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Review

RNA-splicing endonuclease structure and function

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Abstract. The RNA-splicing endonuclease is an evolutionarily conserved enzyme responsible for the excision of introns from nuclear transfer RNA (tRNA) and all archaeal RNAs. Since its first identification from yeast in the late 1970s, significant progress has been made toward understanding the biochemical mechanisms of this enzyme. Four families of the splicing endonucleases possessing the same active sites and overall architecture but with different subunit compositions have been identified. Two related consensus structures of the precursor RNA splice sites and the critical elements required for intron excision have been established. More recently, a glimpse was obtained of the structural mechanism by which the endonuclease recognizes the consensus RNA structures and cleaves at the splice sites. This review summarizes these findings and discusses their implications in the evolution of intron removal processes.

Keywords. tRNA maturation, splicing, endonuclease, intron, phosphoryl bond cleavage, enzyme structure and function, RNA-protein interaction.

Introduction

Transfer RNA (tRNA) is essential to protein synthesis. While providing the physical link between amino acids and messenger RNA (mRNA), tRNAs serve to translate mRNA codons and supply amino acids required for nascent proteins. This process critically depends on the compositional and structural integrity of the tRNA, which is believed to have performed this function since the earliest forms of life emerged [1].

In all three kingdoms of life, tRNAs are matured through a complex pathway. Following transcription, each tRNA may undergo base editing, 5′-end processing, 3′-end processing, base and ribose modifications and removal of intervening sequences or introns [2]. Among these processes, removal of introns is universally conserved and essential to cell growth.

In Archaea and Eukarya, at least 5 % of tRNA genes contain introns and these genes are clustered in a distinct manner. In eukaryotes, tRNA introns are distributed according to tRNA isoacceptor gene families that are found in separate chromosomes and either all or none of the members in a given family contain introns [3–7]. The presence of introns in precursor tRNA interrupts the compact mature structure and thereby presents a challenge to cellular systems that rely upon the mature molecule. Research over the last 30 years has identified and characterized three protein enzymes that are responsible for this splicing process and these earlier works have been extensively reviewed elsewhere [2, 3, 8]. Significant achievements have recently been made which increase our knowledge regarding the structure and function of the splicing endonuclease, which recog-

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nizes and excises the introns. This review will focus on these recent developments and discuss findings in light of the large body of biochemical data already compiled on the splicing endonuclease.

tRNA-splicing pathway

tRNA splicing involves up to three distinct steps with three separate enzymes. It is significant that all of the proteins involved in this process are found to be essential for the growth of yeast cells [9–11], indicating that tRNA splicing is an obligatory prologue to protein synthesis. In the first step of the process (Fig. 1), the splicing endonuclease excises the intron. Second, a ligase joins the two exon halves. Third, in some organisms, a 2′-phosphotransferase removes a 2′-phosphate left at the ligation junction [12–16]. The cleavage step is universally conserved [2] but the same cannot be said for the subsequent steps. Two ligation activities are known that differ in catalysis and reaction products. The eukaryal ligase possesses 2′,3′-cyclic phosphodiesterase, 5′-kinase and ligase activity and produces a ligation junction bearing a 2′-phophate group (Fig. 1). Therefore, the third enzyme, 2′-phosphotransferase, is required in eukaryotes. In Archaea, however, despite the presence of the 2′-phophotransferase homologues in all archaeal genomes [2], the ligation product bears no 2′-phosphate [17]. The fact that the cleavage step has not diverged is demonstrates that the core components involved in cleavage have remained intact despite 'cosmetic' changes to this enzyme.

Among the currently known methods of splicing, tRNA splicing is unique in that it depends solely on protein enzymes. Group I self-cleaving introns, group II self-cleaving introns and mRNA splicing mediated by the spliceosome are all known to involve an RNA catalyst [18]. There is also evidence suggesting that the small tRNA introns were once self-cleaving [19]. Thus, from a comparison of reaction pathways and catalytic mechanisms, investigation of the protein-catalyzed splicing mechanism provides an important parallel view to mechanisms catalyzed by RNA.

