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Review

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Intronic hammerhead ribozymes in mRNA biogenesis

Abstract: Small self-cleaving ribozymes are a group of natural RNAs that are capable of catalyzing their own and sequence-specific endonucleolytic cleavage. One of the most studied members is the hammerhead ribozyme (HHR), a catalytic RNA originally discovered in subviral plant pathogens but recently shown to reside in a myriad of genomes along the tree of life. In eukaryotes, most of the genomic HHRs seem to be related to short interspersed retroelements, with the main exception of a group of strikingly conserved ribozymes found in the genomes of all amniotes (reptiles, birds and mammals). These amniota HHRs occur in the introns of a few specific genes, and clearly point to a preserved biological role during pre-mRNA biosynthesis. More specifically, bioinformatic analysis suggests that these intronic ribozymes could offer a new form of splicing regulation of the mRNA of higher vertebrates. We review here the latest advances in the discovery and biological characterization of intronic HHRs of vertebrates, including new conserved examples in the genomes of the primitive turtle and coelacanth fish.

Keywords: alternative splicing; amniotes; retrotransposon; RNA self-cleavage.

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Introduction

The hammerhead ribozyme (HHR) together with the hairpin, hepatitis-δ virus (HDV), Varkud satellite and *GlmS* ribozymes, belong to a family of small catalytic RNAs (~50–150 nt) capable of performing an endonucleolytic self-cleavage reaction (Ferre-D'Amare and Scott, 2010). The HHR is made up of three double helices (helix I to III) that intersect at a three-way junction containing the catalytic core of 15 highly conserved nucleotides (Figure 1). Originally described as a hammerhead-like fold because of its predicted secondary structure, the motif actually adopts in solution a 'Y'-shaped fold, where helix III coaxially stacks with helix II, and helix I is parallel to the coaxial stack interacting with helix II through tertiary interactions required for efficient self-cleavage *in vivo* (De la Peña et al., 2003; Khvorova et al., 2003; Martick and Scott, 2006: Chi et al., 2008) (Figure 1A). Three different topologies have been described for this ribozyme, named type I, II or III according to the open-ended helix that connects the HHR motif with the flanking sequences (Figure 1B). The first HHR was discovered in 1986 in the satellite RNA of *Tobacco ringspot virus* (sTRSV) (Prody et al., 1986), where it catalyzes the transesterification reaction of self-cleavage required for the rolling-circle replication of these subviral agents (Flores et al., 2004). Almost simultaneously, a type I HHR was reported in the satellite DNA of the newt genome (Epstein and Gall, 1987), which is transcribed in tandem repeats of ~330 nt (Epstein and Coats, 1991) that form part of a small ribonucleoprotein complex with a yet unknown function (Luzi et al., 1997). Similar to the amphibian motifs, other genomic HHRs have been found to reside in DNA tandem repeats of carnation plants (Daros and Flores, 1995), schistosomes (Ferbeyre et al., 1998) and cave crickets (Rojas et al., 2000), suggesting a similar role for these genomic HHRs in the biology of such tandem-repetitive DNA. More recently, different bioinformatic approaches have uncovered a widespread occurrence of the HHR motif among all life kingdoms (De la Peña and García-Robles, 2010a,b; Jimenez et al., 2011; Perreault et al., 2011; Seehafer et al., 2011; for a review see Hammann et al., 2012). Whereas most of the newly detected examples reinforce a role of the HHR in interspersed repetitive DNA, in some other cases, such as in bacteria (intergenic HHRs) or aminiotes (intronic HHRs), new and specific biological functions are hinted for this small catalytic RNA.

