

A newt ribozyme: A catalytic activity in search of a function

(small self-cleaving RNA/transcription regulation/secondary structure)

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ABSTRACT We analyzed the cleavage properties and the transcription regulation of the newt (*Triturus vulgaris meridionalis*) self-cleaving RNA. *In vitro* self-cleavage of model oligoribonucleotides occurs within a double hammerhead structure. In addition, an entire ribozyme molecule, as well as its catalytic domain, “trans-cleaves” *in vitro* appropriate oligoribonucleotide substrates. Signals encoded within the ribozyme DNA sequences regulate the ribozyme transcription, which is RNA polymerase II dependent. Finally, the deduced secondary structure of the self-cleaving RNA appears to be conserved in evolutionarily distant newt species. These features suggest that the newt ribozyme could play some role in the cell, possibly related to its cleavage properties.

The American and the European newts *Notophthalmus* and *Triturus* share a family of repeated DNA sequences, organized in small clusters widely dispersed through the genome (refs. 1 and 2; unpublished data). Strand-specific transcripts of these families, found in either somatic or germinal tissues, are of discrete sizes, equal to a monomer repeat length (≈ 330 bases) and to its progressive multimers (ref. 1; unpublished data; J. M. Varley and H. C. Macgregor, personal communication). As discovered in *Notophthalmus* (3), this RNA undergoes self-cleavage *in vitro* through a reaction similar to that occurring during the replication cycle of several viroid, virusoid, and satellite RNAs of plants (reviewed in ref. 4). However, unlike what has been observed for the infectious agents of plants, the *in vitro* self-cleavage site of the newt ribozyme is located about 50 bases downstream of the RNA 5' end, as found *in vivo* (ref. 3; unpublished data), rendering the involvement of the self-cleavage in monomer formation not obvious. We reasoned that properties of the *in vitro* self-cleavage reaction, as well as structural and functional features of the newt ribozyme, could provide insights into its possible biological significance.

MATERIALS AND METHODS

Cleavage Assays. Nonradioactive and ^{32}P -labeled RNAs were produced by transcription of synthetic DNA templates by T7 RNA polymerase (5) and purified by polyacrylamide gel electrophoresis. RNAs were heated at 80°C for 1 min, allowed to cool to room temperature, and then incubated in 10 μl of cleavage buffer (50 mM Tris-HCl, pH 8.0/50 mM MgCl₂) at 42°C for 120 min (160 min in Fig. 1B). Products were resolved on 8 M urea/20% polyacrylamide gels and revealed by autoradiography. Identity of the self-cleavage products was established by sequencing after digestion with RNase A, T1, and U2 (not shown). The 330-base ribozyme is a T7 RNA polymerase transcript of the pGEM-AluI clone

obtained by subcloning the *Alu* I-digested pSP6-D6 clone (3) into the *Sma* I site of pGEM-3Z (Promega).

Microinjected Clones. The DIM clone (Fig. 2) contains an incomplete dimer, 597 base pairs (bp) long, of the “*Bgl* II” sequences of *Triturus vulgaris meridionalis* (unpublished data), cloned in the *Pst* I site of the pGEM-3Z vector. The DIM clone has a mutation affecting one of the conserved boxes of its most upstream cleavage domain (CUGA \rightarrow CUA: compare with Fig. 1A). Deletion mutants of the DIM clone were constructed using the Promega exonuclease III deletion protocol. Site-directed mutagenesis of the octamer sequence (the distal sequence element; DSE) of the -67 deletion clone was performed according to the protocol provided by Amersham. Oocyte injections were performed using a 30-nl DNA solution of plasmid DNA (final concentration, 200 ng/ μl) per oocyte. In α -amanitin inhibition experiments the DIM and the 5S cloned genes were coinjected in equimolar ratio. When the RNA was also analyzed by direct autoradiography (Fig. 4), the solution contained 0.1–0.5 μCi of [α - ^{32}P]GTP (400 Ci/mmol; 1 Ci = 37 GBq). After injection, the oocytes were incubated for 8 hr at 19°C in Barth’s medium.