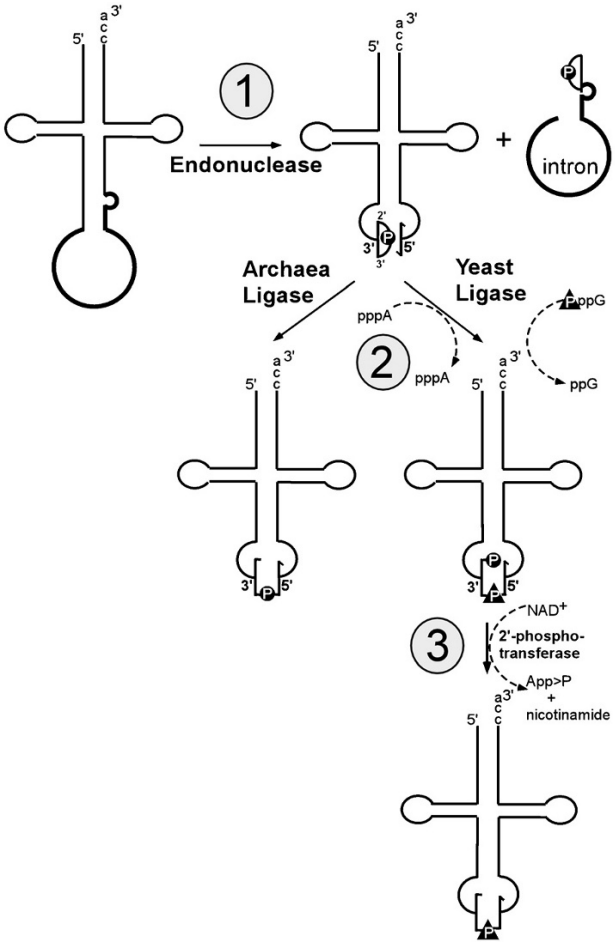

Figure 1. The eukaryotic and archaeal (t)RNA splicing pathway. The three steps are identified by number. The pathways split at the ligation step, where the cyclic phosphates are opened differently. Required co-factors are labeled at the beginning of each step. Gray circles indicate endogenous phosphates while gray triangles indicate exogenous phosphates donated by GTP (yeast pathway). pppA symbolizes ATP, pppG symbolizes GTP, ppG symbolizes GDP, NAD^+ symbolizes nicotinamide adenine dinucleotide and App > P symbolizes ADP-ribose 1″-2″ cyclic phosphate.

tRNA intron structures form the basis of substrate recognition

Introns in tRNA are generally small (~14–106 bases [20]) but share no sequence homology, even within the same organism. Located predominantly in the anticodon stem, introns are believed to minimally interrupt the overall tRNA structure [21]. Most precursor tRNAs, therefore, already have an intact mature domain comprised of the acceptor stem, the D stem and the TψC stem.

Early studies in John Abelson's and Glauco Tocchini-Valentini's laboratories were instrumental in determining the essential elements responsible for the specificity of the intron-exon boundaries in eukaryotes [22–24]. The single most conserved feature among nuclear tRNA introns is their location: they invariably begin at the second nucleotide downstream of the anticodon, suggesting that the cleavage sites (intron-exon boundaries) are recognized by their conserved positions [22, 23]. The mature domain of eukaryotic precursor tRNA facilitates this recognition mechanism by serving as an anchor [22, 25]. Two specific nucleotides were also found to be critical to the recognition of introns. These are referred to as