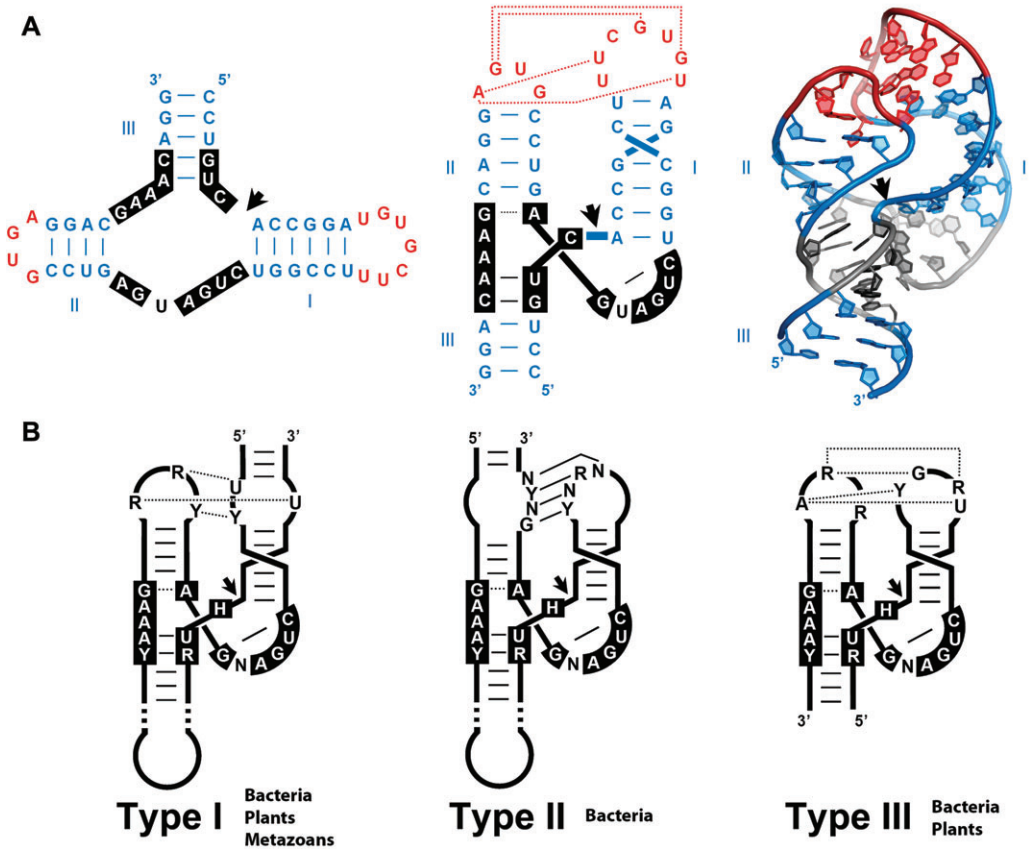

Figure 1 RNA topologies for the hammerhead ribozyme.
(A) Original HHR found in the satellite RNA of Tobacco ringspot virus (Prody et al., 1986) represented either in the classical hammerhead 2D format (left) or in a 3D format (center), showing the real disposition of the helixes based in the crystallographic model (right, PDB 2QUS; Chi et al., 2008). Nucleotides of the catalytic core are boxed. Stems are shown in blue and loops in red. Self-cleavage site is shown by an arrow. (B) Representation of the three possible HHR topologies showing the nucleotides of the catalytic center (boxed) and the conserved loop-loop interactions for each HHR type. Dotted and continuous lines refer to Non-canonical and Watson-Crick base pairs, respectively. The three HHR types can be found in the Prokaryotic/Phage genomes, whereas only type I and III have been described in plants. Metazoan genomes mostly show type I HHRs.

HHRs in vertebrates: variations of a theme

At least three major kinds of HHRs have been described so far in the genomes of vertebrates: (i) retroposon-like HHRs in lower vertebrates; (ii) a 'discontinuous' HHR in some mammals and (iii) intronic HHRs conserved in amniotes (Figure 2). Motifs from the three groups share sequence and structural homology between them, but also with the HHRs previously described in the satellite DNA of trematodes (Ferbeyre et al., 1998; Martick et al., 2008; De la Peña and García-Robles, 2010a), suggesting either a phylogenetic or convergence relationship among them.

The first group of HHRs corresponds to those originally described within tandem repeats of the satellite 2 DNA of newts and salamanders (Epstein and Gall, 1987). Very similar type-I HHRs to these have been recently reported widespread in the genomes of the frog *Xenopus tropicalis* and the lamprey *Petromyzon marinus* (Figure 2A) (De la Peña and García-Robles, 2010a; Perreault et al., 2011; Seehafer et al., 2011). The major feature of this class of HHRs resides in their short and unstable helix III, which is usually capped by a palindromic loop. *In vitro*, such a disposition avoids extensive HHR self-cleavage as a single motif, especially under the physiological Mg^{2+} concentration of ~1 mM (Garrett et al., 1996). However, these HHRs efficiently self-cleave through dimeric motifs thanks to an elongated and more stable helix III (Supplementary Figure 1) in a similar way as described for some plant subviral agents (Prody et al., 1986; Forster et al., 1988). The disposition of tandem HHRs separated by a few hundred nt has been widely detected among many metazoan genomes suggesting that these repeats would be related to SINE-like retrotransposons acting through a yet unknown mechanism (Epstein and Gall, 1987; Ferbeyre et al., 1998;

Rojas et al., 2000; De la Peña and García-Robles, 2010b). Nevertheless, most of the HHR motifs reported in *Xenopus* or lamprey genomes occur as single motifs, within intronic (on either sense or antisense strand) or intergenic regions, with only a few exceptions of multimeric repeats (De la Peña and García-Robles, 2010a).

The second kind of ribozymes are the so-called 'discontinuous' HHR (Martick et al., 2008), an unusual type III ribozyme (Figure 2B) that was found in the 3′ untranslated region (UTR) of some mammalian *Clec-2* genes (for a review see Scott et al., 2009). The specific biological role of this motif is not yet known, although different functions in post-transcriptional gene regulation, like the control of mRNA decay or alternative polyadenilation sites, can be envisaged.

