For the article “Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium,” by Miho K. Furue, Jie Na, Jamie P. Jackson, Tetsuji Okamoto, Mark Jones, Duncan Baker, Ryu-Ichiro Hata, Harry D. Moore, J. Denry Sato, and Peter W. Andrews, which appeared in issue 36, September 9, 2008, of Proc Natl Acad Sci USA (105:13409–13414; first published August 25, 2008; 10.1073/pnas.0806136105), the authors note that in Fig. 7D, Q-PCR data were inadvertently duplicated. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

**CELL BIOLOGY.** For the article “Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium,” by Miho K. Furue, Jie Na, Jamie P. Jackson, Tetsuji Okamoto, Mark Jones, Duncan Baker, Ryu-Ichiro Hata, Harry D. Moore, J. Denry Sato, and Peter W. Andrews, which appeared in issue 36, September 9, 2008, of Proc Natl Acad Sci USA (105:13409–13414; first published August 25, 2008; 10.1073/pnas.0806136105), the authors note that in Fig. 7D, Q-PCR data were inadvertently duplicated. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

**BIOPHYSICS.** For the article “Single-molecule studies of group II intron ribozymes,” by Miriam Steiner, Krishanthi S. Karunatilaka, Roland K. O. Sigel, and David Rueda, which appeared in issue 37, September 16, 2008, of Proc Natl Acad Sci USA (105:13853–13858; first published September 4, 2008; 10.1073/pnas.0804034105), the authors note that Miriam Steiner should have been included among those credited with designing the research. The corrected author contributions footnote appears below.
RNA molecules exhibit very diverse and fascinating folding pathways from random coil to native state. Smaller RNAs fold rapidly and smoothly after an apparent two-state process (1), whereas large RNAs sample intermediate conformations before reaching the active structure (2). Generally, a fast (1–50 ms) overall compaction into a stable but misfolded kinetic trap is found in an early stage of the folding pathway (1, 3–7) followed by slower rate-limiting folding to the native state (2).

Group II introns rank among the largest ribozymes in nature and display remarkable features distinct from other large RNAs (8, 9). These ribozymes are self-splicing multidomain RNA molecules exhibiting a rich evolutionary heritage, the parallels in structure and catalytic mechanism with spliceosomal RNA being only one example (9–11). Their overall secondary structure organization into six modular domains is generally preserved (Fig. 1A) (8). Only nucleotides involved in tertiary contacts are conserved, but these are dispersed throughout the secondary structure, unlike in other large RNAs (12, 13). Another specific property of group II intron ribozymes is the overall high Mg2+ requirement for maximal activity in vitro (14, 15).

The D135 ribozyme is derived from the Saccharomyces cerevisiae group II intron Sc:ai5y and contains the necessary components for catalysis (9), allowing efficient and selective cleavage of RNA substrates with multiple turnover (14). This ribozyme has been used as a model system to study folding and metal ion binding of larger RNAs (14, 16, 17). Until now, the folding pathway of D135 has been described as an apparent two-step process involving one obligatory intermediate (U ↔ I ↔ N) under optimal conditions for splicing (42°C, 500 mM KCl, 100 mM MgCl2) (17–19). The unfolded state U is an extended but structurally defined conformer of D135 in the presence of monovalent ions and possesses extensive secondary structure but no tertiary interactions (17, 19). The first folding step (kobs ~ 1 min−1) consists of a critical rate-limiting compaction within domain 1 (D1, Fig. 1) to a folding intermediate I (17, 19, 20). The second step of domain assembly is not well characterized, but it is generally believed that the remaining domains fold rapidly into their native position onto the D1 scaffold (18).

Unlike other large RNAs, the group II intron folding is unique because the initial tertiary collapse of D135 is slow and I is not a kinetic trap but an obligatory folding intermediate (19). Structural and dynamic information about this intermediate is lacking. To elucidate the structural dynamics of the second folding step by single-molecule Förster resonance energy transfer (FRET), we have designed a fluorophore-labeled D135 construct (D135–L14) by introducing two modular loops (21) into D1 and D4 that specifically bind two DNA-oligonucleotides carrying the FRET pair Cy3 (Cy3-DNA) and Cy5 (Cy5-DNA), respectively [Fig. 1A and supporting information (SI) Figs. S1 and S2]. Our single-molecule experiments reveal three distinct conformations of D135–L14 in dynamic equilibrium under a wide range of Mg2+ concentrations and a previously undetected on-pathway folding intermediate. The native conformation is scarce and becomes apparent only at 20 mM Mg2+ or higher. However, this high-FRET state is stabilized by substrate binding, indicating that this is actually the catalytically active conformation in vitro. The folding rates between these conformations are fast and exhibit only a subtle Mg2+ dependence, in agreement with a recent ensemble averaged study (22), indicating that these conformations are connected by small activation barriers with similar, but not identical, Mg2+ affinities. This is the largest protein-free ribozyme studied by single-molecule fluorescence so far and exemplifies how single-molecule approaches can be applied to characterize the folding pathway of very large ribozymes in detail. Interestingly, the ribozyme dynamics increase with Mg2+ concentration, making the active state accessible.

Results

Fluorophore-Labeled D135–L14 Ribozyme Is Catalytically Competent and Requires Mg2+ for Folding. We tested the activity of the in vitro transcribed (23) D135–L14 construct in a single-turnover cleavage assay with the substrate 17/7 under optimal conditions for self-splicing (17) (Fig. S3A and SI Results). The labeling scheme only minimally affects the cleavage activity of the ribozyme. D135–L14 yields a cleavage rate constant kobs = 0.37 ± 0.02 min−1, which is reduced in the presence of the fluorophore- and biotin-labeled DNA-oligonucleotides by only 27%. The final amount of product formation was 85% for all constructs. kobs depends on [Mg2+] giving a dissociation constant KMg = 52.7 ± 3.4 mM, comparable with reported values (9, 14).

