Local Conformational Changes in the Catalytic Core of the Trans-Acting Hepatitis Delta Virus Ribozyme Accompany Catalysis†

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ABSTRACT: The hepatitis delta virus (HDV) is a human pathogen and satellite RNA of the hepatitis B virus. It utilizes a self-cleaving catalytic RNA motif to process multimeric intermediates in the double-rolling circle replication of its genome. Previous kinetic analyses have suggested that a particular cytosine residue (C75) with a pKₐ close to neutrality acts as a general acid or base in cleavage chemistry. The crystal structure of the product form of a cis-acting HDV ribozyme shows this residue positioned close to the 5′-OH leaving group of the reaction by a trefoil turn in the RNA backbone. By modifying G76 of the trefoil turn of a synthetic trans-cleaving HDV ribozyme to the fluorescent 2-aminopurine (AP), we can directly monitor local conformational changes in the catalytic core. In the ribozyme–substrate complex (precursor), AP fluorescence is strongly quenched, suggesting that AP76 is stacked with other bases and that the trefoil turn is not formed. In contrast, formation of the product complex upon substrate cleavage or direct product binding results in a significant increase in fluorescence, consistent with AP76 becoming unstacked and solvent-exposed as evidenced in the trefoil turn. Using AP fluorescence and fluorescence resonance energy transfer (FRET) in concert, we demonstrate that this local conformational change in the trefoil turn is kinetically coincidental with a previously observed global structural change of the ribozyme. Our data show that, at least in the trans-acting HDV ribozyme, C75 becomes positioned for reaction chemistry only along the trajectory from precursor to product.

The hepatitis delta virus ribozyme is among a class of small endonucleolytic RNAs that catalyze a reversible self-cleavage reaction necessary for the replication and propagation of their satellite RNA genomes. Specifically, the hepatitis delta virus ribozyme is a unique RNA motif found in the human hepatitis delta virus (HDV)† (1). HDV is a satellite of the hepatitis B virus (HBV); coinfection of HDV and HBV results in intensification of the disease symptoms associated with the hepatitis B virus (2). The small RNA genome of HDV replicates through a double-rolling circle mechanism, whereby multimeric units of genomic and antigenomic RNA strands are produced, followed by self-cleavage and ligation into circular monomers (1, 3). Self-cleavage activity in the genomic and antigenomic RNAs resides within continuous 85-nucleotide sequences that both form a nearly identical secondary structure consisting of a nested double pseudoknot (4, 5).

The genomic and antigenomic forms of the HDV ribozyme catalyze self-cleavage by a transesterification reaction, which requires deprotonation of the adjacent 2′-OH group and its nucleophilic attack on the scissile phosphate, resulting in formation of 2′,3′-cyclic phosphate and 5′-OH termini (5).

The reaction mechanism of the HDV ribozyme has been extensively studied. The crystal structure of the self-cleaved genomic ribozyme reveals that the base cytosine 75 (C75) is situated in the active site cleft and, thus, in the proximity of the 5′-OH leaving group (Figure 1a,b). Therefore, C75 in the genomic ribozyme has been proposed to participate directly in reaction chemistry as either a general acid or general base catalyst (6).

Several biochemical and mutagenesis studies support the idea that C75 in the genomic ribozyme and the corresponding αC76 (α used to distinguish antigenomic numbering) in the antigenomic ribozyme are involved in catalysis (7–10). The pH dependence of self-cleavage (or cis cleavage) by the HDV ribozyme reveals a macroscopic apparent pKₐ that approaches neutrality. In a widely accepted model, this pKₐ reflects the ionization equilibrium of N3 in C75 which therefore is strongly shifted in the folded ribozyme compared to that in the free base (pKₐ ≈ 4.2). A decrease in this pKₐ for self-cleavage of an antigenomic ribozyme with an αC76A mutation was observed, consistent with A substituting for C in this position to act as a general base catalyst (8). However, the pH profile of the genomic ribozyme in the presence of 1 M NaCl and 1–100 mM EDTA favors a model where C75 acts as a general acid during catalysis (9, 11). This latter mechanism is in agreement with the crystal structure of the self-cleaved genomic ribozyme, which shows N3 of C75 within hydrogen bonding distance of the 5′-OH leaving group. In addition, C75 is hydrogen bonded to the phosphate group of C22, increasing the local electron density and providing a mechanism for a shift in pKₐ, such that it is...
in the postcleavage crystal structure (red). C75 is positioned deep
ribozyme further evidence for the importance of the ionization of C75
mapping (NAIM) of the genomic ribozyme has provided
transition state (6). Recently, nucleotide analogue interference
studies of the folding pathway of the HDV ribozyme are
key to our understanding of how the RNA positions C75 in
the active site cleft such that it becomes both chemically
activated and poised for catalysis. The crystal structure of
the self-cleaved genomic ribozyme gives hints about how
this positioning occurs. C75 and its two adjacent nucleotides,
G76 and G74, form a “trefoil” turn that pushes C75 deeply
into the catalytic core (Figure 1b) (6). In particular, extrusion
of G76 allows for a backbone geometry that projects C75
toward the 5′-OH leaving group. With this in mind, we have
devised a fluorescence-based technique for monitoring the
unstacking of the nucleotide in position 76 as it extrudes
from neighboring bases into solvent, by substituting 2-amino-
apurine (AP) for G76. AP is a fluorescent guanine analogue
that is highly sensitive to local stacking interactions with
other bases and can be selectively excited in the presence of
the natural bases (17). Mutagenesis studies suggest that the
chosen substitution site can be mutated with only a modest
loss of activity (7). When monitoring conformational changes
at position 76 through AP fluorescence in the trans-acting
HDV ribozyme, we observe a substantial local confor-
mutual arrangement that accompanies ribozyme catalysis. Our
results complement a previous study in which we found,
using fluorescence resonance energy transfer (FRET), that
a significant global conformational change concurs with
catalysis (18). Using AP fluorescence and FRET in concert,
we demonstrate here that the global structural change upon
cleavage is coupled to, and probably the consequence of,
local conformational rearrangements in and around the
catalytic core that lead to proper positioning of C75.

