JOURNAL OF VIROLOGY, Apr. 1993, p. 2221–2227
0022-538X/93/042221-07$02.00/0

Vol. 67, No. 4

RNA-Binding Activity of Hepatitis Delta Antigen Involves Two Arginine-Rich Motifs and Is Required for Hepatitis Delta Virus RNA Replication

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Received 31 July 1992/Accepted 20 January 1993

Hepatitis delta antigen (HDAg) is an RNA-binding protein with binding specificity for hepatitis delta virus (HDV) RNA (J. H. Lin, M. F. Chang, S. C. Baker, S. Govindarajan, and M. M. C. Lai, J. Virol. 64:4051–4058, 1990). By amino acid sequence homology search, we have identified within its RNA-binding domain two stretches of an arginine-rich motif (ARM), which is present in many prokaryotic and eukaryotic RNA-binding proteins. The first one is KERQDHRRRKA and the second is EDEKRERRIAG, and they are separated by 29 amino acids. Deletion of either one of these ARM sequences resulted in the total loss of the in vitro RNA-binding activity of HDAg. Thus, HDAg is different from other RNA-binding proteins in that it requires two ARM-like sequences for its RNA-binding activity. Replacement of the spacer sequence between the two ARMs with a shorter stretch of sequence also reduced RNA binding in vitro. Furthermore, site-specific mutations of the basic amino acid residues in both ARMs resulted in the total loss or reduction of RNA-binding activity. The biological significance of the RNA-binding activity was studied by examining the *trans*-activating activity of the RNA-binding mutants. The plasmids expressing HDAgs with various mutations in the RNA-binding motifs were cotransfected with a replication-defective HDV dimer cDNA construct into COS cells. It was found that all the HDAg mutants which had lost the in vitro RNA-binding activity also lost the ability to complement the defect of HDV RNA replication. We conclude that the *trans*-activating function of HDAg requires its binding to HDV RNA.

Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus and is an etiological agent of certain severe forms of viral hepatitis (32). HDV contains a single-stranded circular RNA genome of 1.7 kb in length (21, 30, 38), which has extensive intramolecular complementary sequences. Its RNA structure resembles that of viroids, virusoids, and plant satellite virus RNAs. HDV RNA has an autocatalytic cleavage and ligation activity (23, 35, 36, 40–42), and appears to replicate by a rolling-circle mechanism (10, 29). It has been shown that HDV RNA replication requires the presence of a virus-encoded protein, hepatitis delta antigen (HDAg) (22). However, the molecular mechanism by which HDAg participates in HDV RNA replication is not clear. HDAg is a phosphoprotein (6) and usually consists of two protein species of 24 kDa (small HDAg, 195 amino acids) and 27 kDa (large HDAg, 214 amino acids) (3, 39). These two proteins are identical in sequence, but the large HDAg contains 19 additional amino acids at the carboxyl terminus (39). The small HDAg is required for HDV RNA replication (22), while the large one inhibits RNA replication (7, 14) and is required for HDV assembly (5, 33). Both HDAgs are localized in the nuclei of HDV-infected cells (27, 44). We have previously shown that HDAg binds specifically to HDV RNA (26). This binding requires a specific RNA structure, rather than specific sequences, of HDV RNA (7, 26). The RNA-binding sites on HDAg have been mapped within the middle one-third region of HDAg (amino acids 79 to 163) (26). Both the large and small HDAg bind to HDV RNA at equal efficiency (20). However, the molecular basis and functional significance of RNA binding by HDAg are not clear.

Two classes of prokaryotic and eukaryotic RNA-binding proteins have previously been identified. One contains RNA recognition motif, which consists of 80 amino acids with two conserved sequences, RNP-1 and RNP-2, of 8 and 6 amino acids, respectively (1, 31). This class of RNA-binding proteins includes proteins which bind to pre-mRNA, mRNA, small nuclear RNA, and pre-rRNA and are involved in the regulation of translation, splicing, and a variety of other activities (1, 31). The other class contains an arginine-rich motif (ARM) with a core of 4 to 8 amino acids, mostly arginines (25). The ARM sequence itself is sufficient for specific RNA recognition. The *tat* and *rev* proteins of human immunodeficiency virus type 1 (HIV-1), the N protein of bacteriophage lambda, and the capsid proteins of some RNA viruses belong to this family (25). In this report, we have demonstrated that HDAg contains two ARM-like sequences, both of which are required for RNA binding. Alteration of either ARM sequence diminished the RNA-binding activity. Thus, HDAg represents a new class of RNA-binding protein. Furthermore, the RNA-binding activity of HDAg is required for its *trans*-activating activity for HDV RNA replication, suggesting that HDAg functions by binding to HDV RNA.