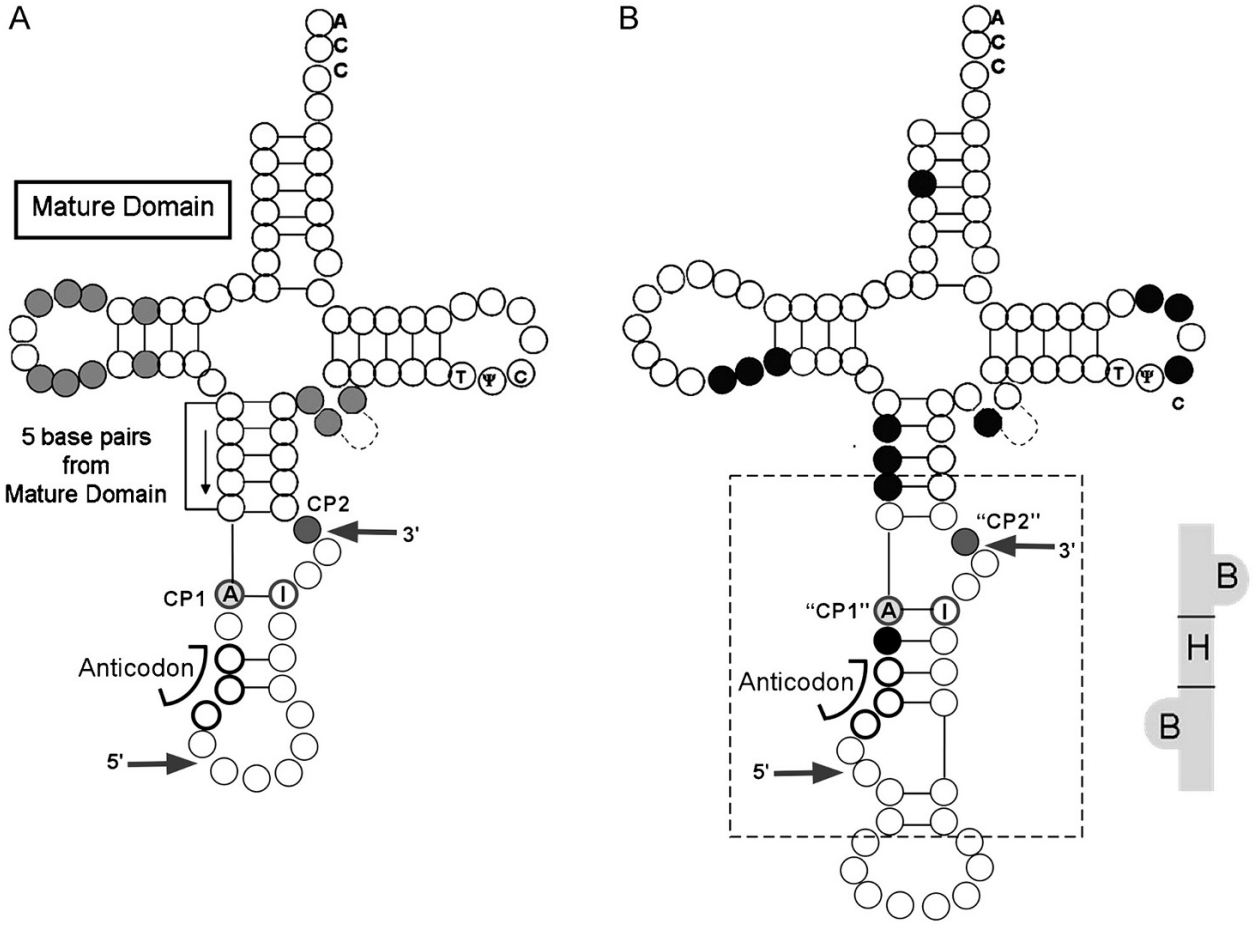

Figure 2. The consensus precursor tRNA substrates of Eukarya (*A*) and Archaea (*B*). The arrows labeled 5′ and 3′ indicate the boundaries of the predominant intron location in most precursors (variable in length). Labels A or I indicate anticodon-intron (A-I) pairs. (*A*) Gray circles in the mature domain indicate additional regions of variable length. Cardinal positions one and two (CP1 and CP2) are labeled. (*B*) Black circles in the mature domain indicate additional intron locations (variable in length and shape). The archaeal CP1 and CP2 analogues are labeled in quotations. The boxed area shows the canonical bulge-helix-bulge motif.

'cardinal positions' 1 and 2 (CP1 and CP2). CP1 is two bases upstream of the anticodon [23, 24, 26] and forms a base pair with a base in introns 3 bases upstream of the 3′ cleavage site; this is referred to as the anticodon-intron pair or A-I pair [23, 24, 27, 28] (Fig. 2a). CP2 is located at the 3′ intron-exon boundary in the anticodon stem, one base downstream of the 3′ cleavage site. Interestingly, the base identity of CP2 co-varies with that of the bases in the A-I pair [26]. It has been demonstrated that the yeast splicing endonuclease uses a 'ruler mechanism' in its native substrates and measures five base pairs from the anchoring mature domain to locate the 5′ cleavage site [22] and relies upon the A-I pair to position the 3′ cleavage site [26] (Fig. 2a).

The substrate specificity of the archaeal splicing endonuclease was initially studied in Charles Daniels' and Roger Garrett's laboratories. In contrast to the eukaryal requirement for the mature domain, intron recognition in Archaea relies upon a small RNA secondary structure called the bulge-helix-bulge (BHB) motif. A canonical BHB motif contains two three-nucleotide bulges separated by four base pairs, although small variations of the BHB motif are observed in certain archaeal organisms [29]. This motif is both necessary and sufficient for the recognition and cleavage of most archaeal splice sites [30–34] (Fig. 2b). Archaeal introns are often found in regions other than the anticodon stem [29], indicating that archaeal splicing enzymes act independently of the pre-tRNA mature domain. In fact, the BHB motif also marks intron boundaries in archaeal ribosomal RNA (rRNA) and mRNA [34, 35].

Despite the differences between eukaryal and archaeal pre-tRNA, some resemblances are also apparent. The BHB motif shares with eukaryal precursors the A-I pair at the 3′ splice site [26]. In addition, an equivalent A-I pair exists in the 5′ splice site of the BHB motif due to its pseudosymmetric nature (Fig. 2b). These analyses of pre-tRNA structures

indicate that RNA structural features – not sequence – are the basis of substrate recognition by the splicing endonuclease.

The splicing endonucleases originate from a common ancestor

Since its 1978 discovery in yeast [2], the splicing endonuclease has been identified in all sequenced archaeal and eukaryotic genomes as well as some parasite genomes. Bacteria, in which splicing is self-catalyzed [36], do not contain the splicing endonuclease. Based on the number of subunits required for the enzyme activity, the endonuclease can be divided into four families: α_4 (homotetramers), α_2 (homodimers), $(\alpha\beta)_2$ (dimers of heterodimers), and $\alpha\beta\gamma\delta$ (heterotetramers) (Fig. 3). Evidence accumulated over several years indicates that the splicing endonucleases of all organisms stem from a common ancestor. Despite their different subunit compositions, the endonuclease families are related by significant sequence homology in individual subunits.

Our fundamental understanding of the splicing endonuclease began when Abelson and colleagues first isolated and characterized the yeast endonuclease. By 1997, the yeast splicing endonuclease was found to contain four different subunits ($\alpha\beta\gamma\delta$) [8]. Meanwhile, two archaeal splicing endonucleases were characterized by Charles Daniels and Roger Garrett, which were found to contain either two or four identical copies of a single subunit (α_2 or α_4) [37, 38]. The yeast endonuclease includes Sen2p, Sen34p, Sen54p and Sen15p, where Sen2p and Sen34p are active and homologous subunits. Furthermore, these subunits are homologous to the archaeal α_2 or α_4 subunits [9, 38]. From these combined findings, it became apparent that all splicing endonucleases must contain two conserved cleavage units. However, the arrangements of these cleavage units in different endonucleases remain a topic of intense interest, as do the functional and evolutionary implications of the widely varied subunit compositions.