MATERIALS AND METHODS

Preparation of RNA Oligonucleotides. All RNA oligo-
nucleotides were obtained commercially from Dharmacon
Research, Inc., with 2′-protection groups (19) and were
deprotected following the manufacturer’s recommendations.
Deprotected RNA was purified by denaturing 20% poly-
acrylamide, 8 M urea, gel electrophoresis, diffusion elution
into 0.5 M NH4OAc, 0.1% SDS, and 0.1 mM EDTA
overnight at 4 °C, chloroform extraction, and ethanol
precipitation, followed by C17 reverse-phase HPLC with a
linear acetonitrile elution gradient in triethylammonium
acetate, as described previously (20). The noncleavable
substrate analogue was modified with a 2′-methoxy group
at the cleavage site (Figure 1a). The 3′-product had the
sequence 5′-GGGUCGG-3′. RNA concentrations were cal-
culated from the absorbance at 260 nm after background
correction. 5′-Fluorescein was incorporated into the RNA
during synthesis by Dharmacon Research, Inc., while tet-
ramethylrhodamine was postsynthetically attached to a 3′-
amin linker as previously described (18). The concentra-
protonated around neutral pH (6). Thus, C75 is in a position
in which it may donate a proton to relieve the developing
charge on the leaving 5′-oxygen ion group in the reaction
transition state (6). Recently, nucleotide analogue interference
mapping (NAIM) of the genomic ribozyme has provided
further evidence for the importance of the ionization of C75
in the cis cleavage reaction, as it is the only cytidine residue
that must be protonated for ribozyme activity (10).

The use of an RNA base to facilitate transition-state
chemistry appears to be surprisingly common among RNA
catalysts. For example, biochemical and structural studies
suggest that the hairpin ribozyme may use two catalytically
essential nucleotide residues, G9 and A38, to function as the
proton acceptor and donor, respectively (12, 13). An
analogous proposal has been made for the large subunit of
the ribosome, where no amino acid side chains or metal ions
but rather a conserved adenosine (A2351 in Escherichia coli
numbering) is found in sufficient proximity to potentially
act as a catalyst in peptidyl transfer (14), although this is
still controversial (15, 16). These discoveries reveal that RNA
bases in ribozymes can participate in catalysis in a manner
similar to that of amino acid side chains in protein enzymes.
Hence, the finding that the HDV ribozyme utilizes one of
its RNA side chains in acid–base catalysis has led the way
to fundamental insights into RNA catalysis.

Studies of the folding pathway of the HDV ribozyme are
preparation annotated according to ref 66 with the tertiary structure
of the postcleavage crystal structure which it models (6). The colored
nucleotides are the ones shown in detail in panel b. The ribozyme
portion consists of two separate RNA strands (A and B). Substrate
strand S3 (outlined) is cleaved at the arrow into the 5′- and 3′-
products (5′P and 3′P, respectively), and 5′P dissociates from the
ribozyme–3′-product complex. (b) Trefoil turn in J4/2 as observed in
the postcleavage crystal structure (red). C75 is positioned deep
in the catalytic core making a single hydrogen bond (orange) with
the 5′-hydroxyl leaving group of G1 (yellow), whereas G76 loops
out exposing it to solvent. Also shown as an overlay (gray) is the
more regular backbone trajectory between G76 and U79. This figure
was generated using VMD 1.7.2 and Pov-Ray 3.1g for Windows.

FIGURE 1: Sequence and structure of the HDV ribozyme. (a) Two-
dimensional representation of our synthetic trans-acting HDV
ribozyme construct D1. The ribozyme–3′-product complex is
shown annotated according to ref 66 with the tertiary structure
of the postcleavage crystal structure which it models (6). The colored
nucleotides are the ones shown in detail in panel b. The ribozyme
portion consists of two separate RNA strands (A and B). Substrate
strand S3 (outlined) is cleaved at the arrow into the 5′- and 3′-
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more regular backbone trajectory between G76 and U79. This figure
was generated using VMD 1.7.2 and Pov-Ray 3.1g for Windows.
of fluorescein- and tetramethylrhodamine-labeled RNAs were corrected for the additional absorbance of the fluorophores by using the relations $A_{260}/A_{392} = 0.3$ and $A_{260}/A_{354} = 0.49$, respectively.

Cleavage Reactions. Activities of ribozymes with G76 modifications were determined using the three-strand HDV ribozyme construct depicted in Figure 1a. All cleavage reactions were conducted under single-turnover (pre-steady-state) conditions and using standard conditions of 40 mM Tris-HCl (pH 7.5) and 11 mM MgCl₂, at 37 °C, unless otherwise stated. The ribozyme was prepared by preannealing strand A and twice the concentration of strand B in standard buffer, heating to 70 °C for 2 min, and cooling to room temperature. After preincubation at 37 °C for 15 min, a trace amount (≤4 nM) of 5′-32P-labeled substrate (also in standard buffer) was added to a saturating concentration of 400 nM ribozyme (based on the strand A concentration), unless otherwise noted. Aliquots (5 µL) were taken at the appropriate time intervals, and the reaction was quenched with 10 µL of 80% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, and 50 mM EDTA. The 5′-cleavage product was separated from the uncleaved substrate by denaturing 20% polyacrylamide, 8 M urea, gel electrophoresis, and was quantified and normalized to the sum of the substrate and product bands using a PhosphorImager Storm 840 instrument with ImageQuant software (Molecular Dynamics).

Time traces of product formation were fit to the single-exponential first-order rate equation $y = y_0 + A_1(1 - e^{-b_1t})$, employing Marquardt–Levenberg nonlinear least-squares regression (Microral Origin 6.0), where $A_1$ is the amplitude and $1/b_1$ the pseudo-first-order rate constant $k_{\text{obs}}$. To yield the cleavage rate constant $k_{\text{cleav}}$ under standard conditions, the apparent ribozyme and magnesium dissociation constants $K_{\text{Mg}}$ and $K_{\text{Mg/1/2}}$, respectively, and a cooperativity coefficient $n$ (21). For the pH dependence, cleavage was assayed in a buffer with 25 mM MES, 25 mM acetic acid, and 50 mM Tris (pH 4.0–8.0) or a buffer with 50 mM MES, 25 mM Tris, and 25 mM 2-amino-2-methyl-1-propanol (pH 8.5–9.5) (8). The pH-dependent rate constants were fit to the following equation (22)

$$k_{\text{obs}} = k_{\text{max}}/(1 + 10^{b_2 - pH}) (9).$$

The reported data are the result of three independent titrations. As a control, the fluorescence intensity of 2-aminopurine 2′-deoxyribose 5′-triphosphate (TriLink Biotechnologies) was measured.