MATERIALS AND METHODS

Computer analysis of HDAg sequence. The HDAg sequence of the Southern California isolate of HDV (30) was used for sequence analysis using the BESTFIT program from the Genetics Computer Group (University of Wisconsin, Madison). Optimal alignment of sequences was made by

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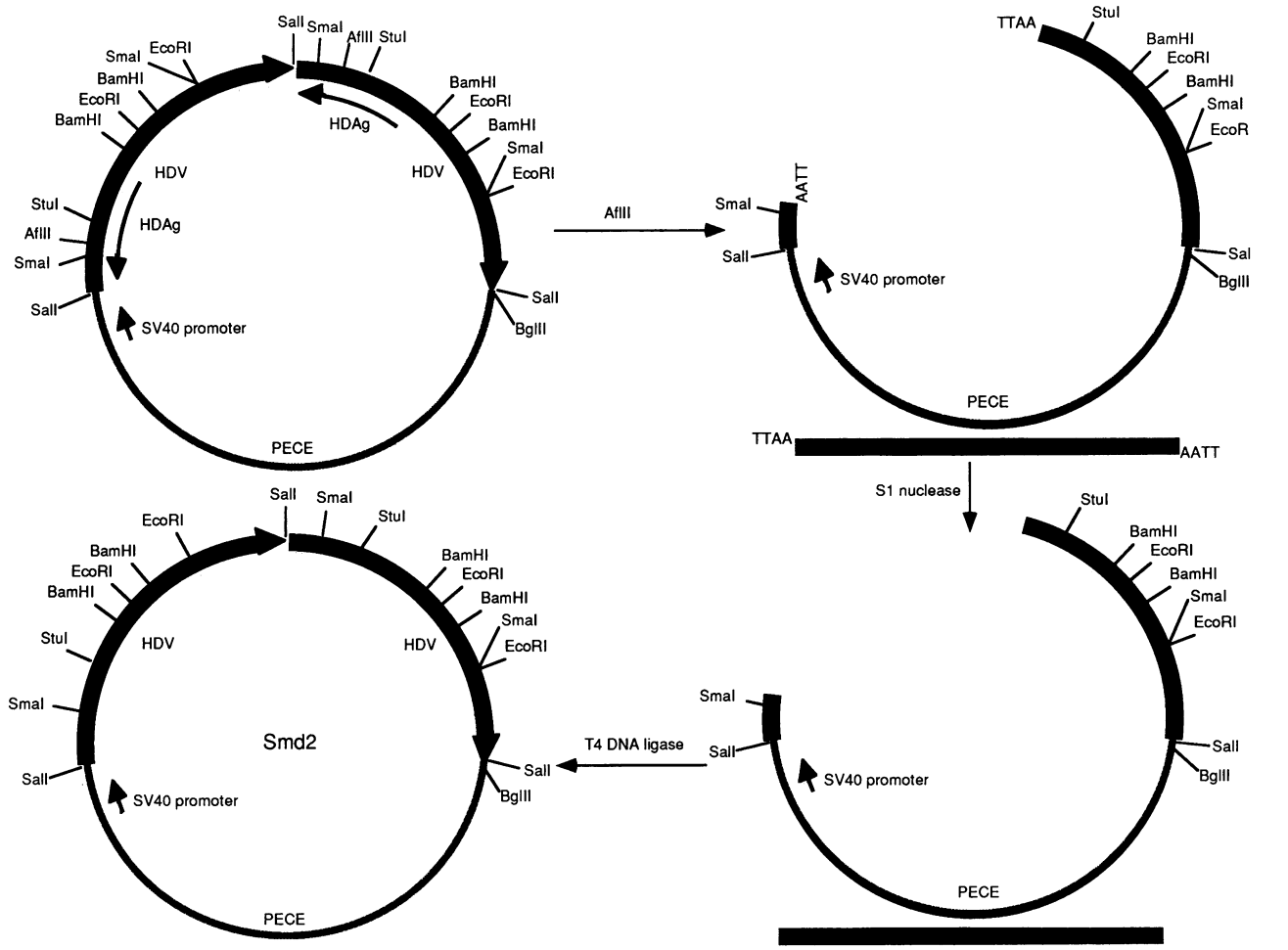

