Selective Strand Annealing and Selective Strand Exchange Promoted by the N-terminal Domain of Hepatitis Delta Antigen*

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We have previously shown that the N-terminal domain of hepatitis delta virus (NdAg) has an RNA chaperone activity in vitro (Huang, Z. S., and Wu, H. N. (1998) J. Biol. Chem. 273, 26455–26461). Here we investigate further the basis of the stimulatory effect of NdAg on RNA structural rearrangement: mainly the formation and breakage of base pairs. Duplex dissociation, strand annealing, and exchange of complementary RNA oligonucleotides; the hybridization of yeast U4 and U6 small nuclear RNAs and of hammerhead ribozymes and cognate substrates; and the cis-cleavage reaction of hepatitis delta ribozymes were used to determine directly the role of NdAg in RNA-mediated processes. The results showed that NdAg could accelerate the annealing of complementary sequences in a selective fashion and promote strand exchange for the formation of a more extended duplex. These activities would prohibit NdAg from modifying the structure of a stable RNA, but allow NdAg to facilitate a trans-acting hammerhead ribozyme to find a more extensively matched target in cognate substrate. These and other results suggest that hepatitis delta antigen may have a biological role as an RNA chaperone, modulating the folding of viral RNA for replication and transcription.

Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus. The genome of HDV comprises single-stranded circular RNA of ∼1700 nucleotides, and HDV RNA replicates through a symmetrical rolling circle mechanism (1). Hepatitis delta antigen is the only protein coded by HDV that is critical for viral replication (2) and virion assembly (3), although the molecular mechanisms have not yet been elucidated. HDV RNA, of both genomic and antigenomic senses, contains a ribozyme domain that can adopt a pseudoknot-like structure and undergo cis-cleavage in vitro (4–7). The cis-cleaving activity is essential for the RNA chaperone activity (16).

In vitro, hepatitis delta antigen can modulate the cis-cleaving activities of HDV genomic RNA fragments and facilitate a trans-acting hammerhead ribozyme to find its target in RNAs of various sequences and lengths (16). Hepatitis delta antigen exerts its effect on these RNA-mediated processes by modifying the conformation of RNA molecules and by promoting strand annealing and strand dissociation. These properties of hepatitis delta antigen parallel many cellular proteins and viral proteins with RNA/nucleic acid chaperone activity (12–15). The functional domain of hepatitis delta antigen appears to locate at the N-terminal region that is rich in basic amino acids and contains the cryptic RNA binding domain, coiled-coil domain, and nuclear localization signal (16). The core of the functional domain overlaps with the coiled-coil domain, whereas the RNA binding domain that binds HDV RNA specifically is not required for the RNA chaperone activity (16).

Here we investigated further the basis of the stimulatory effect of the N-terminal domain of hepatitis delta antigen, named as NdAg, on RNA structural rearrangement. The results confirm and extend our previous model. The results showed that NdAg could promote the annealing of a variety of complementary sequences and stabilize RNA duplexes. NdAg preferentially stimulated the formation of a more extended duplex among competing sequences by facilitating strand annealing and strand exchange in a selective fashion. Moreover, NdAg was able to facilitate a trans-acting hammerhead ribozyme to discriminate a completely matched from a non-completely matched target in a substrate RNA, but may not alter the structure of a stable RNA molecule. The selective strand annealing and selective strand exchange activity of NdAg may be important for modulating the folding of viral RNA for replication and transcription.

EXPERIMENTAL PROCEDURES

Proteins—The N-terminal region (residues 1–88) of hepatitis delta antigen, named as NdAg, was produced in E. coli BL21 (DE3) cells and purified by phosphocellulose column chromatography as described previously (16). Fractions containing NdAg were snap-frozen in liquid nitrogen and stored at −70 °C. The concentration of NdAg was determined by ninhydrin assay with lysine as standard. NdAg was diluted to...
the desired concentrations with protein dilution buffer (50 mM Hepes-NaOH (pH 7.9), 1 mM NaN3, 1 mM EDTA (pH 8.0), and 20% glycerol) and used as 10× stock. T4 phage gene 32 protein (T4gp32) and E. coli single-stranded DNA-binding protein (SSB) were purchased from Amersham Biosciences.