Endonucleases are further related via their catalytic and substrate recognition mechanisms. All splicing endonucleases produce a 2′,3′ cyclic phosphate and a 5′ hydroxyl termini at both cleavage sites [8, 30, 39, 40], suggesting that all families of endonucleases share an identical catalytic mechanism. In addition, works co-published by the Abelson, Daniels and Tocchini-Valentini groups demonstrated that the archaeal and eukaryal tRNA splicing systems are related by their ability to recognize the common BHB RNA motif regardless of their cognate RNA substrates [9, 26, 27, 38, 41, 42].

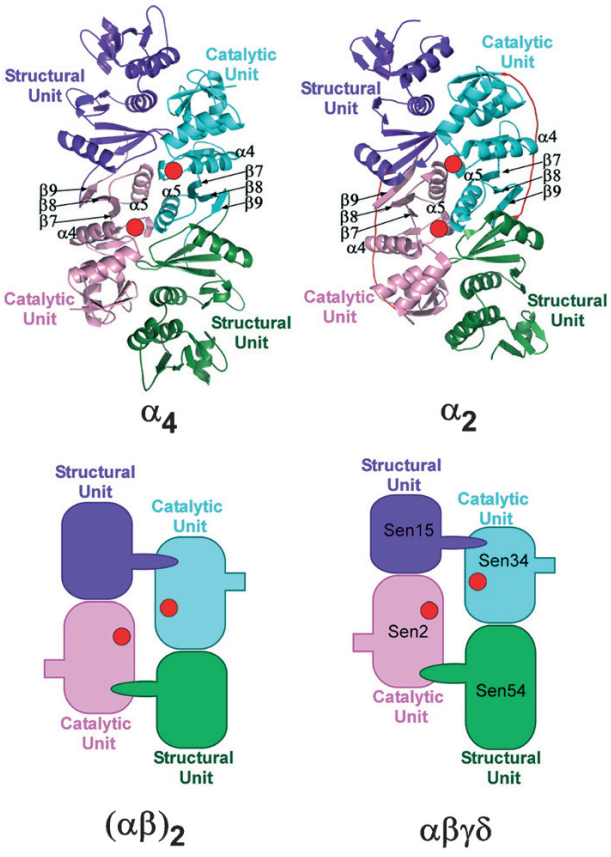

Figure 3. Structural models of the four currently known endonucleases. The α_4 homotetramer and α_2 homodimer structures are known and the schematic drawings of the $(\alpha\beta)_2$ and $\alpha\beta\gamma\delta$ enzymes are proposed structural arrangements. In all cases, the following color scheme is used: pink and cyan units are catalytic, while purple and green units are structural. In the α_4 enzyme, each unit is an identical monomer. In the α_2 enzyme, green and cyan units connected by a red peptide linker form one monomer and the other monomer is pink/purple. In the $(\alpha\beta)_2$ enzyme, each unit is separate with pink and cyan being identical and green and purple also being identical. Green and cyan units form one heterodimer and the other heterodimer is pink/purple. In the $\alpha\beta\gamma\delta$ enzyme, each unit is separate and distinct. Conserved secondary structural features are labeled and red dots indicate the common active sites. The structure of the α_4 enzyme is from PDB id 1A79 [42]; the structure of the α_2 enzyme is from PDB id 1RLV [41].

Subunit composition is correlated with substrate specificity

It was already established that archaeal and eukaryal endonucleases possess different subunit compositions and varied RNA recognition abilities when Marck and Grosjean found another connection between subunit composition and intron-exon structural motifs. Crenarchaeal (but not euryarchaeal) organisms contain unusually high instances of noncanonical BHB motifs (Fig. 2b, black circles) and their splicing endonucleases all belong to the $(\alpha\beta)_2$ family. Subsequent *in vitro* functional studies using purified splicing endonucleases from two crenarchaeal organisms showed that these

endonucleases indeed cleave a broader range of RNA substrates than their euryarchaeal counterparts [43, 44]. Therefore, within one organismal kingdom as well as between kingdoms, subunit composition is correlated to substrate specificity.

An explanation was needed for this observation. A recent study of the new $(\alpha\beta)_2$ family of endonucleases showed that the ability of the endonucleases to recognize a broader range of substrates resides within the catalytic subunit itself. The catalytic subunit of an $(\alpha\beta)_2$ endonuclease was engineered to resemble an α_4 endonuclease (eliminating the need for the β subunit) and was found to retain most of its native recognition properties [45] (Fig. 4b). The demonstration that the catalytic subunit contains all of the features responsible for broad substrate specificity raised the possibility that non-catalytic subunits enhance the ability of the $(\alpha\beta)_2$ endonuclease to regulate and co-ordinate with other processes.