FIG. 1. Schematic diagram of the construction of HDV dimer mutant Smd2 and the relevant restriction enzyme sites. The thick arrow represents HDV monomer sequences of genomic sense. The thin arrow represents the HDAg-coding region, which is on the antigenomic orientation. The thin line represents PECE vector sequence (13).

using the local homology algorithm of Smith and Waterman (37).

Construction of plasmids. All of the constructs were made from the cDNA clones of Southern California isolate of HDV RNA (30). HDAg-truncated mutant plasmids L6 and L14 were derived from pATH-M (26), which encodes the middle one-third (amino acids 79 to 163) of HDAg fused to the TrpE protein of *Escherichia coli*, by the exonuclease digestion method developed by Henikoff (17). For construction of plasmids WD and L10, the full-length and the C-terminal two-thirds (amino acids 108 to 214) of the HDAg-coding region, respectively, were made by polymerase chain reaction to amplify HDAg sequences of the desired regions by using two oligonucleotide primers which contained the appropriate HDV sequences plus *Bam*HI and *Hin*dIII linker sequences. The cDNA products were then inserted into pATH-2 polycloning sites (12). For construction of plasmid D1-2, two cDNAs encoding amino acids 1 to 109 and 136 to 214 of HDAg and containing *Xba*I site at both ends were made by polymerase chain reaction and inserted into pATH-2. The HDAg-coding sequences in A1, A2, and A2′ were made by polymerase chain reaction using the method described by Higuchi et al. (18) and inserted into pATH-2 polycloning sites *Bam*HI and *Hin*dIII.

The replication-defective HDV dimer construct, Smd2, which was used for the testing of the *trans*-acting activity of the HDAg mutants, was constructed by cloning a head-to-tail dimer of full-length HDV cDNA (30) into the *Sal*I site of the PECE vector, which contains a simian virus 40 T-antigen promoter (13). The resulting plasmid was digested into two fragments with *Afl*II; both of the fragments were blunt ended with nuclease S1 and religated (Fig. 1). As a result, a deletion of 4 nucleotides and a frameshift were introduced at the site corresponding to amino acid 130 of the HDAg-coding region. This plasmid transcribed a replication-defective HDV dimer RNA, which could replicate only when a wild-type HDAg was supplied. PECE-Sm, which expresses a wild-type small HDAg, was made by inserting the *Eco*RI-*Bam*HI fragment of the HDV cDNA encoding the small HDAg into the *Eco*RI and *Bgl*II sites of the PECE vector. Other plasmids expressing various HDAg mutants, including PECE-A1, PECE-A2, PECE-A2′, and PECE-D1-2, were made by replacing the *Stu*I-*Sma*I fragment of PECE-Sm with the corresponding fragments from A1, A2, A2′, and D1-2, respectively. The sequences of the resulting plasmids were confirmed by dideoxyribonucleotide chain termination sequencing (34).

Preparation of TrpE-HDAg fusion proteins. Bacteria were grown by previously published procedures (26). Briefly,

cultures of *E. coli* MC1061 cells transformed with plasmids encoding different TrpE-HDAg mutant fusion proteins were induced with 3-indoleacrylic acid (40 μg/ml) for 3 h. Bacteria were lysed by digesting with lysozyme (1 mg/ml) and repeated freeze-thawing. The lysates were further digested with DNase I. The fusion proteins were collected in the insoluble fraction and resuspended in a buffer containing 0.01 M sodium phosphate (pH 7.2), 0.1% β-mercaptoethanol, 1% sodium dodecyl sulfate (SDS), and 6 M urea as previously described (26).