Folding in bulk was also measured by FRET (Fig. 2). Upon addition of Mg2+ ions to fluorophore-labeled D135–L14, the donor intensity decreases while the acceptor increases (Fig. 2A). The overall FRET ratio increases exponentially with kobs = 0.90 ± 0.07 min−1 (100 mM MgCl2, Fig. 2B), in agreement with reported values (14, 18, 19). The FRET increase suggests that a conformational change takes place in the presence of Mg2+, during which the relative distance between the fluorophores on the RNA decreases. The observed FRET increase is Mg2+-dependent, giving again a KMg = 42.3 ± 1.3 mM (Fig. 2C).

Finally, we confirmed efficient and specific binding between the

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**Single-molecule studies of group II intron ribozymes**

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**Abstract**

Group II intron ribozymes fold into their native structure by a unique stepwise process that involves an initial slow compaction followed by fast formation of the native state in a Mg2+-dependent manner. Single-molecule fluorescence reveals three distinct on-pathway conformations in dynamic equilibrium connected by relatively small activation barriers. From a most stable near-native state, the unfolded state is the overall high Mg2+ requirement for maximal activity in vitro (14, 15).

The D135 ribozyme is derived from the Saccharomyces cerevisiae group II intron Sc:ai5y and contains the necessary components for catalysis (9), allowing efficient and selective cleavage of RNA substrates with multiple turnover (14). This ribozyme has been used as a model system to study folding and metal ion binding of larger RNAs (14, 16, 17). Until now, the folding pathway of D135 has been described as an apparent two-step process involving one obligatory intermediate (U ↔ I ↔ N) under optimal conditions for splicing (42°C, 500 mM KCl, 100 mM MgCl2) (17–19). The unfolded state U is an extended but structurally defined conformer of D135 in the presence of monovalent ions and possesses extensive secondary structure but no tertiary interactions (17, 19). The first folding step (kobs ~ 1 min−1) consists of a critical rate-limiting compaction within domain 1 (D1, Fig. 1) to a folding intermediate I (17, 19, 20). The second step of domain assembly is not well characterized, but it is generally believed that the remaining domains fold rapidly into their native position onto the D1 scaffold (18).

Unlike other large RNAs, the group II intron folding is unique because the initial tertiary collapse of D135 is slow and I is not a kinetic trap but an obligatory folding intermediate (19). Structural and dynamic information about this intermediate is lacking. To elucidate the structural dynamics of the second folding step by single-molecule Förster resonance energy transfer (FRET), we have designed a fluorophore-labeled D135 construct (D135–L14) by introducing two modular loops (21) into D1 and D4 that specifically bind two DNA-oligonucleotides carrying the FRET pair Cy3 (Cy3-DNA) and Cy5 (Cy5-DNA), respectively [Fig. 1A and supporting information (SI) Figs. S1 and S2]. Our single-molecule experiments reveal three distinct conformations of D135–L14 in dynamic equilibrium under a wide range of Mg2+ concentrations and a previously undetected on-pathway folding intermediate. The native conformation is scarce and becomes apparent only at 20 mM Mg2+ or higher. However, this high-FRET state is stabilized by substrate binding, indicating that this is actually the catalytically active conformation in vitro. The folding rates between these conformations are fast and exhibit only a subtle Mg2+ dependence, in agreement with a recent ensemble averaged study (22), indicating that these conformations are connected by small activation barriers with similar, but not identical, Mg2+ affinities. This is the largest protein-free ribozyme studied by single-molecule fluorescence so far and exemplifies how single-molecule approaches can be applied to characterize the folding pathway of very large ribozymes in detail. Interestingly, the ribozyme dynamics increase with Mg2+ concentration, making the active state accessible.
DNA-oligonucleotides and D135–L14 by nondenaturing gel electrophoresis (Fig. S2).

Taken together, the above results demonstrate that the fluorophore-labeled D135–L14 behaves like the D135 ribozyme regarding catalysis, folding, and Mg\textsuperscript{2+} requirement.

**Single-Molecule FRET Reveals (smFRET) Three Conformations in Equilibrium.** Single-molecule FRET offers the potential to monitor and identify transient folding intermediates without the need for synchronization and can help elucidate the folding pathways of ribozymes. Here, we have used an approach similar to our earlier studies with the hairpin ribozyme (24, 25) to study the folding dynamics of the much larger D135–L14 ribozyme.

Fluorophore-labeled D135–L14 ribozymes were incubated in the presence of specific amounts of Mg\textsuperscript{2+} at 42°C to induce folding before immobilization on the quartz surface via a biotinylated DNA-oligonucleotide hybridized to the 3’ end tail (Figs. 1A and 3/4 and Fig. S1). Characteristic time trajectories of such surface immobilized D135–L14 ribozymes in 10 mM and 100 mM Mg\textsuperscript{2+} at room temperature are shown in Fig. 3 B and C. The observed FRET ratios jump stochastically between three values \(\approx 0.25\), \(\approx 0.4\), and \(\approx 0.6\). Hence, the D135–L14 ribozyme adopts at least three distinguishable structural conformations [extended intermediate (I), folded intermediate (F), and native (N)] in dynamic equilibrium. We assigned the low-FRET state to I and not U (Fig. 1B) for the following reasons: (i) a minimal FRET for U and low FRET for I are expected based on their hydrodynamic radii (17); (ii) very few molecules, if any, should be in the U state at [Mg\textsuperscript{2+}] > 20 mM because the RNA is incubated with Mg\textsuperscript{2+} before our measurements; (iii) no unfolding from I is observed in accordance with a much slower I to U transition than the duration of our time traces (<200 s, Fig. 3 B and C). In an attempt to assign a FRET value for U and to support our assignments, we measured single-molecule time trajectories of D135–L14 in the absence of Mg\textsuperscript{2+} and in the presence of 10 \(\mu\)M EDTA (Fig. S4). Under these conditions, only the U state is present (20). Indeed, in the absence of Mg\textsuperscript{2+}, nearly all of the observed molecules remain static in a 0.1 FRET state, in agreement with our assignments. However, we cannot distinguish these molecules from a small fraction that lacks the acceptor fluorophore (photobleaching; Fig. 4 Top), making it impossible to quantify the fraction of molecules in the U state. Therefore, we did not include molecules in the U state in subsequent analysis.