Primer Extension and RNase Protection. Approximately 30 μg of total RNA, extracted from injected *Xenopus* oocytes, was used for RNase protection or primer elongation experiments (6). The oligonucleotide used for primer extension is an antisense 36-mer matching the newt self-cleaving RNA sequence from nucleotide 101 to 136 (1). The antisense riboprobe used for the RNase protection experiments was obtained by T7 RNA polymerase transcription of the DIM clone (Fig. 2), linearized with *Hind*III, according to the protocol provided by the supplier (Promega).

RESULTS

Structure of the Catalytic Domain and Trans-Cleavage. Fig. 1A shows a “hammerhead” model (reviewed in ref. 4) for the *T. v. meridionalis* self-cleaving RNA catalytic domain. However, such hammerhead structure should be unstable (4), due to a stem III of 2 bp and a hairpin loop of two nucleotides only (ref. 3; unpublished data) (compare Fig. 1A). In fact, the newt ribozyme may overcome this structural problem by pairing two catalytic domains into an active double hammerhead structure (7, 8) (compare Fig. 1D).

The 40-mer in Fig. 1A undergoes cleavage with a concentration-dependent rate (Fig. 1E, lanes 1–3; results not shown), thus confirming (7, 8) that cleavage may occur through association of two interacting molecules (Fig. 1D). In addition, neither of the two 41-mer oligonucleotides—constructed by inserting an additional nucleotide in the

Abbreviations: PSE, proximal sequence element; DSE, distal sequence element.

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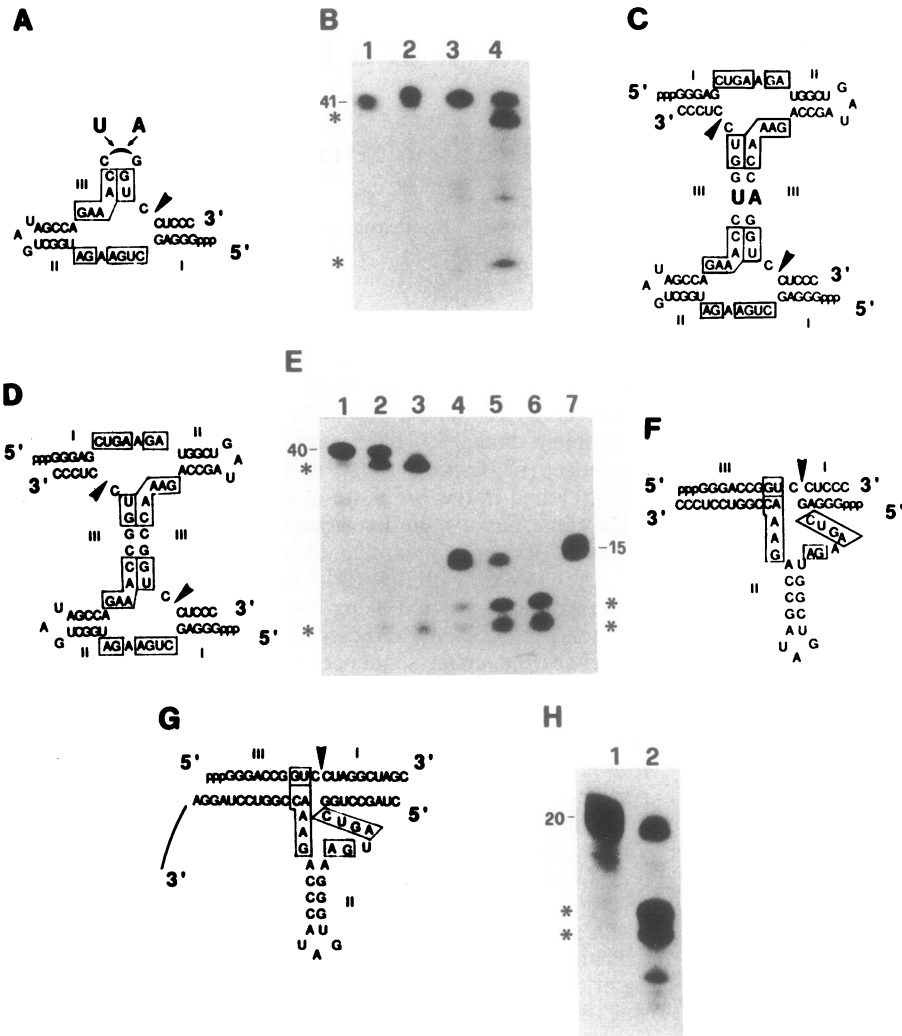