Nucleic Acids—RNA oligos 11, 14, 17, and 22 and DNA oligos A, B, and C were chemically synthesized. Short unlabelled nucleic acid oligos were used directly without purification. DNA oligos A and B were gel-purified. The concentration of each nucleic acid fragment was determined by its absorbance at 260 nm. Carrier-free 5′-end labeled RNA oligo 22 and DNA oligo C were made using [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs). These 5′-end labeled nucleic acid fragments were purified from polyacrylamide gels, and their concentrations were calculated from the radioactivity of each fragment and the specific activity of [γ-32P]ATP.

HH16 and HH163 RNA were synthesized by T3 RNA polymerase (Promega) with synthetic DNAs as templates. HIJ12L RNA, Rz1.H2 RNA, KSS3 RNA, yeast U4 and U6 snRNAs, and F9 RNA were run-off transscripts of the T7 RNA polymerase (Promega) with restriction enzyme-linearized plasmid DNA as templates. These RNAs were internally labeled by incorporating [α-32P]CTP to 10% of total reaction activity, and then purified from polyacrylamide, 7 mM urea gels. The concentration of labeled RNA was calculated from the radioactivity of RNA and the specific activity of CTP. The cellular RNA of E. coli BL21 (DE3) was isolated by TRIzol reagent (Invitrogen) following the instructions of the manufacturer. ΔX174 virion ssDNA and ΔX174 replication form dsDNA were purchased from New England Biolabs. Nucleic acid concentrations were determined according to their absorbance at 260 nm. All nucleic acids were resuspended in TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and stored at −20 °C.

**Strand Annealing/Hybrid Formation Assay**—Complementary RNA oligos, complementary DNA oligos, or U4 and U6 snRNAs were heated separately at 95 °C for 2 min, cooled to room temperature, and incubated at the reaction temperature for at least 5 min before use. In general, 10-μl reaction mixtures contained indicated amounts of nucleic acid fragments and NdAg or other protein in 1× reaction buffer for at least 5 min before use. Reactions were performed at different temperatures; timing initiated by the addition of NdAg or other protein unless otherwise indicated. Reactions were terminated by transfer to ice, and the addition of 2 μl of ice-cold stop solution (50 mM EDTA (pH 8.0), 2.5% SDS, 25% glycerol, 0.01% xylene cyanol, and 0.01% bromphenol blue). Different nucleic acid species were resolved by electrophoresis through a native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 1 mM EDTA) and stained with or without 0.1% SDS at 4 °C. Gels were dried and examined by autoradiography.

**Strand Exchange Assay**—The pre-annealed RNA duplex was made by mixing 0.5 μM [32P]-end-labeled DNA oligo 22 with 2 μM complement in TE buffer. Reaction solution was heated at 95 °C for 2 min, cooled slowly (~1 h) to room temperature, and stored at −20 °C until used. The [32P]-labeled pre-annealed RNA duplex was diluted in TE buffer and mixed with or without a competing RNA oligo, in the presence or absence of NdAg in 10 μl of reaction buffer at room temperature. Timing was initiated upon the addition of NdAg, and the reaction was stopped by the addition of a 0.2 reaction volume of ice-cold stop solution.

All of the reaction mixtures were kept on ice before analysis. Electrophoresis of the RNA samples and the subsequent data analysis were performed as described for the annealing assay.

**Melting Temperature Determination**—Tubes containing the pre-annealed RNA duplex in 10 μl of reaction buffer were transferred to a PCR machine and incubated for 3 min at each indicated temperature. One tube was removed at 5 °C intervals through the temperature range indicated, and 2 μl of ice-cold stop solution was added immediately to terminate the reaction. All of the reaction mixtures were kept on ice before analysis. Electrophoresis of the RNA samples and the subsequent data analysis were performed as described for the annealing assay.