These conformational states could not be resolved in the bulk FRET experiment because it yields an ensemble-averaged result. Furthermore, the single-molecule data show that a large fraction of molecules remain static in the low FRET state (see below), explaining the observed low FRET in bulk.

At low [Mg\textsuperscript{2+}], only the two lower FRET states are observed. The highest FRET state becomes apparent at [Mg\textsuperscript{2+}] > 15–20 mM (Fig. 4) but is populated transiently, and the few molecules that reach it return promptly to the intermediate state. Among the 10,000 total transitions observed, only 1 % go directly from the I state to the N state, consistent with the contribution from very short dwell times in the F state, which we miss because of our 33-ms time resolution. This observation is thus direct evidence that the F state is an obligatory intermediate along the folding pathway and is devoid of any kinetic traps.

**Magnesium Ions Affect Folding Equilibrium of the Group II Intron Ribozyme.** Mg\textsuperscript{2+} ions affect the relative stability of each conformation, as evidenced by the FRET time trajectories and the dwell time spent in each state. At 10 mM Mg\textsuperscript{2+} and below, dynamic molecules stay predominantly in the I state, whereas at 100 mM Mg\textsuperscript{2+} the F state prevails (Fig. 3 B and C). To quantify the effect of Mg\textsuperscript{2+} on the stability of each state, we calculated FRET distributions from \(\approx 10^5\) single-molecule trajectories at Mg\textsuperscript{2+} concentrations ranging from 1 to 100 mM (Fig. 4). At 1 mM Mg\textsuperscript{2+} the D135–L14 ribozyme resides exclusively in the I state, and no structural dynamics are observed. We can easily distinguish this state from complexes lacking the acceptor fluorophore (caused by photobleaching or blinking) because the observed FRET ratio for the latter molecules is \(\approx 0.1\) (Fig. 4, Top). The distributions for the F and N states become apparent only above 10 and 20 mM Mg\textsuperscript{2+}, respectively (Figs. 3 B and C and 4). The N state is never populated for extended times, which is reflected as a low-amplitude distribution in the histograms. The amplitude of the F state distribution...
A syringe pump to increase the Mg⁡²⁺ and 58 s we exchanged the buffer in the microscope slide by using a quartz slide and placed in the microscope. The fluorophores are excited by the laser’s evanescent wave above the critical angle, giving emission signals collected through the objective with a high-quantum yield camera. (A) smFRET analysis of D135–L14 ribozymes. (A) Principle of total internal reflection fluorescence spectroscopy (TIRF). Molecules are immobilized on a quartz slide and placed in the microscope. The fluorophores are excited by the laser's evanescent wave above the critical angle, giving emission signals collected through the objective with a high-quantum yield camera. (B) smFRET time trace and histogram at 10 mM Mg²⁺ showing two conformational states at 0.25 and 0.4. (C) smFRET time trace and histogram at 100 mM Mg²⁺ sampling the three FRET states 0.25, 0.4, and 0.6. (D) (Left) smFRET time trace shows a dynamic behavior only upon increase in MgCl₂ concentration from 10 to 100 mM at 50–58 s. (Right) Distributions of the states before and after the addition of Mg²⁺.

Fig. 3. smFRET analysis of D135–L14 ribozymes. (A) Principle of total internal reflection fluorescence spectroscopy (TIRF). Molecules are immobilized on a quartz slide and placed in the microscope. The fluorophores are excited by the laser’s evanescent wave above the critical angle, giving emission signals collected through the objective with a high-quantum yield camera. (B) smFRET time trace and histogram at 10 mM Mg²⁺ showing two conformational states at 0.25 and 0.4. (C) smFRET time trace and histogram at 100 mM Mg²⁺ sampling the three FRET states 0.25, 0.4, and 0.6. (D) (Left) smFRET time trace shows a dynamic behavior only upon increase in MgCl₂ concentration from 10 to 100 mM at 50–58 s. (Right) Distributions of the states before and after the addition of Mg²⁺.

increases from 0.04 to 0.08 with increasing [Mg²⁺], whereas the amplitude of the low FRET decreases from 0.23 to 0.06. This indicates that the relative stability of the F state increases concomitantly with the decrease of the I state. This antiparallel behavior, however, is not very dramatic because both states are still present between 10 and 100 mM Mg²⁺. The N state distribution amplitude remains constant above ~50 mM Mg²⁺ (see below).

To learn more about the Mg²⁺ requirement for these structural dynamics, we monitored the folding of a single D135–L14 ribozyme while changing the buffer conditions from 10 to 100 mM Mg²⁺ (Fig. 3D). During the first 50 s, the ribozyme kept in 10 mM Mg²⁺ displays a FRET ratio ~0.25 (I state) matching the FRET histogram obtained under steady-state conditions (Fig. 4 Top). Between 50 and 58 s we exchanged the buffer in the microscope slide by using a syringe pump to increase the Mg²⁺ concentration to 100 mM. After a dead time of ~10 s, the FRET ratio jumps to 0.4, indicating that a Mg²⁺-induced conformational change to the F state takes place. The structural dynamics of the ribozyme increase as evidenced by FRET jumps between the 0.25 and 0.4 FRET states. The FRET histogram corresponding to the high-[Mg²⁺] period also matches the FRET histogram obtained in steady-state conditions (Fig. 4 Bottom). This experiment clearly shows that the individual FRET states are directly dependent on the Mg²⁺ concentration and that the D135–L14 ribozyme readily reacts to an increased Mg²⁺ background by reaching a higher FRET state.