Time-Resolved FRET Measurements. The global structure of the HDV ribozyme with AP substitution at position 76 was studied by trFRET analysis of ribozyme complexes doubly labeled with fluorescein and tetramethylrhodamine as previously described (18). Annealed ribozyme complexes (70 µL; 1 µM doubly labeled ribozyme strand B, 3 µM strand A, and 6 µM substrate, noncleavable substrate analogue, or 3′-product) were incubated at 37 °C for at least 15 min in standard buffer (supplemented with 25 mM DTT), prior to collecting time-resolved donor emission profiles using time-correlated single-photon counting, a process similar to previously described procedures (25). A frequency-doubled Nd:YVO₄ laser (Spectra-Physics Millenia Xs-P, operated at with a minimum of a 2-fold excess of unlabeled strand A) was incubated at 37 °C for at least 15 min in standard buffer and then transferred to a 150 µL cuvette. The substrate, noncleavable substrate analogue, or 3′-product was manually added at a (saturating) 5-fold excess, unless otherwise stated. For kinetic experiments, AP was excited at 300 nm (4 nm bandwidth) and fluorescence emission was monitored at 360 nm (8 nm bandwidth). The emission signal was normalized with its value immediately after formation of the ribozyme–substrate complex to obtain the relative AP fluorescence. The resulting time traces were fit similarly to the cleavage time courses, to single- or double-exponential increase and decrease functions of the form $y = y_0 + A_1(1 - e^{-b_1t}) + ...$ and $y = y_0 + A_1 e^{-b_1t} + ...$, respectively, as required.

For the HDV ribozyme substituted with AP76 and labeled with fluorescein and tetramethylrhodamine, the protocol was the following. For AP fluorescence measurements, AP was excited at 300 nm (8 nm bandwidth) and fluorescence emission was recorded at 360 nm (8 nm bandwidth). For FRET measurements, the standard buffer was supplemented with 25 mM dithiothreitol as a radical quencher. Fluorescein was excited at 490 nm (4 nm bandwidth), and fluorescence emission was recorded simultaneously at the fluorescein (520 nm, 8 nm bandwidth) and tetramethylrhodamine (585 nm, 8 nm bandwidth) wavelengths, by shifting the emission monochromator back and forth. A FRET ratio $Q = (F_{585}/F_{520})$ was calculated and normalized with its starting value to obtain the relative FRET efficiency. The resulting time traces were fit to exponential functions as described above.

pH Titration of HDV-AP76. The fluorescence intensity of AP when substituted at position 76 was measured as a function of pH. Annealed ribozyme complexes (200 nM AP-substituted ribozyme strand B, 400 nM strand A, and 1000 nM substrate, noncleavable substrate analogue, or 3′-product) were incubated at 37 °C for at least 15 min in 11 mM MgCl₂, 25 mM MES, 25 mM acetic acid, and 50 mM Tris (pH 4.0–8.0) or 25 mM sodium citrate (pH 2.0–4.0). The pH was titrated with dilute HCl, and solutions were allowed to re-equilibrate for 3 min at 37 °C after each acid addition. The AP emission spectrum was measured between 320 and 500 nm (8 nm bandwidth) upon excitation at 290 nm (8 nm bandwidth). The peak intensity at 360 nm was recorded for all pH values. The fluorescence was corrected for volume changes from the addition of dilute HCl, and the pH₅₀ value was determined by fitting the curves to the equation $F_{\text{obs}} = F_{\text{max}}/(1 + 10^{b_3 - pH})$ (9). The reported data are the result of three independent titrations. As a control, the fluorescence intensity of 2-aminopurine 2′-deoxyribose 5′-triphosphate (TriLink Biotechnologies) was measured.
9.0 W) pumped a frequency-doubled, mode-locked Ti:sapphire laser (Spectra-Physics Tsunami, operated at 1 W) that excited fluorescein at 490 nm by 2 ps width pulses, picked down to 4 MHz. Isotropic emission was detected at 520 nm (10 nm band-pass interference filter) in 4096 sampling channels, with a time increment of 12 ps/channel, up to >40000 peak counts, and under magic angle polarizer conditions. To measure donor—acceptor distances, two time-resolved fluorescence decays were collected, with and without the acceptor in place. The effect of the acceptor on the decay of fluorescein emission in the doubly labeled complex was then used to extract a three-dimensional Gaussian distance distribution between the two fluorophores as previously described in detail (18, 25, 26). To calculate a mean distance, a value of 55 Å for the Förster distance \( R_0 \) of fluorescein and tetramethylrhodamine was used (25), assuming a value of \( I_s^2 \) for the orientation factor as experimentally supported by low fluorophore anisotropies (18).

**FRET Gel Shift Assays.** FRET gel shift assays were conducted to test the homogeneity of the various ribozyme—substrate complexes, as previously described (18). Nondenaturing 10% polyacrylamide (19:1 acrylamide:bisacrylamide ratio) gels containing 40 mM Tris-HCl (pH 7.5) and 11 mM Mg(OAc)\(_2\) were assembled with the electrophoresis unit and equilibrated to 4 °C for at least 2 h. Ten picomoles of doubly fluorophore-labeled ribozyme strand B was annealed to 20 pmol of strand A by heating for 2 min to 70 °C and cooling to room temperature in 40 mM Tris-HCl (pH 7.5), 11 mM MgCl\(_2\), and 10% glycerol. The ribozyme was equilibrated at 37 °C for at least 15 min prior to addition of 50 pmol of substrate, noncleavable substrate analogue, or 3′-product (total volume of 20 μL). These samples were loaded on the gel, and an electric field of 5 V/cm was immediately applied. After electrophoresis for 24 h, the gel was scanned between its low-fluorescence glass plates in a FluorImager SI fluorescence scanner with ImageQuant software (Molecular Dynamics) as described previously (18, 27). A laser excites fluorescein at 488 nm, and the gel is scanned for fluorescence emission using a photomultiplier tube with either a 530 nm band-pass (for the donor fluorescein) or a 610 nm long-pass filter (for the acceptor tetramethylrhodamine). RNAs labeled with only fluorescein and only tetramethylrhodamine were included as color calibration standards. From the volume sampling channels, with a time increment of 12 ps/channel, up to >40000 peak counts, and under magic angle polarizer conditions. To measure donor—acceptor distances, two time-resolved fluorescence decays were collected, with and without the acceptor in place. The effect of the acceptor on the decay of fluorescein emission in the doubly labeled complex was then used to extract a three-dimensional Gaussian distance distribution between the two fluorophores as previously described in detail (18, 25, 26). To calculate a mean distance, a value of 55 Å for the Förster distance \( R_0 \) of fluorescein and tetramethylrhodamine was used (25), assuming a value of \( I_s^2 \) for the orientation factor as experimentally supported by low fluorophore anisotropies (18).

