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Novel processing in a mammalian nuclear 28S pre-rRNA: tissue-specific elimination of an 'intron' bearing a hidden break site

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Splitting and apparent splicing of ribosomal RNA, both previously unknown in vertebrates, were found in rodents of the genus *Ctenomys*. Instead of being formed by a single molecule of 4.4 kb, 28S rRNA is split in two molecules of 2.6 and 1.8 kb. A hidden break, mapping within a 106 bp 'intron' located in the D6 divergent region, is expressed in mature ribosomes of liver, lung, heart and spleen, as well as in primary fibroblast cultures. Testis-specific processing eliminates the intron and concomitantly the break site, producing non-split 28S rRNA molecules exclusively in this organ. The intron is flanked by two 9 bp direct repeats, revealing the acquisition by insertion of a novel rRNA processing strategy in the evolution of higher organisms.

Keywords: mammalian cells/ribosomal RNA processing

Introduction

The information flow from eukaryotic genes to functional RNA molecules involves a panoply of diverse and sometimes sophisticated RNA processing strategies. While 5′ capping, polyadenylation and editing seem to be particular to messenger RNAs, splicing is found in all RNA types. Four major categories of splicing mechanisms are recognized: splicing of group I, group II, nuclear mRNA and tRNA introns. In the two first classes the folded intron participates alone or aided by facilitating proteins in the splicing reaction. The third class is catalyzed by spliceosomes, and the fourth involves nucleases and ligases of protein nature. Group I introns are found in mitochondrial pre-mRNAs from *Neurospora crassa*, yeast and plants, pre-tRNAs from bacteria and plant chloroplasts, and pre-rRNAs from *Tetrahymena thermophila*, *Physarum polycephalum* and *Pneumocystis carinii* nuclei, yeast mitochondria and plant mitochondria and chloroplasts (Cech, 1990; Lambowitz and Belfort, 1993). Group II introns are found in bacteria (Ferat and Michel, 1993), fungal and higher plant mitochondria and in chloroplasts. Most of them are in pre-mRNAs, with a few in precursors of chloroplast tRNA and rRNA (Lambowitz and Belfort, 1993). Both group I and group II introns are mobile genetic elements (Saldanha *et al*., 1993), which is consistent with the fact that they carry their own splicing apparatus, being in consequence able to spread to different cellular compartments and organisms with minimal disrupting effects.

The diversity of splicing mechanisms and their discontinuous distribution suggest that splicing appeared independently in different groups of organisms. Alternatively, it has been proposed that similarities between the spliceosomal process and the self-splicing introns are clues for evolutionary relatedness (Sharp, 1994).

Ribosomal RNAs play fundamental roles providing a scaffold for ribosome assembly and, in the case of the large ribosomal subunit (LSU), the catalytic activity of peptidyl transferase (Green and Noller, 1997). The genes for the rRNA precursor appear in multiple copies organized in tandem arrays separated by non-transcribed spacers along the eukaryotic nuclear genome. Each repeating unit consists of three rRNA genes (18S, 5.8S and 28S) and two internal and one external transcribed spacers. These are segments present in the rRNA precursor that are removed by processing cuts. Each of the 18S and 28S rRNA genes is organized into several highly conserved cores interrupted by divergent domains, also called 'expansion segments' (Clark *et al*., 1984) or 'D domains' (Hassouna *et al*., 1984). Divergent domains evolve rapidly, with substitution rates that are at least two orders of magnitude higher than those of core regions. Unlike core regions, divergent domains show a high rate of insertion and deletion events (Hassouna *et al*., 1984; Olsen and Woese, 1993), which accounts for the great variation in length observed in the LSU rRNA of various organisms (Gonzalez *et al*., 1985). LSU rRNA divergent domains are located in the same places in all eukaryotes studied. The fundamental importance of rRNA in protein synthesis imposes evolutionary constraints to its overall structure. Insertions of sequences that alter this rRNA structure in rapidly evolving divergent domains of the corresponding genes would have negative adaptive value, unless they are accompanied by elimination of the inserted segments from the mature rRNA, similarly to transcribed spacer excision or by cuts that stabilize secondary structures. If excision or single cuts occur in a region that does not require to be covalently continuous to be functional, the result is a 'fragmented' rRNA. While in native conditions fragmented rRNAs show similar electrophoretic behavior to unfragmented rRNAs, in denaturing conditions the hidden breaks are evident by the appearance of rRNA bands of lower molecular weight.

We report here the finding of fragmentation and tissue-specific processing compatible with splicing in the D6

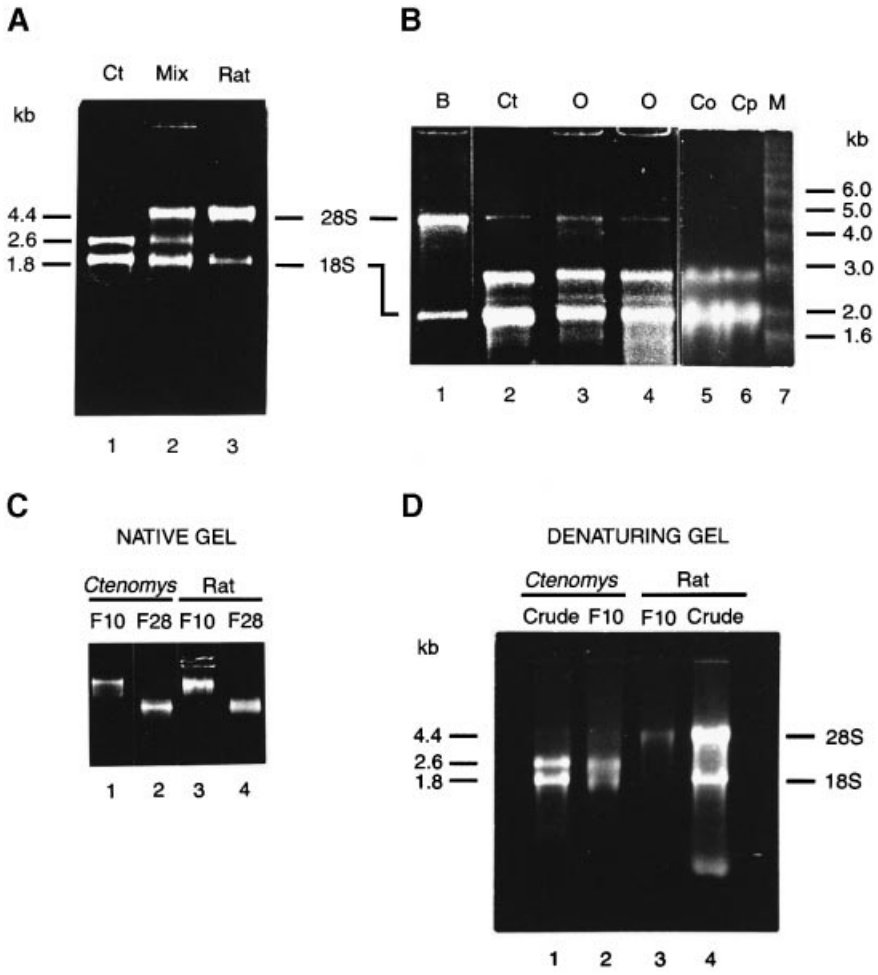

Fig. 1. (**A** and **B**) Denaturing formaldehyde–agarose gel electrophoresis of total RNA from different origins stained with ethidium bromide. Ct, *C.talarum*; Co, *C.opimus*; Cp, *C.porteousi*; O, *Octodontomys gliroides*; B, bovine granulosa cells; M, size markers. All rodent RNA preparations are from liver, except for the one in (B), lane 4, which is from kidney. Faint bands seen in lanes 2–4 of (B) are due to incomplete RNA denaturation. (**C** and **D**) Agarose gel electrophoresis of sucrose gradient fractions of *C.talarum* and rat RNAs. Fractions 10 and 28 correspond to the 28S and 18S peaks, respectively. (C) Native gel. (D) Denaturing formaldehyde gel.

domain of the 28S ribosomal RNA of South American rodents of the genus *Ctenomys* ('tuco-tucos'). Both processes were previously unknown to vertebrate cells and represent important novelties that will contribute to the understanding of the evolution of basic molecular processes in gene expression.

