylamide gels for 2 h. Free (open circle) and complexed (solid circle) RNAs are indicated. (B) Binding curves showing affinity of mutARM I HDAg-160 for 395L and dsRNA. Solid line, 395L; dashed line, dsRNA. Lines are nonlinear regression fits for single-site binding (GraphPad Prism). Apparent dissociation constants from these fits are 0.6 ± 0.1 nM for mutARM I HDAg-160 binding to 395L and 4.8 ± 0.6 nM for mutARM I HDAg-160 binding to dsRNA. Error bars indicate standard deviations.

binding of dsRNA to mutARM I HDAg-160 appeared to produce a slowly migrating complex with mobility less than that exhibited by the longer 395L RNA, as well as more heterogeneous species, which could have arisen due to dissociation of complexes during electrophoresis (Fig. 2). Second, the dissociation constant was about 10-fold higher for binding dsRNA than for binding the unbranched rodlike RNA 395L (Table 1). Thus, although the mutation of ARM I decreased the specificity of RNA binding, the decreased specificity cannot account for the observed binding to unbranched rodlike RNA.

Because nonspecific binding is likely dominated by electrostatic interactions and these interactions are strongly affected by salt, we examined binding of both wt HDAg-160 and mutARM I HDAg-160 to the unbranched rodlike RNA 395L and to dsRNA at low (15 mM) and high (150 mM) salt concentrations. We observed that binding of mutARM I HDAg-160 to dsRNA was strongly affected by salt: the K_d for dsRNA binding by mutARM I HDAg-160 increased from 0.4 nM at 15 mM NaCl to 4.8 nM at 150 mM NaCl. dsRNA was not bound at either salt concentration by wt HDAg-160. The strong salt dependence of the binding of dsRNA by mutARM I HDAg-160 is consistent with binding dominated by electrostatic interactions that could be nonspecific. In contrast, the binding of mutARM I HDAg-160 to the unbranched rodlike RNA 395L was not affected by salt up to 150 mM, a result also observed for wt HDag-160. Thus, binding of both the wt and mutARM I HDAg-160 proteins to unbranched rodlike RNA is not dominated by electrostatic interactions. These results are consistent with the interpretation that mutation of ARM I increased nonspecific RNA binding but had no detectable effect on the ability of the protein to bind unbranched rodlike RNA.

Overall, these data demonstrate that ARM I and ARM II are not required for the binding of HDV unbranched rodlike RNA by HDAg-160 *in vitro*. These results appear to contradict the *in vitro* binding results of Lee et al. (8). As considered in the Discussion, the apparent discrepancy might be explained by the observation that mutation of ARM I increased nonspecific binding, even though this binding was weaker than binding to unbranched rodlike RNA at physiological salt concentration.

ARM I and ARM II are not required for inhibition of RNA editing by full-length HDAg in cells. In order to determine how the above results correlate with RNA binding by full-length HDAg in cells, we used the ability of HDAg to inhibit RNA editing at the HDV amber/W site as an indicator of RNA binding. For HDV genotype 1, the host enzyme ADAR1 edits the amber/W site in the context of the unbranched rodlike structure to produce the packaging form of HDAg (41, 42, 47, 48). Control of editing is a critical aspect of the virus replication cycle (44, 49), and for HDV genotype 1 part of this control occurs through inhibition of amber/W site editing by HDAg-S, the full-length 195-aa form of HDAg (40, 49, 50). This inhibition occurs by HDAg-S binding the unbranched rodlike structure of the RNA, which is required for editing; HDAg-S does not inhibit editing of sites that do not form this structure, such as the amber/W site in HDV genotype 3 RNA or the editing site in the human herpesvirus 8 K12 RNA (41, 44, 48–50).

Cells were cotransfected with an expression construct for a nonreplicating HDV genotype 1 RNA and either expression constructs for HDAg-S or a control expression construct in which the HDAg reading frame was eliminated by a frameshift at codon 7 (42, 43, 47). We note that HDAg-S is the full-length 195-aa form of HDAg with no histidine tag. In the absence of HDAg-S expression, 35% of the nonreplicating RNA was edited at the amber/W site by 3 days posttransfection, in line with previous reports (40, 44, 51). This level of editing is at least 10-fold higher than the level for replicating RNA within the same time posttransfection. As previously shown (40, 49), coexpression of wild-type HDAg-S leads to reduction of editing levels in the nonreplicating RNA by about 95% (Fig. 3). This inhibition is due to HDAg-S binding to HDV RNA. *In vitro*, inhibition of editing and RNA binding are very closely correlated for a genotype 1 RNA segment; however, HDAg-S does not inhibit editing of an RNA derived from the human herpesvirus 8 K12 RNA, which is edited by ADAR1 (R. Chen and J. L. Casey, unpublished data; 33, 52).