**RESULTS**

A Base Substitution of \( G_{76} \) with AP Is Tolerated in a Trans-Acting HDV Ribozyme with an Only Modest Decrease in Catalytic Activity. The trans-cleaving HDV ribozyme used in this study, D1, is a synthetic three-strand construct, which consists of ribozyme strands A and B and substrate strand S3 (Figure 1a). Previously, we characterized in detail the catalytic activity of this trans-cleaving construct under a variety of conditions (18). Our data showed that this construct has cleavage rate constants as well as metal ion, temperature, and pH dependencies similar to those of other trans-acting HDV ribozymes. To evaluate the effect that a \( G_{76} \)/AP mutation has on this construct, we measured its cleavage rate constants, as well as its metal ion and pH dependencies, and compared them to those of the D1 wild type with \( G_{76} \).

First, we measured the cleavage rate constant under standard single-turnover (pre-steady-state) reaction conditions, which consisted of trace amounts of radiolabeled S3 substrate with a saturating excess of 400 nM ribozyme in 40 mM Tris-HCl (pH 7.5) and 11 mM MgCl\(_2\) at 37 °C (see Materials and Methods). In addition, reactions were performed at ribozyme concentrations varying between 25 and 1800 nM to ensure reaching saturation. Figure 2a shows the resulting reaction time courses for the \( G_{76} \)/AP mutant. The observed pseudo-first-order rate constants \( k_{obs} \) were plotted as a function of ribozyme concentration and fit to a simple binding equation (inset of Figure 2a; see Materials and Methods), yielding a \( K_{b} \) of 0.20 min\(^{-1}\) \((K_M = 114 \text{ nM})\). (b) pH dependence of the observed cleavage rate constants. The data were fit [see Materials and Methods; D1 (—) and D1-AP\(_{76}\) (—–)] to yield the two pK\(_A\) values for each construct reported in Table 1.

**FIGURE 2:** Cleavage of substrate strand S3 by HDV ribozyme construct D1-AP\(_{76}\) under single-turnover conditions. (a) Cleavage time courses of D1-AP\(_{76}\) under standard conditions of 40 mM Tris-HCl (pH 7.5) and 11 mM MgCl\(_2\) at 37 °C, with varying ribozyme concentrations ([○] 50, [□] 100, [◇] 200, and [△] 400, (crossed box) 800, (●) 1200, and (tilted triangle) 1600 nM). Data were fit to a single-exponential increase function (—) to yield the rate constants \( k_{obs} \) reported in the inset (total fraction cleaved, ~70%). In the inset, the dependence of \( k_{obs} \) on ribozyme concentration was fit to a binding equation (—) (see Materials and Methods), yielding a \( K_{b} \) of 0.20 min\(^{-1}\) \((K_M = 114 \text{ nM})\). (b) pH dependence of the observed cleavage rate constants. The data were fit [see Materials and Methods; D1 (—) and D1-AP\(_{76}\) (—–)] to yield the two pK\(_A\) values for each construct reported in Table 1.
Local Conformational Changes of the HDV Ribozyme

Table 1: Cleavage Rate Constants, Metal Ion Dissociation Constants, and pK\textsubscript{s} for HDV Ribozyme Construct D1 and Its G76AP Mutant

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D1−S3</th>
<th>D1−AP\textsubscript{76}−S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>k\textsubscript{on} (min\textsuperscript{-1})</td>
<td>1.34 ± 0.07</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>(pH 7.5, 11 mM Mg\textsuperscript{2+}, 37 °C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg\textsubscript{2+} (mM)</td>
<td>9 ± 1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>pK\textsubscript{a1}, pK\textsubscript{e2}</td>
<td>5.7, 8.6</td>
<td>5.1, 8.7</td>
</tr>
<tr>
<td>k\textsubscript{off} (M\textsuperscript{-1} min\textsuperscript{-1})</td>
<td>ND</td>
<td>(2.4 ± 0.2) × 10\textsuperscript{6}</td>
</tr>
<tr>
<td>k\textsubscript{off} (min\textsuperscript{-1})</td>
<td>ND</td>
<td>0.20 ± 0.04</td>
</tr>
</tbody>
</table>

\* The single-turnover cleavage rate constant k\textsubscript{on} was determined with substrate strand S3 and saturating ribozyme as described in the text (standard conditions, 40 mM Tris-HCl, pH 7.5, 11 mM MgCl\textsubscript{2}, and 37 °C). Mg\textsubscript{2+} and pK\textsubscript{s} were derived as described in Materials and Methods, pK\textsubscript{a}s from the data in Figure 2b, and k\textsubscript{off} and k\textsubscript{off} from the data in panels b and c of Figure 3, respectively. ND means not determined.

Table 2: Cleavage Activity of G76 Modifications on the HDV Ribozyme

<table>
<thead>
<tr>
<th></th>
<th>k\textsubscript{obs} (min\textsuperscript{-1})</th>
<th>k\textsubscript{obs} (min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.84 ± 0.02</td>
<td>U</td>
</tr>
<tr>
<td>SU</td>
<td>0.54 ± 0.02</td>
<td>AP</td>
</tr>
<tr>
<td>I</td>
<td>0.53 ± 0.01</td>
<td>A</td>
</tr>
</tbody>
</table>

\* Observed rate constants (k\textsubscript{obs}) were measured under standard conditions (single-turnover, 40 mM Tris-HCl, pH 7.5, 11 mM MgCl\textsubscript{2}, 400 nM ribozyme, and 37 °C) for trans-cleaving HDV ribozyme construct D1 (Figure 1a); errors were estimated from at least three independent measurements.

