A map of the binding site for catalytic domain 5 in the core of a group II intron ribozyme

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Group II introns are ribozymes with a complex tertiary architecture that is of great interest as a model for RNA folding. Domain 5 (D5) is a highly conserved region of the intron that is considered one of the most critical structures in the catalytic core. Despite its central importance, the means by which D5 interacts with other core elements is unclear. To obtain a map of potential interaction sites, dimethyl sulfate was used to footprint regions of the intron that are involved in D5 binding. These studies were complemented by measurements of D5 binding to a series of truncated intron derivatives. In this way, the minimal region of the intron required for strong D5 association was defined and the sites most likely to represent thermodynamically significant positions of tertiary contact were identified. These studies show that ground-state D5 binding is mediated by tertiary contacts to specific regions of D1, including a tetraloop receptor and an adjacent three-way junction. In contrast, D2 and D3 are not found to stabilize D5 association. These data highlight the significance of D1–D5 interactions and will facilitate the identification of specific tertiary contacts between them.

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Introduction

Group II introns are a class of self-splicing RNA molecules and mobile genetic elements that have a complex tertiary structural organization (Michel *et al.*, 1989). Few Watson–Crick base pairing interactions are used to provide long-range tertiary contacts, so the architecture is largely constrained by a network of alternative tertiary interactions, including tetraloop–receptor interactions, backbone-mediated contacts and interactions with the exocyclic amine of G–U wobble pairs (Pyle, 1996). The few conserved nucleotides found in group II introns concentrate within a small hairpin-loop structure designated as domain 5 (D5). This domain is absolutely required for all reactions catalyzed by the intron, and is considered to be one of the primary components of the intron active-site (Pyle, 1996; Qin and Pyle, 1998).

Our understanding of the role of D5 in catalysis has been facilitated by the fact that it can be transcribed as a separate molecule and then added back to other intronic subdomains to catalyze splicing reactions *in trans*. For example, when D5 is added to an RNA consisting of the 5'-exon joined to domains 1, 2 and 3, the 5'-splice site is hydrolytically cleaved in a reaction that has been shown to be a good model for the first step of splicing (Jarrell *et al.*, 1988; Franzen *et al.*, 1993; Pyle and Green, 1994). The D5-catalyzed 5'-splice site hydrolysis reaction obeys standard Michaelis–Menten kinetics, by which the maximum rate is analogous to the chemical step and $K_m = K_d$ for D5 binding (Pyle and Green, 1994). This feature has facilitated detailed studies of D5 function, in which the effects of mutations and single-atom changes in D5 can be attributed to specific roles in the ground-state for binding or the transition-state for catalysis (Peebles *et al.*, 1995; Abramovitz *et al.*, 1996; Konforti *et al.*, 1998).

There is still considerable ambiguity about the positioning of D5 and its tertiary contacts within the group II intron core. One of the most important known constraints is the ζ – ζ' interaction, which places the GAAA tetraloop of D5 squarely within the center of D1 (Costa and Michel, 1995). Crosslinking studies have indicated that the first helical stem of D5 lies near a set of conserved nucleotides at the junction between domains 2 and 3 (Podar *et al.*, 1998). This region also contains nucleotides comprising the γ – γ' element, which helps to specify the 3'-splice site for reaction (Michel and Ferat, 1995), thereby placing D5 near a site of reaction at an intron–exon boundary. Deletion analyses coupled with modification interference studies suggest that the internal bulge of D5 interacts with nucleotides in D3 (Jestin *et al.*, 1997).

In order to analyze globally the likely sites of interaction between D5 and D1, D2 or D3, dimethyl sulfate (DMS) modification footprinting studies were conducted on a variety of deletion constructs from the ai5 γ intron of yeast mitochondria. The footprinting studies were designed to determine the major sites of contact and to lay the foundation for identifying specific D5 pairing partners after incorporating mutations or single-atom changes within D5. Modification footprinting studies were then complemented by quantitative studies of D5 binding to truncated derivatives of the intron. In this way, the study was designed specifically to reveal interactions that make a substantial energetic contribution to the binding of D5. The resulting data therefore serve not only to define the loci for potential D5 contacts with the intron, but to highlight interactions of energetic importance. The ultimate goal of the work is to obtain a set of functional, thermodynamically significant distance constraints that can be used to model the group II intron and reveal the fundamental principles of RNA folding.

Fig. 1. Binding of D5 to exD123 (■) and exD1 (●) RNA. D5 RNA is 32 P-labeled and in trace concentration, while exD1 and exD123 concentrations are shown on the x-axis. Values for K_d were obtained from the fit of the data (Pyle and Green, 1994). For exD123, the K_d was 410 ± 67 nM and the horizontal asymptote of the curve was 0.77; for exD1, the K_d was 460 ± 77 nM and the horizontal asymptote was 0.57. Differences in the final fraction of D5 bound (asymptote) to exD123 and exD1 were probably due to a requirement for different column matrices. The observed binding cannot be attributed to nonspecific association of D5, since single-atom changes in critical regions of D5 (such as the tetraloop or the conserved AGC residues) have been shown to disrupt severely D5 binding (Abramovitz *et al.*, 1996; Konforti *et al.*, 1998).

Results

Interaction of D5 with exD123

DMS modifies a variety of functional groups, including N7 of purines, N1 of A and N3 of C (Moazed *et al.*, 1986; Conway and Wickens, 1989). The latter two modifications are readily visualized by primer extension, as DMS adducts block the base-pairing face of A and C (Inouye and Cech, 1985; Pyle *et al.*, 1992). The first construct studied was exD123, which contains the 5' exon and domains 1, 2 and 3 (Jarrell *et al.*, 1988). This RNA encompasses all of the nucleotides that are likely to play a role in D5 binding and active-site formation (Qin and Pyle, 1998). D5 binds to exD123 with a K_d of 410 ± 67 nM (Figure 1), and the K_m for D5-catalyzed exon hydrolysis from exD123 is 300 nM (Pyle and Green, 1994). Having measured the affinity of D5 for exD123, it was possible to design footprinting experiments in which D5 was present at concentrations that are saturating relative to exD123. Because each molecule of exD123 was bound to D5 upon treatment with DMS, footprints of strong intensity could be observed.

Many sites of D5 protection are observed within domains 1–3 (Figure 2). However the greatest number of protected sites and the regions of most intense protection are found in D1. There are two particularly strong regions of protection in D1, both located in the D1d stem: three nucleotides in the tetraloop receptor (198, 382 and 383), and unpaired nucleotides of an adjacent three-way junction (204–215). Nucleotides of the three-way junction have never before been implicated in D5 interaction, although the receptor (a conserved 11-nucleotide stem-loop motif) has been proposed to interact with the GNRA tetraloop at the terminus of D5 (Costa and Michel, 1995; Costa *et al.*, 1997). Moderate sites of protection in D1 (2–3X) include nucleotides within the asymmetric bulge at the

beginning of the D1 stem (11–13), the D1a stem-loop (38–48), adjacent to the ϵ – ϵ' interaction (83), residues at the D1c1 terminus (93–106), throughout D1c2, at the terminus of D2b (290–294), within the α – α' interaction and adjacent to it (65 and 351–355). Sites of less intense protection (1.5–2X) were observed adjacent to stronger protections, within the D1d''' internal loop (227–229; 368–370), at the large junction between helices D1a, b, c and d, as well as within D1c2.

In D3 there are several regions of strong protection. There is a conserved stem-loop at the base of D3 that has characteristics of a tetraloop receptor both in sequence and in the pattern of DMS footprinting (Figure 2B). A second region of strong protection is found at the terminus of a stem-loop (617–618) that is not highly conserved. Moderate regions of protection are observed throughout other sectors of D3 and in the linker between D2 and D3 (Figure 2B). In D2 there is one site of moderate protection (448–449) and scattered sites of less intense protection (Figure 2B, bottom inset).