**Hepatitis Delta Viruses**—Hepatitis delta viruses HIJ12L and Rz1.H2 were heated at 95 °C for 2 min, cooled to room temperature, and incubated at 37 °C for 5 min before use. RNA was pre-incubated with NdAg in 1× reaction buffer for 30 min at 37 °C, and the cis-cleavage reaction of HIJ12L or Rz1.H2 was initiated by the addition of MgCl2 to a final concentration of 12 mM. The reaction was performed at 30 °C for 15 min, after which samples were treated with 1 μg/ml proteinase K at 37 °C for 25 min. Further cleavage was inhibited by the addition of an equal volume of quench solution containing 50 mM EDTA, 7 mM urea, 0.005% xylene cyanol, and 0.005% bromphenol blue. RNAs in the ribozyme reaction mixture were analyzed on a 10% polyacrylamide, 7 mM urea gel.

Hammerhead ribozyme and the KSS3 substrate RNA were separately heated at 95 °C for 2 min and then cooled to the reaction temperature for at least 5 min. The two RNAs were mixed and pre-incubated with NdAg in 1× reaction buffer for 30 min at 30 °C, and the trans-cleavage reaction was initiated by the addition of MgCl2 to a final concentration of 12 mM. The reaction was performed at 30 °C for 15 min, after which samples were treated with 1 μg/ml proteinase K at 37 °C for 25 min. Further cleavage was inhibited by the addition of an equal volume of quench solution containing 50 mM EDTA, 7 mM urea, 0.005% xylene cyanol, and 0.005% bromphenol blue. RNAs in the ribozyme reaction mixture were analyzed on a 10% polyacrylamide, 7 mM urea gel.

**RESULTS**

**Strategy**—Previously we had shown that the N-terminal domain of hepatitis delta antigen (NdAg) modulates the cis-cleaving activity of HDV genomic RNA fragments and activates the trans-cleavage reaction between hammerhead ribozymes and cognate substrates in vitro (16). Conceivably, NdAg exerts its effect on RNA-mediated processes by acting as an RNA chaperone, promoting RNA re-foiling by facilitating the breakage and reforming of base pairs. To further investigate the basis of RNA chaperone activity, purified NdAg was assayed for the ability to promote the annealing of complementary sequences in RNA oligos and complicated RNA molecules in dilute solutions, and to accelerate strand exchange between an RNA duplex and a competing sequence.

**Strand Annealing Activity of NdAg**—To assay the stimulatory effect of NdAg on strand annealing, complementary oligos of low concentrations were mixed, and duplex formation was monitored. In the case of RNA oligos 22 (0.025 mM) and 11 (0.125 mM) (Fig. 1A), there was only negligible duplex formation in the absence of NdAg, but the extent and the rate of duplex formation were significantly elevated in the presence of 1 μM NdAg (Fig. 2A). Thus, NdAg promoted the annealing of complementary RNA oligos.

It is known that Mg2+ can neutralize the negative charge of phosphate groups and stabilize the structure of RNA molecules. To address the question of the effect of Mg2+ on the strand annealing activity of NdAg, we performed the strand annealing reaction in the presence of MgCl2 and/or NdAg. We found that MgCl2 of 10–40 mM alone facilitated the annealing of RNA oligos 22 and 11, although its stimulatory activity was significantly lower than that of NdAg. Nevertheless, MgCl2 at this concentration range reduced, although it did not eliminate, the strand annealing activity of 1 μM NdAg (Fig. 2A and data not shown).

Annealing assays were done on other complementary nucleic acid pairs of 11–56 nt; NdAg facilitated the annealing of all nucleic acid pairs tested, including DNA-DNA, DNA-RNA, and RNA-RNA pairs (data not shown). In the case of DNA oligos A (43 nt), B (56 nt), and C (18 nt) (oligos A and C are complementary to different regions of oligo B; Fig. 1B), NdAg greatly promoted the formation of the trimolecular duplex and both bimolecular duplexes at low DNA concentrations (~0.5 mM) (B/C and B/CA duplex data are shown in Fig. 2B).