FIG. 1. (A) Hammerhead structure (4) of a 40-mer RNA reproducing the catalytic domain of the *Triturus* self-cleaving RNA. The sequence of the stem I is made consistent with a T7 RNA polymerase promoter (5). In this and in the following schemes the arrowhead points to the site of cleavage; nucleotides conserved between all hammerhead ribozymes are boxed; boldfaced U and A are nucleotides added into the hairpin loop of the stem III to obtain the 41(U)-mer and 41(A)-mer, respectively (see C). (B) Cleavage assay of the 41(U)-mer (lane 1), the 41(A)-mer (lane 2), or a mixture of the two in the absence (lane 3) or the presence (lane 4) of Mg^{2+} . Asterisks mark the cleavage products in this and in the following pictures. Concentration = $0.5 \mu M$ per reaction. (C) Model of the double-hammerhead secondary structure formed through the association of the 41(A) and the 41(U) oligomers, allowing cleavage as shown in B, lane 4. (D) Model of the double-hammerhead secondary structure for the 40-mer RNA. (E) Self-cleavage (lanes 1–3) and trans-cleavage (lanes 4–6) assays performed by the 40-mer: concentrations are 0.036 , 0.36 , and $3.6 \mu M$ nonradioactive 40-mer in each three-lane series, respectively. In addition, lanes 1–3 each contain $0.036 \mu M$ radioactive 40-mer, and lanes 4–6 each contain $0.36 \mu M$ radioactive 15-mer substrate, as shown in F. Lane 7, mock-cleavage of the 15-mer substrate, incubated without ribozyme. (F) Model of a hammerhead structure formed by the 40-mer ribozyme and the 15-mer substrate, possibly allowing cleavage of the latter. (G) Model of a hammerhead structure formed by the monomeric (330 base) ribozyme and the 20-mer substrate, supposed to allow cleavage of the substrate, as shown in H, lane 2. (H) Trans-cleavage assay of the 20-mer substrate in the absence (lane 1) or the presence (lane 2) of the 330-base ribozyme.

middle position of the hairpin loop of stem III (Fig. 1A)—undergoes cleavage when tested separately (Fig. 1B, lanes 1 and 2), whereas an equal mixture of the two performs cleavage efficiently (Fig. 1B, lane 4). Our interpretation is that each 41-mer is unable to perform cleavage in a separate assay because it cannot form either a stable single hammerhead (8) or a double hammerhead structure: in fact, the added nucleotide, facing its twin in the middle of stem III of a double hammerhead, might destabilize the structure, and some inactive conformations might be favored. More significantly, the two 41-mers can perform cleavage in a joint assay because they can arrange into a stable double hammerhead, due to the complementarity of the two added nucleotides (Fig. 1C).

The occurrence of cleavage through interaction of two distinct RNA molecules suggests that the new ribozyme might cut in trans a different RNA species (see Fig. 1F). Evidence of trans-cleavage for other hammerhead ribozymes has been obtained previously (see ref. 4 for references). Fig. 1E, lanes 4–7, shows that the new 40-mer oligo-ribozyme cuts an appropriate 15-mer substrate *in vitro*. Furthermore, a whole monomeric ribozyme transcript, made similar to the ribozyme monomer as found *in vivo*, cleaves *in vitro* an appropriate 20-mer substrate in a site-specific manner (Fig. 1G and H).