Next, we compared Mg\textsuperscript{2+} binding affinities of the wild-type and G76AP mutant D1 constructs. Cleavage activity was measured under standard single-turnover conditions (400 nM ribozyme excess) but at varying magnesium concentrations (2–200 mM Mg\textsuperscript{2+}). The observed rate constants for the G76 wild-type ribozyme increase substantially from 0.075 to 1.47 min\textsuperscript{-1} in this range, while the G76AP mutant shows a more modest increase from 0.26 and 0.393 min\textsuperscript{-1} to 1.47 min\textsuperscript{-1} (Table 1); errors were estimated from at least three independent measurements.

Finally, we compared the pH dependence of cleavage of the wild-type and the G76AP mutant to verify that the latter utilizes a mechanism similar to that of the unmodified D1 construct. Such pH profiles have been critical in establishing the involvement of C\textsubscript{75} in the catalytic mechanism of the HDV ribozyme. Figure 2b shows the resulting curves. Both constructs show bell-shaped pH profiles similar to those of previously characterized trans-acting (22) and cis-acting HDV ribozymes (30). From the pH dependence of the wild-type G76D1 construct, two pK\textsubscript{s} of 5.7 and 8.6 are derived, while the pK\textsubscript{s} of the G76AP mutant are 5.1 and 8.7 (Figure 2b and Table 1). It is noteworthy that both pH profiles suggest that a pH-dependent step is rate-limiting at low pH (5, 31).

Steady-State 2-Aminopurine Fluorescence Assays Reveal Differences between Pre- and Postcleavage Structures and Report on the Folding Kinetics of the HDV Ribozyme. AP is a strongly fluorescent base (excitation maximum at 320 nm, emission maximum at 360 nm, quantum yield of 68%) and is highly sensitive to local stacking interactions with other bases. The fluorescence of AP is quenched when it stacks with other bases, but increases substantially when it becomes fully exposed to solvent (32–34). AP has recently been utilized as a fluorescent probe for local dynamics in other catalytic RNAs, such as the hammerhead and hairpin ribozymes (12, 23, 24). To observe localized structural changes in the catalytic core of the HDV ribozyme, we have monitored changes in AP steady-state fluorescence of our G76AP modified D1 construct (D1−AP\textsubscript{76}) upon addition of either substrate or product. In all fluorescence assays, saturating 2- and 5-fold excesses of the unmodified strand A and substrate (or product), respectively, were added under standard conditions (see Materials and Methods).

The fluorescence changes of AP\textsubscript{76} indicate that a substantial conformational rearrangement around this position accompanies catalysis (Figure 3a). In particular, addition of saturating concentrations of a chemically blocked, noncleavable substrate analogue of S3 (ncS3) to the assembled ribozyme results in a slight (~12%) decrease in relative AP fluorescence (inset of Figure 3a). This fluorescence quenching suggests that AP becomes more stacked as a result of formation of the ribozyme−substrate (precursor) complex. The pseudo-first-order rate constant for this decrease is linearly dependent on the excess concentration of ncS3, indicating that this fluorescence decrease is a direct result of substrate binding. On the basis of this linear dependence, we deduce a second-order substrate binding rate constant k\textsubscript{off} of 2.4 × 10\textsuperscript{6} M\textsuperscript{-1} min\textsuperscript{-1} (Figure 3b and Table 1), similar to a previously determined value for this ribozyme−substrate complex at 25 °C instead of 37 °C (18).

In contrast, when a saturating concentration of the cleavable substrate S3 is added, we observe an ~7-fold increase in AP fluorescence (Figure 3a). The rate constant of 0.27 min\textsuperscript{-1} for this single-exponential increase is similar to the cleavage rate constant obtained in the standard autoradiographic assay (0.17 min\textsuperscript{-1}; see above). This suggests that the observed fluorescence increase is the result of substrate cleavage, which leads to rapid dissociation of its 5′-portion (5, 18) and formation of the ribozyme−3′-product complex. Notably, initial binding of substrate, which is associated with a fluorescence decrease, results in a slight lag phase in the fluorescence increase (inset of Figure 3a).

Direct formation of the ribozyme−3′-product complex was initiated by adding a saturating excess of the 3′-product of the substrate (termed 3P, sequence of 5′-GGGUCGG-3′) to the assembled ribozyme. Previously, it has been shown that the 3′-product remains bound to the ribozyme after cleavage, while the 5′-product rapidly dissociates (4, 18). We find that addition of 3P and formation of the ribozyme−3P complex result in a fast (slightly double-exponential) ~9.5-fold increase in the relative AP\textsubscript{76} fluorescence. We estimate the rate constant for this fluorescence increase to be ~7.1 min\textsuperscript{-1} at 1 μM 3P. This substantial dequenching of its fluorescence implies that AP\textsubscript{76} becomes significantly unstacked and exposed to solvent in the ribozyme−3′-product complex. This observation is consistent with the postcleavage crystal structure of the genomic form of the cis-cleaving HDV ribozyme, which shows G\textsubscript{76} extruding into solvent (Figure 1b). Furthermore, the fluorescence increase following substrate cleavage is approximately 75% of that which occurs upon direct formation of the ribozyme−3′P complex, consistent with the 75% conversion of S3 into 3′P by the G76 AP mutant under standard cleavage conditions (Figure 2a).
due to dissociation of the substrate and replacement with this end, the ribozyme providing an avenue for assessing substrate dissociation. To this end, the ribozyme—ncS3 precursor complex was formed and subsequently chased with a 4 μM excess of 3P. This resulted in a single-exponential increase in AP76 fluorescence due to dissociation of the substrate and replacement with 3P chase (Figure 3c). The observed rate constant \(k_{on}\) of 0.20 min\(^{-1}\) is independent of the concentration of chase (inset of Figure 3c). The substrate dissociation and binding rate constants, \(k_{off}\) and \(k_{on}\), respectively, measured at 37 °C, define the equilibrium dissociation constant \(K_D = k_{off}/k_{on}\) which equals 110 nM at 37 °C, 3-fold higher than a previously determined value at 25 °C (18), as expected for a shift to a higher temperature.