Results

An atypical pattern of rRNA

Tuco-tucos (*Ctenomys*) are the most numerous in species of all mammals. An explosive intrageneric radiation took place during the Pleistocene and led to ~56 living species (Reig *et al*., 1990). When investigating a putative transcription of its major satellite DNA (Rossi *et al*., 1990, 1993; Pesce *et al*., 1994), we unexpectedly found an anomalous pattern of rRNA in denaturing agarose gels: instead of the 28S (~4.4 kb) and 18S (~1.8 kb) bands expected for rodent rRNA (Figure 1A, lane 3), two bands of 2.6 and 1.8 kb were observed (lane 1), the latter being more intense than the former. This pattern is not due to unspecific degradation during RNA preparation. In fact, co-homogenization of equal amounts of *Ctenomys* and rat liver pieces and subsequent RNA extraction resulted in rRNAs with a combined pattern of migration showing three bands in denaturing agarose gels (Figure 1A, lane 2), which indicates that rat 28S rRNA was not degraded during the preparation procedure as a consequence of an unspecific RNase activity present in *Ctenomys* tissues.

The same anomalous pattern was observed in many specimens of *Ctenomys talarum*, and in specimens of other species such as *Ctenomys porteousi*, *Ctenomys opimus*, as well as in the related genus *Octodontomys* (Figure 1B).

A hidden break in LSU rRNA

The presence of two rRNA bands of 2.6 and 1.8 kb in *Ctenomys* could be explained in different ways. One possibility is that the 1.8 kb band corresponds to normal 18S rRNA and the 2.6 kb band corresponds to a full-length LSU rRNA of smaller size than normal mammalian 28S RNA. Another possibility is that the LSU rRNA is cleaved in two molecules of 2.6 and 1.8 kb, the latter co-migrating with normal 18S rRNA. The second hypothesis is consistent with the stronger ethidium bromide staining of the 1.8 kb band in *Ctenomys* (Figure 1A and B). To confirm this, we performed parallel sucrose gradient ultracentrifugations of *Ctenomys* and rat rRNAs in native

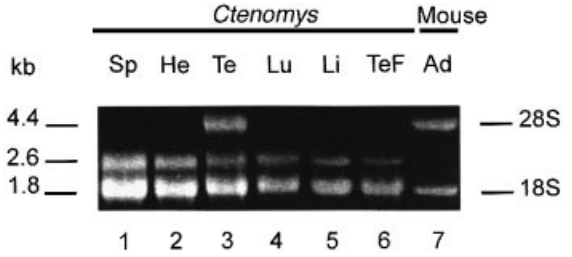

Fig. 2. Tissue specificity of the hidden break. Denaturing agarose gel electrophoresis stained with ethidium bromide of ribosomal RNAs from different *Ctenomys* organs. Sp, spleen; He, heart; Te, testis; Lu, lung; Li, liver; TeF, primary cultures of testis fibroblasts. Lane 7 is a control of rRNA from primary cultures of mouse mammary adenocarcinoma cells (Ad).

conditions and ran the corresponding fractions in native agarose gel electrophoreses. Results shown in Figure 1C indicate that in these conditions *Ctenomys* and rat rRNAs behave similarly, showing two main peaks represented by fraction 10 (towards the bottom of the gradient) and fraction 28 (towards the top of the gradient). When rat fraction 10 was denatured and run in a formaldehyde agarose gel, a single 4.4 kb band corresponding to normal 28S was detected (Figure 1D, lanes 3 and 4). However, when *Ctenomys* fraction 10 was denatured and run in the denaturing gel, two bands of 2.6 and 1.8 kb were observed, with sizes identical to those produced by denaturing crude total RNA (Figure 1D, lanes 1 and 2). These experiments clearly indicate that *Ctenomys* LSU rRNA exists as a 28S species in native conditions. This molecule bears a hidden break that becomes evident upon denaturation, giving rise to the observed 2.6 and 1.8 kb species. The results in Figure 1D show that the LSU rRNA 1.8 kb species indeed co-migrates with the small subunit 18S rRNA species.

Tissue specificity

The break is observed in nearly 100% of the LSU rRNA molecules from spleen, heart, lung and liver (Figure 2, lanes 1, 2, 4 and 5). Primary cultures of *Ctenomys* fibroblasts maintained up to 10 passages also showed complete expression of the hidden break (lane 6). However, rRNA from testis systematically showed a high proportion of an extra band of 4.4 kb, coincident with uncut 28S rRNA (lane 3).

Mapping and sequencing of the break region

The hidden break is asymmetrically positioned within the LSU rRNA molecule. According to the mammalian 28S rRNA gene map outlined in Figure 3, if the hidden break were closer to the 5′ end of the molecule it should map around domain D6, while if closer to the 3′ end, it should map within domain D8. Northern blots of *Ctenomys* total RNA hybridized to different mouse 28S rRNA gene probes (data not shown) demonstrated that the hidden break maps within the D6. Two oligonucleotide primers, named D6F and D6R (Zardoya and Meyer, 1996), designed from the highly conserved regions that flank the D6 were used to isolate genomic *Ctenomys* sequences by PCR. The same primers were used to isolate *Ctenomys* DNA complementary to the uncut, testis-specific rRNA. The genomic DNA product is 762 bp long, while the testis cDNA product is ~100 bp shorter. Sequence analysis (Figure 3) indicates that the size difference is due to a contiguous internal segment of 106 nucleotides (positions 67–172) present in the genomic D6, but absent from the testis cDNA. This segment is also absent from the mouse genomic D6, revealing that the uncut 28S rRNA resembles the typical mammalian structure more than its genomic counterpart.

Two oligonucleotides, named 5′D6 and 3′D6, designed upon the boundaries of *Ctenomys* D6 were used as probes in Northern blots of total RNA from *Ctenomys* testis and liver. 5′D6 lit up two strong hybridization bands of 4.4 and 1.8 kb with testis rRNA. Only one major hybridization band of 1.8 kb was detected in liver by this probe (Figure 4A, lanes 1 and 2). A very faint 4.4 kb band can be detected in liver, suggesting that very low amounts of the uncut species are present in this organ. 3′D6 lit up two strong hybridization bands of 4.4 and 2.6 kb with testis rRNA. Only one hybridization band of 2.6 kb was detected in liver (Figure 4A, lanes 6 and 7). Both oligos hybridized only to the regular 28S band of mouse rRNA (lanes 3 and 8). Hybridization is highly specific since both probes are able to hybridize to sense but not to antisense transcripts (Figure 4A, lanes 4, 5, 9 and 10), obtained by *in vitro* transcription from the 762 bp genomic PCR product inserted into the *Eco*RV site of pBS-SK+.

In summary, these results allow us to conclude that: (i) the hidden break maps within the D6; (ii) the 2.6 kb band observed in all tested tissues corresponds to the 3′ portion of *Ctenomys* LSU rRNA; (iii) the 1.8 kb band, also observed in all tested tissues, co-migrates with uncleaved 18S RNA and corresponds to the 5′ portion of *Ctenomys* LSU rRNA; (iv) the 4.4 kb band observed distinctively in testis corresponds to an uncut version that concomitantly lacks an internal segment of the D6.

rRNA splicing might generate the testis uncut 4.4 kb band

The shorter D6 version of testis LSU rRNA might arise either from a ribosomal gene version lacking the 106 bp internal segment, co-existing with longer gene versions, or alternatively from a single type long gene and a subsequent RNA processing compatible with splicing of the pre-rRNA. Southern blots of *Ctenomys* genomic DNA digested with either *Bam*HI–*Bgl*II or *Nco*I–*Bgl*II reveal single bands of ~1100 and ~420 bp, respectively (Figure 4B), clearly arguing for a single length D6 region which is ~100 bp longer than the region comprised between the same restriction sites in mouse DNA (data not shown and published mouse sequence). Figure 4C shows that PCR analysis is consistent with genomic Southern blots: a genomic band of 762 bp obtained with primers D6F and D6R is the only PCR product observed using either liver or testis *Ctenomys* DNA as templates (lanes 1 and 3). A strong RT–PCR band of 656 bp is the only band observed when testis rRNA is used as template. Liver rRNA also gives rise to a single 656 bp band of much lower abundance. Mouse samples originate single genomic and cDNA bands too, but, unlike *Ctenomys*, both bands are identical in size (661 bp), which roughly coincides with *Ctenomys* cDNA. Similar results were obtained by performing more sensitive radioactive PCR. Figure 4C, bottom, lane 1, shows that the 656 bp PCR band is not generated when the *Ctenomys* rRNA sample is not pretreated with DNase I and reverse transcriptase reaction is not performed. The 762 kb band in this control lane reflects