Of the sites listed above, many are found in regions that are unlikely to represent areas that are important for D5 binding or active-site formation. These nonconserved sectors of the intron include region D1d2b, which can be replaced with a two-nucleotide linker without any effects on the rates of either *cis*-splicing or *trans*-ribozyme reactions, even under nonpermissive low-salt conditions (V.T.Chu and A.M.Pyle, unpublished results). Patterns in regions such as D1d2b indicate that strong sites of protection do not necessarily imply the existence of a direct interaction with D5. Indeed, there are a variety of other possible explanations for any site of protection that is observed in a footprinting experiment (see below).

To understand the roles of individual intron domains and their respective contributions to D5 binding, footprinting and direct D5 binding experiments were conducted on a variety of exD123 deletion constructs. The two that were most extensively studied include exD1 (lacking D2 and D3) and D1 (which also lacks a 5'-exon).

Exploring the effects of D2 and D3: interaction between D5 and exD1

The exD1 RNA is a deletion mutant of exD123, in which D2 and D3 have been removed. ExD1 readily binds D5, having an affinity of 460 ± 77 nM (Figure 1). Since this K_d is not significantly different from that of D5 binding to exD123, D2 and D3 do not appear to be required for D5 binding to D1.

Several regions of D5 protection are apparent on exD1 (Figure 3), all of which correlate well with those observed on exD123 (Figure 2). Some of the corresponding sites of protection are slightly weaker on exD1 than on exD123. This is likely to result from the fact that the smaller exD1–D5 complex has been stripped of peripheral domains, leaving it more exposed to solvent. The major sites of D5 footprinting on exD123 are all observed on exD1, including protections adjacent to the ϵ – ϵ' interaction, region D1c2, the tetraloop receptor and its adjacent three-way junction, within and adjacent to the α – α' interaction (Figure 3). One notable difference, however, is that protections within the tetraloop receptor are significantly less intense on the exD1 molecule than they are on exD123. In particular, the relative intensity of the footprint at

position 198 has decreased [from 7.1 in exD123 (Figure 2) to 3 in exD1 (Figure 3)], and the strong protections previously observed at nucleotides 382–383 of the opposite strand are no longer apparent.

Affinity of a minimal binding motif: interaction of D5 with D1

The D1 molecule contains no 5'-exon sequences or sections of any other domain. Despite this lack of additional sequences, D1 has a strong affinity for D5. The K_d determined from direct binding studies is 530 ± 96 nM (Figure 4). It has been established that D1 can behave as a ribozyme, cleaving oligonucleotides analogous to the 5'-exon/intron boundary when D5 is added *in trans* as a catalytic cofactor. Because this minimal ribozyme system can reflect binding parameters through activity, it was possible to measure the K_m for D5, which was found to be 870 ± 110 nM (Michels and Pyle, 1995). The similarity of this K_m to the K_d reported here indicates that D5 not only binds to D1, but binds productively in an active conformation. This is consistent with deletion studies showing that 5'-exon hydrolysis occurs in *cis*-splicing constructs containing only intron domains 1 and 5 (Koch *et al.*, 1992).

There are clear patterns of D5 protection on the D1 molecule, but there are fewer of them than observed previously with exD123 or exD1 (Figure 5). Nonetheless, the patterns that are observed fall in the major regions already described for D5 footprinting of the other constructs: within and adjacent to the α - α' interaction, at the terminus of D1c1, adjacent to the ϵ - ϵ' interaction, at the tetraloop receptor and its adjacent three-way junction, and at the 3'-terminus of EBS1. The generally less intense protection patterns (with the exception of the tetraloop receptor protections, which are strong on D1) are probably due to the smaller size and more solvent-exposed structure of D1. A more exposed structure will increase the rate at which solvent-based probes can move in and out of the breathing molecule, making it more difficult to footprint even a very strong interaction. Notable sites that are not protected in D1, despite their presence in exD123 and exD1, include the internal loop at the base of D1, the D1a region and the D1c2 region (Figure 5). It is formally possible that these protections are observed only in exD1 and exD123 because each bears a 5'-exon. However, the footprints that are observed in D1, and their proximity to those seen in the other constructs, indicate that D2, D3, the linker between them and the 5'-exon are not requirements for D5 association with D1.

It is important to consider whether the D5–D1 interaction is influenced by EBS1–IBS1 and EBS2–IBS2 pairings. These phylogenetically covarying pairings are necessary for 5'-splice site recognition and are potentially important elements for maintaining the overall fold of D1 (Jacquier and Michel, 1987). In the absence of a 5'-exon or a substrate oligonucleotide with complementarity to the EBS sequences, they are stretches of unpaired structure. This is actually evident from the DMS sensitivity of the EBS regions (nucleotides 331–333, Figure 5, and nucleotides 238–243, data not shown). Formation of the two EBS–IBS helices is likely to rigidify sections of D1, potentially causing significant alterations of the structure. Nonetheless, direct binding studies of D5 to D1 in the

presence and absence of substrate result in binding curves that are virtually superimposable (Figure 4). In addition, most regions of D5 protection do not differ in the presence or absence of substrate (Figure 3, legend). The results indicate that binding of D5 to D1 is largely independent of EBS–IBS base pair formation and support the assertion that substrate binding does not radically alter the overall fold of the D1 scaffold (Qin and Pyle, 1997).

The role of D3 in D5 function: a kinetic analysis

The direct binding and modification protection experiments presented here suggest that D5 can bind D1 independent of any other intron domains. These findings contrast with a previous report that D3 is specifically required for D5 association, and that D3 forms important tertiary contacts with the conserved internal loop of D5 (Jestin *et al.*, 1997). In that study, modification interference studies compared D5 binding to exD1 and exD123. However, in those experiments, the K_d for the D5–exD1 interaction (~ 25 μ M) was reported to be at least 50 times higher than that observed for the D5–exD123 interaction (~ 0.5 μ M). Because binding in those experiments was so compromised, differences in interference patterns for the D5–exD1 and D5–exD123 complexes are difficult to interpret. Since there is now an abundance of data supporting the fact that D5 binds to D1 without requiring additional ground-state contacts to D3, there is presently insufficient data for drawing conclusions about the regions of D5 that interact with D3 at any point during catalysis.

While there does not appear to be a strong ground-state interaction between D3 and D5, it remains formally possible that D5 binds to exD1 (or D1) and exD123 differently, with a net association energy that appears the same when measured by a direct binding assay. This is contraindicated by the fact that D5 protection patterns on exD123, exD1 and D1 are very similar. It might be expected that an alternative method for examining D5–D1 binding affinity would be to determine whether D1 is a competitive inhibitor of D5-catalyzed exD123 cleavage. However, when D1 was tested as a competitive inhibitor of the D5/exD123 hydrolysis reaction, a modest but reproducible stimulation of exD123 cleavage was observed as the concentration of D1 was increased (data not shown). This is reasonable, since D1 (or exD1, for that matter) is not simply an unreactive inhibitor: rather, it can catalyze hydrolysis of 5'-splice site sequences in the presence of D5 (see below). Therefore, any competitive effects of D1 (or exD1) on D5 binding to exD123 will be offset by the extent to which the 'inhibitor' cleaves the 5'-exon of exD123, particularly at the high competitor concentrations that would be required for inhibition of D5 (proportional to the K_d of D5). Therefore, competitive inhibition effects are overshadowed by the intrinsic catalytic activity of D1 molecules.