Furthermore, NdAg accelerated the hybridization of more complicated RNAs in addition to simple complementary nucleic acid pairs. 50–500 nM NdAg facilitated 0.5 mM U4 snRNA and 2.5 mM U6 snRNA to form U4/U6 hybrid that potentially contains two intermolecular base pairing regions (Figs. 1C and 2C). All of the aforementioned simple complementary nucleic acid pairs hybridize spontaneously; however, U4 snRNA and U6 snRNA did not hybridize spontaneously at even up to 25 mM in the absence of NdAg (data not shown). This suggests NdAg influences the ability of complex RNAs to interact. Therefore, in addition to short complementary oligos, NdAg also promotes...
the annealing of the complementary sequences reside in longer or more complicated RNAs.

Strand Annealing/Hybrid Formation in the Presence of Non-homologous Nucleic Acid or Other Proteins—To test whether non-homologous nucleotide sequences perturb the acceleration of strand annealing or hybrid formation stimulated by NdAg, we analyzed the hybridization of a constant amount of U4 snRNA (0.5 nM 161-nt RNA, 80.5 nM nucleotide) and U6 snRNA (2.5 nM 112-nt RNA, 280 nM nucleotide)) in the presence of increasing amounts of a non-homologous P9 RNA (a 160-nt run-off transcript of pET15b vector) at 100, 250, or 500 nM NdAg. As shown in Fig. 3A, for a 360 nM nucleotide concentration of U4 snRNA (0.5 nM RNA, 80.5 nM nucleotide) and U6 snRNA (2.5 nM RNA, 280 nM nucleotide), U4/U6 hybridization was not inhibited until the molar ratio of total nucleotide (the sum of two snRNAs and P9 RNA) to NdAg exceeded 5:1, and at higher molar ratios (>/=5:1), U4/U6 hybrid did not form. The result suggests that there is a finite amount of NdAg available to facilitate duplex hybridization with a limiting ratio of one NdAg to five nucleotides. To confirm this finding, we performed strand annealing reactions of DNA oligos A and B at low concentrations (total nucleotide was =5 nM) in the presence of a relatively high concentration of NdAg (1 mM) and increasing concentrations of three kinds of nucleic acid: E. coli cellular
RNA, φX174 ssDNA and φX174 dsDNA. As shown in Fig. 3B, B/A duplex formation was not affected by the E. coli cellular RNA or φX174 ssDNA until the total nucleotide concentration exceeded 5 μM, whereas the presence of φX174 dsDNA did not fully inhibit the annealing of oligos A and B at even 12 μM total nucleotide. These results support the notion that the binding site size of an NdAg monomer is ~5 nucleotides, and suggests that complementary strand annealing can occur in the presence of a 100–1000-fold excess of non-homologous sequence as long as the amount of NdAg is sufficient to coat the non-homologous sequences and the complementary nucleic acid pair.

The acceleration of strand annealing/hybrid formation associated with NdAg is not specific for any particular type of nucleic acid. Hence, NdAg interacts with nucleic acid with broad specificity. We tested whether two general nucleic acid-binding proteins share similar strand annealing properties with NdAg. We found that E. coli SSB (single-stranded nucleic acid-binding protein) and T4 phage gp32 protein at a concentration range of 1–10 μM failed to promote U4/U6 hybrid formation or to stimulate DNA or RNA duplex formation (data not shown). In addition, the stimulatory effect of 50 nM NdAg on U4/U6 hybrid formation was not perturbed in the presence of a 20-fold excess of T4 phage gp32 protein, a 50-fold excess of E. coli SSB, or a 200-fold excess of bovine serum albumin (Fig. 4). Thus, NdAg can facilitate strand annealing/hybrid formation in the presence of these two nucleic acid-binding proteins. These results indicate that the stimulation of strand annealing is not a general property of nucleic acid-binding proteins.