Structural studies

High-resolution crystal structures of the α_4 and α_2 endonucleases and the catalytic subunit of an $(\alpha\beta)_2$ endonuclease are known. The crystal structure of an α_2 splicing endonuclease bound to the BHB substrate RNA is also available. These structural studies have shed significant light on the methods of subunit assembly and RNA splice site recognition and are described below.

Conserved four-unit construct

Crystal structures of the splicing endonucleases from the euryarchaeal organisms *Methanococcus jannaschii* (MJ) [42] and *Archaeoglobus fulgidus* (AF) [41] were first determined by John Abelson and co-workers. MJ endonuclease is a small protein of 197 amino acids and AF endonuclease is a tandem repeat of the MJ protein, where the first repeat lacks the putative catalytic residues. Accordingly, MJ endonuclease forms a homotetramer (α_4) equivalent to the AF endonuclease homodimer (α_2). Therefore, the apparent difference in subunit compositions diminishes at the level of enzyme quaternary structure. Because of its simplicity, the α_4 family is commonly believed to be the earliest form of the endonuclease [37, 46, 47], and the arrangement of the four MJ endonuclease subunits in space becomes the phenotypical assemblage for all endonucleases. This arrangement is anticipated to also represent the architecture of the eukaryotic splicing endonucleases, with the four identical subunits replaced by four different ones. Therefore, regardless of subunit composition, the fundamental units of all known endonucleases are of two distinct types: structural and catalytic.

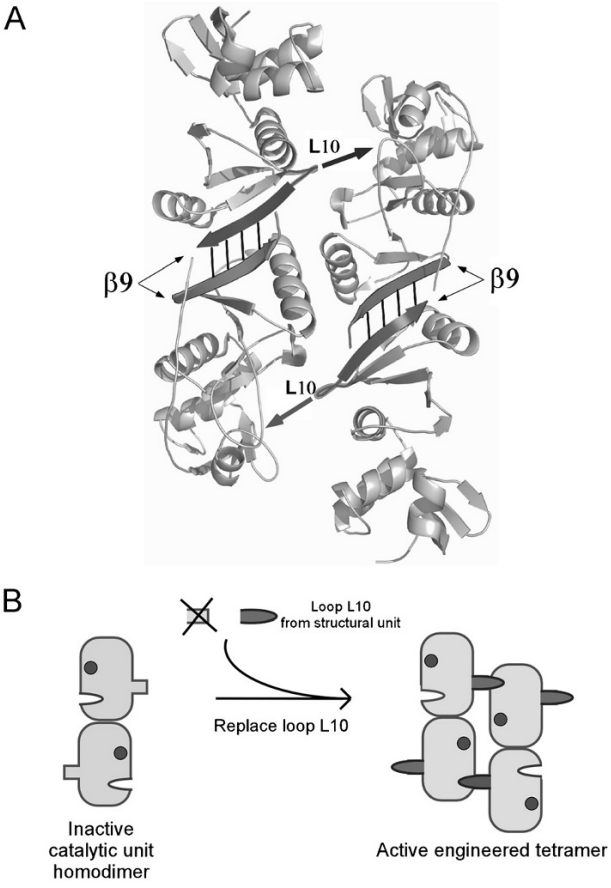

Figure 4. Conserved endonuclease secondary structural features essential to assembly and their importance in RNA-splicing activity. Units are separated for clarity. (*A*) Oligomerization interfaces. The β9 regions are highlighted in each unit and the dimerization interfaces between β9 strands are marked with black lines. The loop L10 of each structural unit is labeled and arrows indicate the pockets into which they insert to form the tetramerization interface. The structure is adapted from [41]. (*B*) A protein engineering experiment [45] which demonstrates that the conserved loop L10 is essential to cleavage activity through its role in tetramer formation.

In the structures of known endonucleases, the four fundamental units were seen organizing two active sites poised to interact with the BHB RNA. The two active sites within the catalytic units are approximately 27 Å apart, the same distance between the cleavage sites in BHB RNA modeled with the enzyme [42]. More importantly, the structural elements responsible for formation of the four-unit construct are conserved throughout evolution (Fig. 3). Although structural information for the fully assembled $\alpha\beta\gamma\delta$ and $(\alpha\beta)_2$ endonucleases is still lacking, yeast two-hybrid studies on the yeast endonuclease subunits [9] and biochemical studies on a $(\alpha\beta)_2$ endonuclease [43, 45] also support this conserved architecture.

Each catalytic unit contains a highly conserved C-terminal domain. This C-terminal domain contains a central β sheet (β7, β8 and β9) sandwiched by two α helices (α4 and α5) (Fig. 3, α_4 and α_2). β strand 9 also takes part in dimerization with the neighboring structural unit, leading to a tail-to-tail isologous dimer stabilized by a cross-unit β sheet (Fig. 4a). The dimerization interface buries ~ 2400 Å^2 of surface area through main-chain hydrogen bonding and hydrophobic interactions. This tail-to-tail dimer is anticipated to be analogous to the interactions detected in yeast two-hybrid studies that showed dimerization between Sen34p and Sen15p and between Sen2p and Sen54p [9]. These data corroborate previous protein cross-linking data showing that the most stable oligomeric states of the MJ endonuclease were dimers and tetramers [37].