Although catalysis by D1 has been studied extensively, there have been no analogous studies on the reactivity of exD1. To examine *cis*-cleavage of exD1 in the presence of D5, trace quantities of 32 P-labeled exD1 molecules (<1 nM) were incubated with saturating concentrations of D5, and the evolution of 5'-exon and D1 products was monitored. Unlike the related exD123 molecule, exD1 molecules do not undergo autocatalytic hydrolysis of the 5'-exon in the presence of D5 (Figure 6). To determine

the raw data for DMS protection (rather than the D5-dependent differences in protection intensity) can reveal whether the secondary structure of a molecule is consistent with published models. To this end, we focused on regions of putative secondary structure that showed a high level of DMS susceptibility (rather than the converse, which would be difficult to interpret due to the potential involvement in tertiary interactions). Like most group II introns, the secondary structure of ai5 γ has been deduced primarily from phylogenetic data and a limited set of chemical

modification experiments (Michel *et al.*, 1989; Kwakman *et al.*, 1990). Here, a map of DMS susceptibility (Figure 2B, gray bars) shows that the base-pairs adjacent to the ϵ' element (C82, A83) are DMS-sensitive, as are the majority of adenosines throughout the entire D1c2 region. A section of the D1d'' helix (A218–219), together with the bent helix adjacent to the α' motif (A351–355) are susceptible to DMS, suggesting that these regions are either unpaired or unusually dynamic in the absence of D5. This sensitivity to DMS diminishes in the presence

Fig. 2. DMS modification footprints of D5 binding to exD123. (A) High-resolution sequencing gels showing sites of protection by D5 after primer extension. Open arrows indicate sites of moderate protection (1.5- to 2-fold) and closed arrows indicate sites of intense protection (2- to 7-fold). Extended regions of protection are indicated by brackets. From left to right, the panel numbers are 1–4, respectively. DMS modification was conducted at room temperature. Limited additional DMS modification experiments were also performed on ice [inset, where (+) and (–) indicate the presence and absence of D5] to visualize modification of cytosine. At room temperature, only adenosine residues were found to be modified by DMS, while on ice both adenosine and cytosine residues were modified by DMS. The same adenosine residues were modified under both conditions. In panels 3 and 4, strong stops at the top of the gels [D5 (+) lanes] indicate D5-catalyzed RNA cleavage products (the 5'-splice site and secondary intron cleavage site, respectively). The strong band located immediately above position A198 (panel 2) corresponds to a uridine that has been modified by DMS. Uridine occasionally becomes modified by DMS and acts as a primer extension stop, for reasons that are unknown. (B) The secondary structure of exD123 RNA, showing sites of DMS protection by D5 (●). Intronic nucleotides are indicated by positive numbers, starting at the 5'-splice site (●). Established tertiary interactions α – α' , β – β' , η – η' , θ – θ' , ζ – ζ' , EBS1–IBS2 and EBS2–IBS2 are indicated. Domains 2 and 5 are shown as insets. D5 is not connected to exD123, and is merely shown here for reference, with dashed lines indicating positions of alternative pairing. Data on modifications at positions C65, C82 and C102 were obtained from experiments conducted on ice (Figure 2A, inset). Gray bars indicate regions where DMS modification patterns are different from published secondary structure in the absence of D5 (see text).

of D5 (Figure 2B), suggesting that D5 binding may help stabilize certain secondary structural features or that D5 forms tertiary contacts with regions formerly thought to be helical.

It is notable that the vast majority of secondary and tertiary structural features that have been proposed to exist in group II introns are observed in DMS protection and nucleotide analog interference (Boudvillain and Pyle, 1998) maps of intron ai5 γ . This fact, together with hydroxyl radical footprinting studies showing that ai5 γ is tightly folded and encloses elements important for the catalytic core (J.Swisher and A.M.Pyle, in preparation), underscores the fact that ai5 γ behaves in a manner that is characteristic of a large, uniformly folded ribozyme that is suitable for both enzymological and biophysical studies.

Discussion

The physical basis for DMS protection patterns

In this study, a collection of DMS modification footprints were obtained on group II intron subdomains in the presence and absence of catalytic D5. Given the marked reproducibility of the protection patterns, particularly on severely cropped deletion constructs, it is tempting to speculate that all sites of D5 interaction with the intron have now been elucidated. However, any individual protection pattern could represent one of four types of effects: (i) a direct contact with D5; (ii) steric occlusion by D5, thereby protecting nucleotides that are in close proximity; (iii) a D5-induced conformational change near a bona-fide D5 tertiary interaction; and (iv) a D5-induced conformational change in one section of a molecule, which causes it to interact with another section. The latter effect is the most difficult to interpret, as it is the most indirect.

The interpretations described above can apply to both DMS footprinting and interference experiments.

Despite the complexity of interpreting D5 protection patterns, it was nonetheless possible to narrow down a group of sites that are the most likely to represent the critical sites of D5 interaction. This was accomplished using the following approach: strong sites of D5 footprinting were first obtained on exD123, a molecule known to contain all the nucleotides important for catalytic function in group II introns. Deletion constructs of exD123 were then made and a set of truncated variants was identified that still bound to D5 with high affinity (exD1 and D1). These deletion constructs were analyzed for DMS protection patterns in the presence or absence of D5. Those sites that are present on each variant (exD123, exD1 and D1) are likely to represent the most important sites of D5 interaction with the intron. However, even for these common sites of protection, one cannot exclude the possibility that some of them represent any one of the indirect effects described above.

Although many sites have been identified by DMS footprinting, it is also important to reiterate that this method provides information only about A and C residues (and only their base-pairing faces). It is likely that new sites, or expanded views of the ones reported here, will become evident upon probing with other modifying reagents. Therefore, the DMS footprints described here may represent only a subset of potential interactions between D5 and the rest of the intron. It is also important to recognize that group II introns are dynamic molecules which have multiple conformational states, particularly between the first and second steps of splicing (Chanfreau and Jacquier, 1996). The set of potential tertiary interactions shown herein may not exist in all conformations

of the intron, at all times during the catalytic cycle, and they may not be important for all of its functions. Indeed, certain motifs within the intron may actually find new tertiary interaction partners as a result of conformational change. Thus, the potential contacts described here need not necessarily fit a single model that incorporates all known group II intron tertiary interactions.

Major sites of D5 interaction with the group II intron core

A set of six strong DMS protections are consistently observed upon footprinting of all three exD123, exD1 and D1 constructs (Figures 2B, 3B and 5):

(i) *The three-way junction in region D1d'' (nucleotides 204–215).* The most extensive set of strong protections occurs in this unusual substructure, which has not been identified previously as a region critical for intron function or, more specifically, for interaction with D5. It is difficult to assess the phylogenetic conservation of this area because its morphology appears to be variable; it is represented in some introns as an unstructured loop. Within the three-

way junction region of intron ai5 γ , internal loop nucleotides 204, 214 and 215 are the residues most likely to be involved in the D5 tertiary contact, based on their high mutational sensitivity (B.B.Konforti and A.M.Pyle, unpublished results; Boudvillain and Pyle, 1998) and the intensity and reproducibility of their D5-dependent protections. Strong D5 footprints in this region are consistent with the fact that it has recently been implicated in a novel tertiary interaction with stem 1 of D5 (Boudvillain and Pyle, 1998). This κ - κ' interaction joins D1 with a section of D5 containing the conserved, catalytically critical 'AGC' triad, and may therefore represent an essential architectural element of the group II intron active site. Although the molecular structure of this tertiary interaction is still under investigation, it is significant that nucleotides G213, A214, A215 and A204 can be classified as a form of GN_n/RA loop, which consists of a GAAA tetraloop structure that is split or imbedded in an alternative structural context such as an expanded loop, internal loop or other substructure (Abramovitz and Pyle, 1997; Massire *et al.*, 1998).

Strong footprints are also observed at residues 208 and

Fig. 3. DMS modification footprints of D5 binding to exD1 RNA. (A) Representative high-resolution sequencing gels showing sites of D5 protection on exD1. The gel is marked as described in Figure 2A. (B) Secondary structure of exD1, showing sites of protection on both exD1 (colored circles) and D1 RNA (colored stars). Since these RNA molecules are 3'-truncated, primer extension did not permit visualization of nucleotides from the 3'-termini to nucleotide 400. Note also that the 3' end of D1 extends for an additional 13 nucleotides beyond the terminus shown for exD1. All DMS modification was performed at room temperature, although protections at C65, C82 and C102 were only visualized on ice. Note that for D1, all D5 footprints were observed in the same positions whether or not substrate oligonucleotide was also bound. The one exception to this is A336, which is more accessible to DMS when EBS1 is base-paired to substrate.