When expression constructs for HDAg-S containing the same ARM I or ARM II mutations used in the *in vitro* binding assays (Fig. 1) were coexpressed with the nonreplicating HDV RNA expression construct, we observed strong inhibition of editing (Fig. 3). Mutation of all 4 arginines in ARM II had no observable effect

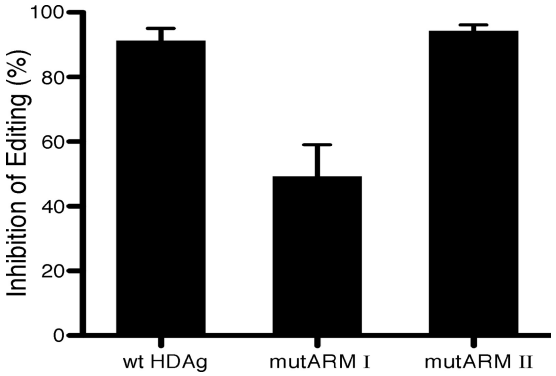

FIG 3 Mutation of HDAg ARM I or ARM II does not prevent HDAg inhibition of editing at the amber/W site in cells. Huh7 cells were cotransfected with plasmids expressing a nonreplicating HDV RNA expression construct and constructs expressing either no HDAg, or wild-type or mutant HDAg. RNAs were harvested 3 days posttransfection and analyzed for levels of editing at the amber/W site (40). In independent transfection experiments performed on different days, editing in the absence of HDAg was between 27 and 43% and was reduced to less than 2% in the presence of HDAg. Percent inhibition of editing was determined relative to levels of RNA editing in the absence of HDAg.

on the ability of HDAg-S to inhibit amber/W site editing. This result suggests no requirement for ARM II for binding to HDV RNA in cells, a conclusion in agreement with the *in vitro* binding result (Fig. 1). Mutation of the three arginines in the core of ARM I decreased inhibitory activity to about 50% (Fig. 3). However, substantial inhibitory activity remained, suggesting that ARM I is not absolutely required for RNA binding activity. Overall, our results suggest that neither ARM I nor ARM II is essential for binding HDV RNA in cells. This conclusion is consistent with the observations of Chou et al. (12), but contradicts the packaging studies of Wang et al. (11).

The N-terminal and middle regions of HDAg bind RNA nonspecifically. The results presented in Fig. 1 and 3 raise questions about the roles of ARM I and ARM II in binding HDV RNA, both *in vitro* and in cells. Although Lin et al. (25) and Lee et al. (8) concluded that RNA binding activity is limited to the middle region of HDAg, which contains these ARMs, other studies implicated the N-terminal region of the protein in binding HDV RNA. We attempted to determine whether specific binding could be attributed to either the N-terminal or middle region of HDAg using the *in vitro* binding assay with soluble, bacterially expressed, truncated forms of HDAg containing only the N-terminal domain (aa 1 to 78) or only the middle domain (aa 79 to 160) of HDAg. We observed that both of these proteins bound the unbranched rodlike RNA 395L (Fig. 4). The amino-terminal region bound the RNA more tightly than the middle region.

However, the connections between these binding activities and the specific binding exhibited by HDAg-160 are unclear. First, there were significant differences among the complexes formed by these protein segments and those formed by HDAg-160. Complexes formed with HDAgN78 were not discrete, and the magnitude of the mobility shift that occurred upon binding HDAgM79-160 was much smaller than the one that occurred upon binding HDAg-160 (Fig. 1 and 4). Because HDAgM79-160 lacks the coiled-coil domain, HDAgM79-160 binding to HDV RNA likely occurred as successive binding of monomers rather than preformed oligomers (37). Second, both protein fragments exhibited levels of nonspecific RNA binding that were indistinguishable from specific binding (Fig. 4). These results suggest that the structural integrity of the protein is important for binding specificity. Particularly considering the high positive charge of HDAg and the tendency of the protein to be structurally disordered, it seems likely that it will be difficult to identify regions of RNA binding by such large deletions.