The last family of vertebrate HHRs is composed of a group of ultraconserved motifs (Figure 2C) that occur within the introns of a few specific genes of amniotes (De la Peña and García-Robles, 2010a). These intronic ribozymes are type I HHRs similar to those found in amphibians, with the main difference being that the amniota HHRs show an elongated helix III with at least four Watson-Crick base pairs instead of only one. This arrangement results in a robust *in vitro* self-cleavage activity of the amniota HHRs acting as single motifs (k_{obs} of 2.4/min for the human HHR under low Mg^{2+} concentration) and in about the same

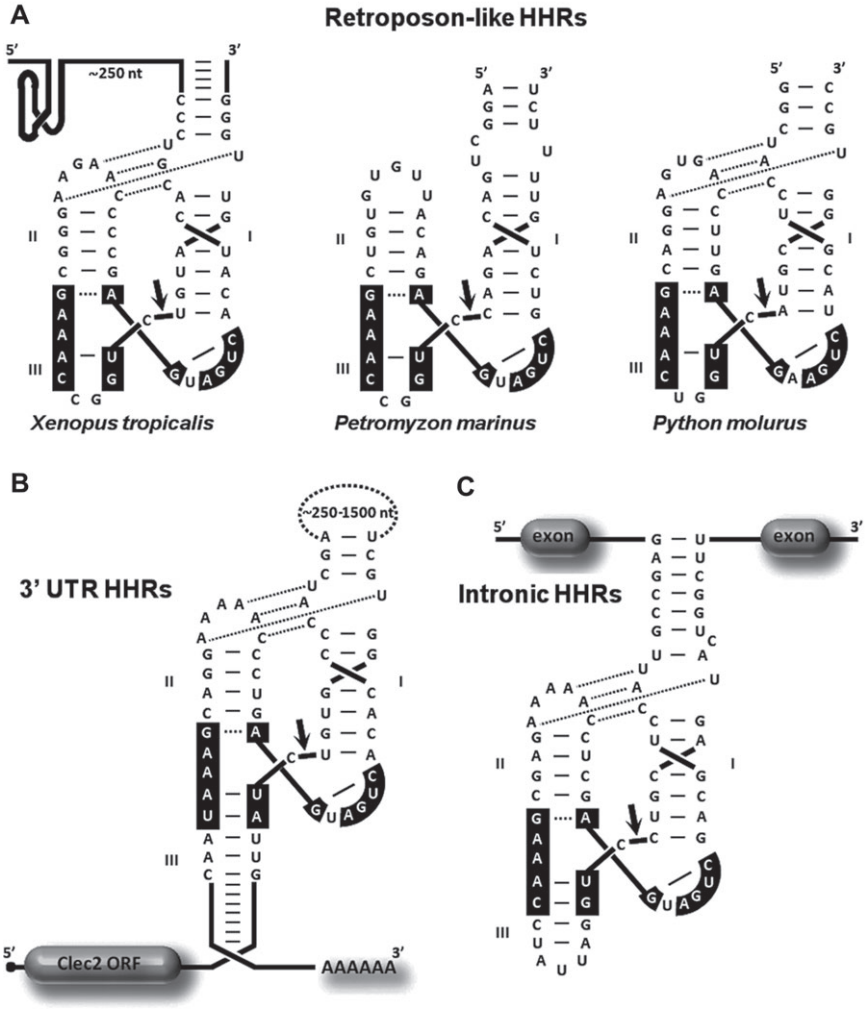

Figure 2 Three main classes of HHRs are found in the genomes of vertebrates.
(A) Type I HHRs found in amphibians (left; GenBank AC157678), lampreys (center; GenBank CO547793) or the python snake (right; GenBank AEQU010519677) showing a small helix III capped by a palindromic loop characteristic of ribozymes from tandem-repeat DNA. (B) Discontinuous HHR found in the 3′ UTR of some Clec-2 genes of rodents, platypus and some other mammals. (C) Intronic ribozyme HH9 of the human *RECK* gene. Similar intronic HHRs to this one have been detected in all aminota genomes. Putative tertiary interactions between helix I and II are depicted by dotted lines.

order as the fastest natural HHRs (De la Peña et al., 2003; De la Peña and García-Robles, 2010a). In other words, these intronic HHRs should efficiently catalyze the intron self-cleavage during *in vivo* transcription, disrupting the continuity of the pre-mRNA. Nevertheless, the two cleavage products could remain connected through helix I base pairing, and this interaction may depend on the stability of that helix I and/or the cellular conditions during the process of transcription and splicing, like temperature, RNA helicases, hnRNPs or any other RNA chaperones (see below).

It can be presumed that discontinuous and intronic HHRs found in the genomes of highly evolved organisms, like amniotes, would have originated from a domestication or exaptation process of those retroposon-like HHRs occurring in less evolved metazoans (Gould and Vrba, 1982; Okada et al., 2010), either after a simple event of helix III extension (intronic HHRs) or an intermediate situation where one of the two distant halves of a dimeric motif would have remained ('discontinuous' HHR).

Intronic HHRs in amniotes: the HH9- and HH10-like motifs

Our previous studies revealed the occurrence of at least two related variants of intronic HHRs in the genomes of amniotes, hereafter HH9- and HH10-like ribozymes based in the two first human HHRs found in chromosomes 9 and 10, respectively (De la Peña and García-Robles, 2010a). These motifs show very similar nucleotide sequence and tertiary interactions, with the main difference located at the helix III, whose size range from ~10 nt to ~45 nt for HH9-like and HH10-like ribozymes, respectively. Examples of these conserved HHRs were detected in the genomes of all amniota organisms (reptiles, birds or mammals) sequenced so far, and more precisely, in the sense strand of large introns (>10 kb) of a few specific genes (De la Peña and García-Robles, 2010a).