The 2-Aminopurine Fluorescence as a Function of pH Reveals Significant Differences in the Chemical Environment of the Catalytic Core before and after Cleavage. Our steady-state AP76 fluorescence assays indicate that there are structural differences between the precursor and postcleavage conformations near the catalytic core of the HDV ribozyme. The AP fluorescence in the ribozyme and ribozyme—ncS3 complex is significantly quenched, presumably as a result of local stacking interactions. In contrast, significant unquenching (i.e., unstacking) of AP76 is observed upon formation of the 3'-product complex (Figure 3a). On the basis of these observations, we decided to monitor the protonation state of the N1 position of AP76 in the ribozyme and its substrate and 3'-product complexes by measuring the fluorescence intensity as a function of pH. At low pH, the relative fluorescence intensity of 2-aminopurine is reduced as a result of N1 protonation (i.e., unstacking) of AP76 by 30% (17, 35). First, we measured the pH titration curve of 2-aminopurine 2'-deoxyribose 5'-triphosphate in solution as a control, which resulted in a \(pK_a\) of 3.9 (Figure 4), consistent with a previously reported value of 3.8 (17). Next, we measured the pH titration curve of the AP76 in the ribozyme, the ribozyme—ncS3 complex, and the ribozyme—3P complex. These curves indicate clear differences in titration of AP76 that relate to its local environment (Figure 4). From the pH titration curve of the ribozyme—3'-product complex an AP76 \(pK_a\) of 4.8 is derived. Although this experimentally determined \(pK_a\) is nearly 1 pH unit higher than that of free AP in solution, the AP76 fluorescence intensity in this complex essentially titrates as the free base. This result further supports the idea that position 76 in the product complex is looped out and exposed to solvent. Conversely, the fluorescence in the ribozyme and the ribozyme—ncS3 complex is strongly quenched throughout the whole pH range. Although the low intensity of AP76 fluorescence in these constructs impedes extraction of reliable \(pK_a\)s from the titration curves, these curves show that the local environments in the free ribozyme and the ribozyme—
ncS3 complex are very different from that in the ribozyme—3’P complex. These observations are consistent with the notion that significant local structural changes around the catalytic core occur upon cleavage in the HDV ribozyme.

Synchronous 2-Aminopurine Fluorescence and Steady-State FRET Kinetics Suggest that Local and Global Conformational Changes Occur Simultaneously. Previously, we have shown by fluorescence resonance energy transfer (FRET) that this trans-acting HDV ribozyme undergoes a global conformational change upon substrate cleavage (18). However, these experiments could not reveal whether this global conformational change is accompanied by local structural rearrangements. In light of our success with AP76 as a local conformational probe near the catalytic core of the HDV ribozyme, we sought to test whether the local and global conformational changes are coupled. To this end, we utilized AP76 and FRET in concert, by employing a modified strand B of our D1 construct with the G76AP substitution and terminal 5’-fluorescein and 3’-tetramethylrhodamine labels as donor—acceptor FRET pair (Figure 1a). To ensure that all fluorophore-labeled strands are converted into complexes, we added 2- and 5-fold excesses of the unmodified strand A and substrate (or product), respectively (see Materials and Methods). Similar results were obtained when AP76 and the FRET fluorophores were incorporated separately into strands B and A, respectively, and used at equimolar concentrations (data not shown).

Figure 5a shows the changes in relative AP76 fluorescence and FRET efficiency upon addition of a 1000 nM excess of noncleavable substrate analogue, ncS3, to the assembled AP76 and FRET-labeled ribozyme. The synchronous decrease in both signals was fit to single exponentials that yield rate constants of 1.3 and 1.4 min⁻¹, respectively. These values compare well with the rate constant for the AP76 fluorescence decrease under identical conditions, but in the absence of the FRET probes (1.0 min⁻¹, Figure 3a).

The AP fluorescence and FRET changes after addition of a saturating concentration of 1000 nM of either cleavable substrate S3 or 3’P to the ribozyme show characteristics expected for formation of the ribozyme—3’-product complex. Specifically, when adding a saturating concentration of S3, we observed an increase in the relative AP fluorescence and a decrease in the relative FRET efficiency (Figure 5b). The two fluorescence changes are again synchronous and, when fit to single-exponential increase and decrease functions, respectively, yield rate constants of 0.21 and 0.24 min⁻¹, respectively. These fluorescence changes are indicative of substrate cleavage and their rate constants comparable to the ones obtained from the radioactive cleavage assay (0.17 min⁻¹) and monitoring of AP76 fluorescence in the absence of FRET fluorophores (0.27 min⁻¹).

Finally, when we directly initiated formation of the ribozyme—3’-product complex by adding 3’P to the ribozyme, a substantial increase in the relative AP fluorescence and a synchronous decrease in relative FRET efficiency were observed (Figure 5c), with rate constants of 5.2 and 5.7 min⁻¹, respectively. These values are in reasonable agreement with the value obtained from AP76 probing in the absence of FRET probes (7.1 min⁻¹). These results demonstrate that local conformational changes of the trans-acting HDV ribozyme as monitored by AP76 fluorescence occur simultaneoussly with the global conformational changes observed by FRET.

A FRET Gel Shift Assay Demonstrates the Homogeneity of the Various, Structurally Distinct Ribozyme Complexes. We utilized a previously described FRET gel mobility assay (18) to examine the homogeneity of our various ribozyme complexes. To study the effect that the G76AP substitution has on the overall folding of the ribozyme and its complexes, the doubly labeled G76AP mutant was electrophoresed on a
FRET ratio (green-shifted). This decrease in FRET efficiency shows a slower migrating band with a slightly decreased complex. Lane 4, loaded with the ribozyme substrate cleavage and formation of the ribozyme-ribozyme cleavable substrate complex, shows a band that comigrates with the ribozyme-ribozyme cleavable substrate complex, consistent with our solution assays (Figure 5a). These findings further support the idea that the precursor conformation is structurally distinct from the postcleavage ribozyme complex.