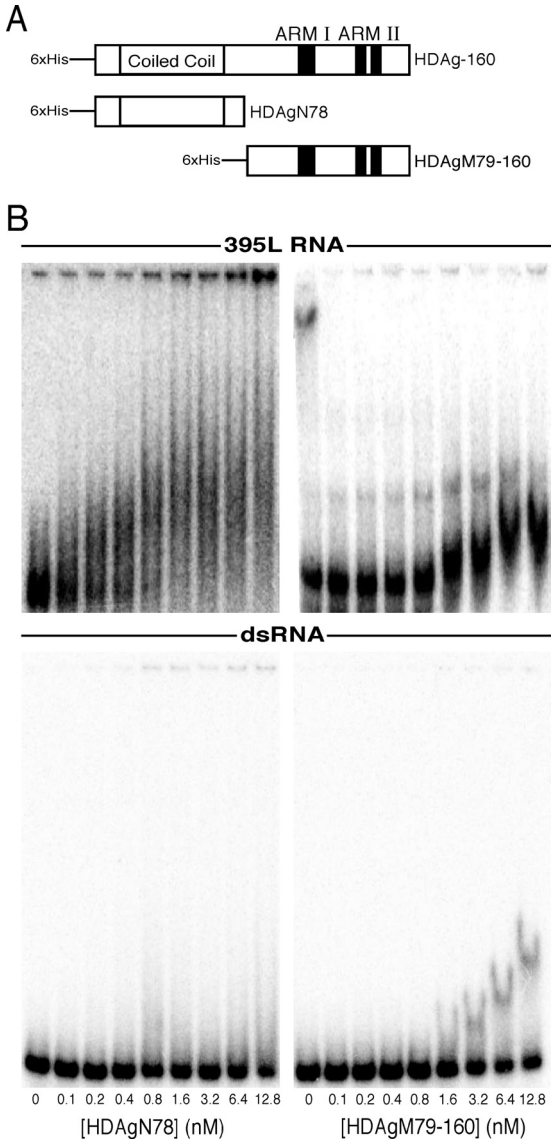

FIG 4 The N-terminal and middle domains of HDAg bind RNA nonspecifically. (A) Schematic of bacterially expressed, His-tagged wt and truncated HDAg proteins. (B) Electrophoresis of 395-nt unbranched rodlike HDV RNA segment 395L (top) and dsRNA (bottom) after incubation with different concentrations of HDAgN78 or HDAgM79-160. RNAs (10 pM) were incubated with indicated concentrations of proteins for 30 min at 37°C prior to electrophoresis in native 6% polyacrylamide gels for 2 h.

The N-terminal domain of HDAg-160 is cross-linked to HDV RNA *in vitro*. To ascertain the relative roles of the amino-terminal and middle regions of HDAg in RNA binding in a more native context, we employed cross-linking of the protein to radiolabeled RNA. Ryu et al. (38) previously showed that HDAg can be