NdAg Facilitates Strand Annealing and Strand Exchange in a Selective Fashion—

We next examined the stimulatory effect of NdAg on the annealing of RNA oligos of different lengths to a common sequence. RNA oligos 11, 17, and 22 were used for this study (Fig. 1A). Both oligos 11 and 17 are complementary to oligo 22. Oligo 17 has the same sequence as oligo 11 but also has a 2-nucleotide extension at the 5’ terminus and a 3-nucleotide extension at the 3’ terminus. Hence, oligo 17 can form a 17-base pair duplex with oligo 22, whereas oligo 11 can form an 11-base pair duplex with oligo 22. In the presence of 1 μM NdAg, the kinetics of annealing of oligos 22 and 17, and oligos 22 and 11 were quite similar, and the duplex formation reaction of both pairs of oligos reached an equilibrium within 10 min at 30, 37, and 42 °C (data not shown). The melting temperatures of the 22/11 duplex and 22/17 duplex were ~50 and ~65 °C, respectively, and oligo 22 annealed more favorably to oligo 17 at 30 and 37 °C, and exclusively to oligo 17 at 42 °C (Fig. 5B). Moreover, the relative amount of 22/17 and 22/11 duplexes at each incubation time varied between 10 and 180 min (data not shown). Thus, NdAg promotes the formation of a more extended duplex among competing sequences, with the activity higher at 42 °C than 30 and 37 °C. Therefore, hypothetically NdAg facilitates strand annealing or stimulates duplex dissociation in a selective manner.

We next monitored the ability of NdAg to promote strand exchange between an RNA duplex and a competing RNA oligo (Fig. 6). The pre-annealed 32P-labeled 22/11 duplex (0.25 nM) was mixed with a 100-fold excess of oligo 17 (25 nM). In the absence of NdAg, the pre-annealed duplex gradually dissociated over a 30-min period, with the released 32P-labeled oligo 22 concurrently forming a duplex with oligo 17. Consequently, the 32P-labeled 22/11 duplex was converted to the 32P-labeled 22/17 duplex (Fig. 6A). In the presence of NdAg, the conversion of 22/11 duplex to 22/17 duplex occurred at an elevated rate at least during the first 10 min of the reaction (Fig. 6A). Thus, in this experiment, NdAg facilitated strand exchange. However, a strikingly different result was obtained when the assay was performed using two other combinations of pre-annealed RNA duplex and a competing RNA oligo in the presence of NdAg. The data in Fig. 6 (B and C) show that strand exchange between a pre-annealed 22/14 duplex (0.25 nM) and oligo 11 (25
After each step of incubation, one tube was transferred to ice and 2 \text{nM} NdAg were mixed with snRNA, and 50 \text{nM} NdAg were mixed with snRNA, 2.5 \text{nM} internally labeled U6 22/17 and 22/11 duplexes. The 10-min annealing in the presence of other proteins.

We found that 1 \text{M} NdAg speeded up the strand exchange reaction between 22/11 duplex and RNA oligo 17 in the presence of 0–5 \text{mM} MgCl$_2$ but failed to facilitate the conversion of 22/11 duplex to 22/17 duplex in the presence of 10 or 20 \text{mM} MgCl$_2$ (Fig. 6D).

\textit{NdAg May Not Alter the Structure of a Stable RNA—}The studies with the synthetic nucleic acid oligos disclose that NdAg promotes strand annealing and strand exchange in a selective fashion, with this property allowing NdAg to stimulate the formation of the most stable duplex among complementary sequences. This finding led us to speculate that NdAg could assist a contiguous sequence in adopting a stable structure, which may not be biologically active, and that NdAg would not alter the structure of a stable RNA. Here we investigated these speculations by studying the effect of NdAg on an RNA that contains the autolytic domain of the HDV genome and a cis-acting hepatitis delta ribozyme mutant that has a more stable structure than its wild type counterpart. Because hepatitis delta ribozyme has to adopt a specific catalytic structure, the alteration of autocatalytic activity upon NdAg treatment reflects ribozyme structure change.