The presence of both the F and N states only above 20 mM Mg²⁺ coincides with the onset of catalytic activity (compare Fig. 4 and Fig. S3), suggesting that one of these two conformations corresponds to the catalytically active structure. It has been suggested that D135 collapses directly to the active state with a midpoint of ~20–40 mM Mg²⁺ (14).

Peaks of the FRET Histogram Distributions Decrease Slightly with Mg²⁺. Close inspection of the histograms (Fig. 4) reveals that the peak distributions of the I and F states shift to slightly lower FRET values with increasing Mg²⁺. The I state distribution shifts from ~0.27 (1 mM Mg²⁺) to 0.24 (20 mM Mg²⁺), whereas the F state distribution shifts from 0.45 (10 Mg²⁺) to 0.40 (≥40 mM Mg²⁺). At lower Mg²⁺ concentrations, the F and N states are possibly in fast dynamic equilibrium (lifetime <30 ms), and as a result we observe only a weighted-average FRET ratio between F and N. Alternatively, the I and F states each become structurally less compact above 1 mM and 20 mM Mg²⁺, respectively, giving rise to a shorter distance between the two fluorophores. Neither of the two hypotheses can be ruled out at the moment, but this result supports the idea that the group II intron conformations are connected by relatively small activation barriers.
The double-exponential growth fit is shown as a solid line. (H9261 and 52x333) in the 0.25 FRET state at 50 mM Mg2+

The occurrence of the 0.4 state only at higher [Mg2+] clearly assigns the I and F state to folded D1 and compact D135, respectively.

**Mg2+ Ions Increase Structural Dynamics to Form the Active State.** It is generally assumed that the addition of Mg2+ to RNA initiates folding and leads to a compact and more rigid structure. Here, we detect the opposite and so far unobserved behavior. Although Mg2+ clearly initiates folding of D135 leading to the more compact I, F, and N states, the structural dynamics increase considerably with higher [Mg2+]. At very low [Mg2+] the molecules remain static in the I state, but at higher concentrations an increasing number of transitions among the three states is observed. The static molecules depict constant FRET values of \( \approx 0.25 \) (i.e., the I state) at all Mg2+ concentrations \( > 1 \) mM tested (compare Fig. 4 and Fig. S5). It is possible that these static molecules correspond to a subpopulation of molecules that display a very slow \( k_1 (\approx 0.005 \text{ s}^{-1}) \), slower than our \( \approx 200 \text{ s} \) observation window, but we cannot distinguish between these two possibilities. The fraction of dynamic molecules versus [Mg2+] (Fig. 6A) yields a dissociation constant \( K_{Mg} = 41.4 \pm 1.4 \) mM, which coincides with the values for bulk folding and cleavage (Fig. 2C and SI Results). Even at high [Mg2+], the fraction of dynamic molecules does not exceed 50% in apparent discrepancy with the cleavage experiments of the same D135–L14 construct (see above). However, the cleavage assays are conducted at 42°C, whereas the smFRET experiments are done at 22°C for technical reasons. This finding suggests that the higher temperature is needed to enable the remaining molecules reach the active conformation. Taken together, these two results clearly link the structural dynamics of D135 to its function, raising the interesting possibility that domain motion contributes to successful reactivity.

**Dwell-Time Analysis Determines the Rates of Folding.** To quantify the relative stability of each conformation, we determined the rates of folding \( k_1, k_{-1}, k_2, \) and \( k_{-2} \) for the ribozyme. The dwell time in each state between two conformational transitions from \( \approx 150 \) single-molecule time trajectories was measured, and dwell time distributions for each state were calculated. Static molecules in the I state could not be included in this analysis. Dwell times in the 0.4 FRET state were divided into two distributions depending on the final state (I or N) to determine \( k_{-1} \) and \( k_2 \), respectively. Fig. 6B shows a characteristic dwell time (\( t_\text{d} \)) distribution for the I state in 50 mM Mg2+. The distribution decays exponentially as expected for a stochastic process. To obtain \( k_1 \), we integrated the distribution into a cumulative time distribution (Fig. 6C), which was then fit to an exponential growth curve, as described (24, 25, 27). The integrated distribution reveals folding heterogeneity in the form of two distinguishable exponentials, as observed for other ribozymes (25, 28–30). The major component exhibits an exponential rate \( k_1 = 0.66 \text{ s}^{-1} \). The folding rate \( k_{-1} = 0.51 \text{ s}^{-1} \) obtained similarly also exhibited heterogeneity. The measured rates in 100 mM Mg2+ are in agreement with the FRET histogram distributions (Fig. 4A).

**Substrate Binding Stabilizes the 0.6-FRET Conformation.** To determine which state (F or N) represents the active conformation, we performed smFRET studies in the presence of the substrates 17/7 and 17/7-dC (2′-deoxy at cleavage site) (26). The slow cleavage of 17/7-dC \( \left( k_{\text{obs}} = 0.05 \pm 0.01 \text{ min}^{-1} \right) \) enabled us to observe the substrate-bound D135–L14 molecules for longer periods of time. The presence of either 17/7 or 17/7-dC clearly stabilizes N (Fig. 5), suggesting that the high-FRET state corresponds to the active conformation. Consequently, F represents a hitherto undetected obligatory intermediate, or near-native state, from which only few molecules reach the active state at a time. The formation of the \( \kappa\alpha\gamma\) and \( \lambda\alpha\lambda \) tertiary contacts and the two intron and exon binding sequences (IBS-EBBS) interactions between the substrate and D1 appear to be essential to stabilize the active conformation. The wild-type (WT) substrate 17/7 leads to a higher occurrence of the 0.6 state compared with the 17/7-dC oligonucleotide (Fig. 5A). A plausible explanation for this observation could be that the 2′-OH at the cleavage site makes contacts that contribute to assembly of the catalytic core and hence also to the overall architecture of the ribozyme.