The HH9 ribozyme (Figure 2C and 3A) maps in the sixth intron of the *RECK* gene (Reversion-inducing Cysteine-rich protein with Kazal motifs), a gene coding for a tumor suppressor factor that inhibits the metalloproteinases involved in remodelling the extracellular matrix, a key step during embryogenesis and vasculogenesis (Takahashi et al., 1998; Oh et al., 2001). One of the most striking features of the HH9 is its occurrence as an ultraconserved element in the *RECK* intron of all the warm-blooded amniotes examined (four birds and 47 mammals; Figure 3A), but not in the *RECK* orthologues of cold-blooded amniotes or any other metazoan examined so far. Only minor sequence variations are detected in the loop of helix III, which predictably does not affect their self-cleavage activity. But HH9-like ribozymes do not seem to be restricted to the *RECK* example. A highly similar motif to HH9 (>90% sequence identity for 63 nt) was also described in the first and large intron of the *DTNB* (dystrobrevin beta) gene of birds and reptiles (Figure 3B; De la Peña and García-Robles, 2010a). More recently, we have also found the unexpected occurrence of hundreds of HH9-like ribozymes in the genome of the West Indian Ocean coelacanth (*Latimeria chalumnae*) (Figure 3B and 4A; M. de la Peña, unpublished results). This primitive lobe-finned fish is considered as a living fossil that led up to the origin of early tetrapods ~390 million years ago (Zardoya and Meyer, 1997). Some of the coelacanth HHR motifs show very high similarity in sequence and topology to the amniota HHRs, with a stable helix III that, predictably, allows efficient self-cleavage as single ribozymes. For many of the coelacanth motifs, however, the presence of mutations at the catalytic boxes or the helixes are expected to deeply affect the self-cleavage capabilities of the ribozyme. Some of the coelacanth HHRs putatively map within introns of few specific genes, suggesting that they could perform similar roles to the intronic HHRs of amniotes. Furthermore, these coelacanth motifs would indicate that this particular ribozymal innovation is older than expected and even predates the origin of tetrapods. It is noteworthy that amphibian genomes only show retroposon-like HHRs (Figure 2), whereas the genome of the coelacanth, which preceded the tetrapods, shows many examples of intronic HHRs strikingly similar to the ultraconserved ones found in amniotes. This puzzling situation indicates a more complex landscape for the evolutionary relationships between vertebrate HHRs.

Conversely, the HH10 ribozyme has very similar helixes I and II to HH9, but a longer helix III (Figure 5). HH10 was originally found in the first intron of the human *C10orf118* gene that codes for the CTCL (cutaneous T-cell lymphoma) tumor antigen L14-2. Highly similar ribozymes to the human HH10 were also detected in the *C10orf118* orthologues of most mammals, with the notable absence of glires (rodents and lagomorphs) and a few other species. Recent data mining in diverse mammalian *CTCL L14-2* genes has revealed the presence of six different copies of the HH10 ribozyme in the genome of the marsupial wallaby, as well as a much larger version of HH10 in the genome of the common shrew *Sorex araneus*, which shows a possible helix III of 128 nt instead of the typical 45 nt (Figure 5). Actually, not only mammals but other vertebrates seem to contain HH10-like ribozymes. At least two different motifs