HJ12L contains the autolytic domain of the HDV genome, and Rz1.H2 is a hepatitis delta ribozyme mutant that has had its helix 1 substituted, the helix 2 extended from 5 to 8 base pairs, and the helix 4 replaced by a stable hairpin loop (Fig. 7A). Rz1.H2 cis-cleaved efficiently and rapidly in the absence of NdAg, whereas HJ12L was much less active under the same conditions (Fig. 7B). The extended helix 2 rather than the alterations in helixes 1 and 4 of Rz1.H2 may account for the elevated cis-cleaving activity under native conditions, because we showed previously that the elongation of helix 2 enhances the resistance to formamide and stabilized the catalytic core of hepatitis delta ribozymes (17). Moreover, in the experiment shown in Fig. 7B, we found that pre-mixing with NdAg prior to the initiation of cis-cleavage did not alter the cis-cleavage reaction of Rz1.H2, whereas the same treatment attenuated the cis-cleaving activity of HJ12L. The results indicate that NdAg modified the structure of HJ12L but not Rz1.H2 and that the interaction with NdAg does not alter the catalysis of Rz1.H2. The finding supports the hypothesis that NdAg does not modify the structure of a stable RNA.

\textit{Effect of NdAg on the Reaction of a Trans-acting Hammerhead Ribozyme—}We then used the trans-cleavage reactions of a hammerhead ribozyme HH16 to further study the RNA chaperone activity of NdAg. The trans-cleavage reaction of a hammerhead ribozyme involves at least two steps. First, the ribozyme anneals to its target in a substrate RNA for the assembly of a hammerhead catalytic domain; then, cleavage occurs at a specific location by the catalysis of divalent cations (18). KSS3 is a 107-nt RNA containing three targets for the

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\caption{\textbf{A.} The double strand to single strand transition reaction of 22/17 and 22/11 duplexes. The 10-\textmu l reaction mixture contained 0.25 \text{nM} pre-annealed \text{32P}-labeled 22/17 duplex in 1\times reaction buffer. Incubation was carried out at 25 °C for 3 min, and the temperature increased every 3 min by an increment of 5 °C to a final temperature of 75 °C for 3 min. After each step of incubation, one tube was transferred to ice and 2 \text{µl} of stop solution was added immediately. Samples were analyzed on an 18\% polyacrylamide, 0.1\% SDS gel at 4 °C. B. NdAg promotes the formation of a more extended duplex. 0.025 \text{nM} \text{32P}-labeled oligo 22 and two competing complementary oligos 11 and 17 (0.5 \text{nM} amounts of each) were incubated with or without 1 \text{µM} NdAg in 1\times reaction buffer at the indicated temperature for 30 min; Samples were analyzed on an 18\% polyacrylamide, 0.1\% SDS gel at 4 °C.}
\end{figure}
hammerhead ribozyme HH16 (Fig. 8A). The first target has a C to A substitution; hence, upon binding to HH16, the helix I of the reconstituted hammerhead catalytic domain contains an AG mismatched pair. The second and the third targets are completely matched to HH16; each can form 16 base pairs with HH16. HH163 is a mutant of HH16 and has a G to U substitution to compensate for the mutation of the first ribozyme target of KSS3 (i.e. no mismatch; Fig. 8A). We showed previously that NdAg promotes the trans-cleavage reaction of HH16 and all its targets in KSS3 under ribozyme excess conditions, with NdAg appearing to exert its effect by elevating the mutual accessibility of two RNAs (16). Here, we found that >95% of KSS3 was cleaved by HH16 and HH163 when 1 nM KSS3 and 10 nM of each ribozyme were pre-incubated with 1 μM NdAg prior to the initiation of trans-cleavage (Fig. 8B). Furthermore, the major products of the two ribozymes were the same; they were the 49-, 23/22-, and 13-nt RNAs, with none of them containing any ribozyme target (Fig. 8B; the 22- and 23-nt RNAs were not resolved on the gel). Therefore, with an excess amount of ribozyme and the facilitation of NdAg, each ribozyme target of KSS3 is available for ribozyme binding and cleavage. The mismatched base pair in the helix I region of the hammerhead catalytic domain does not prevent cleavage under these reaction conditions.