Regulation of Transcription. Signals for transcription promotion are encoded within the repeated DNA sequences

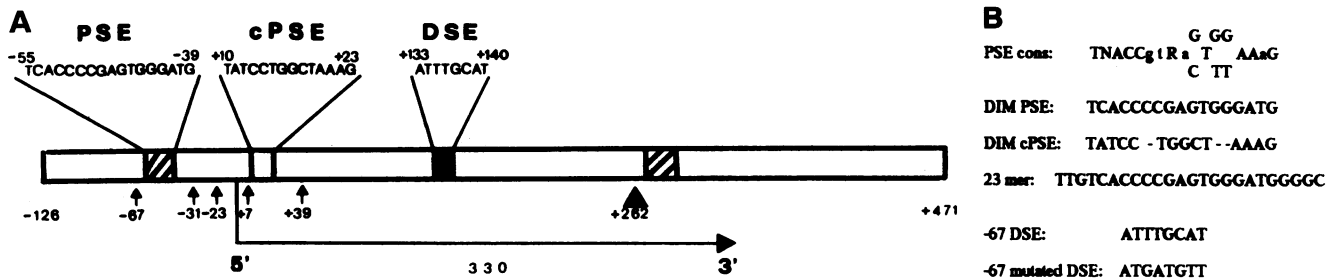


FIG. 2. (A) Scheme of the DIM clone; 5' and 3' mark the ends of the oocyte RNA monomer (unpublished data). The arrowhead marks the 3' end of the +262 deletion clone, from which all 5' deletion clones (small arrows) were derived. (B) The DIM PSE and cPSE are compared with a consensus PSE sequence (9); the 23-mer, containing a PSE sequence, is used in the competition experiment shown in Fig. 3C; the DSE and the mutated DSE sequences of the 5' -67 clones, used for the experiment in Fig. 4A, are compared.