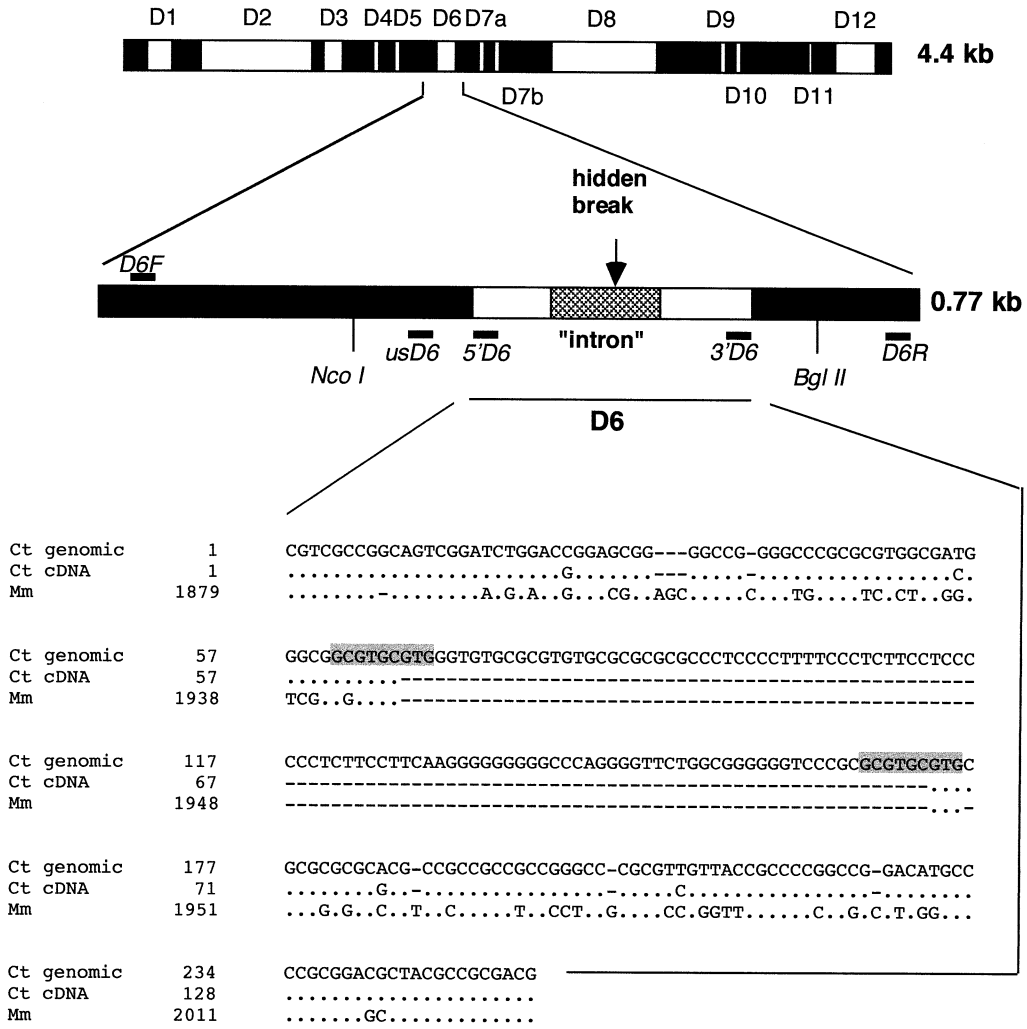

Fig. 3. Top: overall structure of the mouse LSU RNA gene. Black boxes, conserved regions. White boxes, divergent domains D1–D12. Bottom: nucleotide sequence comparison of the genomic and testis cDNA D6 segments from *Ctenomys* and the genomic D6 from mouse. Identical bases are indicated by dots. Hyphens indicate gaps. Gray sequences highlight two direct repeats. Mouse sequence numbering corresponds to the full-length published sequence (Hassouna *et al*., 1984). DDBJ/EMBL/GenBank accession Nos of the *Ctenomys* genomic and cDNA sequences are AF119340 and AF119339, respectively.

that the rRNA sample is contaminated with genomic DNA. This contamination is eliminated by DNase I treatment, since lane 2 only shows the 656 bp radioactive cDNA product. Figure 4C, lane 5, shows that a single radioactive PCR product is formed using *Ctenomys* genomic DNA as template. Most importantly, no bands of lower molecular weight are detected when 20 times more of this PCR product was loaded in the gel (Figure 4C, lane 7).

To demonstrate that the 656 bp band was not an artifact of RT–PCR, it was subcloned into the *Eco*RV site of pBS-SK+. The clone obtained, named pCtcD6, was used to prepare a *Nco*I–*Sac*I 472 bp probe, labeled with ^{32}P at its *Nco*I site. This site is internal to the rRNA sequence while the *Sac*I site is located 50 bp downstream of the insert 3′ end, in the vector polylinker. This probe was used in S1 mapping of *Ctenomys* rRNA from testis and liver. Both rRNAs gave rise to a protected band of 412 nucleotides, but the intensity is much stronger in testis than in liver (Figure 5A, lanes 4 and 5). The size of the protected band is that expected for the distance between the *Nco*I site and the 3′ end of the clone insert, which is corroborated by running in parallel the input probe cut with *Eco*RI, whose cleavage site is located only 4 bp from the cloning site (Figure 5A, lane 3). This experiment indicates that the RT–PCR product corresponds to a bona fide rRNA that is shorter than the corresponding genomic region and is more abundant in testis than in liver.

S1 mapping with a 424 bp *Nco*I–*Bgl*II genomic probe labeled at its *Nco*I site (Figure 5B, lane 2, band c) exhibits two protected bands of 215 (band d) and 170 (band e) nucleotides when hybridized to testis rRNA. These bands reveal the split and processed LSU rRNA molecules respectively, mapped from their 5′ ends. The 215 nucleotide band allows location of the break between approximately bases 121 and 122 of the sequence in Figure 3.

The results described above rule out any putative gene rearrangement occurring in testis as the cause for the shorter uncut version and strongly support the idea that there is a single ribosomal gene structure containing the 106 bp ‘intron’ and that the shorter testis-specific RT–PCR product represents a processed LSU rRNA lacking