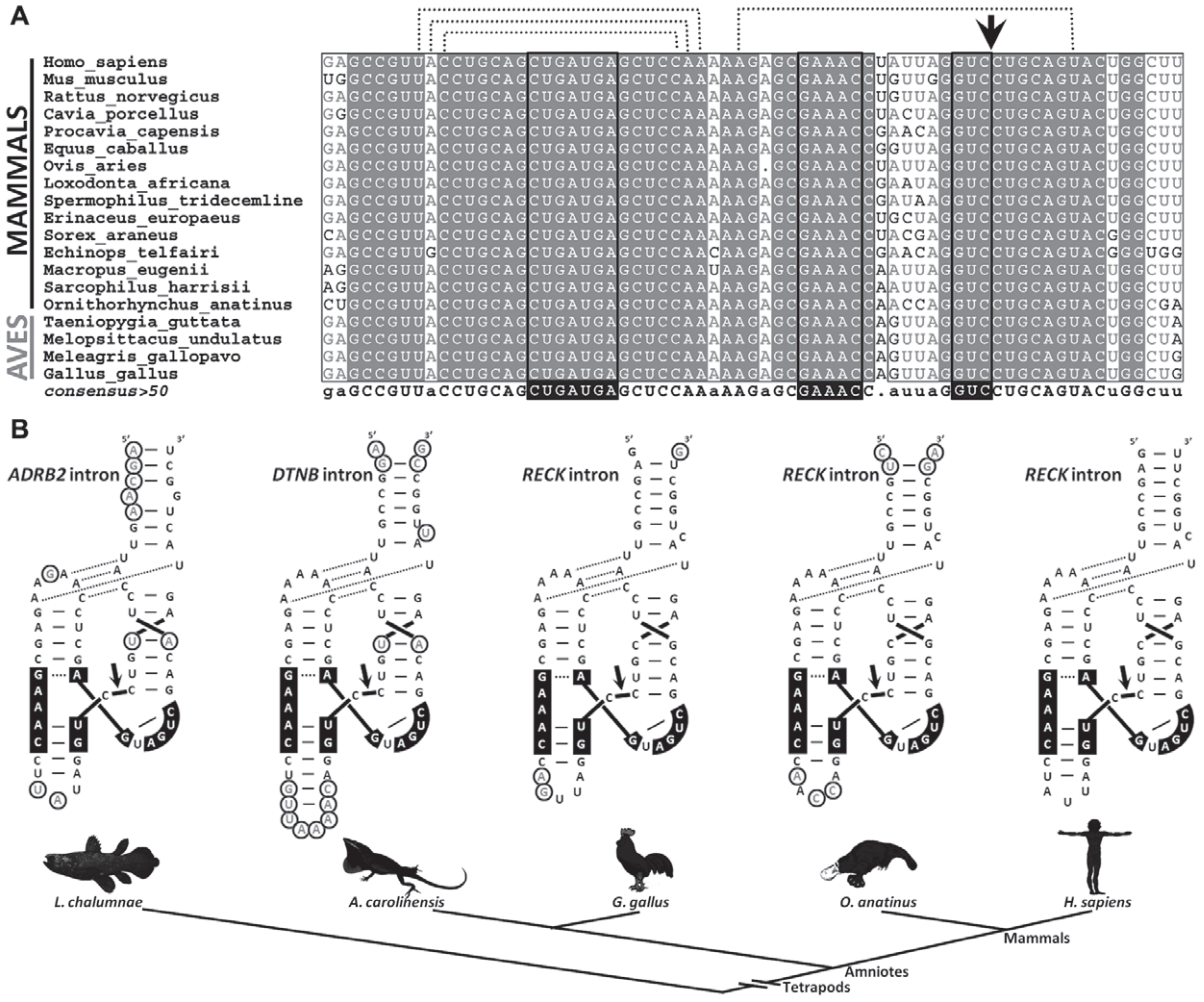

Figure 3 The ultraconserved ocurrence of the HHR in higher vertebrates.
(A) Alignment of representative HH9 ribozymes found in the *RECK* gene of endothermic vertebrates (mammals and aves). Sequence heterogeneity is mostly restricted to helix III as shown in the consensus sequence (bottom). (B) Examples of HH9-like ribozymes detected in the genomes of different vertebrates, from West Indian Ocean coelacanth to humans. Sequence heterogeneity with respect to the human HH9 is shown (circled). Conserved nucleotides of the catalytic core are boxed. Tertiary interactions between helix I and II are depicted by dotted lines. Site of self-cleavage is shown by an arrow.

have been detected in the painted turtle *Chrysemys picta*, as well as a sequence related, but presumably inactive, version of HH10 in the intron of the *NGLY1* (N-Glycanase 1) gene of several birds (Figure 5) (M. de la Peña, unpublished results). Altogether, these data suggest that many more instances for this particular catalytic RNA can be found widespread among the genomes of vertebrates.

Intronic ribozymes, snRNAs and pre-mRNA splicing

Most eukaryotic genes are interrupted by introns that must be removed during transcription through the splicing pathway to give the mature mRNAs. In recent years, it has become clear that introns and their splicing are key elements of the mRNA biogenesis of eukaryotes, not only to improve the whole process of gene expression (Le Hir et al., 2003) but also as a major source of protein and RNA diversity that results from the genomes of pluricellular eukaryotes through alternative splicing. In the human genome, for example, almost any multi-exon gene (>95%) is processed to yield multiple mRNAs and protein isoforms (Pan et al., 2008; Wang et al., 2008). How alternative splicing is regulated constitutes an exciting topic in the field that will require intensive work to fully understand the capabilities of eukaryotic genomes. In that way, the presence of RNA domains like ribozymes and riboswitches mapping to non-protein-coding regions like introns and UTRs opens a