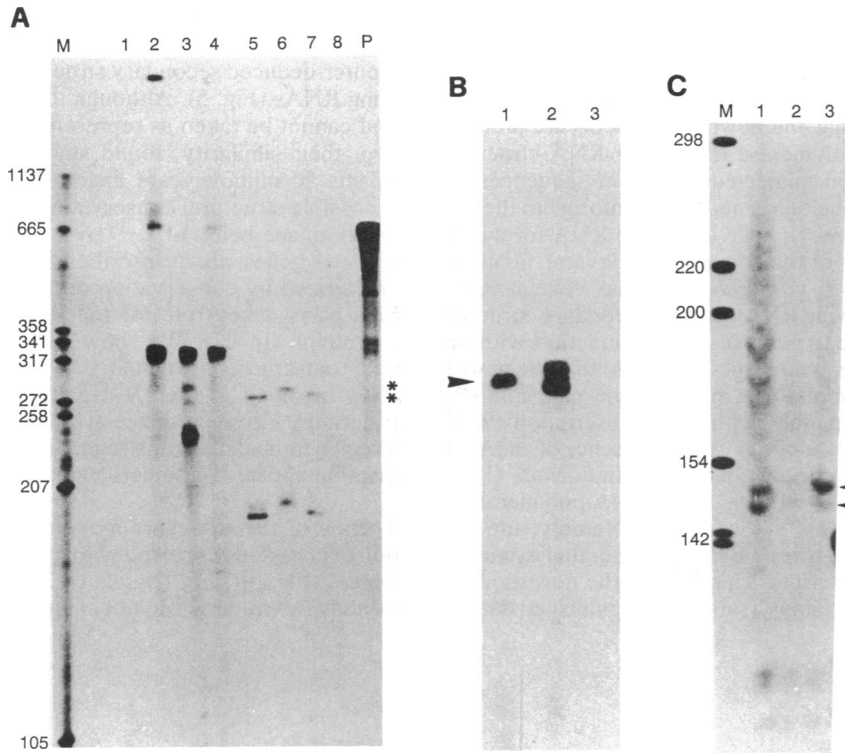


FIG. 3. (A) Autoradiography of RNase protections performed by using the antisense transcript of DIM as a probe (P). M is a ³²P-labeled *Sau* IIIA digest of pBR322. Lane 1, negative protection of total RNA from noninjected *Xenopus* oocytes. The remaining lanes present the results from oocytes injected as follows. Lanes 2-4, DIM clone either alone (lane 2) or coinjected with the -67 (lane 3) or the +39 (lane 4) deletion clone, in an equimolar ratio. Lanes 5-8, deletion clones -31, -23, +7, and +39, respectively. The asterisks mark artifactual bands (notice that the band produced by the +7 clone is longer than the entire insert). (B) Primer elongation products (arrowhead) from *Xenopus* oocytes injected with the -67 clone (lane 1) as compared to *Triturus* (lane 2) or *Xenopus* (lane 3) oocyte RNA. (C) Primer elongation products (arrowheads) after injection of the -31 deletion clone alone (lane 3) or with a 10-fold molar excess of the double-stranded 23-mer shown in Fig. 2 (lane 2) or of a double-stranded 17-mer representing the T7 prokaryotic promoter as a control (lane 1). M is a marker of lengths (1-kilobase ladder, BRL).

coding for the newt ribozyme (*Bgl* II sequences in *T. v. meridionalis*; to be reported elsewhere). A search for these signals has revealed a sequence motif similar to the "proximal sequence element" (PSE) (9, 10) at position -55/-39 with respect to the 5' end of transcripts (Fig. 2). We tested the ability of the *Bgl* II PSEs to promote transcription by injecting the deletion clones shown in Fig. 2 into *Xenopus*

oocytes and analyzing the resulting transcripts by RNase protection (Fig. 3A) and primer extension (Fig. 3B). All clones were derived from a 3' deletion clone (at position +262) of a dimeric (DIM) clone to obtain clones provided with one PSE only (Fig. 2) as well as to distinguish transcripts from the 3' +262 clone, by their shorter sizes, from transcripts from the entire DIM clone in coinjection experiments (see Fig. 3A).

A 5' -67 deletion clone, containing the PSE, produces transcripts of about 250 nucleotides (Fig. 3A, lane 3) with a 5' end corresponding to that found *in vivo* in the newt oocytes (Fig. 3B). The 5' deletion clones -31, -23, and +7, all lacking the canonical PSE, generate, with a lower efficiency, RNA molecules about 200 nucleotides in length (Fig. 3A, lanes 5-7); the 5' ends of the -31 clone transcripts fall at about -15 with respect to the 5' ends of either the *in vivo* or the DIM and the -67 clone transcripts (Fig. 3C, lane 3). The occurrence of a second, degenerated PSE at position +10/+23 (Fig. 2, cPSE) might explain these results: in the absence of the canonical PSE such a cryptic PSE may promote transcription starting from anomalous initiation sites. Indeed, a 5' +39 deletion, which eliminates the cPSE as well, completely abolishes transcription (Fig. 3A, lanes 4 and 8). Consistent with this interpretation are results of competition experiments performed by coinjecting the 5' -31 deletion clone with a PSE oligonucleotide, where the transcription level of the -31 clone is reduced (compare lanes 2 and 3 of Fig. 3C).

On the whole, these results suggest that the PSE promotes transcription of the newt ribozyme. Transcription termination also appears to be regulated by *Bgl* II DNA sequences alone (see Fig. 3A, lanes 2-4).