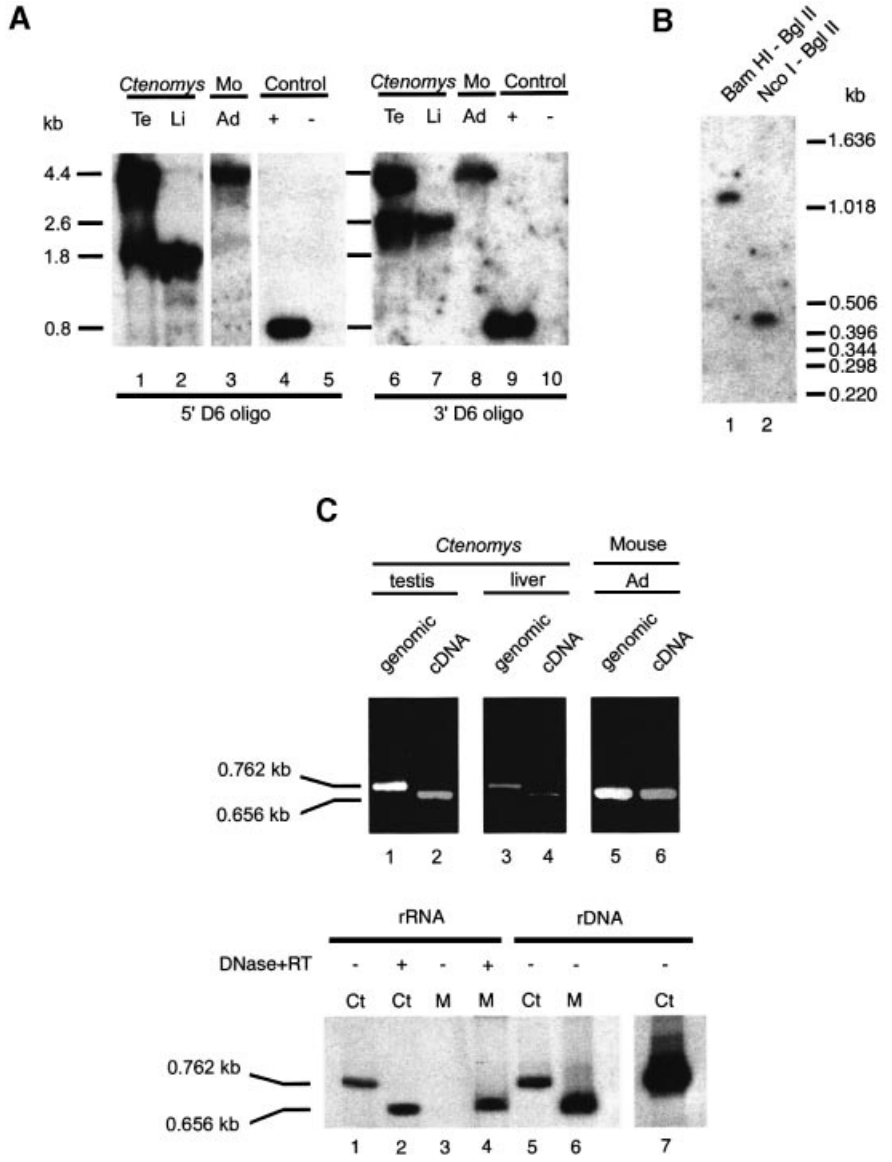

Fig. 4. (**A**) Northern blots of *Ctenomys* (lanes 1, 2, 6 and 7) and mouse (lanes 3 and 8) rRNA probed with oligonucleotides mapping at the 5′ and 3′ ends of the D6. Locations of the oligonucleotides 5′D6 and 3′D6 are shown in Figure 3. Positive and negative hybridization controls are sense (lanes 4 and 9) and antisense (lanes 5 and 10) RNA transcribed *in vitro* from pCtgD6. (**B**) Southern blot of *Ctenomys* genomic DNA cut with two pairs of restriction enzymes flanking the D6 and probed with oligonucleotide usD6 (see location in Figure 3). (**C**) Top: agarose gel electrophoresis of PCR (lanes 1, 3 and 5) and RT–PCR (lanes 2, 4 and 6) products using primers D6F and D6R, whose locations are shown in Figure 3. Templates for PCR: genomic DNA from *Ctenomys* testis (lane 1), *Ctenomys* liver (lane 3) and mouse adenocarcinoma cells (lane 5). Templates for RT–PCR: rRNA from *Ctenomys* testis (lane 2), *Ctenomys* liver (lane 4) and mouse adenocarcinoma cells (lane 6). Bottom: autoradiograms of agarose gel electrophoresis of PCR amplifications performed in the presence of [α-^{32}P]dCTP, using either rRNA (lanes 1–4) or genomic DNA (lanes 5–7) as templates. In lanes 2 and 4, samples were pre-treated with DNase I and subsequently used as templates for reverse transcriptase, prior to the PCR. Ct (lanes 1, 2, 5 and 7): samples from *Ctenomys* testis. M (lanes 3, 4 and 6): samples from mouse liver. Lane 7 corresponds to a 20-fold excess of the same PCR product as that of lane 5, respecting the same exposure time.

the intron. The intron has a GC content of 84% and bears no significant identity with other published sequences. No consensus sequences for 5′ or 3′ splicing sites for introns processed by spliceosomes could be recognized at the boundaries. Noticeably, the intron is flanked by two 9-nt direct repeats (5′-GCGTGCGTG-3′; Figure 3).

The hidden break maps within the intron

Since both the intron and the hidden break map within the D6, and the spliced testis molecule lacks the hidden break, it was very likely that the break occurred within the intron sequence. This was indeed confirmed by a series of RNA mappings. An 870 nucleotide genomic riboprobe labeled internally (Figure 6A, lane 3, band a), made from plasmid pCtgD6 yields two protected fragments of ~465 (band b) and 297 (band c) nucleotides in an RNase A protection assay of liver rRNA. This is consistent with a single break located within the intron. The same position is mapped by S1 mapping from the 3′ end side using as probe the same 424 bp *Nco*I–*Bgl*II of Figure 5B, but labeled at its *Bgl*II site (Figure 6B, band d for the input probe and band e doublet for the protected fragment).

The precise mapping of the break within the intron explains why the intron-less molecule is contiguous: the