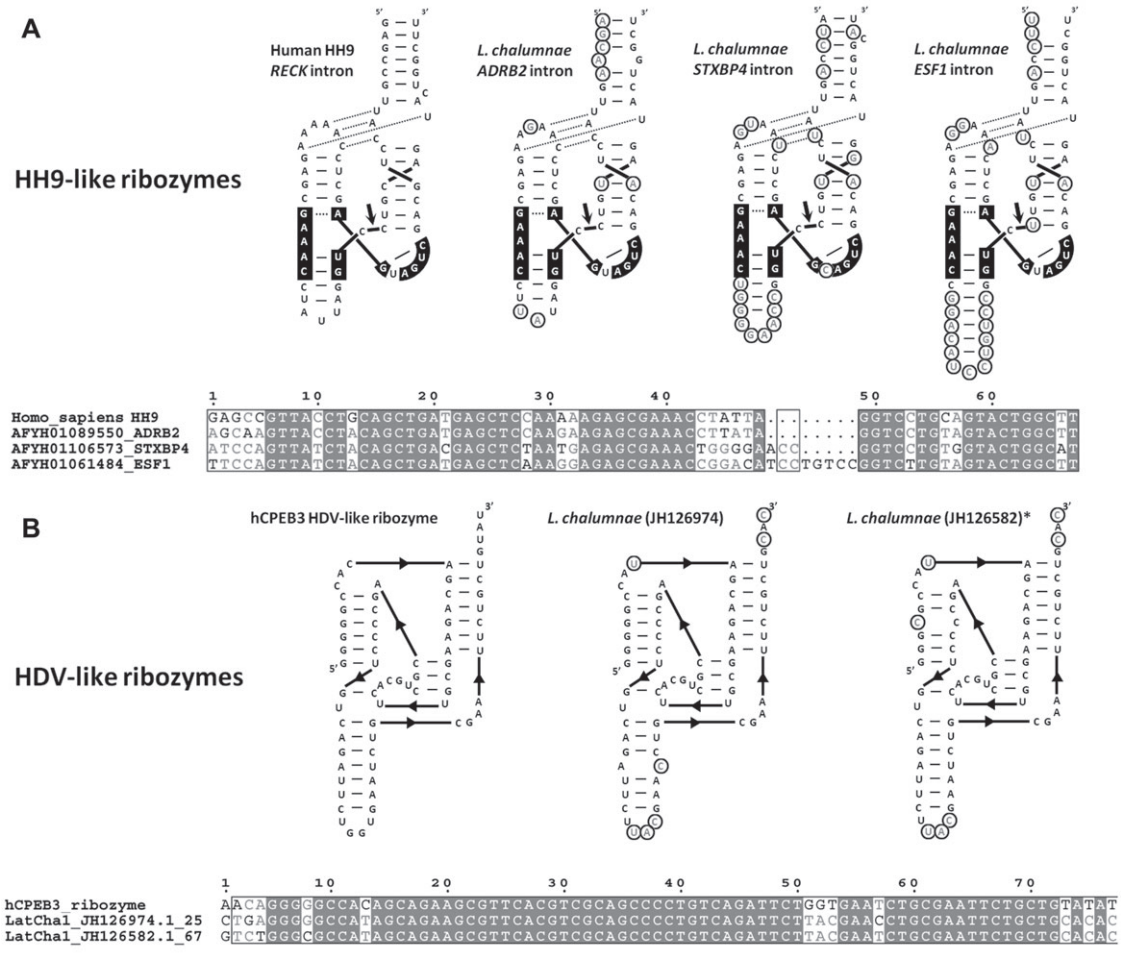

Figure 4 Small self-cleaving ribozymes in the genome of the West Indian Ocean coelacanth.
(A) Comparison of three representative examples of coelacanth HHRs with the human HH9. Putative annotation of the gene introns containing the HHRs is shown. Nucleotide differences with human HH9 are shown (circled). Tertiary interactions are depicted by dotted lines. (B) Comparison of the human CPEB3 HDV-like ribozyme with two HDV-like ribozymes from the coelacanth genome with the nucleotide differences shown (circled). Alignments of the corresponding DNA sequences are shown at the bottom of both panels for clarity.

new and interesting area of research. Concerning intronic HHRs, our previous analysis revealed two EST sequences isolated from *Bos taurus* neural tissues that mapped to the *RECK* intron containing the HH9 ribozyme (De la Peña and García-Robles, 2010a). Both sequences were chimeric molecules that have resulted from fusion events of two RNAs; the 5′ side of the ESTs corresponded to snRNAs U5 or U6, whereas the 3′ side corresponded to the 3′ fragment resulting from the intron self-cleavage through HH9. Therefore, these ESTs indicate that: (i) the intron would self-cleave *in vivo* and (ii) the resulting 3′ product of the intron cleavage could eventually interfere with the splicing machinery. In principle, this particular RNA-RNA fusion would not involve an HHR catalyzed ligation because the catalytic portion of the HH9 ribozyme capable of RNA-ligase activity (Canny et al., 2007) remains at the 5′ and not at the 3′ fragment of the intron. So, most probably, ligation of the two RNA molecules would be due to either the spliceosome or any other RNA-ligase activity in the cell. It is also possible that these RNA-RNA fusion events could represent artifactual events during ESTs synthesis. Nevertheless, initial experiments performed in our lab have revealed that very similar chimeric molecules (snRNA U6 fused to a HHR self-cleaved intron) can be detected by RT-PCR in transgenic plants transformed with a reporter gene harboring an intronic HH9 (J. Sánchez-Navarro and M. de la Peña, unpublished results). The biological significance of these RNA-RNA fusions is under study, but the presence of identical RNA intermediaries in distantly related organisms such as mammals and plants suggests that intronic HHR self-cleavage could be involved in a conserved mechanism in eukaryotes.