209 of an adjacent GAAA tetraloop. These residues are less likely to be critical for D5 binding because they are not protected in the D1 RNA construct and mutational studies show that this GAAA loop can be mutated to UUCG without reducing the rate of the first step of splicing (Boudvillain and Pyle, 1998).

Fig. 4. Binding of D1 to D5 RNA in the presence (□) and absence (○) of oligonucleotide substrate. The D1–D5 K_d in the absence of substrate was 530 ± 96 nM with an asymptote of 0.65; the D1–D5 K_d in the presence of substrate was 500 ± 91 nM with an asymptote of 0.64.

(ii) *The conserved tetraloop receptor, ζ .* This region is involved in one of the best-characterized interactions with D5: the GAAA tetraloop of D5 (ζ') docks into the phylogenetically conserved helix–bulge–helix motif (ζ) of the D1d stem (Figure 2; Costa and Michel, 1995). The protections observed at receptor positions 198, 382 and 383 are consistent with crystallographic studies on a similar tetraloop–receptor interaction found in group I introns (Cate *et al.*, 1996): protection of receptor nucleotide A383 can be attributed to a sterically inaccessible stacking interaction with the second nucleotide of the D5 tetraloop (A830). The N1 of receptor nucleotide A382 also becomes protected from methylation by stacking within the tetraloop–receptor helix, as reported in a group I intron (Murphy and Cech, 1993). Protection of A198 is due to formation of a reverse Hoogsteen base pair with U381, which is involved in a triple-base interaction with the second nucleotide of the D5 tetraloop. In fact, these hydrogen bonds between A198 and D5 nucleotide A830 are the only direct contacts made between the tetraloop and the receptor that involve N1 of A; all the other receptor protections are due to D5-induced conformational change. Based on the D5 protection patterns observed here (and in a potential receptor located in D3, see below), we propose that the ‘signature’ DMS protection pattern of a bound stem–loop receptor motif involves two strong protections on the three-nucleotide side of the bulge and

Fig. 5. DMS modification footprints of D5 binding to D1 RNA. Representative high-resolution sequencing gels showing sites of D5 protection on D1. Sites of protection are mapped on the secondary structure shown in Figure 3B, where D5 protections on D1 are shown as stars. All the DMS modification experiments with D1 were performed at room temperature, with the exception of that shown in the third panel from the left, in which DMS modification was performed on ice.

Fig. 6. Pseudo first-order plots of 5'-exon hydrolysis in the presence of D5. Four RNA molecules, each of which contains a 5'-exon and one or more intron domains, were incubated with saturating D5 (3 μ M), and the evolution of hydrolyzed products was monitored with time. No products for 5'-splice site hydrolysis by the exD1 and exD12 RNA molecules were evident, even after extended exposure of autoradiographs.

a particularly strong protection on the two-nucleotide side of the receptor bulge (Figure 2B). The footprinting results reported herein are qualitatively consistent with modification interference data which show that modification of positions 382 and 383 had 2- to 3-fold effects on D5 binding while effects of modification of position 198 were >3-fold (Jestin *et al.*, 1997).

(iii) *Adjacent to the conserved ϵ - ϵ' interaction.* There is a set of nucleotides (82–84) that are consistently protected upon D5 binding. The ϵ - ϵ' interaction facilitates exon definition during *cis*-splicing (Jacquier and Michel, 1990) and is likely to be critical for activity of group II introns (Suchy and Schmelzer, 1991; Podar *et al.*, 1995b). The proximity of D5 to the ϵ - ϵ' substructure is therefore reasonable, since both are critical elements of the catalytic core. DMS susceptibility patterns in the absence of D5 reveal that nucleotides 82–84 may be unpaired, which is inconsistent with common secondary structural arrangements of group II introns. This implies that these nucleotides, or their 'pairing partners', may actually be free to form tertiary contacts with other molecules, such as triple-base interactions with D5. If this occurs, the molecular contacts are likely to involve backbone atoms of C82, A83 and A84, since their base sequences are not conserved. Alternatively, the binding of D5 may contribute to stabilization of the C82–G119, A83–U118 and A84–U113 pairings, and this may facilitate proper orientation of the adjacent ϵ - ϵ' interaction.

(iv) *The nucleotide immediately 3' of EBS1 (nt 336).* This is an isolated protection that is consistently observed. This nucleotide is most readily modified when EBS1 is paired (see Figure 3B legend), suggesting an induction of strain that makes A336 more accessible to DMS, and potentially more accessible for interaction with D5. The exact position of this footprint is surprising given that the 5' and not the 3' end of EBS1 pairs with the cleavage-site nucleotide of IBS1. This suggests that D5 may form ground-state interactions with the EBS1–IBS1 pairing at the helical terminus opposite that of the cleavage site, where D5 contributes functional groups which are important in the

transition state (Abramovitz *et al.*, 1996; Konforti *et al.*, 1998). That the two helical ends may be relatively close in space is suggested by the highly bent 'kissing hairpin' structure (Marino *et al.*, 1995), which may share topological features with the EBS1/IBS1 site.

(v) *The adenosines adjacent to and including the α - α' interaction (nts 351–355 and 65–66).* The nucleotides immediately downstream of α' (351–355) are susceptible to DMS in the absence of D5 (a fact which is inconsistent with published secondary structure models) and protected in its presence. Likewise, a D5-induced protection is observed at the 3'-end of α in region D1b (C65 or A66). The α - α' pairing is an essential tertiary interaction in D1 (Harris-Kerr *et al.*, 1993), specifically required for establishing a folded structure that can bind 5'-exon substrates (P.Z.Qin and A.M.Pyle, in preparation). Consideration of the 'kissing hairpin' model (Marino *et al.*, 1995) and inspection of the intron secondary structure (Figures 2B and 3B) suggests that formation of the α - α' and EBS–IBS interactions will induce considerable strain on nucleotides adjacent to and within the α - α' helix, rendering them more sensitive to DMS. D5-induced protection of these residues can be attributed to several possible factors: D5 may help to stabilize α - α' and surrounding substructures, but this is unlikely to be an essential function, since α - α' readily forms in the absence of D5 (Qin and Pyle, 1997). Alternatively, residues A351–A355 may form contacts with D5 that help orient it with respect to α - α' , the 5'-exon and/or other core substructures.

(vi) *The terminus of the D1c1 stem.* This contains a set of protections ranging from nucleotides A92–A106. Nucleotides A100 and A101 are more accessible in exD1 (Figure 3A) than in exD123 (Figure 2A). This finding is consistent with a proposed interaction between the GNRA tetraloop that caps D1c1 (θ) and a receptor at the base of D2 (θ' , residues 422, 423, 582 and 583) (Costa *et al.*, 1997). However, sequences surrounding the tetraloop display patterns of D5 footprinting whether or not D2 is present. The D1c1 hairpin may therefore represent an important core structural element that binds D5 exclusive of D2, binds them both or binds them at different times in the splicing cycle. This is consistent with reports that the D1c1 stem stimulates reaction when it is added as a separate molecule to deletion constructs of the intron (Suchy and Schmelzer, 1991).