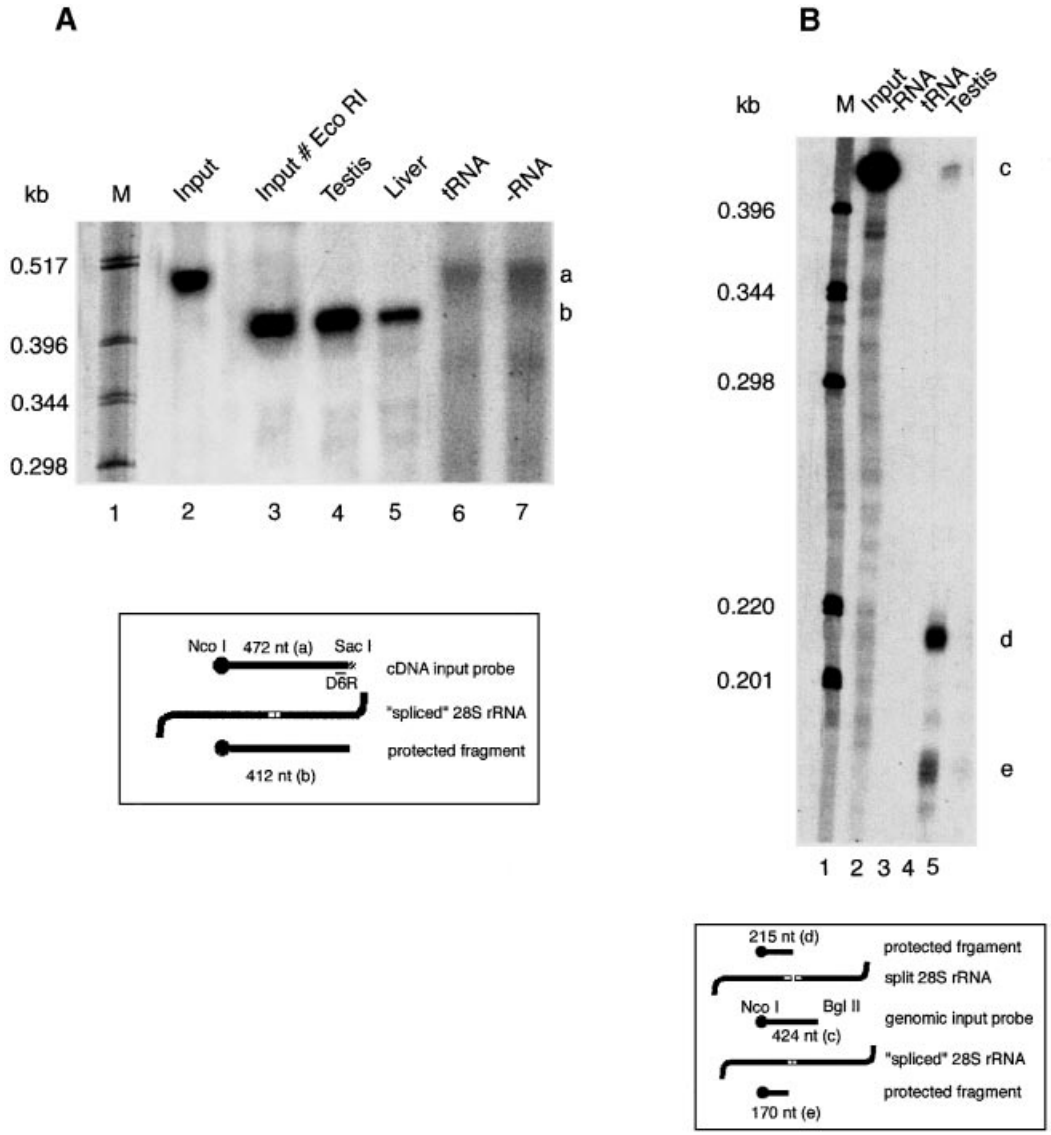

Fig. 5. (**A**) Evidence for a bona fide spliced rRNA. S1 nuclease mapping of *Ctenomys* rRNA using a 472 bp *Nco*I–*Sac*I probe end-labeled at its *Nco*I site prepared from the cDNA clone pCtcD6. The *Sac*I site belongs to the vector polylinker and maps 60 bp away from the *Eco*RV cloning site. In consequence, the protected fragment is 60 bases shorter than the input probe. Lane 2 shows the size of the input probe cut with *Eco*RI, a polylinker enzyme located only 4 bp away from the cloning site. Controls in which only yeast tRNA or no RNA were present in the hybridization reaction are shown in lanes 6 and 7, respectively. (**B**) S1 nuclease mappings of *Ctenomys* testis rRNA using a 424 bp *Nco*I–*Bgl*II genomic probe labeled at its *Nco*I site. Controls in which no RNA or only yeast tRNA were present in the hybridization reaction are shown in lanes 3 and 4, respectively. Diagrams showing the probes, rRNA species and protected fragments are at the bottom of each autoradiogram.

processing that eliminates the intron also eliminates the target sequence for the break.

Secondary structures

Figure 7 shows the predicted secondary structures for the putatively spliced (Figure 7A) and split (Figure 7B) *Ctenomys* D6 regions. Conservation of the GC-rich intron in the split molecule makes it extremely stable, with a calculated ΔG of –145.2 kcal/mol. The spliced D6 is much less stable with a ΔG of –81.5 kcal/mol. These theoretical differences in stability are consistent with primer extension experiments depicted in the same figure. Using liver rRNA as template, a single band of 178 nucleotides generated by reverse transcriptase with primer D6R reveals a full stop for cDNA synthesis located ~14 nucleotides upstream of the 3′ end of the D6. However, when testis rRNA is used as template, an additional band of ~2.0 kb is detected. This band might correspond to the distance between the primer and a site further upstream near the 5′ end of the LSU rRNA. These results clearly indicate that the lower ΔG of spliced molecules offers no structural constraints for cDNA synthesis to proceed towards the 5′ end. Extension of the same primer using mouse rRNA as template produces only one band of ~2.0 kb (data not shown) which is in agreement with the sequence and length similarities between the spliced LSU rRNA of *Ctenomys* and the normally contiguous mammalian LSU rRNA. In fact, the free energies of the mouse upstream D segments (D5 = –19.6 kcal/mol; D4 = –1.5 kcal/mol; D3 = –61.8 kcal/mol) are much lower than the free energy

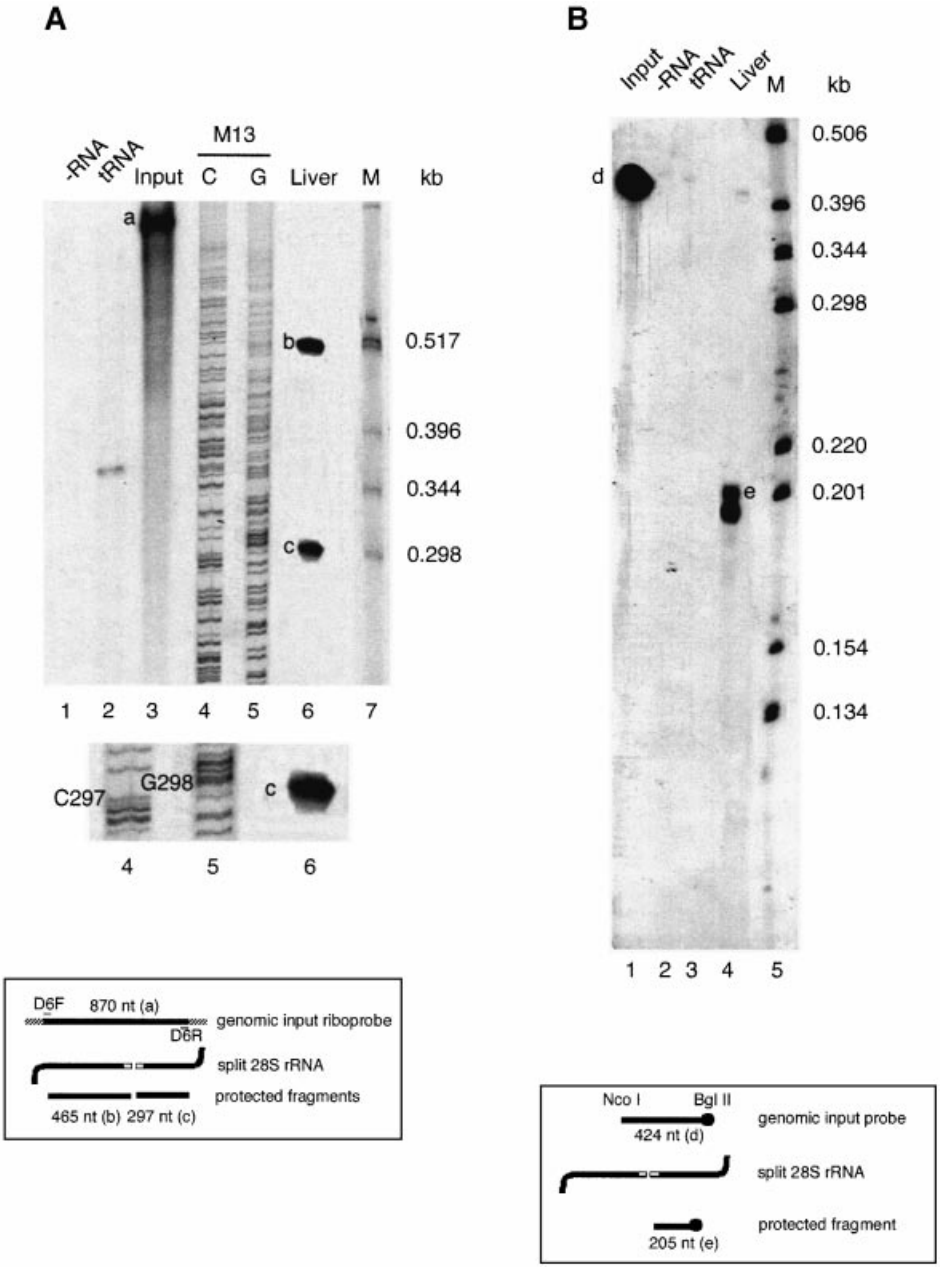

Fig. 6. (**A**) RNase A protection analysis of *Ctenomys* liver rRNA with a 870 nucleotide, internally labeled riboprobe spanning the D6 region. Controls in which no RNA or only yeast tRNA were present in the hybridization reaction are shown in lanes 1 and 2, respectively. A chain termination sequencing ladder corresponding to the C and G reactions of M13 phage DNA using a 'universal' primer is shown in lanes 4 and 5. (**B**) S1 nuclease mappings of *Ctenomys* testis rRNA using a 424 bp *Nco*I–*Bgl*II genomic probe labeled at its *Bgl*II site. Controls in which no RNA or only yeast tRNA were present in the hybridization reaction are shown in lanes 2 and 3, respectively. Diagrams showing the probes, rRNA species and protected fragments are at the bottom of each autoradiogram.

of the *Ctenomys* fragmented D6 (–145.2 kcal/mol). Only the D2 is more stable. If RT stopped at the D2, a high mol. wt extension product would appear, consistent with the ~2.0 kb band in Figure 7.