In the DIM clone a perfect octamer signal, or DSE (9), is present at position +133/+140 downstream of the RNA first 5' end (Fig. 2). When the octamer sequence of the -67 clone was disrupted by site-specific mutagenesis, the level of transcription was paradoxically increased (Fig. 4A; compare lanes 2 and 4 with lanes 1 and 3, respectively). We are unaware of any similarity between the mutated octamer and any known enhancer. These results suggest that the octamer may play an inhibitory role in transcription of its same

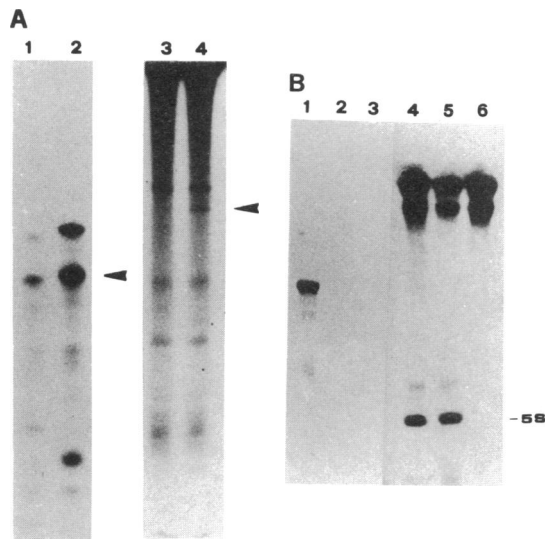


FIG. 4. (A) Products of RNase protection of transcripts after oocyte injections with the 5' -67 deletion clone (see Fig. 2) (lane 1) or the same clone containing a mutated octamer sequence (ATGATGTT; lane 2). Lanes 3 and 4, direct autoradiography of the total RNA used for the RNase protections of lanes 1 and 2. The arrowheads point to the transcript produced by the 5' -67 clone with the mutated DSE. (B) α -Amanitin inhibition experiments. Lanes 1-3, RNase protections of RNA from oocytes coinjected with the DIM clone and a 5S RNA *Xenopus* cloned gene in the absence (lane 1) or the presence (0.2 μ g/ μ l, lane 2; 5 μ g/ μ l, lane 3) of α -amanitin. Lanes 4-6, total RNA from the injected oocytes, used for the RNase protections of lanes 1-3, respectively, as directly visualized by autoradiography.

transcription unit, while not excluding that it might enhance transcription of different transcription units.

Regulation of transcription by PSE and DSE may operate through either RNA polymerase II or III (9). α -Amanitin inhibition experiments (11) provide evidence that the newt ribozyme transcription is performed by RNA polymerase II (Fig. 4B); although the 5S gene is transcribed when coinjected with 0.2 μ g of α -amanitin per μ l (lane 5), the DIM clone is not transcribed at this α -amanitin concentration (lane 2).

Models of Secondary Structure. The similarity of the computer-deduced (12) secondary structures of the *T. v. meridionalis* and *Notophthalmus viridescens* self-cleaving RNAs is noteworthy (Fig. 5). This similarity is partly due to compensatory changes of the nucleotides paired in the stem structures; in addition, variations of only one residue of a pair in a putative helical region are in general compatible with pairing.

DISCUSSION

In this paper we present unusual features of the *Triturus Bgl* II tandemly repeated sequences and of their transcripts. This sequence family is 80% homologous to a DNA family (sat-

ellite 2) of the American newt *Notophthalmus*, whose transcripts undergo self-cleavage *in vitro* in a site-specific manner (1, 3). We present the computer-deduced secondary structure models of the corresponding RNAs (Fig. 5). Although these models are preliminary and cannot be taken as representing the RNA structure *in vivo*, their similarity, found starting from sequences in organisms 50 million years apart (13), conforms to the view of a possible structural conservation of the RNA for a cellular function (see below).