In addition to the sites described above, there are four prominent protections that are seen upon D5 footprinting of exD123 and exD1, but not D1. This may indicate that a *cis*-exon is required for certain tertiary interactions, or that D1 is simply too exposed for the footprints to be evident. Since they are not seen in all constructs, the possibility that these protections represent indirect effects is significantly greater. (a) At the base of the D1 stem, there is protection of an internal loop containing the sequence 5'-AAAG (nucleotides 11–13, Figure 3B). The protection observed in this region is consistent with phosphorothioate and nucleotide analog interference effects (Jestin *et al.*, 1997; Boudvillain and Pyle, 1998). Moreover, this portion of the D1 stem cannot be deleted without destroying activity (A.M.Pyle and K.Chin, unpublished results) and may therefore be an important active-

site element. (b) A strong set of footprints is observed in the D1a region, within a stem-loop structure. Although most group II introns contain a D1a stem of some type, the sequence itself is not overtly conserved. Careful inspection of the region of nucleotides 34–47 reveals that it strongly resembles the tetraloop receptor motif (Costa *et al.*, 1997), which normally occurs as a set of G–C pairs flanking an A-rich internal loop rather than a terminal loop as shown here. That the D1a region could serve as a receptor is consistent with the recent finding that similar RNA structural motifs can occur in different stem-loop contexts (Abramovitz and Pyle, 1997). Because the only tetraloop in D5 is already involved in an interaction with receptor ζ (Figure 2B) (Costa and Michel, 1995), protection observed in D1a is likely to represent a D5-induced conformational change in this region. Further sites of protection are (c) the three-way junction of D1c, c1 and c2 (nucleotides 76–77 and 124–127), and (d) the D1c2 region, particularly the internal loop, containing strong protection of nt 176. These protections were surprising given that the complete absence of D1c2 does not diminish the rate of *cis*-splicing (V.Chu and A.M.Pyle, unpublished results). In addition, certain regions of DMS susceptibility (162–167, 180 and 181) are inconsistent with published secondary structures, indicating that, even in the absence of D5, secondary structural elements of D1c2 are either very weak or unformed (Figure 2B, gray bars).

There are other positions of D5 protection on exD123 that may represent interesting sites of interaction or D5-dependent conformational change. Examples include sites at positions 253–254, 290–294, 368–370 and 391–395. The protections in the internal loop between regions D1d'' and D1d''' are significant, since the region A227–A229 has been shown previously to become DMS-sensitive upon binding of Mg^{2+} to D1 (Qin and Pyle, 1997). A metal-ion-dependent conformational change was therefore predicted for this region, and this may play a role in the binding of D5. The D5-induced protections 5' of β' in subdomain D1d2a (253–254) suggest that the β – β' pairing may be involved in binding of D5. However, the fact that all of the protections mentioned above occur only in exD123 suggests that they are among the least likely to represent critical tertiary interactions with D5.

Protections in D3, D2 and the linker between them

Footprints in D3. Some of the most intense protections of exD123 occur within D3. This would seem to suggest that D3 is in close contact with D5, despite the fact that it does not provide significant levels of additional D5 binding stabilization and it does not markedly alter patterns of protection observed within D1. Although the data provided herein establish that D3 is not necessary for stabilizing the ground-state docking of D5, they are consistent with the idea that D3 may play a very important role in D5 function. Close proximity of D3 to D5 is reasonable given that D3 has been shown to enhance the chemical rate of catalysis (Griffin *et al.*, 1995; Xiang *et al.*, 1998), without changing ground-state binding parameters of other substructures. Furthermore, D3 molecules have been shown to bind strongly *in trans* ($K_d = 130$ nM) to a construct containing D1 and D5, exerting powerful effects on the rate of splicing reactions (Podar *et al.*,

1995a). Thus D3 is likely to be tightly packed within or adjacent to the catalytic core. The DMS protection patterns that are observed upon D5 binding are likely to represent steric protections of D3 as it packs near D5, or they may represent tertiary contacts with D1 or D2 that form in the presence of D5.

The most interesting protection of D3 occurs within a conserved internal loop (nts A596–A599 and A661–A662, Figure 2B). Particularly when one considers that nts A600 and U660 might be unpaired, and that A598–C663 can form a wobble pair, the motif closely resembles a tetraloop receptor. The protections observed in this receptor sequence cannot, however, be attributed to interactions with the D5 tetraloop because the latter is engaged with a receptor in D1. Either the D3 receptor substructure makes energetically neutral contacts to a different region of D5 (perhaps becoming significant only in the transition state), it is sterically protected by D5 or it undergoes a D5-dependent conformational change and interacts with regions of D1, D2 or D3. The GN_nRA loop at residues 615–618 is also strongly protected from DMS upon binding of D5. Modification interference studies have implicated both of these regions in ground-state contact with D5 (Jestin *et al.*, 1997), although the data reported herein are inconsistent with this interpretation. The contacts made to the D3 stem-loop, and the significance of other protections in D3 (including residues 627–653), cannot be interpreted adequately without more systematic mutagenesis and nucleotide modification studies.

Protections in the J2/3 linker and in D2. It is highly significant that strong protections are observed from nucleotides A584–587, in the linker between D2 and D3 (J2/3) (Figure 2B). This highly conserved region undergoes efficient photocrosslinking to 4-thiouridine residues incorporated at the base of D5, and nucleotides G588 and A589 are known to be required for the first step of *cis*-splicing (Podar *et al.*, 1998). It has therefore been proposed that J2/3 may make direct tertiary contacts with D5. Although D5 footprinting data on exD123 are consistent with a D5–J2/3 contact, the binding studies reported herein, together with footprints observed on D1 and exD1, suggest that any interaction of this type does not make an energetic contribution to ground-state binding of D5. Since footprints and crosslinks can result from spatial proximity rather than the formation of direct hydrogen bonds, the footprints observed in J2/3 may result instead from steric protection by a closely associated D5 molecule. The latter model does not exclude the notion that J2/3 is an important active-site component that is close to D5 and is necessary for cleavage of 5'-exons that are present *in cis* (the first step of splicing).

D5 protections are also observed in D2, particularly at residues 448–449. These positions of D5 protection are not found in any region that has ever been implicated in D5 function. Position A466, which is protected upon D5 binding, is adjacent to a tandem G–C pair receptor that has been proposed to interact with the GUAA tetraloop that caps D6 (forming the η – η' interaction) (Costa *et al.*, 1997).

Mechanistic implications of the D5 binding and protection data

Taken together, D5 binding and protection data on three different constructs indicate that D1 contains the sites

where D5 forms most (if not all) of its energetically significant ground-state tertiary interactions. Furthermore, a localized part of D1 (the d-d'' region) containing a three-way junction and a tetraloop receptor are likely to be the primary sites of D5 binding in group II introns. In addition to providing essential active-site constituents (such as ϵ - ϵ'), D1 therefore serves as a docking center for stabilizing, orienting and presenting D5 in a manner that allows it to potentiate chemical catalysis.

The protection and activity data confirm that D3 plays an important role in the mechanism of group II intron catalysis (Griffin *et al.*, 1995; Podar *et al.*, 1995a; Xiang *et al.*, 1998), even if it is not critical for stabilizing the association with D5. If one assumes that none of the protections listed below result from long-range D5-induced conformational changes, then the footprinting patterns suggest a set of motifs that coalesce to form the group II intron active site: D5 is surrounded by the residues of the three-way junction, tetraloop receptor, ϵ - ϵ' , D1c1, α - α' and EBS1 regions of D1, the J2/3 linker nucleotides and two major regions of D3. Each of these motifs probably plays an important role in the mechanism, whether or not direct contacts are made with D5.