Time-Resolved FRET Reveals the Global Architectures of the Precursor and Product Complexes. To quantify the global structural changes in the trans-acting G76AP mutant ribozyme upon substrate cleavage, we used time-resolved FRET (tr-FRET) to measure the donor–acceptor distances in the FRET-labeled ribozyme and its substrate and 3′-product complexes as previously described (18). To this end, we recorded the time-resolved donor (fluorescein) decay curves of the ribozyme (D1-AP3′S3), ribozyme–noncleavable substrate complex (D1-AP3′ncS3), and ribozyme–3′-product (D1-AP3′P) complex, each of them singly labeled with donor as well as doubly labeled with donor and acceptor. The fluorescence decay curves of the donor-only-labeled complexes are nearly identical, and all show mean lifetimes of ~4 ns (data not shown). We have shown previously that the anisotropies of the terminally attached donor and acceptor fluorophores in all HDV ribozyme complexes are low and similar in magnitude (18). This suggests that a decrease in donor lifetime in the doubly labeled complex is due to FRET and can be used to calculate donor–acceptor distances. Our tr-FRET data analysis (see Materials and Methods) confirms our results from steady-state FRET in solution and in gels, indicating that there are significant differences in the global architectures, particularly between the ribozyme–substrate and ribozyme–3′-product complexes. In particular, in the free D1-AP76 mutant ribozyme, the donor–acceptor distance distribution is centered around a mean distance of 48 Å (full width at half-maximum, fwhm, of 25 Å), in the D1-AP76–ncS3 complex around 52 Å (fwhm of 21 Å), and in the D1-AP76–3′P complex around 65 Å (fwhm of 33 Å) (Table 3). The full width at half-maximum, which is a measure of structural flexibility, suggests that a relatively rigid conformation is acquired prior to cleavage, while the ribozyme–3′-product complex adopts a more flexible conformation upon product formation. Similar results were obtained when the measurements were carried out at 25 °C rather than under standard conditions at 37 °C (data not shown). These results confirm that our preparations are indeed structurally homogeneous, since in all three cases a single distance distribution fits the decay data well, as judged by the residuals and the reduced χ² values (<1.2). The tr-FRET data directly support our steady-state fluorescence measurements in that the ribozyme becomes slightly more extended (by 4 Å) when it binds substrate, while it becomes significantly more extended (by 13 Å) upon cleavage and formation of the 3′-product complex.

**DISCUSSION**

Biological catalysis depends on the proper positioning of functional groups of an enzyme to increase their “effective
loop quenched when it stacks on any of the natural nucleobases responsive element (interactions in J4/2. Replacement of G76 is particularly becomes a probe for rearrangements in local stacking trans-acting HDV ribozyme construct D1 (Figure 1a), it becomes a probe for complex, the trefoil turn forms and the global conformational changes indicated by fluorescence resonance energy transfer (FRET), respectively. 2-Aminopurine in position 76 (AP76) becomes strongly quenched as a consequence of folding of the trefoil turn in J4/2 (red). At the same time, the distance between a terminal donor (D) and acceptor (A) fluorophore pair increases by ~25%, and we observe a faster migrating, green-shifted FRET gel shift band (bottom).

concentration” and lower the entropic cost of their interaction with the substrate (31). RNA catalysts are no exception, as exemplified in the ribozyme from the human hepatitis delta virus. The crystal structure of the self-cleaved product form of the genomic HDV ribozyme (6) shows a tightly interwoven nested double-pseudoknot structure with five Watson–Crick base-paired segments (P1, P1.1, P2, P3, and P4) (Figure 1a). In addition, it reveals a trefoil turn in the single-stranded region connecting P4 and P2 (termed J4/2) that pushes the catalytically functional C75 within hydrogen bonding distance (~2.7 Å) of the 5′-OH leaving group of the transesterification reaction (Figure 1b). The sequence upstream of the leaving group and including the scissile phosphate (the 5′-product, 5′P; Figure 1a) is absent from the crystal structure, and there is no high-resolution structural information available yet for the reaction precursor (5). Our data demonstrate that a trans-acting form of the HDV ribozyme, which allows us to site-specifically incorporate fluorophores as structural probes, undergoes a substantial conformational rearrangement in J4/2 from the precursor to the product complex. In the course of this local rearrangement, the trefoil turn forms and the global conformational changes, as observed by fluorescence resonance energy transfer and summarized in Figure 7.

AP76 Reports Formation of the Trefoil Turn in J4/2 upon Cleavage. 2-Aminopurine is a fluorescent guanine analogue that has previously been used to monitor local structural changes in a number of RNAs, including the hammerhead (23, 36) and hairpin ribozymes (12, 24), the HIV Rev responsive element (37), aptamers (38), hairpins (39), and loop–loop kissing complexes (40). Its fluorescence is quenched when it stacks on any of the natural nucleobases (33, 34). When AP is incorporated in place of G76 of our trans-acting HDV ribozyme construct D1 (Figure 1a), it becomes a probe for rearrangements in local stacking interactions in J4/2. Replacement of G76 is particularly appealing since (a) it only modestly perturbs the catalytic activity of the ribozyme (Tables 1 and 2) as expected from previous mutational analyses (7) and (b) in the postcleavage crystal structure this nucleotide is unstacked and extrudes into solvent, forming the trefoil turn motif that positions the adjacent C75 for general acid (or base) catalysis (Figure 1b).

That is exactly what we observe with AP76. Upon formation of the ribozyme–3′-product complex through substrate cleavage or direct 3′P binding, the fluorescence of AP76 becomes unquenched (Figure 3a); i.e., the base becomes solvent-exposed. In addition, the fluorescence of AP76 strongly increases with pH, like that of the free nucleoside triphosphate, but with an upward shift in pKₐ of 0.9 unit (Figure 4), as expected for incorporation of 2-aminopurine into a highly negatively charged RNA (41–43).

In contrast, the ribozyme–noncleavable substrate analogue (ncS3) complex shows the AP76 fluorescence strongly quenched, i.e., the base stacked (Figure 3a), independent of pH (Figure 4). Since AP is quenched by any nucleobase, we cannot derive direct structural information from these data, but it is reasonable to assume that AP76 stacks between the adjacent C75 and A77 (Figure 1a). Without the extrusion of nucleotide 76 into solvent, the trefoil turn of J4/2 with its sharp double reversal in the RNA backbone trajectory cannot form, and presumably, a more regular backbone trajectory will be adopted, as suggested in Figure 1b. As a result, C75 will not be within hydrogen bonding distance of the scissile phosphodiester or the phosphate backbone of P1.1 (Figure 1) and cannot be expected to have the strongly shifted pKₐ that this cytosine would need to act as a general acid in phosphodiester transfer (5, 6, 9, 10). This conclusion is in accord with a recent NMR study that showed that the microscopic pKₐ of C75 in the precursor of the genomic HDV ribozyme is not shifted toward neutrality (44). The same NMR study showed an upward pKₐ shift for C75 in the product form of only up to 0.6 unit compared to the free nucleoside. This is similar to the 0.9 unit upward shift in pKₐ that we observe by pH titration of AP fluorescence for the adjacent AP76 in the ribozyme–3′-product complex (Figure 4) and suggests that, electrostatically, the local environments of the two bases are rather similar.