Discussion

We report here the discovery of two unexpected processing events in the LSU rRNA in South American rodents of the genus *Ctenomys*. Both processes were previously unknown in vertebrates.

First, we show that instead of being formed by a conventional single molecule of 4.4 kb, LSU rRNA is split in two molecules of 2.6 and 1.8 kb, corresponding to the 3′ and 5′ segments, respectively. This rRNA exists as a single 28S species stabilized by extensive base pairing in native conditions. A hidden break is evident upon denaturation, giving rise to the two shorter segments. Cleavage occurs in domain D6 and is present in all *Ctenomys* organs analyzed, including liver, lung, heart, testis and spleen, as well as in primary fibroblast cultures.

Secondly, we show the existence of an RNA processing mechanism in *Ctenomys* LSU rRNA that provokes the elimination of a 106-nt intron with a very high GC content (84%), located in the middle of the D6 domain. Moreover, this putative splicing event is cell type specific.

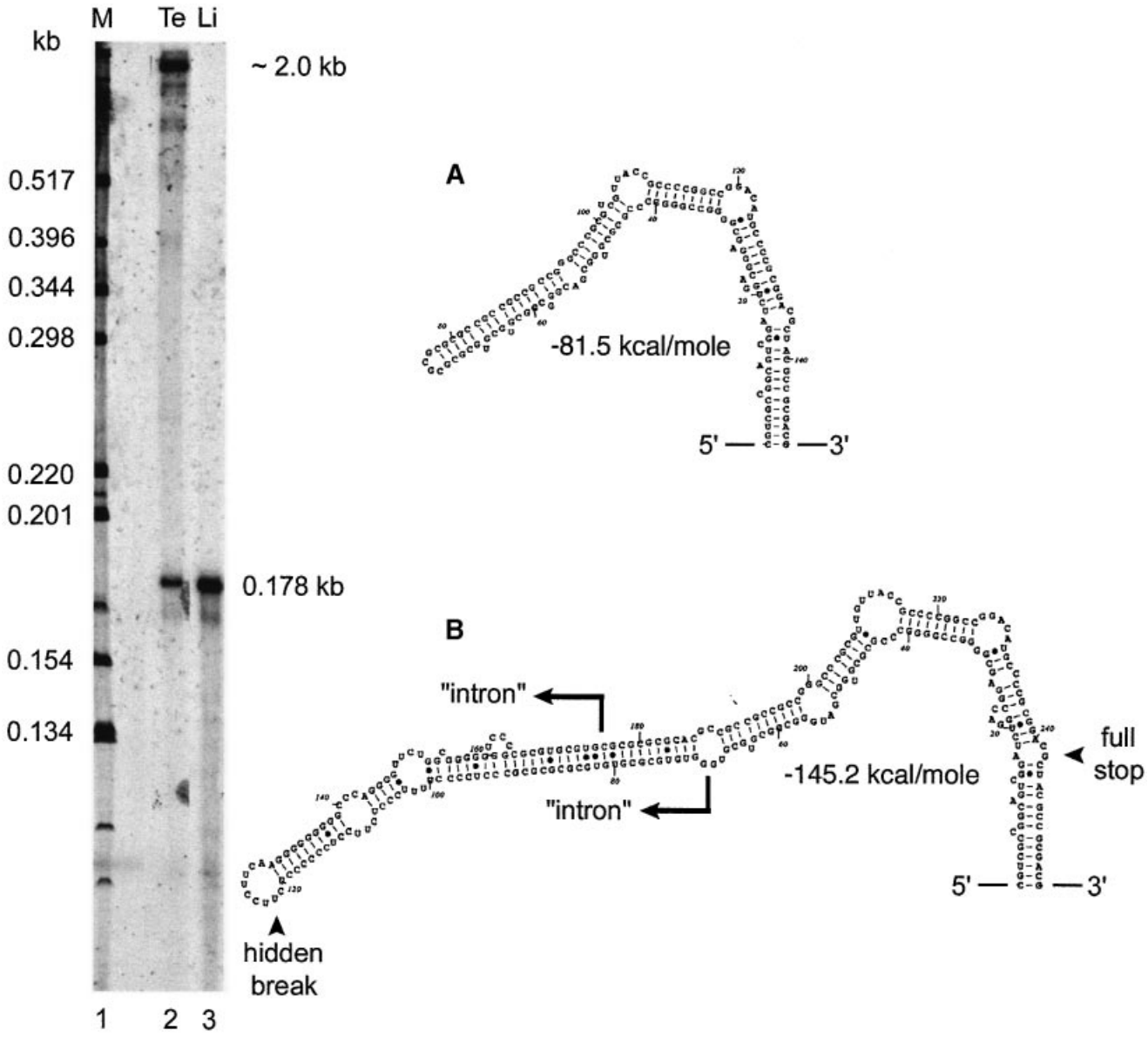

Fig. 7. Left: reverse transcriptase reactions extending primer D6R on testis (lane 2) and liver (lane 3) *Ctenomys* rRNAs used as templates. Right: predicted secondary structures for intron-less (**A**) and split (**B**) D6 domains of *Ctenomys* LSU rRNA. Structures were estimated by using the programs mfold, version 2.3, according to Zuker and Turner (http://www.ibc.wustl.edu/~zuker/rna/form1.cgi) and loopDloop, version 1.2a63. Arrows mark the estimated positions of the hidden break and the full stop for primer extension. These positions might be affected by an error of ±5 nucleotides, due to the resolution of the mapping experiments.

Because the putative splice sites do not follow the consensus for one of the known classes of RNA splicing, it is important to consider other possible mechanisms as the source of the intron-less form. For instance, intron skipping by RNA polymerase I during transcription, perhaps aided by the extremely tight secondary structure of the D6. We have no formal proofs for either mechanism. However, if intron skipping were the cause of *Ctenomys* intron-less rRNA, its testis-specificity would make it even more surprising and novel than splicing.

The hidden break lies within the intron, which allows the 'spliced' LSU rRNA to remain uncut. Figure 8 illustrates a model for the generation of the split and spliced 28S rRNA molecules from a common precursor.

Although to date, the existence of a single break and tissue-specific intron elimination in the D6 region of LSU rRNA are unique features of the particular group of organisms studied here, our findings reveal that mechanisms underlying gene expression are more diverse than expected and reinforce the idea of independent acquisition of RNA processing strategies in different groups throughout evolution.

Fragmentation

Split rRNAs have been found in prokaryotes (*Salmonella typhimurium*, Burgin *et al*., 1990) and in several lower eukaryotes ranging from protozoa (*Euglena gracilis*, Rawson *et al*., 1971; *Acanthamoeba castellani*, Stevens and Pachler, 1972; *Tetrahymena pyriformis*, Eckert *et al*., 1978; *Trypanosoma cruzi*, Castro *et al*., 1981; *Crithidia fasciculata*, Spencer *et al*., 1987) to worms (*Schistosoma mansoni*, van Keulen *et al*., 1991; *Trichinella spirallis*, Zarlenga and Dame, 1992) and arthropods (*Bombyx mori*, Applebaum *et al*., 1966; *Galleria mellonella*, Ishikawa