It is significant that an exD1 construct cannot cleave its own 5'-exon, despite the fact that both exD1 and D1 readily cleave short 5'-splice site oligonucleotides in the presence of D5. This suggests that the first step of splicing (where the 5'-exon is present *in cis*) is mechanistically different from reactions in which the intron binds and cleaves oligonucleotides *in trans* (Podar *et al.*, 1995b). The latter reaction is common for free group II intron molecules (either as linear or lariat species) which cleave or integrate into separate polynucleotide substrates. This *trans*-cleavage reaction is the basis for intron mobility, and it is formally a reverse-splicing event. Thus, cleavage of a 5'-exon *in cis* (by hydrolysis or transesterification) represents a true example of the first step of splicing (Podar *et al.*, 1995b), whereas cleavage of an oligonucleotide *in trans* (by hydrolysis or transesterification) is likely to represent a reversal of the second step of splicing. *Trans*-ribozyme reactions (such as oligonucleotide cleavage by D1, D13, etc.) are therefore not necessarily models for the first step of splicing. This is supported by the fact that these ribozymes show a strong (30-fold, Q.Xiang and A.M.Pyle, in preparation) preference for the cleavage of Rp phosphorothioate diastereomers (as in second-step reversal reactions of lariat introns), while cleavage of exD123 by D5 (or D56) occurs with Sp stereospecificity (as in the forward first step of splicing) (Podar *et al.*, 1995b). The ability of exD1 and D1 to react with separate, but not covalently connected, substrates in the presence of D5, indicates that D1 and D5 are necessary and sufficient for the *trans*-cleavage reaction. In contrast, the finding that exD123 and exD13 undergo efficient 5'-exon hydrolysis whereas exD12 and exD1 do not suggests that exD12 lacks sequences which contribute to the proper recognition and/or positioning of a *cis* 5'-exon. Thus, D3 alone, or perhaps together within D2 or the linker between D2 and D3, is important for *cis*-cleavage of the 5'-exon, and therefore plays a role in catalyzing the first step of splicing. However, it has been previously observed that the requirement for D3 in *cis*-exon cleavage can be alleviated under high salt conditions (1.5 M KCl) (Koch

et al., 1992). If *cis*- and *trans*-cleavage reactions represent models for the first and second steps of splicing, respectively, then the similarities of D5 protection on all three constructs indicate that the major difference between active-sites for the two steps of splicing can be linked to the involvement of D3, and that massive conformational rearrangements of the D1 scaffold or its interactions with D5 do not occur. This is consistent with previous studies on D5 functional groups, suggesting that the step 1 and step 2 active-sites are very similar (Chanfreau and Jacquier, 1994).

Perspective

By combining DMS footprinting with quantitative binding studies, the most likely sites of contact between catalytic D5 and the rest of the intron have been identified. Despite the significance of these findings, they represent only the first step in mapping the functional tertiary contacts that stabilize the folding of group II intron RNAs. Previous studies have identified many important functional groups on D5 that are involved in tertiary contacts with other intronic domains (Chanfreau and Jacquier, 1994; Abramovitz *et al.*, 1996; Konforti *et al.*, 1998). Having identified these atoms on D5 and created a map of critical D5 footprints on D123, it should now be possible to identify the precise location of individual tertiary contacts between them. This will be accomplished by applying the techniques of single-atom modification footprinting (Pyle *et al.*, 1992) and nucleotide analog interference (Strobel and Shetty, 1997; Strobel *et al.*, 1998). These approaches efficiently screen large RNA molecules for atoms of specific contact to an RNA functional group of known importance, and they have been successfully applied to the identification of tertiary interactions in group I introns (Pyle *et al.*, 1992; Strobel and Shetty, 1997; Strobel *et al.*, 1998), tRNA (Yap and Musier-Forsyth, 1995) and now group II introns (Boudvillain and Pyle, 1998). The functional distance constraints generated from these experiments will ultimately be combined with crosslinking, and phylogenetic and crystallographic data in an effort to model the active site architecture of a group II intron.

Materials and methods

RNA transcripts, oligonucleotides and DNA primers for reverse transcription

The exD123 RNA consists of 293 nts of 5'-exon and the first 712 nucleotides of the ai5 γ intron, which encompasses Domains 1–3. The ai5 γ intron is the fifth intron from the third subunit of the mitochondrial cytochrome oxidase gene of *Saccharomyces cerevisiae*. It was transcribed with T7 RNA polymerase under standard conditions from *Hind*III-digested plasmid pJDI3'-673 (Jarrell *et al.*, 1988), which was kindly provided by Dr Philip S.Perlman. The exD13 RNA is identical to exD123 except that D2 has been replaced by a 10-nucleotide stem-loop (5'-CCCUUCGGG-3'), which still contains nucleotides capable of participating in the θ - θ' interaction. This RNA was transcribed from a *Hind*III digest of plasmid pQL29, which was made by site-directed mutagenesis of plasmid pJDI3'-673 (Kunkel *et al.*, 1991). The exD12 RNA was generated by cleaving exD123 with a 44-nucleotide DNAzyme (Santoro and Joyce, 1997), yielding a homogeneous 3'-end at nucleotide 588. The exD1 RNA contains 293 nucleotides of the 5'-exon and the first 416 nucleotides of the intron, which encompasses D1. This RNA was transcribed from an *Eco*RV digest of plasmid pQL32, which was made by site-directed mutagenesis of plasmid pJDI3'-673. D1 RNA contains nucleotides 1–429, and was transcribed from plasmid pT7D1 (also pWM01) cut with *Eco*RI as described previously (Michels and Pyle, 1995). D5 RNA is 58 nucleotides in length, comprising 34 nucleotides of

D5 and flanking vector sequences, and was transcribed as described previously, from a *Hpa*II digest of plasmid pJD15'-75 (Jarrell *et al.*, 1988), which was also provided by Dr Philip S.Perlman. The substrate RNA oligonucleotide (S) corresponds to the first 17 nucleotides of the 5'-exon and seven nucleotides of intron sequence (5'-CGUGGUGGG-ACAUUUUCGAGCGGU-3').

DNA oligonucleotides for primer extension were designed so that there was extensive overlap between regions of RNA analyzed. Primers C2 (5'-CTGTCAAGAATATGGATACTTTTGTGTC) or EBS2 (5'-CAATTA-GTGGTGAAGTA) allowed visualization from intron position 120 through the first 50 nucleotides of the 5'-exon. Primer D2a (5'-AATAAT-TGAATATCAGAC) permitted visualization from intron nucleotides 240–110. Primer D1stem (5'-GGGTTTATTCTGTTTATC) permitted visualization from nucleotides 400–190. Primer H3 (5'-TGCATG-CCTGCAGGTCGACTC) allowed visualization from vector sequences lying past the 3' terminus of D3 (~670) to nucleotide 380. Primer D2 (5'-GGTATTATTATTATTTTATTAT) is redundant, but permitted additional visualization of the regions spanning nucleotides 430–300.

DMS modification and reverse transcription

All stock solutions of RNA were stored in ME buffer (10 mM MOPS pH 6.5, 1 mM EDTA). Prior to reaction, solutions were thawed, and aliquots of the RNA were removed, added to a buffer of MOPS 40 mM pH 7.5 and then heated at 95°C for 1 min. After quickly spinning the sample, the RNA was then placed in a water bath at the reaction incubation temperature of 45°C. Samples of exD123 or exD1 RNA (50 nM) were simultaneously combined with D5 RNA (9 µM) and a salt mix (0.5 M KCl and 100 mM MgCl₂) in a volume of 20 µl for 15 min. All concentrations indicated are final. Samples of D1 RNA (50 nM) were preincubated with (or without) substrate oligonucleotide (500 nM) for 10 min, and then combined simultaneously with D5 (9 µM), sufficient to fully saturate exD123, exD1 and D1 RNAs) and the salt mix described above (except that the buffer was 80 mM MOPS pH 7.5). To ensure that the extent of modification was the same in reactions with and without D5, the total RNA concentration was adjusted with 4 µg/µl 16S/23S ribosomal RNA (Boehringer Mannheim). In reactions without D5, 12 µg of rRNA was added, but in reactions containing D5, the amount of rRNA concentration was lowered to 6.8 µg in a 20 µl reaction volume. It is important to note that rRNA was used as a carrier in these experiments because tRNA was found to be an inhibitor, binding various intronic constructs with micromolar affinity (data not shown). This has important implications for experiments in which tRNA was used during activity assays or even during precipitations of group II intron RNA.

After RNA components had been preincubated so that exD123, exD1 or D1 would be fully bound to D5, the samples were treated with DMS: a DMS mix comprised 1 µl DMS in 80 µl ethanol. Two microliters of this mix were added to 20 µl of RNA (in complex with D5 as above) and incubated at 25°C for 5 min. The reaction was stopped by addition of three vols of quench buffer as described previously (Murphy and Cech, 1993), followed by ethanol precipitations and resuspension in 20 µl of ME buffer. In some instances, DMS modification experiments were also conducted on ice, instead of at 25°C, which results in a different pattern of nucleotide sensitivity to the modification reagent (increasing attack of C relative to A).