Immunoblot and RNA-protein blot procedures. TrpE-HDAg fusion proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide) and electrotransferred to nitrocellulose membranes. For immunoblot analysis, the membranes were first incubated with 5% nonfat milk and then with anti-HDAg antibody (26) followed by ^{125}I-labeled protein A according to the published procedures (9). For RNA-protein blot (Northwestern) analysis, the membranes were incubated in standard binding buffer (SBB; 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 50 mM NaCl, 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) containing 10 μg of yeast transfer RNA per ml and 100 μg of cellular RNA from DBT cells per ml for 1 h and then incubated with ^{32}P-labeled HDV genomic RNA in the same buffer for another hour according to the published procedures (26). After washing three times with SBB at room temperature for 15 min each, the membranes were exposed to X-ray films.

RNA mobility shift assay. RNA mobility shift assay was carried out as previously described (26). Briefly, 20 μg of partially purified TrpE-HDAg proteins was incubated with 10 ng of ^{32}P-labeled HDV genomic RNA in 10 μl of buffer containing 2.5 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol with 40 U of RNasin (Promega), 10 μg of tRNA, and 10 μg of total cellular RNA at 37°C for 30 min. After incubation, the samples were directly analyzed by electrophoresis under nondenaturing conditions. Electrophoresis was carried out on a 0.6% low-melting agarose gel at 25 V for 15 h at 4°C (26). After electrophoresis, the gel was dried and visualized by autoradiography.

DNA transfection. COS 7 cells (16) at 50% confluency in 6-cm petri dishes were incubated with 5 μg of PECE plasmids containing replication-defective HDV dimer DNA, plasmids encoding either the wild-type or mutant small HDAg, and 25 μg of Lipofectin (Bethesda Research Laboratories) for 12 h. The cells were washed with Dulbecco modified Eagle medium (DMEM) and then incubated with DMEM containing 5% fetal calf serum for another 5 days. The total cellular RNA was then extracted by acid guanidinium isothiocyanate (11) at 6 days posttransfection. The RNA was analyzed by Northern blot analysis using ^{32}P-labeled anti-genomic HDV RNA as a probe (43).

RESULTS

RNA-binding motifs of HDAg. To identify the amino acid residues responsible for the RNA binding of HDAg, we first searched for the presence of the known RNA-binding motifs in HDAg. Since the RNA-binding region is located within the middle one-third domain (amino acids 79 to 163) of HDAg (26), we examined whether this region contained an RNA recognition motif or an ARM. No RNA recognition motif was found in this region. However, two stretches of amino acid sequences have different degrees of similarity with a battery of the known ARMs (25). The first one was

KERqdhRRRKa	1st ARM of HDAg (aa 97-107)
eDeKRERRiag	2nd ARM of HDAg (aa 136-146)
qTRRRERRaEK	N of bacteriophage λ
RKKRRgRRRap	Tat of HIV-1
sQRRnrRRRwK	Rev of HIV-2
BOBRBJRRZZB	Consensus

FIG. 2. Conserved ARMs in representative RNA-binding proteins. Abbreviations: B, basic; O, nonbasic polar; Z, charged; and J, acidic amino acids; HIV, human immunodeficiency virus. Part of the data are from reference 25.

KERQDHRRRKA (from amino acids 97 to 107), and the second was EDEKRERRIAG (from amino acids 136 to 146) (Fig. 2). The presence of two stretches of ARM-like sequences in the same protein is unique among the known RNA-binding proteins.

RNA-protein-binding assays of HDAg deletion mutants. To determine whether either of the ARM-like sequences was involved in RNA binding, we made a series of deletion mutants starting from the C-terminal end of the middle domain (M) of HDAg (amino acid residues 89 to 163), which includes the necessary and sufficient RNA-binding sequences (26). These mutants were expressed in *E. coli* as TrpE fusion proteins. The partially purified fusion proteins were separated by SDS-PAGE, transferred to nitrocellulose filter membrane, and incubated with ^{32}P-labeled HDV genomic RNA by a Northwestern procedure, under conditions shown to allow specific binding between HDAg and HDV RNA (6, 26). Among these deletion mutants, L6 contains the longest HDAg sequence (amino acid residues 89 to 143) and was found to bind HDV RNA (Fig. 3B). Further truncation at the C-terminal end resulted in the complete loss of the RNA-binding activity. For examples, mutant L14 (amino acids 89 to 135) and smaller mutants did not bind HDV RNA (Fig. 3B and data not shown) when the same