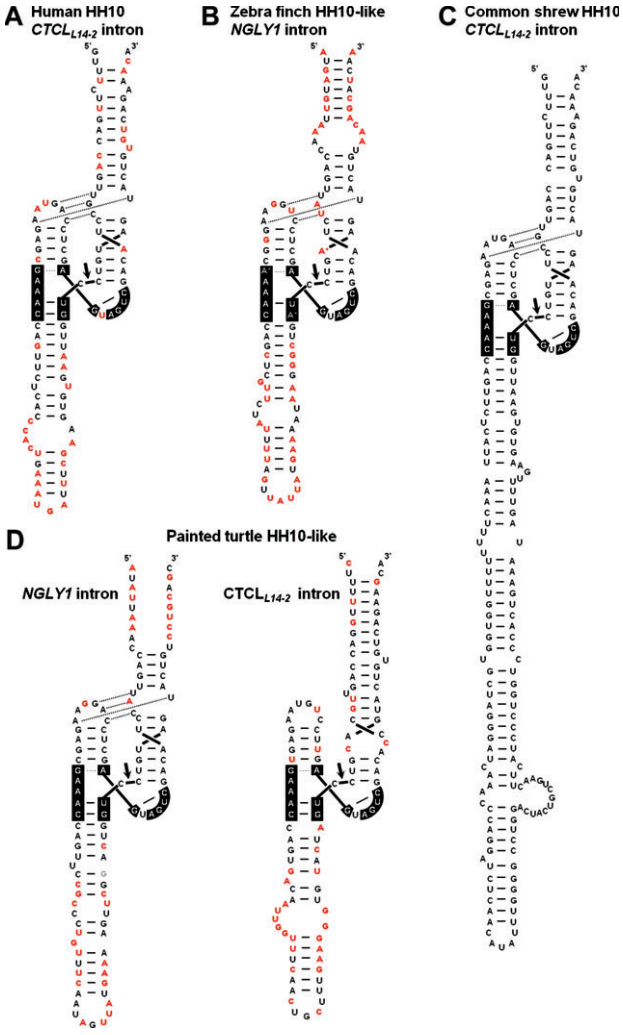

Figure 5 Different examples of HH10-like ribozymes.
(A) HH10 ribozyme found in the intron of the human *CTCL tumor antigen L14-2* gene. Nucleotide positions known to change in some mammals orthologues are shown in gray. (B) HH10-like sequence found in the genome of the Zebra finch bird (*Taeniopygia guttata*). Differences with respect to the human HH10 are shown in gray. Mutations that are predicted to disrupt self-cleavage are marked with an asterisk. (C) Atypical version of HH10 with a much larger helix III found in the *CTCL tumor antigen L14-2* gene of the common shrew (*Sorex araneus*). (D) Two examples of HH10-like ribozymes found in the genome of the painted turtle (*Chrysemys picta*). Differences with respect to the human HH10 are shown in gray. Tertiary interactions are depicted by dotted lines.

Recent bioinformatic mining has revealed another intriguing coincidence among the two human genes harboring intronic HHRs. Both *RECK* and *CTCL tumor antingen L14-2* genes are known to have different transcripts or splice variants, and a main alternative splicing event concerns a cassette exon that could be alternatively spliced in or out. For the two genes, that cassette exon precedes the large intron carrying either the HH9 or HH10 ribozymes (Figure 6). Although these observations offer just circumstantial support, previous experimental data obtained with intronic HHRs has revealed that certain intronic regulatory elements involved in processes of alternative splicing have to be covalently attached to exons, and the presence of intronic HHRs disrupt their usual behavior during splicing (Gromak et al., 2008; Pastor et al., 2011).

Closely connected with the intronic HHRs of amniotes is the mammalian Hepatitis-δ like (HDV) ribozyme (Salehi-Ashtiani et al., 2006), another exceptional example of intronic self-cleaving motif that offers many parallelisms with the HHR and could shed some light on the biological role of both motifs. Like the HHR, self-cleaving HDV-like ribozymes have been recently found widespread in genomes from all life kingdoms and usually associated with autonomous LINE retroelements (Webb et al., 2009; Eickbush and Eickbush, 2010; Ruminski et al., 2011). In mammals, however, only one example of HDV-like ribozyme has been described and, similarly to the amniota HHRs, specifically conserved in a large intron (~47 kb) of the *CPEB3* (cytoplasmic polyadenylation element binding protein 3) gene of most mammalian genomes. The human *CPEB3* ribozyme was shown to have a low self-cleavage activity (k_{obs} ~0.01/min; Salehi-Ashtiani et al., 2006), although recent data suggest a faster and more complex kinetic behavior for this motif under co-transcriptional conditions (k_{obs} ~0.5–2.5/min under non-physiological Mg^{2+} concentration; Chadalavada et al., 2010). Another similarity with the HHRs resides in the striking sequence identity (95% for 72 nt) found between the *CPEB3* ribozyme and a group of HDV-like ribozymes detected in the *L. chalumnae* genome (Figure 4B; M. de la Peña, unpublished results), which strongly suggests a close evolutionary relationship between mammalian and the coelacanth ribozymes. In this line, Lupták and coworkers recently described several HDV-like ribozymes rather similar to the *CPEB3* one in a retrotransposable element of the *Latimeria menadoensis* coelacanth (Ruminski et al., 2011) and some other metazoans (Webb and Lupták, 2011), which reinforces the idea of an exaptation process for these self-cleaving motifs. The biological role of the *CPEB3* ribozyme is still under study, although a relationship of a faster self-cleavage activity with poorer performance in an episodic memory task has been reported (Vogler et al., 2009). At molecular level, the *CPEB3* intron harboring the HDV ribozyme is not known to follow alternative splicing events through a cassette exon like those found in the genes with intronic HHRs, although a competing 3′ splice site does seem to occur. Therefore, though both HHR and HDV ribozymes catalyze the same reaction of self-cleave transesterification, the final product of the reaction is slightly different