The DMS reaction was divided into aliquots, each of which was annealed to different primers for examining different portions of the intron. For a given primer extension reaction, 5 µl of RNA/DMS reaction (from above) was combined with primer (25 nM ³²P-5'-end-labeled and 75 nM unlabeled), heated at 95°C for 1 min and then cooled to room temperature. This RNA/primer solution was then combined with an equal volume of RT mix containing dNTPs (0.5 mM, final concentration), RT buffer (Boehringer Mannheim) and RNasin (Boehringer Mannheim). Finally, AMV RT (0.625 U/µl final concentration, Boehringer Mannheim) was added and the solution was incubated at 55°C for 15 min, followed by addition of an equal volume of urea/sucrose loading buffer. Prior to loading on an 8% denaturing polyacrylamide gel, the sample was heated to 95°C for 1 min. Gels were dried and then subjected to autoradiography. For comparison of relative band intensities, gels were analyzed on a PhosphorImager (Molecular Dynamics) or an Instantimager (Packard Instruments) and subjected to quantitation. Integral values for the relative intensity of individual protection patterns were obtained by determining the ratio of counts for corresponding bands in the (–D5) relative to the (+D5) lanes. In several cases, all counts in an individual lane were normalized to correct for unequal loading relative to other lanes (never more than a 15% difference from lane to lane).

DMS modifications were performed at 25°C rather than at 45°C due to the extreme reactivity of DMS at the higher temperature. However,

it is important to note that the exD123–D5 complex is still catalytically reactive under the conditions used for DMS footprinting (preincubation of the components at 45°C, followed by incubation of the complex at 25°C). Like most chemical reactions, catalysis is significantly slowed at 25°C (B.B.Konforti and A.M.Pyle, unpublished results), complicating complete kinetic analysis at this temperature. However, there are many lines of evidence indicating that the conformation of the complexes is not altered by lowering the temperature for DMS reactions: (i) DEPC modification patterns of D5 molecules are identical at 45 and 25°C (B.B.Konforti and A.M.Pyle, unpublished results). (ii) Reducing the temperature from 45 to 25°C does not significantly decrease the fraction of D5 molecules bound to exD123 (B.B.Konforti and A.M.Pyle, unpublished results). (iii) The patterns of DMS accessibility within D1 at 42°C (Qin and Pyle, 1997) and 25°C (Figures 2A, 3A and 5) are similar and are consistent with known tertiary interactions (such as α – α' and β – β'). (iv) Binding of substrate, as evidenced by protection of the EBS sequences, is observed at 42°C (Qin and Pyle, 1997) and at 25°C (Figure 5).

Direct D5 binding studies

Binding of ³²P-labeled D5 to unlabeled exD123, exD1 or D1 was monitored using a gel filtration spin column method that was adapted from published methods (Beebe and Fierke, 1994; Konforti, 1998), quantitated and fitted as previously described (Pyle and Green, 1994). Briefly, the RNAs were incubated separately at 95°C and then cooled to 45°C before being combined together with reaction buffer (40 mM MOPS pH 7.5, 100 mM MgCl₂, 500 mM KCl). After incubating for 10–15 min to achieve equilibrium, bound and free D5 molecules were separated on spin columns as previously described (Konforti *et al.*, 1998). For exD1 and D1 it was important to test a variety of different column matrices and elution times to determine conditions that would be most appropriate for partitioning exD1 (or D1) in the eluate and D5 in the matrix. For exD1 and D1, Sephadex G-75 was the optimal column matrix, while Sephacryl S-100 was used for exD123 (different matrices are necessary for eluting complexes of different sizes). Samples were loaded onto the column, spun for 15 s, chased with 10 µl buffer and then spun again for 15 s. Under these conditions, ~80% of exD1, ~70% of D1 and <5% of D5 were eluted. These values were used to correct the value for the fraction D5 bound. Each experiment was conducted at more than six different concentrations and performed in triplicate.

In the case of D1, binding was also conducted under standard conditions for reactions with D5 and oligonucleotide substrate (80 mM MOPS pH 7.5, 100 mM MgCl₂ and 1 M KCl at 42°C) (Michels and Pyle, 1995). The *K_d* obtained under these conditions was identical (within margin of error) to that obtained under the above reaction conditions. Binding of D5 to D1 was conducted in the absence and presence (500 nM) of oligonucleotide substrate by preincubating D1 and S for 10 min at 42°C and then combining with D5 and the salt mix. Separate preincubations in the presence of salts (D1 and S in one, and D5 in the other) were not found to affect the *K_d* (data not shown).

Kinetic analysis

For *cis*-exon cleavage experiments, single-turnover reactions were performed using trace concentrations (~1 nM) of exD123, exD13, exD1 or exD12 which had been transcribed in the presence of [α -³²P]ATP (as substrate) and an excess (3 µM) of D5 RNA (as enzyme) as described previously (Pyle and Green, 1994). The exD12 RNA was generated by the DNase reaction described above. Data were plotted and quantitated as described previously (Pyle and Green, 1994).

To determine whether exD1 could hydrolyze the 5' splice site of an oligonucleotide (substrate) provided *in trans*, exD1 was tested under the same reaction conditions described previously for D1, D5 and substrate (Michels and Pyle, 1995). Briefly, exD1 (100 nM), D5 (3 µM) and 5'-³²P-labeled substrate (0.5 nM) were heated separately to 95°C for 2 min in buffer (80 mM MOPS pH 7.5). The RNAs were cooled to 42°C and the reaction was initiated by adding a salt mix to final reaction concentrations of 100 mM MgCl₂, 1 M KCl and 80 mM MOPS pH 7.5 in a reaction volume of 10 µl. Reactions were monitored and quantitated as described previously (Michels and Pyle, 1995). Under these conditions, rates for D1 and exD1 were 0.026 and 0.0023/min, respectively. To maximize sensitivity (Doudna and Cech, 1995), competitive inhibition experiments were conducted under conditions in which the concentration of D5 was well below *K_m* (~50 nM) and the inhibitor was present 500, 1000 or 3000 nM.