The assignment of the 0.25 state to I (D1-folded), the 0.4 state to F (compact D135), and the 0.6 state to the native structure is further supported by a recent study (22). Under low-salt conditions, D1 folds to the near-native state to which D3 and D5 can dock. However, docking is not stable, and the folding pathway arrests primarily at the I state. This lack of structural stability can be overcome by increasing the Mg2+ concentration in vitro or the participation of proteins in vivo (9). The occurrence of the 0.4 state only at higher [Mg2+] clearly assigns the I and F state to folded D1 and compact D135, respectively.

**Fig. 5.** Effect of substrate binding on the dynamics and native state stabilization. (A) Histograms of FRET efficiency of fully labeled D135–L14 alone (Top), in the presence of WT substrate 17/7 (Middle), and in the presence of the slower cleaving substrate 17/7-dC (Bottom). The 0.6 state is clearly stabilized by substrate binding. (B) Typical smFRET time trace in the presence of 17/7-dC. The 0.6 state occurs at high frequency (compare with Fig. 3). The drop to 0.25 at 35 s might indicate cleavage. (Right) Distribution.

**Fig. 6.** Mg2+ dependence of single-molecule dynamics and the observed rates \( k_{\text{obs}} \). (A) The percentage of molecules on a slide showing dynamics are plotted versus the Mg2+ concentration. The ratio of dynamic molecules increases from 7 to 49% with \( K_{Mg} = 41.4 \pm 1.4 \) mM. (B) Representative histogram of dwell times in the 0.25 FRET state at 50 mM Mg2+ showing the number of events at a particular dwell time range. The solid line corresponds to the double exponential decay fit characterizing the rate. (C) The number of dwell times in low FRET state N(0 shorter than time t) obtained by integration of the dwell time histogram in A. The double-exponential growth fit is shown as a solid line. (D) Effect of Mg2+ concentration on the observed single-molecule folding rates \( k_1 \) (●) and \( k_{-1} \) (○).
Bottom). The ratio $k_1/k_{-1} = 1.3$ corresponds with the ratio of distribution amplitudes for the F and I states, $A_{0,1}/A_{0,2} = 0.78/0.56 = 1.4$. Please note that in an ensemble experiment the observed folding rate would be slower than those reported here because of averaging between the static and dynamic subpopulations. This illustrates the strength of single-molecule experiments, which enable us to uncover heterogeneity in folding and thus elucidate details of the mechanism otherwise hidden by ensemble averaging.

Lacking a statistically significant number of I to N transitions because $k_{2,-2} < k_{1,-1}$ and the high FRET conformation is only rarely reached, $k_2$ and $k_{-2}$ could not be determined with dwell time distributions. To estimate their values, we measured the average dwell time in I before jumping to N, and in N before jumping back to I for 19 molecules ($t_{av} = 15.3 \pm 0.6$ s, respectively). We calculated $k_2 = 0.07 s^{-1}$ and $k_{-2} = 1.7 s^{-1}$ as the inverse of these values (Fig. 1B). The ratio $k_2/k_{-2} = 0.04$ is in reasonable agreement with the ratio of relative amplitudes of the 0.6 and 0.4 FRET distributions ($A_{0,2}/A_{0,4} = 0.008/0.078 = 0.10$, Fig. 4), confirming the low abundance of N in absence of the substrate. We also measured $k_2$ and $k_{-2}$ in presence of 17/7 and 17/7-dC from 6 and 17 single-molecule trajectories, respectively. Substrate binding increases $k_2$ by nearly 10-fold (0.8 and 0.7 s$^{-1}$, respectively) and decreases $k_{-2}$ by $\sim50\%$ (1.1 and 1.2 s$^{-1}$, respectively), further indicating that the substrate stabilizes folding into the N state.

Forward Rates Are Independent of Mg$^{2+}$. We determined the folding rates in the absence of substrate between 5 and 100 mM [Mg$^{2+}$] (Fig. 6D). Below this concentration, the ribozyme is mostly static in the I state, and thus, not enough dynamic events were observed to quantify the rates with confidence. We found evidence of folding heterogeneity for $k_1$ and $k_{-1}$ at all concentrations (Fig. 6S and Tables S1 and S2). At 5 mM Mg$^{2+}$, the major component of $k_1$ is 1.5-fold larger than the major component of $k_{-1}$, consistent with I being more stable than F at low Mg$^{2+}$ (Fig. 6D). $k_1$ remains approximately constant, whereas $k_{-1}$ increases first and then decreases at [Mg$^{2+}$] > 40 mM, shifting the equilibrium from I toward F (Tables S1 and S2). This finding is consistent with the change in relative stability observed in the FRET histogram distributions (Fig. 4). The rate constants $k_2$ and $k_{-2}$ were also found to be approximately constant over the Mg range tested.

Discussion

Folding of Large Multidomain RNAs. Here, we have used the group II intron ribozyme as a model system to study folding of large multidomain ribozymes. Our single-molecule experiments reveal important concepts for RNA folding in general resolve long-standing questions for group II intron catalysis.