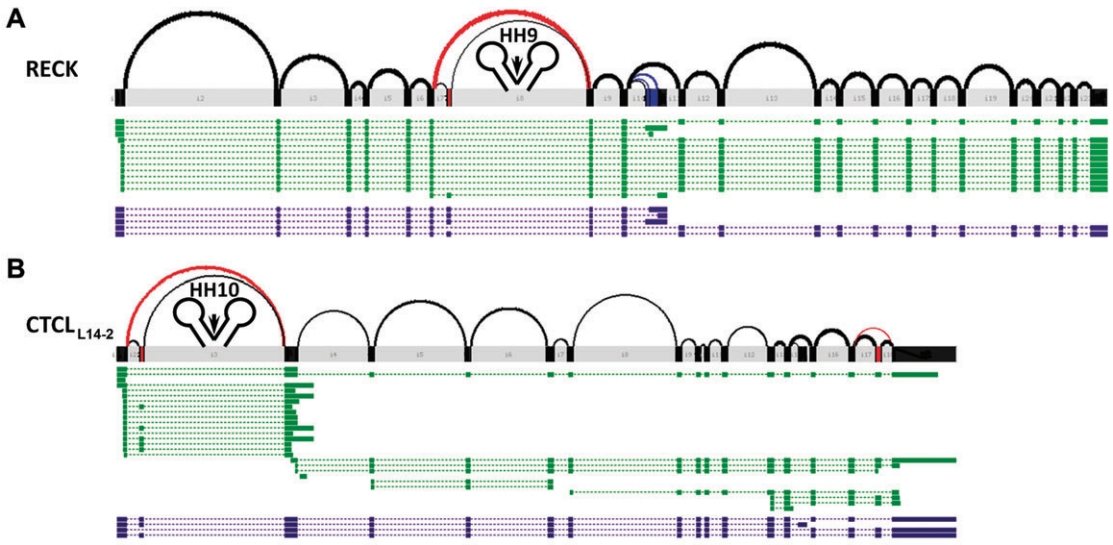

Figure 6 Splicing graphs of the human *RECK* (A) and CTCL *tumor antigen L14-2* (B) genes obtained through the ASG web (Leipzig et al., 2004).
Introns and exons are shown in gray and black, respectively, with the exception of cassette exons (in red) and competing 3′ splice sites (in blue). Representative ESTs (green) and ENSEMBL transcript assemblies (purple) are shown under each gene. A schematic representation of HH9 and HH10 ribozymes are drawn on their corresponding introns.

in each case. In the HDV ribozyme, the 5′ side of the cleavage product corresponds to the preceding sequence of the ribozyme, keeping the HDV ribozyme moiety in the 3′ product. In contrast, self-cleavage of the intronic HHRs results in a 5′ product that keeps most of the ribozyme moiety, whereas the 3′ product corresponds to one of the helix I strands. In consequence, whereas the HHR is expected to stably keep 5′ and 3′ products through the helix I base pairing and other interactions (Canny et al., 2007; Shepotinovskaya and Uhlenbeck, 2010), no interaction is expected to happen between the two products of the HDV-like ribozymes after cleavage. These differences, together with differences in the self-cleavage efficiency or even the RNA ligation capabilities only described for the HHR, suggest that not only RNA self-cleavage itself but some other features could define distinct biological roles for these intronic ribozymes.

Conclusions

We now know that small self-cleaving RNAs are much more frequent in DNA genomes than previously thought. A relationship with the biology of genetic mobile elements in eukaryotes has been advanced for both the HHR and the HDV-like ribozymes, either with non-autonomous SINE retroelements (Epstein and Gall, 1987; Ferbeyre et al., 1998; Rojas et al., 2000; Hammann et al., 2012) or with autonomous LINE retroelements (Eickbush and Eickbush, 2010; Ruminski et al., 2011), respectively. The similarities between both ribozymes seem to reach the genomes of higher vertebrates, where just a few examples of the two self-cleaving motifs have been so far detected as exceptionally conserved motifs (Salehi-Ashtiani et al., 2006; De la Peña and García-Robles, 2010a). As the most feasible scenario, we could assume that both ribozymes would have followed an exaptation process from a role in retrotransposition to a new biological task during the evolution of vertebrates, in a similar way as described for other SINEs (Bejerano et al., 2006; Okada et al., 2010). In consequence, whereas those particular retrotransposons harboring self-cleaving motifs seem to be absent or currently unrecognized in amniotes, their ribozymes would have been preserved to add an extra level of complexity in the genomes of these organisms. With regards to the specific role of the intronic ribozymes, although different scenarios can be envisaged (see before), it is clear that their high level of conservation point to a molecular and biological innovation involving cleavage of the nascent pre-mRNA transcript. Future research will dissect this and new other roles for these small ribozymes.

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