Several families of repeated genes are transcribed into small cellular RNAs characterized by conservation of their secondary structure, which plays a key role for the RNA interaction with specific proteins (14–17). We show (Figs. 2–4) that the newt ribozyme transcription is regulated by the same elements (PSE, DSE) involved in the U1–U5 RNA transcription (9, 10); furthermore, in accordance with the absence of an (A+T)-rich region immediately upstream of the initiation site (18), transcription appears to be performed by RNA polymerase II.

Notably, the 5' and 3' ends of the ribozyme monomers generated by microinjection experiments correspond to those of the newt oocyte monomers (Fig. 3B; Fig. 3A, lanes 2–4; unpublished data). The monomer extremities do not coincide

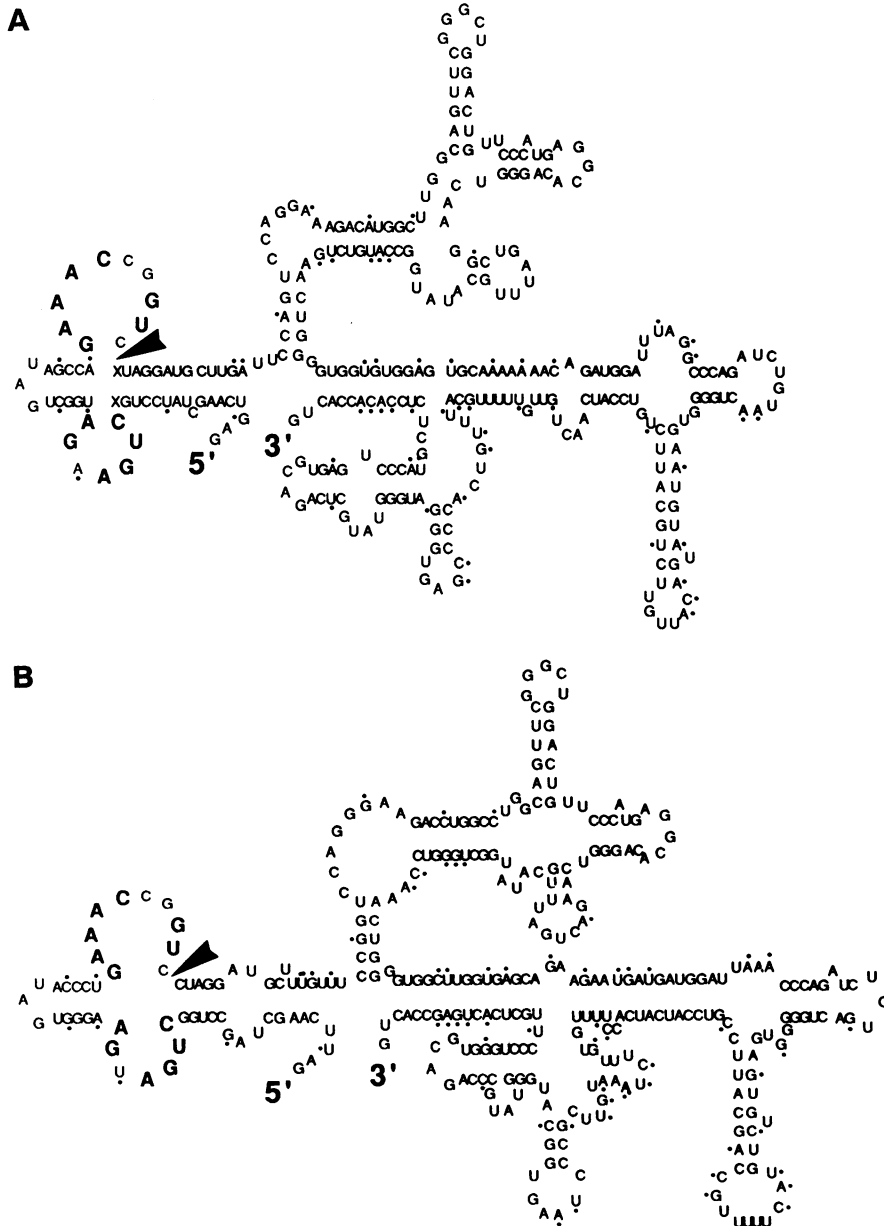


FIG. 5. Secondary structures of the self-cleaving RNA from *T. v. meridionalis* (A) and *N. viridescens* (B) as deduced by computer analysis (12). The sequences derive from the corresponding DNA consensus sequences (unpublished data; ref. 1). Dots mark base changes between the RNAs of the two species. Arrowheads point to the self-cleavage sites. Boldfaced letters represent the nucleotides that are perfectly conserved in the self-cleaving viroid, virusoid, and satellite RNAs of plants (4).

with the *in vitro* self-cleavage site, which is located about 50 bases downstream of the 5' end (ref. 3; unpublished data and present work). Therefore, as suggested by Epstein and Gall (3), an *in vivo* cleavage generating the monomer extremities should occur within a structure different from that catalytically active *in vitro*, allowing cleavage in a different site. Although this remains a formal possibility, as discussed below, alternative possibilities might be conceivable.

Our studies with model oligoribonucleotides (Fig. 1, A–E) show that the newt ribozyme may perform cleavage *in vitro* by arranging two distinct catalytic domains in a double hammerhead structure (7, 8). Concentration-dependence experiments (Fig. 1E; also, results not shown), as well as S1 and V1 nuclease digestion analyses (results not shown), support this view. Since the site of *in vitro* cleavage is the same regardless of the occurrence of cleavage in a single or in a double hammerhead structure (Fig. 1A and D), these studies provide no clues on the question of how the RNA ends originate *in vivo*. We think that a different observation pertains to this issue. In the microinjection experiments a correct 5' end of