The group II intron ribozyme folds sequentially in three steps from the unfolded to the native state (Fig. 1B), with a previously uncharacterized on-pathway folding intermediate, extending earlier studies that suggested a folding pathway devoid of kinetic traps. High [Mg$^{2+}$] (>10 mM) is required in vitro to populate the near-native state F significantly. We propose that the newly discovered N state corresponds to the active state, but it can only be distinguished from F above 20 mM Mg$^{2+}$. Because N is a transient conformer in the absence of substrate, it was not observed in bulk experiments.

We observe no Mg$^{2+}$ dependence for the I to F transition (Figs. 4 and 6D), similarly to the *Tetrahymena* ribozyme (29) and the RNase P C domain (30). A possible explanation is that this step is dominated by a conformational capture mechanism, in which the F conformation is unstable and must be trapped by Mg$^{2+}$ coordination. A recent study showing that the rate of D1 compaction does not depend on Mg$^{2+}$, whereas the compaction amplitude does (20), supports this idea. Hydroxyl radical footprinting experiments also show that D5 becomes fully protected only above 100 mM Mg$^{2+}$, in agreement with our observations (14). Mg$^{2+}$ ions are, therefore, essential to capture D3 and D5 in their active conformation and to stabilize the D135 complex in vitro. Interestingly, we observed increased RNA structural dynamics upon Mg$^{2+}$ binding (Fig. 6A), linking domain motion to catalysis.

The relative stability of the F and N states remains constant above 40 mM Mg$^{2+}$, ruling out a capture mechanism involving Mg$^{2+}$ for this step. Instead, substrate binding stabilizes the N state, making it the catalytically active species (Fig. 5). The FRET increase from F to N indicates that a major conformational change accompanies D135 compaction. Because the IBS-EBS and e-e’ interactions are located within D1 and thus, cannot be observed with our labeling scheme, it must be the $\lambda\lambda’-\lambda’$ interaction, which links the intronic 5’ end with central nucleotides in D1 and the catalytically crucial D5 (31), that causes this major rearrangement of domains. A recently published crystal structure of a group II intron further supports our findings (32). This structure shows that the product form of the RNA folds into a highly compacted state where domains 1–4 interact with the catalytic domain 5. We propose that formation of this highly compacted state might correspond to our F to N transition. However, a quantitative structural comparison with our data is not possible because the loops where the fluorophores are attached are not present in the crystallized group II intron from *Oceanobacillus iheyensis*. In addition, there is no structural information available on the loop where the donor is located, which could easily introduce an error of 10–20 Å in any distance estimation from the crystal structure.

The transient nature of the native state raises the question of whether the rarely occurring transition from F to N, regulated by $k_2$, is limiting for cleavage. To address this question we measured the cleavage kinetics of the ribozyme at room temperature and 100 mM Mg$^{2+}$. We found a cleavage rate $k_{obs} = 0.024 \pm 0.001$ min$^{-1}$ (see Fig. S7), 180-fold slower than $k_2$. This suggests that the transition from F to N alone is not rate-limiting. Alternatively, a combination of folding, catalysis, and activation of the static population may be rate-limiting for group II intron catalysis. Testing this hypothesis will be the scope of future work.

Implications for Group II Intron Folding and Function in Vivo. Our results show that large multidomain RNAs, such as D135–L14, can fold in a highly ordered stepwise pathway consisting only of on-pathway intermediates without kinetic traps. This strategy is very advantageous for a retroelement such as a group II intron that must fold cotranscriptionally in vivo. It allows folding to start in D1, which is transcribed first. Then, the slow transition from U to I changes proper folding of this domain and subsequent docking of the downstream domains as soon as they are transcribed (e.g., D3 and D5).

Although Sc.ai5 requires high salt and temperature to function in vitro, Mss116, a DEAD-box ATPase with helicase activity, promotes splicing in vivo (33, 34). It has been a matter of debate as to whether Mss116-unwinding activity is needed to resolve kinetic traps (35, 36). The absence of folding kinetic traps in vitro supports a model where Mss116 is needed only to stabilize the native fold at low [Mg$^{2+}$] and not to help the RNA escape from misfolded intermediates.

Materials and Methods

Preparation of RNA. The D135 ribozyme sequence from *S. cerevisiae* intron Sc.ai5 on plasmid pTD135 was modified by insertion of two loops with the length of 18 nt each. Sequence L1 (3’-CCCAAUAUAUAAGCGUCUGGUAGGGG-5’) was placed between residues 276 and 305 on the d2b stem of D1 (Fig. S1), and L2 (3’-CCCAAUAUAUAUGCACUGAGGAGG-5’) replaced the residues 681–806 in D4, giving the plasmid pTD135-L14. Digestion with HindIII and subsequent in vitro transcription with home-made T7 RNA polymerase under standard conditions (23) gave D135–L14 RNA, which was stored in water at –20°C. Biotinylated DNA strands, Cy3-DNA, Cy5-DNA, and the RNA substrates 17/7 (3’-UGGCGAG(dC)UUUUACAGGGUGGUGC-5’–protected) and 17/7-dC (3’-UGGCGAG(dC)UUUUACAGGGUGGUGC-5’–protected) were purchased from Howard Hughes Medical Institution Biopolymer/Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). DNA

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strands were purified by denaturing 18% PAGE and subsequent C8 reversed-phase HPLC as described in ref. 37. The RNAs were deprotected as suggested by the manufacturer and subsequently gel- and HPLC-purified (37).

Design of a Fluorophore-Labeled Group II Intron Ribozyme for Single-Molecule Studies. The D135 ribozyme is a 618-nt-long single-stranded RNA (Fig. S1) (16, 17, 21). The resulting construct D135–L14 (0.25 M), D135–L14 (0.25 μM), and Cy5-DNA (0.6 μM) were denatured at 90°C for 1 min in reaction buffer and subsequently incubated at 42°C for 15–20 min. Reaction mixtures were excited at 555 nm, and emission was recorded at 655 nm and 665 nm on a Cary Eclipse fluorescence spectrometer. Slits were set at 5 nm, and sample was thermostatted at 42°C. Emission time courses were measured for 15 min after the addition of varying amounts of MgCl2 in the range of 0–100 mM.