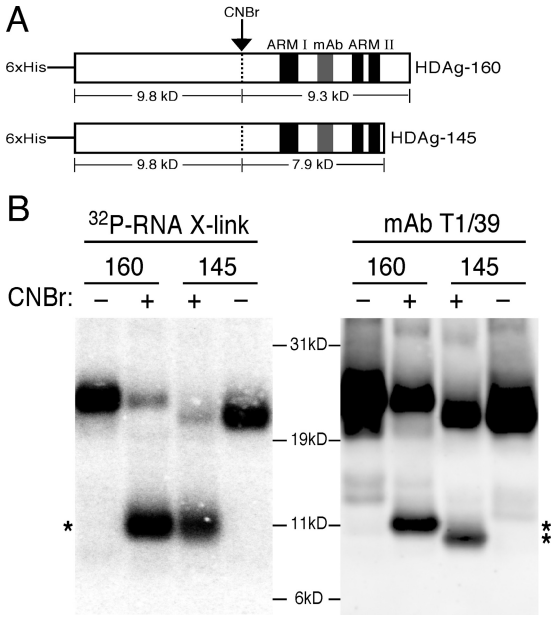

FIG 5 HDV RNA contacts the N terminus of HDAg. (A) Diagram of bacterially expressed His_6-tagged HDAg-160 and HDAg-145 (33, 37). The cyanogen bromide cleavage site at Met79 is indicated as a dashed line. The T1/39 monoclonal antibody (mAb) recognition epitope (45, 63) is shaded gray. The core arginines of ARM I and ARM II are indicated by black shading. (B) HDAg cross-linked to radiolabeled HDV RNA containing 4-thiouridine. Complexes formed by ^{32}P-labeled HDV RNA containing 4-thiouridine and HDAg-160 or HDAg-145 were cross-linked with long-wavelength UV, digested with RNase A, and separated by SDS-PAGE. After gel purification, a fraction of the cross-linked proteins was reacted with cyanogen bromide, which cleaves at the sole methionine residue in the HDAg sequence. Undigested (−) and digested (+) products were separated by SDS-PAGE and transferred to nitrocellulose. The blot was exposed to a phosphorimager screen (left) to detect ^{32}P-labeled proteins, then immunoblotted with monoclonal antibody T1/39 to detect the C-terminal digestion fragment; the latter was done on the same phosphorimager (right). All scaling and cropping manipulations of the two images were the same. The cross-linked undigested proteins migrate with clearly different mobilities; however, following CNBr digestion the cross-linked species had the same mobility (left, asterisk), even though the migrations of the C-terminal fragments were clearly distinguishable (right, asterisks).

FIG 6 Clusters of HDAg basic amino acids mutated to alanine. The sequence shown is amino acids 1 to 160 of HDAg. Roman numerals above the sequence indicate the 7 HDAg site-directed mutants in which clusters of basic amino acids in the amino-terminal region of the protein were mutated to alanine. mutARM I and mutARM II indicate the site-directed mutants in which the core arginines in ARM I and ARM II were mutated to alanine. Amino acids mutated are shown in boldface.

cross-linked to HDV RNA in virion RNPs. We therefore incubated radiolabeled 395L RNA containing 4-thiouridine with HDAg-160 or the more truncated HDAg-145, which binds unbranched rodlike RNA with the same affinity and specificity, and cross-linked the RNA and protein with long-wavelength UV irradiation (37). The use of 4-thiouridine allows cross-linking to be performed at less-damaging wavelengths and with high efficiency (53, 54). Moreover, photoactivated 4-thiouridine reacts rapidly and with less biased reactivity for different amino acids than unsubstituted RNA and is therefore considered to favor cross-linking to positions directly participating in binding (55). Both proteins were cross-linked to the RNA by UV irradiation (Fig. 5); the different mobilities observed during SDS-gel electrophoresis are related to the differences between their molecular weights.

After cross-linking, proteins were cleaved at the single internal methionine (aa 79) by cyanogen bromide (CNBr) in order to determine which regions were cross-linked. This cleavage generates identical N-terminal fragments but different C-terminal fragments for HDAg-145 and HDAg-160. As shown in Fig. 5, following CNBr treatment, the major cross-linked species from both HDAg-145 and HDAg-160 comigrated in an SDS-NuPAGE gel. The C-terminal CNBr cleavage fragments of these proteins have different sizes and were readily distinguished by electrophoresis followed by immunoblotting with a monoclonal antibody that recognizes an epitope located between aa 106 and 123 (Fig. 5). While the CNBr cleavage products of HDAg-160 are too close in size (9.8 kDa and 9.3 kDa for the N- and C-terminal fragments, respectively) to separate clearly on the gel, the size difference between the CNBr digestion fragments of HDAg-145 is greater. Indeed, the labeled CNBr cleavage product of HDAg-145 is clearly distinguished from the C-terminal fragment detected by the monoclonal antibody and migrates with faster mobility. We draw two conclusions from these observations: (i) the RNA is cross-linked to the amino-terminal region of HDAg; (ii) cross-linking to the C-terminal half of the protein, which includes the middle region containing the ARMs, was not detected. These results indicate that the RNA is in close contact with the N-terminal region of HDAg and suggest that these contacts dominate the RNA-protein interface.