The isologous catalytic-structural dimer further dimerizes into the final four-unit construct. This process is mediated predominantly by electrostatic interactions. The acidic L10 loops of the structural units protrude into basic pockets of the opposing catalytic units (Fig. 4a). The L8 loops also participate in these electrostatic interactions. Based upon previous biochemical studies, these electrostatic interactions are expected to be less stable than the interactions at the isologous dimer interfaces [9, 37, 42]. This expectation has led to the hypothesis that the catalytic and structural units pair first to form the isologous dimers, followed by the electrostatic interactions that produce the tetramer, but this hypothesis has yet to be proven experimentally. Overall, among the structural units, only the β8-L10-β9 region is strongly conserved, consistent with its primary role in tetramerization [41, 45]. These findings have established the foundation for what is believed to be the architecture common to all splicing endonucleases.

The universal methods of RNA recognition by the endonuclease

The recent co-crystal structure of AF endonuclease bound to BHB RNA (along with the complementary enzymatic studies) has provided significant insight into how the endonuclease recognizes and binds RNA (Fig. 5) [48]. It was predicted from structural studies and extensive biochemical characterization of the enzyme alone that each of the two catalytic units interacts with each splice site. This is made possible by the locations of the two splice sites on the RNA. The BHB RNA motif, due to its progression within the central helix, presents the two splice sites on one side of the RNA. A relatively flat surface formed jointly by the two catalytic subunits docks directly onto the two bulges that contain the splice sites. The detailed structural information from the endonuclease-RNA complex illustrates clearly how the enzyme recognizes the substrate independently of nucleotide sequence. The bulge nucleotides are critical to recognition and binding. The phosphate backbones of the bulge nucleotides are twisted into open knots stabilized by both protein and RNA functional groups. As a result, these backbone regions rise above the helical surface of the RNA and dock inside the protein. The three bulge nucleotides are completely flipped out of their usual stacking positions to interact with the endonuclease (Fig. 5). Despite a very large buried solvent-accessible surface area between the endonuclease and RNA (3208 Å^2), the 'hot spots' of interactions are, surprisingly, confined to the bulge regions.

Specifically, the first bulge nucleotide is sandwiched by two arginine residues (Fig. 5). One of the arginine residues is strictly conserved and the other may be replaced by aromatic residues in some organisms. The orientations of these side chains relative to one another indicate cation-π interactions. Mutation of the strictly conserved arginine abolished the endonuclease activity, suggesting the importance of this cation-π interaction in binding. This interaction is probably the most crucial recognition mechanism between endonuclease and RNA.

RNA functional groups of the BHB motif also stabilize the sharply bent bulge backbone. The nucleotides immediately flanking each bulge come together to form important close contacts that stabilize the bulge in its bound conformation. Specifically, the base pairs in the central helix adjacent to each bulge correspond to the A-I base pair defined in the yeast pre-tRNAs (Fig. 2a). Not only the Watson-Crick base pairing of the A-I equivalent base pairs but also their interactions with the third bulge nucleotides lend credence to the critical importance of this local RNA structure in enzyme activity (Fig. 5). The third bulge nucleotides form A-minor interactions with these A-I equivalent base pairs. Interestingly, the third bulge nucleotide corresponds to the yeast CP2 nucleotide position and, therefore, its involvement with the A-I pair provides the structural basis for the well-described A-I and CP2 interdependence in eukaryal splicing activity [23, 24].

The overall mode of RNA recognition also reveals the basis for co-operativity between the two splice sites. Remarkably, the arginine pair of one catalytic subunit creates the cation-π interaction with the bulge that is cleaved by the opposing catalytic subunit (Fig. 6a). Disruption of the cation-π interaction at the 5′ splice site reduced the enzyme activity on the 3′ splice site, strongly supporting this model of co-operative binding [48]. Because archaeal catalytic sites are identical, the reciprocal effect would also apply (Fig. 5). Interestingly, in eukaryotes disruption of each cation-π