Normalized absolute FRET changes (FRET = FRET/ (I + I)) were plotted against Mg2+ concentrations for analysis of overall Mg2+ dependence of folding by the Hill equation (15).

Steady-State FRET Kinetic Assay. Ratios of Cy3-DNA to ribozyme to Cy5-DNA were chosen to be 1:2:5 to minimize unbound amount of Cy3-DNA and maximize Cy5 binding to ribozyme for optimized fluorescence transfer. Cy3-DNA (0.1 μM), D135–L14 (0.25 μM), and Cy5-DNA (0.6 μM) were denatured at 90°C for 1 min in reaction buffer and subsequently incubated at 42°C for 15–20 min. Reaction mixtures were excited at 555 nm, and emission was recorded at 655 nm and 665 nm on a Cary Eclipse fluorescence spectrometer. Slits were set at 5 nm, and sample was thermostatted at 42°C. Emission time courses were measured for 15 min after the addition of varying amounts of MgCl2 in the range of 0–100 mM.

Normalized absolute FRET changes (FRET = FRET/ (I + I)) were plotted against Mg2+ concentrations for analysis of overall Mg2+ dependence of folding by the Hill equation (15). Time courses of FRET ratio (defined as in Methods) were fitted with a single exponential expression.

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Supporting Information

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SI Text

Results. The fluorophore-labeled D135–L14 ribozyme is catalytically competent. The activity of the in vitro transcribed (1) D135–L14 construct was measured in a single-turnover cleavage assay with the substrates 17/7 under optimal conditions for self-splicing (2) and compared with the unmodified D135 construct (Fig. S3A), clearly indicating that our labeling scheme only minimally affects the cleavage activity of the ribozyme. The unmodified ribozyme yields a cleavage rate constant $k_{\text{obs}} = 0.40 \pm 0.02 \text{ min}^{-1}$, whereas the D135–L14 ribozyme, which includes the modular loops but not the fluorophore labels, resulted in $k_{\text{obs}}$ values up to 0.37 $\pm$ 0.02 min$^{-1}$. In the presence of the fluorophore- and biotin-labeled DNA-oligonucleotides, the cleavage rate was only reduced by 27% ($k_{\text{obs}} = 0.27 \pm 0.01 \text{ min}^{-1}$). The final fraction of product formation was 85% and remained comparable for all constructs. These results correspond well with earlier values for the unmodified D135 ribozyme (2), giving evidence that our D135–L14 ribozyme behaves accordingly.

Group II introns are known to require high ionic strength for optimal activity in vitro (3, 4), which is thought to be caused by high Mg$^{2+}$ requirement for compaction of D1 and stabilization of the native state (5). We measured the Mg$^{2+}$ requirement for the fluorophore- and biotin-labeled D135–L14 ribozyme using cleavage assays in the presence of Mg$^{2+}$ ranging from 0 to 100 mM. The observed cleavage rate constants are shown as a function of Mg$^{2+}$ concentration in Fig. S3B. This experimental data fit to the Hill equation (S2) to yield the dissociation constant, $K_{Mg} = 52.7 \pm 3.4 \text{ mM}$, which is comparable to reported values ($K_{Mg} = 20$–40 mM) (6, 7), illustrating that our fluorophore-labeled D135–L14 performs catalysis much like D135. The fluorophore-labeled D135–L14 ribozyme requires Mg$^{2+}$ ions for folding. We characterized the folding behavior of freely diffusing fluorophore-labeled D135–L14 in solution to determine the role of Mg$^{2+}$ in folding independent of catalysis at 42°C. The fluorescence emission spectrum in absence of Mg$^{2+}$ shows two bands for the donor and acceptor fluorophores, respectively (Fig. 2.4). The donor intensity at 565 nm is higher than the acceptor at 665 nm, and the observed FRET ratio is 0.275. Upon addition of 100 mM Mg$^{2+}$, the donor intensity decreases slightly, whereas the acceptor increases. As a result, the overall FRET ratio increases exponentially to 0.290 with a rate constant $k_{\text{obs}} = 0.90 \pm 0.07 \text{ min}^{-1}$ (Fig. 2B), indicating the presence of a (sub)portion of compacted molecules in which the fluorophores draw nearer. This rate is in very good agreement with the $k_{\text{obs}}$ $\approx$ 1 min$^{-1}$ for the rate-limiting step of D1 compaction obtained from hydroxyl radical footprinting, NAIM studies, and ribozyme activity assays (5, 7, 8).

The FRET increase to 0.295 in this bulk experiment suggests that a conformational change takes place in the presence of Mg$^{2+}$ ions, during which the relative distance between the fluorophores on the RNA decreases. To determine the dissociation constant, the observed FRET ratio was plotted as a function of Mg$^{2+}$ concentration (Fig. 2C) and fit with Eq. S2, yielding $K_{Mg} = 42.3 \pm 1.3 \text{ mM}$. This value is again in very good agreement with the $K_{Mg}$ from the cleavage reaction described above as well as reported values for D135 (6, 7).