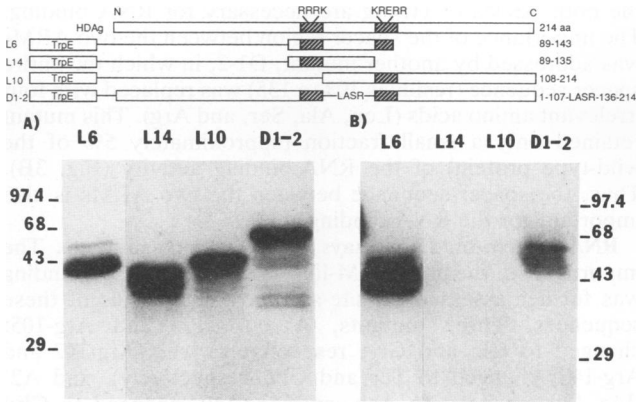

FIG. 3. RNA-protein binding assays of the HDAg deletion mutants. The various TrpE-HDAg fusion proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, and incubated with an anti-HDAg antibody for the quantitation of the fusion proteins by immunoblot analysis (A) or incubated with ^{32}P-labeled HDV genomic RNA for RNA-binding assays by Northwestern blot analysis (B) as described in Materials and Methods. The schematic diagram of the structures of the TrpE-fusion proteins is shown above. The molecular mass markers (in kilodaltons) are indicated. The shaded areas represent the ARM-like sequences.

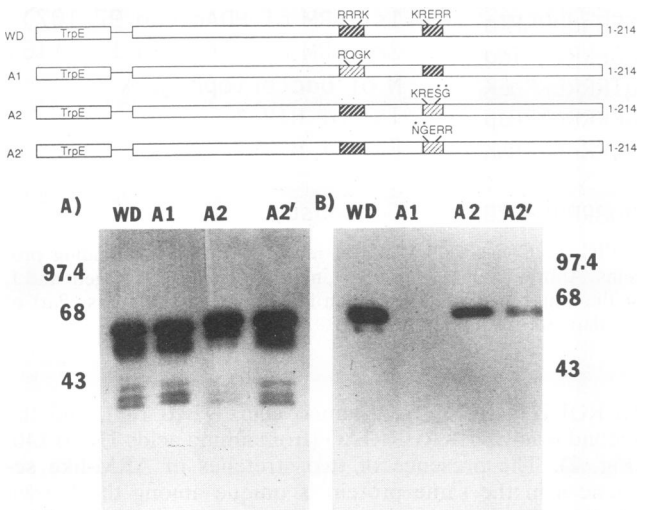

FIG. 4. RNA-protein binding assays of the site-specific mutants of the HDAg ARM sequences. The different TrpE-HDAg fusion proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, and incubated with anti-HDAg antibody for immunoblot analysis (A) or incubated with ^{32}P-labeled HDV genomic RNA for Northwestern blot analysis (B). The schematic diagram of the structures of the TrpE-fusion proteins is shown above. The molecular mass markers (in kilodaltons) are indicated. The mutated ARM sequences are identified by lighter shaded boxes, and the substituted amino acids are indicated.

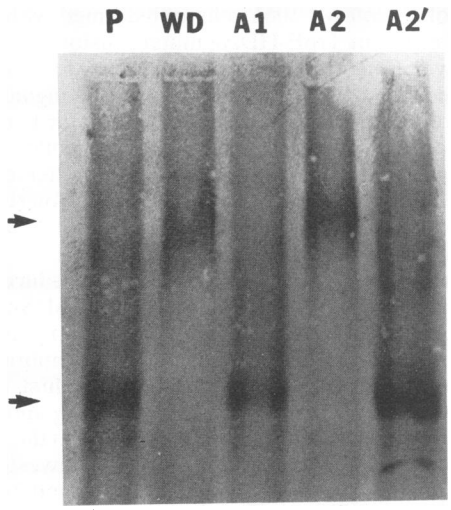

FIG. 5. RNA mobility shift analysis of the arginine mutants of the two ARMs. Different mutant HDAg proteins as described in the legend to Fig. 4 were incubated with ^{32}P-labeled HDV genomic RNA and separated by electrophoresis on 0.6% low-melting-point agarose gels under nondenaturing conditions (26). P, HDV RNA. Arrows indicate the free RNA and RNA-protein complexes.