transcripts (i.e., identical to that found *in vivo* in the newt oocyte molecules) is achieved after injection either of clones possessing perfectly conserved cleavage domains (unpublished data) or of the DIM clone, which possesses a mutation affecting one of the conserved boxes of its most upstream cleavage domain (present work; see *Materials and Methods*). It could be argued that one intact cleavage domain (the downstream one in DIM) is sufficient to ensure a 5' end formation of transcripts by cleavage. However, the DIM-derived, –67/+262 deletion clone, which possesses only one, mutated, cleavage domain (Fig. 2), is well transcribed and gives rise to RNA molecules with a correct 5' end (Fig. 3A, lane 3; Fig. 3B, lane 1). Thus, a mutation in the conserved cleavage domain has no effect on either transcription or 5' end location. Transcription is decreased and the 5' end is displaced only when derivatives of the DIM clone where the PSE is deleted are used as templates (Fig. 3A, lanes 5–7); however, even in these instances the 5' end does not coincide with the cleavage site (Fig. 3C, lane 3). It seems to us that the simplest interpretation of these data is that the monomeric ribozyme molecules of oocytes are generated directly by transcription promoted by the PSE sequence and not by cleavage. Perhaps the generation of multimeric molecules is due to mutations affecting the transcription regulation elements of contiguous units.

The high level of sequence and structure conservation, the *in vivo* occurrence of discrete sized, strand-specific transcripts with precise 5' ends, the accuracy of transcription regulation, and the presence of regulatory elements within the repeat units suggest to us that the ribozyme sequences might have been selected during evolution because of a defined function in the cell. Like other hammerhead ri-

bozymes (4), a newt oligo-ribozyme cuts in trans an appropriate RNA substrate (Fig. 1E and F). More significantly, a whole monomeric molecule, made similar to the oocyte monomer, also cleaves *in vitro* a substrate RNA in a specific site (Fig. 1G and H). We suggest that the cleavage properties of the newt ribozyme could be employed *in vivo* to cut in trans different RNA molecules. In this view, taking into account the similar transcription regulation of the ribozyme and the URNAs, the newt ribozyme could be conceived as an enzymatic core of ribonucleoproteins, involved in some events of oocyte RNA processing.

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