Methods: Single-Turnover Cleavage Assay. The substrates 17/7 and 17/7-dC were 5’-labeled with [$\gamma$-32P]ATP and T4 DNA ligase using standard procedures. DNA-oligonucleotides were in 6-fold excess over D135–L14 to avoid unbound ribozyme in the experiments. Cy3-DNA, Cy5-DNA, and T-biotin-DNA (600 nM) were heat-annealed to D135–L14 (100 nM) at 90°C for 1 min in reaction buffer [80 mM Mops (pH 6.9), 500 mM KCl] followed by Mg$^{2+}$ addition and incubation at 42°C for 15 min in varying amounts of MgCl$_2$. 32P-labeled 17/7 or 17/7-dC (1 nM) was separately incubated in reaction buffer and MgCl$_2$ and then added to annealed, biotinylated, and Cy3/Cy5-labeled ribozyme to start the cleavage reaction. Cleavage reactions were carried out at 42°C as well as at room temperature. Aliquots (1 µl) were removed from the reaction mixture (20 µl) at specific points in time and analyzed on 18% polyacrylamide gels. Reaction rate constants were determined by quantification of the gels and fitting to single-exponential expression:

$$frac{[\text{product}]}{[\text{substrate}]} = (1 - A_1) - A_2 \times e^{-kt} \quad [S1]$$

with $A_1$ being the fraction of uncleaved substrate, $A_2$ the fraction of product formed, and $k$ the first-order rate constant in minutes$^{-1}$.

The first-order rate constants were plotted with respect to increasing [Mg$^{2+}$] and fit to a modified Hill equation

$$y = y_0 + \frac{x^n}{K_{Mg} + x^n} \quad [S2]$$

where $K_{Mg}$ is the [Mg$^{2+}$] at half-maximum rate and $n$ represents the cooperativity constant.

Fig. S1. Primary and secondary structure of the D135–L14 ribozyme derived from the aI5γ group II intron from the cox 1 gene in S. cerevisiae. The two modular loops, which bind two oligonucleotides with the fluorophores Cy3 (green marker) or Cy5 (red marker) at their 5’ end, are inserted in the d2b hairpin in D1 and into D4 as indicated. The numbering corresponds to the WT sequence, and the tertiary interactions are indicated by Greek letters. The 3’ end of the intron is elongated to bind to a 5’-biotinylated complementary DNA-oligonucleotide for attachment on the quartz surface carrying the counterpart streptavidin. Cy3 label is depicted in green, Cy5 label in red and biotin in gray.
Fig. S2. Hybridization of Cy3-DNA and Cy5-DNA to D135–L14 detected on nondenaturing gel by fluorescence. The RNA–DNA hybrids (indicated by black bar on right) are shifted relative to the free DNA-oligonucleotides (Cy3-DNA, green circle; Cy5-DNA, red circle). Amount of Cy3-DNA is kept at 10 nmol and Cy5-DNA is constant at 10 nmol except for lanes 6 and 10, where it is doubled to 20 nmol. D135–L14 is present at 15 nmol, except for lane 7, where it is reduced to 1.8 nmol to simulate ratios used in bulk FRET experiments. KCl concentration is at 0.5 M, apart from lane 13, where it is reduced to 0.1 M. MgCl₂ concentration is constantly 6 mM. Samples were prepared analogously to FRET measurements before native gel (6% wt/vol) electrophoresis run at 4°C [3 mM MgOAc and 66 mM Hepes, 34 mM Tris (pH 7.4) (room temperature)] (9).
Fig. S3. Splicing activity of the D135–L14 ribozyme. (A) Product formation in single-turnover cleavage assays with 5′-labeled 17/7 substrate together with the experimental fit for D135 (**, solid line), D135–L14 (●, dashed line) as well as FRET-labeled and biotinylated D135–L14 (▲, dotted line). Rate constants $k_{\text{obs}}$ are $0.40 \pm 0.02$, $0.37 \pm 0.02$, and $0.27 \pm 0.01 \text{ min}^{-1}$, respectively. (B) Mg$^{2+}$ dependence of substrate cleavage by fully labeled D135–L14 and fit with Hill equation (S2) giving $K_{M_{\text{G}}}=52.7 \pm 3.4 \text{ mM}$ and $k_{\text{obs, max}}=0.18 \pm 0.01 \text{ min}^{-1}$. Error bars stem from two independent assays.
Fig. S4. FRET value of D135–L14 molecules in the U and I states. Molecules at conditions of 0 mM MgCl$_2$, 10 µM EDTA (dashed bars) show a FRET distribution at a mean peak value of ~0.1 (U state). The FRET histogram at 1 mM MgCl$_2$ (white bars, see also Fig. 4) is shown for comparison (I state). The experiments were performed in 80 mM Mops (pH 7.0), 500 mM KCl.
Fig. S5. FRET distribution of static D135–L14 molecules. All static molecules at 1, 10, 20, 40, 60, 80, and 100 mM show a FRET value ≈0.25.
Fig. S6. Calculation of rates $k_1$ (Left) and $k_{-1}$ (Right). The number of dwell times shorter than time $t$ obtained from the integration of dwell time histograms at MgCl$_2$ concentration as indicated to the right together with double-exponential fits (black lines) is shown.
Fig. S7. D135–L14 cleavage activity at room temperature after incubation at 42°C. The cleavage rate obtained is \( k_{\text{obs}} = 0.024 \pm 0.001 \text{ min}^{-1} \).
Table S1. Rates $k_1$ and amplitudes $A_1$ from double-exponential fit at tested Mg$^{2+}$ concentrations

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<th>$A_1$ (1)</th>
<th>$k_1$ (2)</th>
<th>$A_1$ (2)</th>
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<tr>
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<tr>
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<tr>
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<tr>
<td>60</td>
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<tr>
<td>100</td>
<td>0.66 ± 0.06</td>
<td>434 ± 34</td>
<td>0.06 ± 0.04</td>
<td>256 ± 11</td>
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</table>

Values are corrected as described in SI Methods ref. 10. The error limits correspond to 1 SD.
Table S2. Rates $k_{-1}$ and amplitudes and $A_{-1}$ from double-exponential fit at tested Mg$^{2+}$ concentrations

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<th>$k_{-1}(2)$</th>
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<tr>
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Values are corrected as described in SI Methods ref. 10. The error limits correspond to 1 SD.