amounts of the various fusion proteins were used (Fig. 3A). Thus, residues 135 to 143 appear to contain sequences necessary for HDV RNA binding. This stretch of sequences corresponds to a major portion of the second ARM-like sequence (Fig. 2). To delineate the contribution of the first ARM, another mutant, L10, which spans amino acids 108 to 214 and contains only the second ARM but not the first, was constructed. This protein did not bind HDV RNA (Fig. 3B). Thus, mutants without either the first or second ARM showed no binding to HDV RNA. This result indicates that the both ARMs of HDAg are necessary for RNA binding. The importance of the spacer region between the two ARMs was addressed by another mutant, D1-2, in which the entire spacer sequence (residues 108 to 135) was replaced with four irrelevant amino acids (Leu, Ala, Ser, and Arg). This mutant retained only a small fraction (approximately 5% of the wild-type protein) of the RNA-binding activity (Fig. 3B). Thus, the spacer sequence between the two ARMs is also important for the RNA binding.

RNA-protein-binding assays of site-specific mutants. The importance of the both ARM-like sequences in RNA binding was further assessed by site-specific mutagenesis of these sequences. Three mutants, A1 (Arg-104 and Arg-105; changed to Gln and Gly, respectively), A2 (Arg-142 and Arg-143; changed to Ser and Gly, respectively), and A2′ (Lys-139, changed to Asn, and Arg-140, changed to Gly) were made on the full-length HDAg-TrpE fusion protein. Their RNA-binding activities were examined by Northwestern blot with ^{32}P-labeled HDV genomic RNA. The same amounts of the fusion proteins were used for each mutant, as evidenced by immunoblot analysis (Fig. 4A). The result showed that the substitutions of Arg in the first ARM (A1) completely eliminated the RNA-binding activity (Fig. 4B). The effects of mutations in the second ARM were more variable. A2′, which had substitutions of the first Lys and Arg, had a 10-fold-lower binding activity than the wild-type HDAg. In contrast, A2, which had substitutions of the two downstream arginines, had a fourfold lower binding activity (Fig. 4B). The specificity of this binding assay has previously been demonstrated by competition with the excess cold RNA (26). The RNA-binding activities of the mutant HDAgs were further examined by RNA mobility shift assays (Fig. 5). The results showed that the wild-type HDAg and A2 mutant protein formed an RNA-protein complex with HDV genomic RNA. In contrast, only negligible amounts of A1 or A2′ mutants formed the RNA-protein complex with HDV RNA under the same conditions. Again, the specificity of this assay has previously been demonstrated (26). Thus, the two upstream basic amino acids in the second ARM-like sequence are more important than the downstream ones for the RNA binding. These studies indicated that both of the ARM-like sequences are necessary for the RNA-binding activity of HDAg.

Biological significance of the RNA-binding activity of HDAg. To determine the biological significance of the RNA-binding activity of HDAg, we examined whether the RNA-binding activity of HDAg was required for its *trans*-acting activity for HDV RNA replication (22). We constructed a plasmid expressing a defective HDV dimer which had a frameshift mutation in the HDAg-coding region. When this plasmid was transfected into COS 7 cells, very little HDV monomer RNA was detected, indicating that this plasmid could not replicate (Fig. 6, lane Smd2). However, when this construct was cotransfected with a plasmid (PECE-Sm) expressing a wild-type small HDAg, an HDV monomer RNA could be detected (Fig. 6, lane Sm), suggesting that HDAg could activate RNA replication in *trans*. This result was in agreement with the published finding that HDAg is required for HDV RNA replication (22). When the HDV dimer construct was cotransfected with the different plasmids expressing an HDAg with various mutations in the RNA-binding motif, only mutant A2, which retained most of the in vitro RNA-binding activity, could restore the RNA replication of Smd2. The level of HDV RNA replication was