Mutation of additional clusters of basic amino acids did not reduce RNA binding. The above results suggest not only that the ARMs do not act as primary RNA binding motifs for binding to HDV RNA but also that the amino-terminal domain of HDAg might be in closer contact with the RNA. In an attempt to identify amino acids that play important roles in this interaction, we created 7 additional site-directed mutated versions of HDAg-160 in which a total of 18 of the 23 basic amino acids in the N-terminal 78-aa region were changed to alanine (Fig. 6). Alanines were substituted for basic amino acids in 5 clusters of 2 or 3 basic amino acids that are more than 95% conserved among HDV genotype 1 isolates and for the 4 highly conserved arginines near the N terminus of the protein (28). We also substituted alanines for K25 and K26, which, according to a crystal structure, could be positioned to interact with bound RNA (35).

All of these mutated HDAg-160 proteins formed discrete complexes with 395L RNA that migrated with little or no variation in mobility or affinity compared with wild-type HDAg-160 (Table 1). The abilities of these mutated proteins to bind dsRNA were simultaneously assessed to determine whether the observed binding was specific for the unbranched rodlike RNA. Several of the mutated forms of HDAg-160 did bind dsRNA; however, in all cases the dissociation constant determined for dsRNA binding was more than 10-fold higher than that for binding the unbranched rodlike RNA (Table 1) and no discrete bands were observed. Thus, the high-affinity interactions with the unbranched

rodlike RNA were highly specific. Hence, although the amino-terminal region is in close contact with the unbranched rodlike RNA, no single cluster of basic amino acids, including the ARMs, plays a dominant role in binding the RNA.

DISCUSSION

The results presented here suggest a revision of the model for specific binding of HDV unbranched rodlike RNA by HDAg. Using a C-terminally truncated form of bacterially expressed HDAg as a model for *in vitro* binding, we found that the two previously described ARMs in the middle region of HDAg are dispensable for RNA binding. The conclusion that neither ARM is required for RNA binding is supported by the observation that full-length HDAg can inhibit site-specific editing of HDV RNA in cells even when the ARMs are heavily mutated. Finally, cross-linking followed by protein fragmentation indicated that only a 78-aa fragment from the N terminus of the protein, which does not contain the ARMs, was in direct contact with the RNA. Although our results regarding the ARMs appear to conflict with previous observations (8, 11), some of these discrepancies may be explained by our findings, as discussed below.

The ARM model for RNA binding by HDAg is based heavily on the *in vitro* results of Lee et al., who found that RNA binding by bacterially expressed protein was abolished by mutation of either ARM I or ARM II (8). The mutations used in the current study are more extensive than those employed by Lee et al.: 3, rather than 2, arginines replaced by alanine in ARM I and 4, rather than 2, basic residues replaced by alanine in ARM II. Yet, we observed that the affinity of the mutant HDAg-160 forms for an HDV unbranched rodlike RNA segment was indistinguishable from that of the wild-type protein. Moreover, the RNA and protein formed discrete complexes with mobilities similar to those for complexes formed by the wild type.

We suggest that the lack of binding observed by Lee et al. (8) for their HDAg ARM mutants was due to factors other than elimination of required RNA binding elements. For example, the Northwestern blot procedure used in that study required that the protein be properly refolded following SDS-PAGE; this process is not trivial (6), and no analysis of refolding was performed. We have shown that multimerization is critical for binding specificity (37), but there is no way of knowing in the previous study by Lee et al. (8) whether multimers were re-formed or whether the trpE fusion used for expression and purification affected multimerization. The mutations introduced could have affected the refolding process or even the overall structure of the protein. An additional possibility suggested by our findings is that an increase in nonspecific binding activity, such as occurs in some HDAg preparations and with certain mutations, could appear as a decrease in specific binding under the experimental conditions used by Lee et al. (8), which included a more-than-1,000-fold excess of nonspecific competitor RNA. Thus, an increase in nonspecific binding, such as we observed for the ARM I mutation, could have led to saturation of the protein by the nonspecific competitor and the apparent loss of specific RNA binding. Finally, we note that the protein concentrations used by Lee et al. (8) in mobility shift assays were at least about 50 μM. Lee et al. (8) did not perform titrations, but it is possible that the interactions between their fusion protein preparation and HDV RNA were much weaker than those analyzed in the current study. Although HDAg might eventually reach micromolar concentrations in cells (56), highly specific, high-affinity interactions between HDAg and HDV RNA are likely to be critical for establishing virus replication; whether additional low-affinity interactions occur and play important roles later in the replication cycle is unknown.