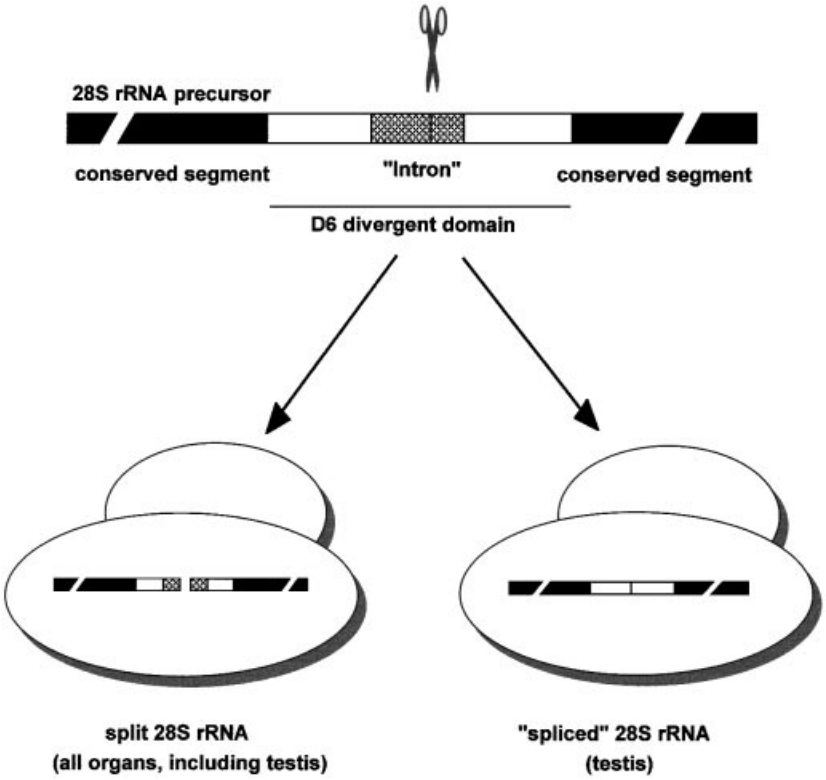

Fig. 8. Model for the generation of split and putatively spliced 28S ribosomal RNAs from a single precursor molecule. Black boxes, conserved regions; white box, D6 domain; cross-hatched box, D6 intron. The scissors point out the cleavage that gives rise to the hidden break in mature rRNA.

and Newburgh, 1972; *Aedes aegypti*, Shine and Dalgarno, 1973; *Musca carnaria*, Lava–Sanchez and Puppo, 1975; *Drosophila*, Jordan *et al*., 1976; *Sciara coprophila*, Ware *et al*., 1985). rRNAs of *Chlamydomonas reinhardtii* mitochondria (Boer and Gray, 1988) and higher plant chloroplasts (Fujiwara and Ishikawa, 1986) are also fragmented. However, it has been assumed that rRNAs of deuterostomes (echinoderms and chordates) are not internally fragmented in mature ribosomes (Fujiwara and Ishikawa, 1986). Our findings conclusively demonstrate that specific breaks may also occur in vertebrates.

Fragmentation in arthropods takes place only in the D7a domain, while in trypanosomatids the central D7b/D7c region is involved among other sites. In both cases, AU-rich segments are eliminated from mature LSU rRNAs by double excisions (de Lanversin and Jacq, 1989), which marks a clear difference with the GC richness and the single cut at the *Ctenomys* D6 domain. No apparent consensus sequences have been found in the invertebrates, which explains why a given region is preserved in or excised from mature LSU rRNA molecules. What is clear is that fragmentation always occurs in divergent regions and that they might show higher accessibility to nucleases (de Lanversin and Jacq, 1989).

Little is known about the mechanisms that generate split rRNAs in prokaryotes and lower eukaryotes. Cleavage of *Ctenomys* LSU rRNA could be caused by the activity of a species-specific ribonuclease, a non-specific ribonuclease that recognizes the particular sequence or conformation of D6, or alternatively by a ribozyme activity residing in the D6 itself.

Intron sequence and origin

The 106 bp intron spliced out in testis bears no significant identity with other known sequences. The intron boundaries lack the consensus sequences for splice sites of spliceosomal introns (5′AG/GU...PynXCAG/G3′ rule). A sequence that resembles a spliceosomal 3′ splice site (‘acceptor’ site) can be found in the middle of the intron: a row of 37 pyrimidines (bases 92–128 in Figure 3) followed by AAG/G. However, the site does not seem to be used since we did not find sequences downstream of it in the cDNA clones.

The intron is flanked by two 9 bp direct repeats: 5′-GCGTGCGTG-3′, highlighted in gray in Figure 3. This supports an insertion origin for the intron as a mobile element. Many introns are spread through homing endonucleases. The insertion site sequence of the *Ctenomys* rRNA intron does not match completely any of the known homing sites, listed in the REBASE (Restriction Enzyme Data Base). Nevertheless, the direct repeat contains the sequence 5′-GTGCG-3′ found in the cleavage site of PI-*Sce*I, a protein intron homing endonuclease from *Saccharomyces cerevisiae* (Lambowitz and Belfort, 1993). In addition, cleavage and intron insertion via PI-*Sce*I both take place between the C and the G, which would generate a direct repeat at the boundaries of the inserted element. Coincidence between cleavage and insertions sites is not the rule for homing; in many cases insertion occurs within the target sequence but away from the cleavage site.

The existence of the direct repeats allows nine possible alignments of the cDNA and genomic sequences, which define nine possible exon–intron boundaries. In Figure 3

we arbitrarily chose the alignment that fits the PI-*Sce*I insertion site. If this were the case, the intron would begin by GUG and end by a PuC, which partially complies with the conserved 5′ and 3′ boundary sequences (GUGPyG and APy) reported for group II introns (Michel *et al*., 1989).

Independently of the intron nature, *trans*-acting factors (RNA or proteins) with or without catalytic activity seem to be required for either cleavage or putative splicing, because a precursor rRNA, synthesized *in vitro* from plasmid pCtgD6, remains unprocessed when incubated alone, following the *in vitro* splicing protocol described by Partono and Lewin (1988) (data not shown).

It is worth noting that the D6 domain, where the intron is inserted, is one of the three LSU rRNA regions that show extreme size and sequence variation among species, ranging from a length of 27 bases and a GC content of 55% in yeast to a size of ~200 bases and a GC content of 84% in humans (Gonzalez *et al*., 1985). Figure 3 shows that there are four single-base differences between the *Ctenomys* D6 genomic and cDNA sequences outside the intron. This is not surprising since high sequence variability has been observed among D6 segments: three different D6 sequences have been detected in humans, two of which have been obtained from the same organ, indicating heterogeneity among the ribosomes of the same individual (Gonzalez *et al*., 1985). In fact, it is now assumed that no two 28S rRNA gene copies of the same individual are identical at their D segments (Leffers and Andersen, 1993; Kuo *et al*., 1996). In addition, Ruiz Linares *et al*. (1991) have shown that in insect D2 and D10 divergent domains, the secondary structure is conserved despite considerable variation in its sequence. In this context, the subtle sequence differences shown in Figure 3 do not contradict the key point of our findings: since all *Ctenomys* genomic copies contain the intron there must be some kind of RNA processing that gives rise to the abundant intron-less 28S rRNA found in testis.

The fact that the intron, and consequently its splitting or elimination, are not found in other rodents is indicative of an horizontal acquisition in a common ancestor to *Ctenomys* and *Octodontomys*. Homogenization processes such as gene conversion must have spread the intron in all copies of rRNA genes of the ancestor before or during vertical transmission.

Functional significance

There is a general consensus that breaks are tolerated because they have little effect on the structure of ribosomes (Clark, 1987). However, the existence of a precisely defined break in all organs of four different species of a group of higher organisms, together with an apparent splicing mechanism that is able to eliminate the segment where the break lies, argues for biological significance. Subtle primary sequence changes in rRNA such as breaks or point mutations can lead to drastic secondary structure alterations that are known to affect the binding of relevant ribosome proteins. For example, mutations that prevent the formation of two base pairs in domain III of the LSU rRNA of *S.cerevisiae* abolish completely the binding of protein L25, involved in the early assembly of ribosomes (Kooi *et al*., 1993). Similar tertiary interactions seem to be important in mammalian cells where an homologue to L25 exists, which is able to duplicate L25 function in yeast (Jeeninga *et al*., 1996). In our system, cleavage and/or splicing of *Ctenomys* 28S rRNA might help by preventing the presence in the ribosomes of the intact GC-rich sequence of insertion origin, because it might indirectly affect protein binding to sites in conserved regions, resulting in loss of ribosome function. This would explain why we never detected the intact intron sequence in mature rRNA: it is not tolerated and must therefore be cleaved or eliminated. In this hypothesis, cleavage is not merely incidental but has an important adaptive value. In any case, there might be a relationship between the presence of 'extra' sequences in eukaryotic LSU rRNA and the fact that eukaryotic ribosomes contain more ribosomal proteins than prokaryotic ribosomes (de Lanversin and Jacq, 1989).