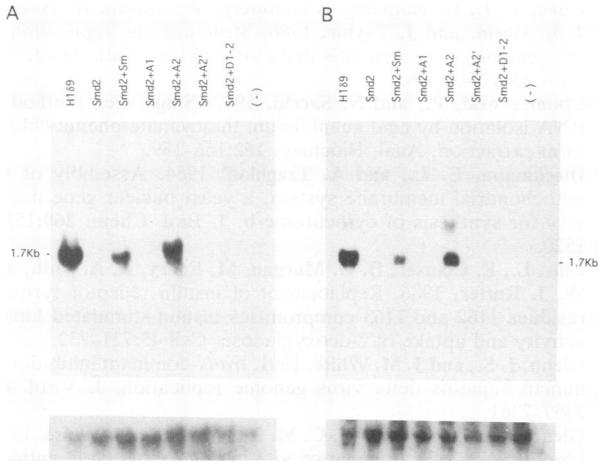

FIG. 6. Analysis of the *trans*-activating activity of mutant HDAgs with a defective HDV dimer and mutant HDAg-expressing plasmids. COS 7 cells were cotransfected with HDV dimer DNA (Smd2) and various mutant HDAg DNA as described in Results. Cellular RNA was extracted 6 days posttransfection, separated in formaldehyde gels, transferred to nylon membrane, and incubated with ^{32}P-labeled HDV antigenomic-sense (A) or genomic-sense (B) RNA. The membrane was also incubated with ^{32}P-labeled ribosomal RNA (lower panels) for the quantitation of RNA. The arrow indicates monomeric HDV genomic RNA. HIδ9 is an HDV stably transformed COS 7 cell line which serves as a size marker for monomer HDV RNA. All constructs are in PECE plasmid with the simian virus 40 early promoter (13).

similar when either the wild-type HDAg or A2 mutant was used. The remaining mutants, A1, A2', and D1-2, which had no or only minimal RNA-binding activities, did not complement the defects of replication of Smd2 RNA (Fig. 6A). This result was confirmed by the relative amounts of antigenomic RNA synthesized in cells transfected with Smd2 and the different HDAg mutants (Fig. 6B), while the amounts of RNA used for Northern blot analysis were shown to be the same for all of the samples. We thus conclude that the RNA-binding activity is crucial for the *trans*-activating activity of HDAg.

DISCUSSION

HDAg is an RNA-binding protein with a binding specificity for HDV RNA (26). The results presented in this report showed that the RNA-binding properties of HDAg require two stretches of the ARM-like sequence working in concert. Furthermore, a spacer sequence separating the two ARM-like sequences also contributes to the binding activity. Although the ARM-like sequence has previously been detected in several prokaryotic and eukaryotic RNA-binding proteins (25), this is the first protein shown to require two of these sequences for RNA binding. Thus, HDAg likely represents a new type of RNA-binding protein which has a unique specificity for HDV RNA sequence or structure. We have previously shown that HDAg binds specifically to HDV RNA but not to other viral or cellular RNA (26). The binding specificity appears not to be dependent on nucleotide sequence but more likely on secondary structure of RNA (26). Chao et al. further demonstrated that HDAg binds only to the rod structure of HDV RNA (8), which is generated by intramolecular base pairing (4, 38). However, a simple double-stranded RNA structure derived from an unrelated

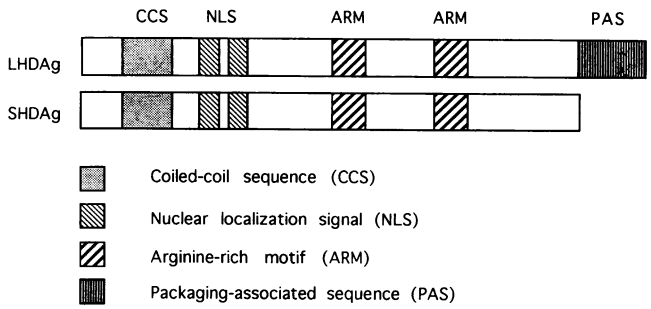

FIG. 7. Schematic diagram of the functional domains of HDAg.