Our results also appear to contradict the observation that mutation of ARM I (the effect of mutating ARM II was not reported) abolished the ability of HDAg to incorporate HDV RNA into secreted virus-like particles (VLPs) (11). In contrast, we observed that a more extensive mutation of ARM I did not abolish another critical intracellular biological activity that depends on RNA binding, namely, the ability of HDAg to inhibit RNA editing of HDV genotype 1 RNA in cells (40, 49, 50). It is obvious that the analysis of binding in the intracellular environment is more complex than *in vitro* studies performed using purified components. An increase in nonspecific binding activity similar to what was observed for HDAg-160 *in vitro* and binding of the protein by cellular RNAs could result in a decrease in apparent specific binding activity. Such an effect might explain the partial reduction in the ability of HDAg to inhibit editing and could also have contributed to the reported abolishment of packaging activity. When considering the effects of HDAg mutations on RNA incorporation into VLPs, one must also consider the possibility that modifications of RNP structure could be important for packaging. Although the structural constraints for packaging HDV RNA are not known, it is clear that some constraints exist: packaging of greater-than-full-length RNAs is about 10-fold less efficient than that of genome-length RNAs (57). The slight shift in the mobility of complexes formed by mutARM I HDAg-160 and the unbranched rodlike RNA segment 395L suggests an alteration in the structure of the RNP that could affect packaging efficiency even though RNA binding is unaffected.

Other previous observations are consistent with our results. Chou et al. (12) found that ARM I and ARM II were not necessary for transport of HDV RNA to the nuclei of permeabilized cells, an activity dependent on RNA binding by HDAg. We note that the expressed fusion proteins used by Chou et al. were similar to HDAg-160, in that they were soluble and contained nearly the same length of amino acid sequence, while those in the Lin and Lee studies required solubilization from inclusion bodies. The observation (Fig. 5) that the N-terminal region of HDAg is in close contact with RNA bound in a discrete complex goes beyond previous observations that N-terminal fragments of HDAg can bind RNA (26–28). Our analysis of HDAg fragments from both the N-terminal and middle regions of the protein indicated that the nature of the complexes formed by these fragments was considerably different from RNPs formed by HDAg-160, particularly regarding the lack of specificity for unbranched rodlike HDV RNAs (Fig. 4).

Three properties of HDAg—high net positive charge, an oligomeric structure, and the high degree of disorder for more than half of the protein (34)—likely contribute to a tendency for nonspecific nucleic acid binding. This tendency, which varies according to the length of the protein synthesized and which can be affected by site-directed mutations (Table 1), has posed challenges to the identification of the regions of the protein involved in RNA binding. Although such nonspecific interactions might occur at high HDAg concentrations reached late in the replication cycle (56), the significance of these potential interactions is not known. For reasons that are not yet clear, full-length HDAg expressed in

bacteria exhibits considerable nonspecific binding, whereas HDAg-160 does not.

Overall, the results presented here suggest a dominant role for the N-terminal region of HDAg in interacting directly with HDV RNA. The failure to eliminate binding activity by mutation of highly conserved clusters of basic amino acids is consistent with distribution of binding activity over a large number of positions, likely involving both basic and nonbasic amino acids. This pattern of RNA recognition has been observed in the nucleoproteins of several negative-strand RNA viruses (58–61). The observation that the HDV RNP formed with a 311-nt RNA is protected against micrococcal nuclease (33) suggests extensive contacts between the RNA and the protein and is also consistent with a wide distribution of contacts. The cross-linking results suggest that these amino acids are concentrated in the N-terminal region but do not exclude the possibility that some RNA contacts are in the middle region. The results presented here indicate that the proposed ARMs are not likely to form direct contacts with the unbranched rodlike RNA and therefore might not function as typical RNA-binding ARMs (62). The observation that HDAg recognizes the RNA secondary structure but not the primary sequence, *per se* (Griffin and Casey, unpublished), suggests a type of interaction different from those involving most ARMs, which are generally sequence specific (62), and may be more consistent with numerous amino acid contacts in the binding of HDV RNA by HDAg.

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