We then used the same system to analyze the ability of NdAg to aid HH16 and HH163 in discriminating different ribozyme targets in KSS3 under a condition with the ribozyme target in excess of the ribozyme. We performed the trans-cleavage reaction of 1 nM KSS3 and 1 nM HH16 or HH163 (each KSS3 contains three ribozyme targets; therefore, the molar ratio of ribozyme target to ribozyme is 3). We found that the pre-incubation of two RNAs with 0.2 or 1 μM NdAg promoted trans-cleavage significantly; the overall extent of cleavage of KSS3 was elevated from less than 5% to >40% for each ribozyme. However, three ribozyme targets in KSS3 were not equally accessible to each ribozyme. In the case of HH16, the amount of the 72-nt RNA, which contains the first target, was dominant over the amount of all other cleavage products, indicating
cating most of the cleavage events occurred at the second and third targets that are completely matched to HH16. In the case of HH163, the major cleavage products were the 58-nt RNA, which contains the second and third targets, and the 49-nt RNA, indicating the cleavage process occurred predominantly at the first target that is completely matched to HH163. These results illustrate that, when ribozyme is limiting, NdAg selectively promotes the annealing of ribozyme to its completely matched target in the substrate RNA.

**DISCUSSION**

We showed previously that NdAg is able to modify the structure of HDV RNA and non-HDV RNAs (16). This property is known as RNA chaperone activity (11). In this study we set up model systems to further analyze the RNA chaperone activity of NdAg. We found that NdAg can facilitate rearrangements of RNAs into more stable structures by selectively accelerating the annealing of complementary sequences, as well as by selectively promoting strand exchange.

How Does NdAg Stimulate Strand Annealing?—Many nucleic acid chaperones identified so far, such as the nucleocapsid protein of human immunodeficiency virus (NCp7) (19, 20), the prion protein (PrP) (21), the ORF1 protein of mouse LINE-1 retrotransposon (22), heterogeneous nuclear ribonucleoprotein A1 (23, 24), and the major messenger ribonucleoprotein particle protein p50 (25), facilitate the annealing of a variety of complementary sequences. These proteins and NdAg do not have a common structural domain or sequence motif for this strand annealing activity except that most of them are rich in basic residues. Basic residues can participate in nonspecific, electrostatic interaction with nucleic acids, so the binding of NdAg and other nucleic acid chaperones may facilitate complementary strand annealing by charge shielding. Nevertheless, the extent of strand annealing stimulated by each of these proteins appears to be too great to be explained solely by a reduction in electrostatic repulsion.

NdAg contains the coiled-coil domain of hepatitis delta antigen. A synthetic peptide corresponding to aa 12–60 of hepatitis delta antigen forms anti-parallel coiled-coil dimers with its surface lined with basic side chains, and dimer can associate with other dimers to form doughnut-like octamers (26). We speculate that NdAg has a structure similar to that of 12–60 peptide and NdAg binds nucleic acids as coiled-coil dimers in a nonspecific manner mainly through charge-charge interactions. After this, a dimer may interact with other dimers to form multimers of NdAg, consequently, bringing complementary nucleic acid strands together via protein-protein interaction. Alternatively, NdAg may form multimers with each multimer interacting with several nucleic acid molecules simultaneously. Because NdAg remains associated with nucleic acids after facilitating strand annealing, nucleic acid molecules must be able to change positions rapidly in the nucleic acid-NdAg complex to find their pairing partners. Therefore,
NdAg could facilitate annealing by increasing the local concentration of complementary strands.

**How Does NdAg Facilitate the Formation of a More Stable Structure?**—In this study we found that NdAg promotes RNA to adopt a more stable structure via several methods. First, NdAg catalyzes annealing differentially among competing sequences; second, NdAg facilitates strand exchange selectively between a duplex and a single-stranded nucleic acid; and third, NdAg stabilizes a duplex if a competing nucleic acid, for the formation of a more stable duplex, is absent. Moreover, NdAg can modify the structure of short oligonucleotides and long RNAs either intermolecularly or intramolecularly (this study and Ref. 16). We suspect that NdAg catalyzes selective strand annealing and selective strand exchange by allowing the single strand ↔ double strand transition to reach an equilibrium more rapidly than normal. However, the molecular mechanisms underlying the formation of a more stable structure remain to be elucidated, e.g. because all short duplexes for the strand exchange analysis of this study are perfect duplexes with single-stranded overhangs, factors that determine the efficiency of the strand exchange process, such as internal mismatches, the length/sequence of the initial duplex and the role of the single-stranded overhangs, remain to be identified. In addition, whether the strand exchange process requires the complete dissociation of the initial duplex or whether partial melting of the initial duplex is sufficient to initiate strand exchange remains to be studied.