virus did not bind to HDAg (26), suggesting that some RNA sequence specificity is also required for the protein-RNA interaction. Furthermore, we have demonstrated that a small RNA fragment (110 nucleotides) containing the ribozyme domain of HDV genomic RNA can also bind to HDAg (unpublished observation). This fragment does not form the rod structure but forms a multiple stem-and-loop structure (42). These results suggest that HDAg might recognize a double-stranded RNA structure unique to HDV RNA. Conceivably the two ARM-like sequences may bind to different RNA strands or different regions of such an RNA structure, thus explaining the requirement for a spacer sequence. The precise RNA structure of the HDAg-binding sites will require additional studies.

HDV RNA replication requires a *trans*-acting function of HDAg (22), although the precise role of HDAg is not clear. A previous study suggests that HDAg is required for the nuclear transport of HDV RNA but not directly involved in RNA transcription per se (28). However, this interpretation cannot explain the failure of large HDAg to *trans* activate HDV RNA replication since both large and small HDAgs are transported to the nuclei (44). Conceivably, HDAg may serve as a transcription factor to enhance the efficiency of HDV RNA replication. The result presented in this report showed that the RNA-binding activity of HDAg is required for its *trans*-activating activity, suggesting that HDAg has to bind to HDV RNA to fulfill whatever role HDAg plays in HDV RNA replication. However, the binding of HDAg to HDV RNA is not sufficient for HDV RNA replication, since both the large and small HDAgs bind to HDV RNA at equal efficiency (20); yet only the small one can *trans*-activate RNA replication while the large one inhibits it (7, 14, 22). Conceivably, HDAg binds to RNA, allowing other functional domains of HDAg to interact with components of the transcriptional machinery. This interaction may be possible only with the small HDAg. This possible mechanism of action of HDAg is similar to that of several other RNA-binding proteins which contain an ARM-like sequence and play regulatory roles in transcription. For example, bacteriophage lambda N protein, when it binds to the *nut* site, may engage RNA polymerase and modify it in a way to prevent termination (2, 19). Also, the *tat* protein of HIV-1 increases transcription processivity through interaction with the TAR element (24). Both of these proteins recognize specific RNA sequences through a single ARM (25). HDAg may use a similar mechanism to redirect host cell RNA polymerase for HDV RNA replication.

Several functional domains have been identified in HDAg (Fig. 7). The nuclear localization signal (NLS) of HDAg directs HDV RNA into the nuclei (44) so that RNA replication can utilize the nuclear machinery (28). The coiled-coil or

leucine-zipper sequence permits HDAg oligomerization and is also required for RNA replication (43). It appears that HDAg forms an oligomer to participate in RNA replication; however, oligomerization of HDAg is not required for its RNA binding, since the middle domain of HDAg, without the coiled-coil sequence, is sufficient for RNA binding (26). Finally, the C-terminal 19 amino acids of the large HDAg have an isoprenylation site (15) and probably contain the sequences (packaging-associated sequences) required for the packaging of HDV RNA during the assembly of HDV particles (5, 33). The incorporation of HDV RNA into virus particles most likely requires the RNA-binding activity of HDAg as well. Indeed, HDV RNA and HDAg have been shown to form a complex inside the virion particles (26). Thus, HDAg-HDV RNA binding is required not only for RNA replication but also for assembly of infectious virus particles. It should be noted that some of the mutant HDAgs (A2′ and D1-2) retained a residual amount of RNA-binding activity and yet no HDV RNA replication could be detected when they were cotransfected with the mutant HDV dimer. Thus, the region containing the RNA-binding motifs may have other activities as well. Sequence analysis suggests that there is a helix-loop-helix structure between the two ARM-like sequences (data not shown). Conceivably, the mutations in the ARM sequences may also affect this and other neighboring structures. This possibility would explain the total lack of the *trans*-activating activity of mutant D1-2, which had deleted the spacer sequence and thus, the helix-loop-helix structure. The properties of these additional sequences will require further studies.

ACKNOWLEDGMENTS

This work was supported in part by a Public Health Service Research grant (AI 26741) from the National Institutes of Health. M.C. is a research associate and M.M.C.L is an investigator of Howard Hughes Medical Institute.

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