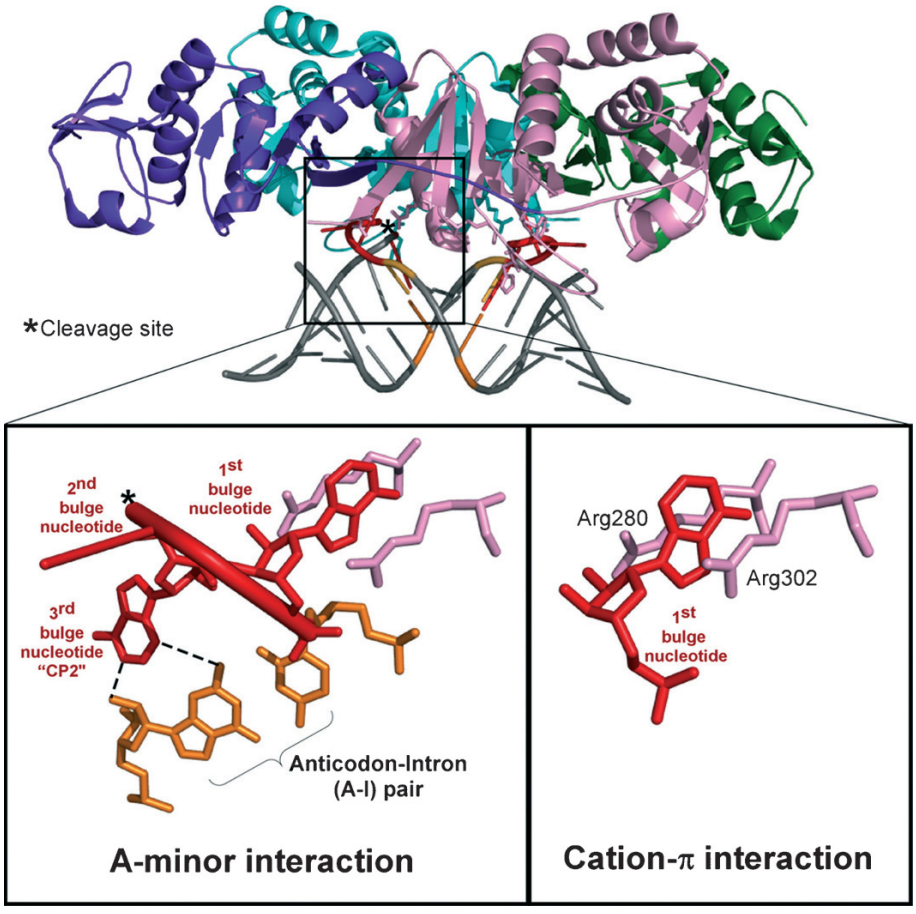

Figure 5. Conserved features critical to RNA recognition by the splicing endonuclease. The structure of the splicing endonuclease-RNA complex [48]. Details reveal the specific key interactions involved in recognition and binding.

interaction has a distinct effect, disrupting cleavage of the 5′ site but not the 3′ site (Fig. 6b). This suggests that the eukaryal 3′ cleavage site orientation is accomplished solely by the A-I pair [49].

The catalytic mechanism

Sequence alignments of all available splicing endonucleases identify four invariant amino acid residues (AF numbering): a histine (His257), a lysine (Lys287), a tyrosine (Tyr246) and an arginine (Arg280). While the arginine residue has been observed to play the key role in binding to substrate via the cation-π interaction, the other three residues are closely clustered in space around the scissile phosphate group, suggesting their critical roles in catalysis [48].

The splicing endonuclease has been compared to RNase A in the context of cleavage chemistry [50] because they both (1) contain a histidine and a lysine residue in their catalytic triad and (2) produce 2′,3′-cyclic phosphate and 5′-hydroxyl termini. RNAse A is extremely well-characterized and its catalytic mechanism is an archetypal example of general acid-base catalysis. In RNase A, His12 acts as a general base and deprotonates the 2′OH of the ribose at the scissile phosphate. The resulting $2'O^-$ is the nucleophile in an in-line attack on the adjacent phosphodiester bond producing a pentavalent transition state stabilized by Lys41. His119 is the general acid which protonates the 5′ leaving group thereby producing the 2′, 3′-cyclic phosphate and 5′-hydroxyl group (Fig. 7) [50]. In the enzyme-substrate structure of Xue et al. [48], RNA-endonuclease interactions at the active sites are consistent with the involvement of catalytic triad residues in cleavage reactions; however, Tyr246 replaces His12 in this mechanism (Fig. 7). Tyr246 would be a weaker base than histidine for the depronation of 2′-OH. This observation is the first departure of the endonuclease catalytic mechanism from that of RNase A.

The placement of the endonuclease catalytic residues suggests a mechanism where Tyr246 deprotonates the nucleophilic 2′-oxygen, His257 protonates the leaving 5′-hydroxyl oxygen and Lys287 stabilizes the developing negative charge of the transition state, in a manner similar to the His12, His119 and Lys41, respectively, of RNase A [50]. These proposed roles are supported by the distances observed in the structure: the pro-S_p nonbridging oxygen of the 5′