The conspicuous organ-specificity of the intron elimination event (Figure 2) remains intriguing and at the same time fascinating to investigate. Why testis? Which testis cell type is involved? Is this 'splicing' developmentally regulated? If this splicing is not catalyzed by spliceosomes, what *trans*-acting factors determine cell specificity? Which nuclear compartment is involved?

Our findings are a good indication that the horizontal acquisition of novel gene expression mechanisms during evolution is an active process that can even affect families of higher organisms of recent origin. We must therefore be cautious in assuming uniformity in the molecular biology of higher organisms such as mammals, when such uniformity has thus far been based in the study of a limited number of laboratory and domesticated species.

Materials and methods

Specimens

With the exception of laboratory rats and mice, all specimens were caught in the wild using live traps. Geographic localities of the collected specimens, all from Argentina, are as follows: *C.talarum* (Necochea, Buenos Aires), *C.porteousi* (Bonifacio, Buenos Aires), *C.opimus* and *O.gliroides* (Tres Cruces, Jujuy).

Primary cultures

Testes were decapsulated and dispersed in a medium containing 1000 U/ml collagenase, 0.1% (w/v) trypsin inhibitor, 0.1% (w/v) hyalouronidase, 0.4% (w/v) bovine serum albumin, 0.1% (w/v) DNase II, 1 mM glucose, 2.5 ng/ml amphotericin B, 1 U/ml penicillin/streptomycin at 37°C for 30 min. After pelleting, cells were plated in high glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), 10% (v/v) fetal calf serum, 100 g/ml penicillin/streptomycin.

RNA preparation

Total RNA was routinely prepared by the acid guanidinium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi (1987).

Sucrose gradients

Total RNA (120 μg) was run in 10–30% (w/v) sucrose linear gradients prepared in 10 mM Tris–HCl, 1 mM EDTA at 24 000 r.p.m. in a SW55 Beckman rotor for 13 h at 4°C. Gradients were fractionated in ~45 fractions of 100 μl each.

Oligonucleotides

The following oligonucleotides were used in this paper:
D6R: 5′-GACTGACCCATGTTCAACTGCTGT-3′;
D6F: 5′-GGTAAAGCGAATGATTAGAGGTCTT-3′ (Zardoya and Meyer, 1996);
5′D6: 5′-AGATCCGACTGCCGGCGACG-3′;
3′D6: 5′-GTCGCGGCGTAGCGTCCGCGG-3′;
usD6: 5′-CAACTCACCTGCCGAATCAA-3′.

Northern blots

RNA was electrophoresed in 1% (w/v) agarose–formaldehyde gels (Lehrach *et al*., 1977) and blotted to Hybond N+ membranes (Amersham) in 50 mM NaOH overnight. Oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Hybridizations were performed at 56°C in 0.9 M NaCl, 0.09 M Tris–HCl pH 7.5, 6 mM EDTA, 0.5% (v/v) NP-40, 100 μg/ml herring sperm DNA. Washings were performed in 6× SSC, 0.1% (w/v) SDS, at 56°C for 30 min.

Southern blots

DNA was restricted with *Bam*HI–*Bgl*II or *Nco*I–*Bgl*II using 4 U of enzyme per microgram of DNA. After a double phenol extraction and ethanol precipitation, digested DNA was electrophoresed in 1% agarose gel and after denaturing with 0.5 N NaOH in 1.5 M NaCl blotted to Hybond N membranes (Amersham) in 10× SSC overnight. Probe labeling, hybridization and washing conditions were identical to those used for Northern blots.

Primer extension

Approximately 1 μg of total RNA was denatured at 95°C for 10 min in 5 μl H_2O. If denaturation was performed at 65°C, as most protocols indicate, no cDNA was synthesized, confirming the need to unfold the extremely stable secondary structure of *Ctenomys* D6. Annealing and cDNA synthesis were performed in 20 μl final volume containing 25 pmol of oligonucleotide primer plus 8 nmol of dNTPs, 5 μCi of [α-^{32}P]dCTP, 20 U of human placental ribonuclease inhibitor and 300 U of MMLV reverse transcriptase (Promega) in the buffer provided by the manufacturer. The mix was incubated at room temperature for 10 min to allow RNA–primer annealing and then at 35°C for 60 min. Nucleic acids were ethanol-precipitated in 2.5 M NH_4OAc. Pellets were resuspended in 10 μl of formamide-containing loading buffer and run in 8% polyacrylamide sequencing gels.

PCR, RT–PCR, subcloning and sequencing

PCR of rDNA was performed in 50 μl containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM primers, 10% (v/v) dimethylsulfoxide, 100 ng of genomic DNA and 1 unit of *Taq* DNA polymerase (Life Technologies) in the buffer provided by the manufacturer, with or without the addition of 2 μCi of [α-^{32}P]dCTP. A 30-cycle reaction was preceded by incubation at 95°C for 5 min and ended by incubation at 72°C for 10 min. Each cycle consisted of incubations at 95°C for 1 min, 50°C for 30 s and 72°C for 90 s. RT–PCR was performed following the same primer extension (without labeled nucleotides, and adding a final incubation at 95°C for 10 min after the polymerization) plus PCR protocols specified above. Two microliters of each RT reaction were taken as template for the PCR. PCR and RT–PCR products (762 and 656 bp, respectively) were phosphorylated with T4 polynucleotide kinase, blunt-ended with Klenow, eluted from 1.8% (w/v) low melting point agarose gels, and subcloned into the *Eco*RV site of the plasmid pBS-SK+ (Stratagene). Direct sequencing of the PCR and RT–PCR products or their subclones was performed using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit provided by Perkin Elmer in an Applied Biosynthesis 373 DNA sequencer. Genomic and cDNA clones used for further studies were named pCtgD6 and pCtcD6, respectively.

RNase protection assay

pCtgD6 was linearized with *Xho*I and a 872-nt anti-sense riboprobe was obtained by incubation with T3 RNA polymerase in the presence of 40 μCi of [α-^{32}P]UTP (3000 Ci/mmol). This probe was purified by elution from a 6% denaturing polyacrylamide gel. The probe (10^5 c.p.m.) and 1 μg of total RNA from *Ctenomys* liver or mouse adenocarcinoma cells were denatured in 40 mM PIPES pH 6.7, 400 mM NaCl, 1.25 mM EDTA, 80% (v/v) deionized formamide, at 95°C for 10 min, in a final volume of 25 μl. Subsequent annealing was carried out at 60°C for 15 h. Then, RNase A was added to a final concentration of 3.7 μg/ml and incubation proceeded for 30 min at 30°C. The reaction was stopped by addition of SDS and proteinase K to final concentrations of 0.6% (w/v) and 0.3 mg/ml, respectively, and incubation at 37°C for 15 min. After phenol extraction and ethanol precipitation in the presence of yeast tRNA as carrier, the samples were denatured and electrophoresed in 6% sequencing gels.

S1 mapping

The 424 bp *Nco*I–*Bgl*II probe labeled only at its *Nco*I end was prepared by digesting pCtgD6 with *Nco*I, labeling by fill in with [α-^{32}P]dCTP and Klenow, heat inactivation of the Klenow enzyme and re-cutting with *Bgl*II. The 424 bp *Nco*I–*Bgl*II probe labeled only at its *Bgl*II end was prepared by digesting pCtgD6 with *Bgl*II, labeling by T4 PNK with [γ-^{32}P]ATP after dephosphorylation, heat inactivation of the kinase enzyme and re-cutting with *Nco*I. The 472 bp *Nco*I–*Sac*I probe labeled at its *Nco*I site was prepared from plasmid pCtcD6 as described above. Probes were eluted from 6% native polyacrylamide gels. Probes (showing 200–3000 c.p.m.) and RNA samples were co-precipitated with ethanol and 20 μg of glycogen as carrier. After resuspending the pellet in 13 μl of R-loop buffer [80% (v/v) deionized formamide, 10 mM PIPES pH 6.5, 1 mM EDTA, 0.4 M NaCl] and denaturing at 80°C for 10 min, hybridization was carried out at 55°C overnight. Subsequent steps were as described previously (Kornblihtt *et al*., 1984).

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