The **Effect of Mg**\(^{2+}\) on the RNA Chaperone Activity of NdAg—In this report we showed that, in the presence of \(\geq 10 \text{mM} \text{Mg}^{2+}\), the strand exchange activity of 1 \(\mu\text{M} \text{NdAg}\) is attenuated. We suspect that NdAg may not exert its effect on RNA structural modification in the presence of \(\geq 10 \text{mM} \text{Mg}^{2+}\). This speculation is supported by our previous observations; the ability of NdAg to modulate the cis-cleaving activity of HDV subgenomic fragments and to stimulate the catalytic activity of hammerhead trans-acting ribozymes relies on the pre-incubation of NdAg with the relevant RNA molecules in the absence of \(\text{Mg}^{2+}\) prior to the initiation of cleavage by the addition of \(\text{Mg}^{2+}\) (16, 27), and 1 \(\mu\text{M} \text{NdAg}\) in conditions of limiting hammerhead trans-acting ribozyme, with a \(\text{Mg}^{2+}\) concentration of 12 \text{mM}, resulted in a very low rate of hammerhead ribozyme turnover, i.e. the release of cleavage products from ribozyme for the binding of new substrate (16). Thus, \(\text{Mg}^{2+}\) attenuates the RNA chaperone activity of NdAg.

**What Is the Possible Role of Hepatitis Delta Antigen in Vivo?**—The finding that NdAg possesses RNA chaperone activity strongly emphasizes the importance of this activity in the life cycle of HDV. Hepatitis delta antigen stimulates viral genome replication and HDV mRNA transcription (27–29). One end of the rodlike structure of the HDV genome, which has a highly ordered structure preserved between HDV isolates, contains the promoters to direct the synthesis of viral antigenomic RNA and the 0.8-kb mRNA (30–33). Because hepatitis delta antigen stimulates the rearrangement of RNAs into more stable structures, this protein may allow the HDV genome to adopt an active promoter structure to recruit cellular factors for replication and transcription.

The cis-cleaving activity of HDV RNA is involved in the processing of multimeric RNA during viral replication (8, 9). The ribozyme domain requires a pseudoknot structure for catalysis, but because of the highly self-complementary nature of HDV RNA, the catalytically active structure is not expected to exist in multimeric size RNA. How is the ribozyme conformation formed on multimeric size RNA molecules? Because hepatitis delta antigen can modulate the ribozyme activity of HDV subgenomic fragments in *vitro* (16) and can enhance ribozyme activity in *vivo* (10), hepatitis delta antigen appears to be a facilitator of the processing reaction, although the mechanism remains unsolved. Moreover, in addition to modulating the activity of HDV ribozyme during viral replication, hepatitis delta antigen may stabilize the HDV genome by preventing circular monomers from adopting thermodynamically unstable and catalytically active pseudoknot structure.

The core of the RNA chaperone domain of hepatitis delta antigen has been located at aa 24–59 (16). Therefore, the arginine-rich sequence (aa 2–27) and the arginine-rich motifs (aa 97–107 and aa 136–146) of hepatitis delta antigen that bind specifically to HDV RNA in a rodlike structure in *vitro* (34, 35) are not required for the RNA chaperone activity. However, these “HDV RNA binding motifs” may allow hepatitis delta antigen to interact preferentially with HDV RNA in virus-infected cells.

**Applications**—In this report, we have demonstrated that NdAg improves the specificity of hammerhead trans-acting ribozymes. The selective strand annealing activity and the selective strand exchange activity of NdAg may have other practical applications. The ability of NdAg to promote the formation of a more stable duplex might be employed to elevate the accuracy of nucleic acid annealing for primer extension reactions, polymerase chain reactions, reverse transcription reactions, and all kinds of hybridization reactions, such as *in situ*, Southern, and Northern hybridizations. Whether NdAg is useful in these applications remains to be explored.

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