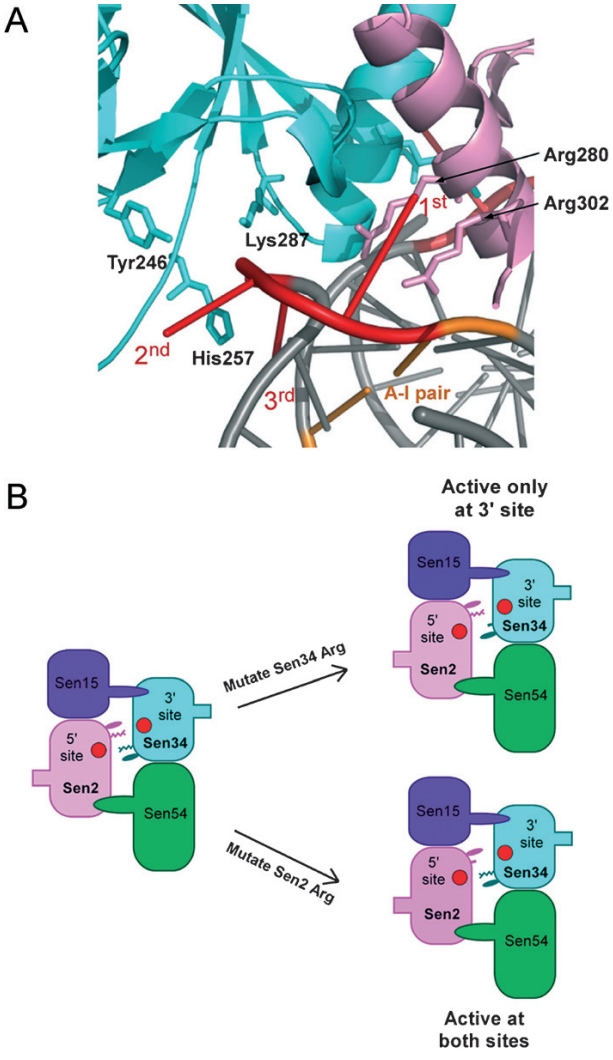

Figure 6. Co-ordinated RNA recognition and cleavage via a composite active site. (*A*) The splicing endonuclease-RNA complex [48] demonstrates co-operativity between pink and cyan catalytic units. Amino acid residues are labeled in black, RNA residues are labeled in red and the A-I pair is labeled in orange. (*B*) A site-directed mutagenesis experiment which demonstrates the elements of co-operativity in the eukaryotic splicing endonuclease [49].

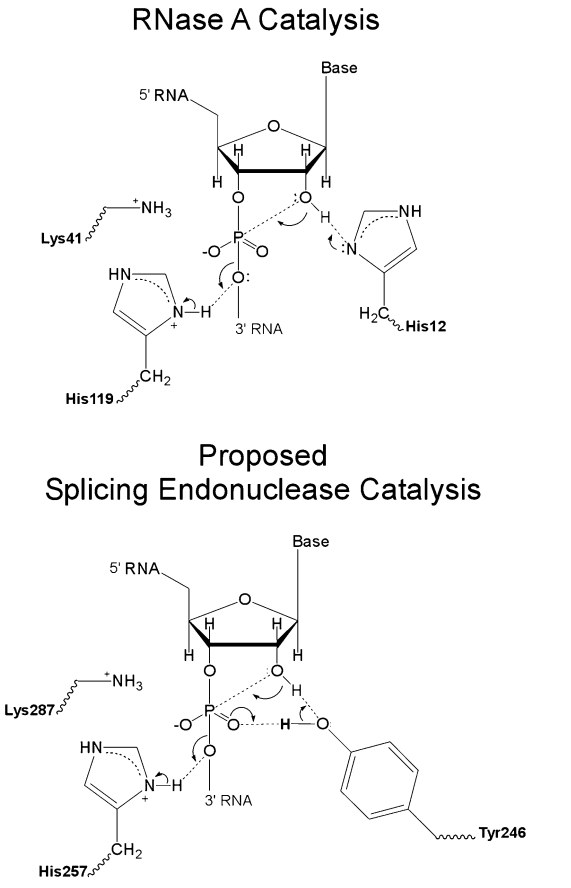

Figure 7. The catalytic mechanism of RNase A and a proposed similar mechanism for the RNA splicing endonuclease.

splice site is 2.6 Å from Lys287, and the leaving 5′ oxygen is 2.7 Å from His257 [48] (Fig. 7). Further enzyme kinetics and theoretical studies are required to completely decipher this catalytic mechanism.

Organisms find innovative uses for the splicing enzymes

This review has focused on the splicing endonuclease, one of the three enzymes involved in tRNA splicing, and discussed recent findings regarding endonuclease assembly and mechanisms of substrate recognition and cleavage. While the findings summarized here have provided several interesting results, more questions remain to be investigated, especially with regard to the three splicing enzymes' evolution and connections to other cellular processes. Over the course of evolution, organisms have demonstrated the propensity for innovation by recruiting these proteins for other purposes. For example, it has been shown that bacteria have a 2′ phosphotransferase enzyme even though they have no other splicing enzymes. The bacterial protein can even substitute for the yeast protein *in vivo* [2]. What the bacteria use this protein for remains a mystery. In eukaryotes, the splicing ligase seals the exon junctions of HAC1 mRNA during the expression of this transcription factor in the unfolded protein response [51]. Plant ligases have been shown to work on a variety of substrates *in vitro*, indicating the possibility of additional unidentified functions *in vivo* [52]. The splicing endonuclease is also multi-tasking. Non-tRNA substrates in mouse cells have been the target of both *cis* and *trans* splicing by the endonuclease [53]. Additionally, the Sen2p catalytic subunit of the human splicing endonuclease was shown to be involved in pre-mRNA 3′ end formation [54]. What other functions exist for these

proteins? Is (t)RNA splicing their original function or an innovation? The answers to these intriguing questions will improve our understanding of how RNA splicing and other cellular processes are interconnected.

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