Thus, our data indicate a substantial rearrangement of the catalytic core of the HDV ribozyme along the cleavage reaction trajectory from the precursor to the product form (Figure 7). The trefoil turn in J4/2, as detected by a strongly unquenched AP76 fluorescence, is absent from the precursor complex of our trans-acting construct, but clearly formed in the product complex. The unusual pKₐ shift of C75 as derived from pH profiles of the cleavage kinetics (8–10, 30, 45, 46) may then relate to a rate-limiting, short-lived pKₐ shift in the transition state of the reaction, as proposed previously (5, 44). It is important to note that our FRET gel shift (Figure 6) and time-resolved FRET data (Table 3) suggest that both our precursor and product complexes are structurally homogeneous. In addition, we have independent footprinting data indicating that paired regions P1–P4 are properly formed in both structures, and that P1.1 is formed in the 3′-product complex (S. Jeong and N. G. Walter, unpublished data), as observed in the 3′-product crystal structure (Figures 1a and 7). Rapid structural dynamics which may control access to the transition state, however, will not be resolved by these techniques.

Formation of the Trefoil Turn Coincides with a Previously Observed Change in Global Conformation; Do These Conformational Changes Limit the Overall Reaction Kinetics? Global and local conformational changes in RNA are often coupled. Due to the hierarchical nature of RNA folding [secondary structure forms before tertiary structure (47)],
independently folding secondary structures often have to readjust locally (by induced fit) to establish a global tertiary structure. Examples are the hairpin ribozyme (13, 24, 48, 49) or the P5abec domain of the Tetrahymena group I intron ribozyme (50). In previous work, we have shown that the trans-acting HDV ribozyme undergoes a global conformational change upon cleavage, leading to an increase in the distance between the termini of helices P4 and P2 that can be observed as a decrease in the extent of FRET between a donor—acceptor fluorophore pair (18). Here, we have expanded this analysis by employing AP fluorescence and FRET in concert and have shown that the formation of the trefoil turn in J4/2 kinetically coincides with the global change in the P4—P2 distance (Figures 5 and 7). Similar measurements on the hairpin ribozyme have demonstrated the kinetic coincidence of local secondary structure readjustments upon formation of long-range tertiary contacts between its domain A and its domain B (24). This induced fit involves base flipping of G_{24} immediately 3′ of the cleavage site in domain A to make a Watson—Crick base pair with C_{25} in domain B, which positions the cleavage site backbone for catalysis (13).

In the absence of a high-resolution structure for the precursor of the HDV ribozyme, it is less evident how the local and global conformational changes observed here relate to placement of the scissile phosphate for catalysis. It has been pointed out previously that the 5′-hydroxyl leaving group is deeply buried within the catalytic cleft of the 3′-product crystal structure and that accommodation of a sequence 5′ to the cleavage site may be sterically strenuous (51). Consistent with this notion, Been and co-workers have found that sequence components immediately 5′ to the cleavage site destabilize ground-state binding. This ground-state destabilization appears to contribute up to 2 kcal/mol cleavage site destabilize ground-state binding. This ground-state destabilization appears to contribute up to 2 kcal/mol.

The P4—P2 distance between the termini of helices P4 and P2 can be observed as a decrease in the extent of FRET between a donor—acceptor fluorophore pair (18). Our capture of significant conformational changes along the reaction trajectory from the precursor to the product forms of the trans-acting HDV ribozyme raises the possibility that these may limit the observed cleavage rate constant. This notion certainly has precedence in other small catalytic RNAs, as cleavage by the hairpin ribozyme is rate-limited by a combination of global conformational transitions and reversible chemistry (49), while hammerhead ribozyme activity appears to be limited by a local base flipping event at the cleavage site (52). Access to a structurally constrained transition state may also limit the rate of product formation in the trans-acting HDV ribozyme. Our pH profiles in Figure 2b present indirect evidence for this idea; our ~5-fold slower G_{76}AP mutant has an apparent pK_a that is 0.6 unit lower than that of the G_{76} wild type (5.1 vs 5.7, Table 1). This is consistent with a pH-dependent step being rate-limiting at low pH and a pH-independent conformational change (5-fold slower for the G_{76}AP mutant than for the wild type) becoming rate-determining above pH 5.1 and 5.7, respectively. Interestingly, single-turnover rate constants of all trans-acting HDV ribozymes (0.1—0.9 min⁻¹ at 37°C; see this work as well as refs 4, 29, 53, and 54) fall into the same range as those for the hairpin (55—57) and most hammerhead ribozymes (58—60) which both are rate-limited by conformational changes. In contrast, cleavage in the cis-acting genomic and antigenomic HDV ribozymes is considerably faster with rate constants of ~8—20 min⁻¹ (5, 8, 9), typically after elaborate preannealing protocols (30, 61). Faster cleavage by the cis-acting ribozymes does not imply that the conformational change observed in the trans-acting ribozymes is absent, but only that it is considerably faster and/or occurs in a fast pre-equilibrium, perhaps due to a higher probability of proper positioning of all functional groups for catalysis. This may be accomplished by the helical crossover at the top of helix P1 that distinguishes cis-acting from most trans-acting HDV ribozymes.

Finally, there is evidence that other local conformational changes in the catalytic core of the HDV ribozyme may occur when it folds into a catalytic structure. For example, an NMR structure of the isolated closing loop of P3 (Figure 1a) revealed a base paring pattern very different from that observed in the 3′-product crystal structure (62); formation of P1.1 will be critical to shifting the equilibrium from one base pairing scheme to the other. In fact, a more recent NMR study has found evidence for local conformational changes around many nucleotides in two trans-acting HDV ribozymes (63). In addition, differential photo-cross-links to J4/2 and P3 and its closing loop were observed in monovalent versus divalent cations, suggesting a metal ion-dependent conformational change near the cleavage site (64). Similarly, the accessibility of the closing loop of P3, as probed by an oligonucleotide hybridization assay, decreased upon addition of magnesium, presumably due to the formation of P1.1 (65). Further experimental work will have to clarify whether structural changes in this loop are linked to the local rearrangement of J4/2 upon cleavage as described here.

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