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References

- Abramovitz, D.L. and Pyle, A.M. (1997) Remarkable morphological variability of a common RNA folding motif: the GNRA tetraloop-receptor interaction. *J. Mol. Biol.*, **266**, 493–506.
- Abramovitz, D.L., Friedman, R.A. and Pyle, A.M. (1996) Catalytic role of 2'-hydroxyl groups within a group II intron active site. *Science*, **271**, 1410–1413.
- Beebe, J.A. and Fierke, C.A. (1994) A kinetic mechanism for cleavage of pre-tRNA Asp catalyzed by the RNA component of *Bacillus subtilis* Ribonuclease P. *Biochemistry*, **33**, 10294–10304.
- Boudvillain, M. and Pyle, A.M. (1998) Defining the functional groups, core structural features and inter-domain tertiary contacts essential for group II intron self-splicing: a NAIM analysis. *EMBO J.*, **17**, 7091–7104.
- Cate, J.H., Gooding, A.R., Podell, E., Zhou, K., Golden, B.L., Szewczak, A.A., Kundrot, C.E., Cech, T.R. and Doudna, J.A. (1996) RNA tertiary structure mediation by adenosine platforms. *Science*, **273**, 1696–1699.
- Chanfreau, G. and Jacquier, A. (1994) Catalytic site components common to both splicing steps of a group II intron. *Science*, **266**, 1383–1387.
- Chanfreau, G. and Jacquier, A. (1996) An RNA conformational change between the two chemical steps of group II self-splicing. *EMBO J.*, **15**, 3466–3476.
- Conway, L. and Wickens, M. (1989) Modification interference analysis of reactions using RNA substrates. *Methods Enzymol.*, **180**, 369–379.
- Costa, M. and Michel, F. (1995) Frequent use of the same tertiary motif by self-folding RNAs. *EMBO J.*, **14**, 1276–1285.
- Costa, M., Deme, E., Jacquier, A. and Michel, F. (1997) Multiple tertiary interactions involving domain II of group II self-splicing introns. *J. Mol. Biol.*, **267**, 520–536.
- Doudna, J.A. and Cech, T.R. (1995) Self-assembly of a group I intron active site from its component tertiary structural domains. *RNA*, **1**, 36–45.
- Franzen, J.S., Zhang, M. and Peebles, C.L. (1993) Kinetic analysis of the 5'-splice junction hydrolysis of a Group II intron promoted by Domain 5. *Nucleic Acids Res.*, **21**, 627–634.
- Griffin, E.A., Qin, Z.-F., Michels, W.A. and Pyle, A.M. (1995) Group II intron ribozymes that cleave DNA and RNA linkages with similar efficiency and lack contacts with substrate 2'-hydroxyl groups. *Chem. Biol.*, **2**, 761–770.
- Harris-Kerr, C.L., Zhang, M. and Peebles, C.L. (1993) The phylogenetically predicted base-pairing interaction between α and α' is required for group II splicing *in vitro*. *Proc. Natl Acad. Sci. USA*, **90**, 10658–10662.
- Inouye, T. and Cech, T.R. (1985) Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: A technique for RNA structure analysis using chemical probes and reverse transcriptase. *Proc. Natl Acad. Sci. USA*, **82**, 648–652.
- Jacquier, A. and Michel, F. (1987) Multiple exon-binding sites in class II self-splicing introns. *Cell*, **50**, 17–29.
- Jacquier, A. and Michel, F. (1990) Base-pairing interactions involving the 5'- and 3'-terminal nucleotides of group II self-splicing introns. *J. Mol. Biol.*, **213**, 437–447.
- Jarrell, K.A., Dietrich, R.C. and Perlman, P.S. (1988) Group II Intron Domain 5 facilitates a *trans*-splicing reaction. *Mol. Cell. Biol.*, **8**, 2361–2366.
- Jestin, J.-L., Deme, E. and Jacquier, A. (1997) Identification of structural elements critical for inter-domain interactions in a group II self-splicing intron. *EMBO J.*, **16**, 2945–2954.
- Koch, J.L., Boulanger, S.C., Dib-Hajj, S., Hebbard, S.K. and Perlman, P.S. (1992) Group II introns deleted for multiple substructures retain self-splicing activity. *Mol. Cell. Biol.*, **12**, 1950–1958.
- Konforti, B.B., Abramovitz, D.L., Duarte, C.M., Karpeisky, A., Beigelman, L. and Pyle, A.M. (1998) Ribozyme catalysis from the major groove of group II intron domain 5. *Mol. Cell*, **1**, 433–441.
- Kunkel, T.A., Bebenek, K. and McClary, J. (1991) Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.*, **204**, 125–139.
- Kwakman, J.H., Konings, D.A., Hogeweg, P., Pel, H.J. and Grivell, L.A. (1990) Structural analysis of a group II intron by chemical modifications and minimal energy calculations. *J. Biomol. Struct. Dyn.*, **8**, 413–430.
- Marino, J.P., Gregorian, R.S., Csankovszki, G. and Crothers, D.M. (1995) Bent helix formation between RNA hairpins with complementary loops. *Science*, **268**, 1448–1454.
- Massire, C., Jaeger, L. and Westhof, E. (1998) Derivation of the three-dimensional architecture of bacterial Ribonuclease P RNAs from comparative sequence analysis. *J. Mol. Biol.*, **279**, 773–793.
- Michel, F. and Ferat, J.-L. (1995) Structure and activities of group II introns. *Annu. Rev. Biochem.*, **64**, 435–461.
- Michel, F., Umesono, K. and Ozeki, H. (1989) Comparative and functional anatomy of group II catalytic introns—a review. *Gene*, **82**, 5–30.
- Michels, W.J. and Pyle, A.M. (1995) Conversion of a group II intron into a new multiple-turnover ribozyme that selectively cleaves oligonucleotides: elucidation of reaction mechanism and structure/function relationships. *Biochemistry*, **34**, 2965–2977.
- Moazed, D., Stern, S. and Noller, H.F. (1986) Rapid chemical probing of conformation in 16S ribosomal subunits using primer extension. *J. Mol. Biol.*, **187**, 399–416.
- Murphy, F.L. and Cech, T.R. (1993) An independently folding domain of RNA tertiary structure within the *Tetrahymena* ribozyme. *Biochemistry*, **32**, 5291–5300.
- Peebles, C.L., Zhang, M., Perlman, P.S. and Franzen, J.F. (1995) Identification of a catalytically critical trinucleotide in Domain 5 of a group II intron. *Proc. Natl Acad. Sci. USA*, **92**, 4422–4426.
- Podar, M., Dib-Hajj, S. and Perlman, P.S. (1995a) A UV-induced Mg²⁺-dependent cross-link traps an active form of Domain 3 of a self-splicing group II intron. *RNA*, **1**, 828–840.
- Podar, M., Perlman, P.S. and Padgett, R.A. (1995b) Stereochemical selectivity of group II intron splicing, reverse-splicing and hydrolysis reactions. *Mol. Cell. Biol.*, **15**, 4466–4478.
- Podar, M., Zhou, J., Zhang, M., Franzen, J.S., Perlman, P.S. and Peebles, C.L. (1998) Domain 5 binds near a highly-conserved dinucleotide in the joiner linking domains 2 and 3 of a group II intron. *RNA*, **4**, 151–166.
- Pyle, A.M. (1996) Catalytic reaction mechanisms and structural features of Group II intron ribozymes. In Eckstein, F. and Lilley, D.M.J. (eds), *Nucleic Acids and Molecular Biology*. Vol. 10. Springer Verlag, New York, pp. 75–107.
- Pyle, A.M. and Green, J.B. (1994) Building a kinetic framework for group II intron ribozyme activity: quantitation of interdomain binding and reaction rate. *Biochemistry*, **33**, 2716–2725.
- Pyle, A.M., Murphy, F.L. and Cech, T.R. (1992) Ribozyme substrate binding site in the catalytic core of the *Tetrahymena* ribozyme. *Nature*, **358**, 123–128.
- Qin, P.Z.F. and Pyle, A.M. (1997) Stopped-flow fluorescence spectroscopy reveals that domain 1 of a group II intron is an independent folding unit. *Biochemistry*, **36**, 4718–4730.
- Qin, P.Z. and Pyle, A.M. (1998) The architectural organization and mechanistic function of group II intron structural elements. *Curr. Opin. Struct. Biol.*, **8**, 301–308.
- Santoro, S.W. and Joyce, G.F. (1997) A general purpose RNA-cleaving DNA enzyme. *Proc. Natl Acad. Sci. USA*, **94**, 4262–4266.
- Strobel, S.A. and Shetty, K. (1997) Defining the chemical groups essential for *Tetrahymena* group I intron function by nucleotide analog interference mapping (NAIM). *Proc. Natl Acad. Sci. USA*, **94**, 2903–2908.
- Strobel, S.A., Ortoleva-Donnelly, L., Ryder, S.P., Cate, J.H. and Moncoeur, E. (1998) Complementary sets of noncanonical base pairs mediate RNA helix packing in the group I intron active site. *Nature Struct. Biol.*, **5**, 60–65.
- Suchy, M. and Schmelzer, C. (1991) Restoration of the self-splicing activity of a defective group II intron by a small *trans*-acting RNA. *J. Mol. Biol.*, **222**, 179–187.
- Xiang, Q., Qin, P.Z., Michels, W.J., Freeland, K. and Pyle, A.M. (1998) The sequence-specificity of a group II intron ribozyme: Multiple mechanisms for promoting unusually high discrimination against mismatched targets. *Biochemistry*, **37**, 3839–3849.
- Yap, L.-P. and Musier-Forsyth, K. (1995) Transfer RNA aminoacylation: Identification of a critical ribose 2'-hydroxyl-base interaction. *RNA*, **1**, 418–424.

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