Two different snoRNAs are encoded in introns of amphibian and human L1 ribosomal protein genes

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ABSTRACT

We previously reported that the third intron of the X.laevis L1 ribosomal protein gene encodes for a snoRNA called U16. Here we show that four different introns of the same gene contain another previously uncharacterized snoRNA (U18) which is associated with fibrillarin in the nucleolus and which originates by processing of the pre-mRNA. The pathway of U18 RNA release from the pre-mRNA is the same as the one described for U16: primary endonucleolytic cleavages upstream and downstream of the U18 coding region produce a pre-U18 RNA which is subsequently trimmed to the mature form. Both the gene organization and processing of U18 are conserved in the corresponding genes of X.tropicalis and H.sapiens. The L1 gene thus has a composite structure, highly conserved in evolution, in which sequences coding for a ribosomal protein are intermingled with sequences coding for two different snoRNAs. The nucleolar localization of these different components suggests some common function on ribosome biosynthesis.

INTRODUCTION

Recently, it has been discovered that genes for a new class of snoRNAs reside in introns of protein coding genes. All these RNAs are localized in the nucleolus and are found in introns of nucleolar proteins such as the ribosomal and hsc70 proteins (1–3). The only exception is represented by the U17 snoRNA which is encoded in introns of the human cell cycle regulatory gene RCC1 (4). In addition to the peculiar gene organization, these snoRNAs show an unusual biosynthesis: they originate by processing of the pre-mRNA in which they reside and not by independent transcription. Two different approaches led to this discovery: on one side the study of the genomic arrangement of already known snoRNAs (1,3,4,5) and on the other the study of conserved intron sequences (2,6,7). The latter case applies for the U16 RNA which was first characterized as a phylogenetically conserved sequence present in the third intron of L1 r-protein genes of amphibia and humans (2,8). When microinjected into oocytes, this intron underwent site specific cleavages with the release of the internal conserved region. This region was shown to correspond to a previously uncharacterized small nucleolar RNA that was called U16. This RNA, as expected for a processed RNA, does not possess the trimethylguanosine (m3G) cap, such as all the other intron-encoded snoRNAs. The absence of the m3G cap and the intron localization have now become diagnostic for snoRNAs that originate by processing.

In this paper we characterize another snoRNA (U18) encoded in four different introns of the L1 r-protein gene. Both sequence and gene localization are conserved in the corresponding introns of the X.tropicalis (L1t) and H.sapiens (L4h) L1-homologous genes. U18 RNA originates by processing of the pre-mRNA following a pathway conserved in evolution and corresponding to the one already described for U16 RNA. This RNA is also uncapped and is associated with fibrillarin in the nucleolus.

MATERIALS AND METHODS

Cloning of second intron sequences from L1 genes

The second intron of the L1b gene copy of X. laevis (L1b, see ref. 9) and of the single copy of X. tropicalis (L1t, see ref. 10) were isolated by PCR using primers derived from the sequence of the L1b cDNA (11). The forward oligo includes 25 nucleotides of the 3' portion of the second exon plus an EcoRI site (5'-GGGAATTCCCTTGCCAGAAGCTTCAGCAGGC-3'), while the backward primer includes 23 nucleotides of the second exon of the third intron plus an Xhol site (5'-CGCTCAAGCAAGCTGCCCTCGAGTCTTCACATTT-3'). The second exon of the human homologue gene (L4h) was amplified from genomic DNA utilizing two oligonucleotides derived from the human L4 cDNA clone (12). The forward oligo includes 23 nucleotides of the second exon plus an Xhol site (5'-CGCTCAAGCAAGCTGCCCTCGAGTCTTCACATTT-3'), while the backward primer includes 24 nucleotides of the third exon with an EcoRI site (5'-GGGAATTCCCTTGCCAGAAGCTTCAGCAGGC-3'). PCR reactions were carried out in 50 µl of 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl2, 250 µM dNTP, 1 µM of each primer, 4.5 units of Taq polymerase and 1 µg of genomic DNA. Each amplification cycle consisted of 1 min. denaturation at 94°C, 2 min. annealing at 58°C and 3 min. extension at 72°C. Thirty cycles were performed. The EcoRI and Xhol sites of the primers allow the cloning of the amplified DNA in the corresponding sites of the Bluescript vector (Stratagene). The recombinant plasmids were sequenced according to the Sanger method (13).

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In vitro RNA synthesis and oocyte injection

The templates for the pre-RNA containing the second introns are hereafter described. L1a gene copy (002a): HinfI digestion of plasmid 00234 (14) and transcription with SP6 polymerase produce an RNA with 20 bases of the upstream polylinker, 100 nucleotides of exon 2, 210 nucleotides of intron and 20 nucleotides of exon 3. L1b gene copy (002b): XhoI digestion of the corresponding plasmid and transcription with T7 polymerase produce an RNA with 67 bases of the T7 upstream polylinker, 112 nucleotides of exon 2, 199 nucleotides of intron and 50 nucleotides of exon 3. L1t gene copy (002t): XhoI digestion of the corresponding plasmid and transcription with T7 polymerase produce an RNA with 67 bases of the T7 upstream polylinker, 112 nucleotides of exon 2, 187 nucleotides of intron and 50 nucleotides of exon 3. L4h gene copy (002h): EcoRI digestion of the corresponding plasmid and transcription with T3 polymerase produce an RNA with 35 bases of the T3 upstream polylinker, 84 nucleotides of exon 2, 193 nucleotides of intron 2 and 54 nucleotides of exon 3.

The 32P-labelled transcripts were gel purified and injected into the nuclei of stage VI oocytes. Total RNA was extracted from manually purified nuclei as described by Caffarelli et al. (14) and an equal number of injected counts were loaded on 10% polyacrylamide gels.

Northern blot analysis

The immunoprecipitation of the different RNP complexes and the subcellular fractionations (15) were performed as described in Fragapane et al. (2). The extracted RNAs were electrophoresed on 6% acrylamide—7M urea gels, electrotransferred to Amersham's Hybond-N paper and UV crosslinked. The U18 (5'-GGGTTCAAGAAACCGGACCATC-3') and U3 (16) oligo probes were 5' 32P-end-labelled and hybridized according to Caizergues-Ferrer et al. (16). The hybridization with the U1 probe (17) was carried out in standard conditions.

Southern blot analysis

Total genomic X.laevis DNA was digested with EcoRI and BamH1 and 15 μg were loaded on single slots of a 0.8% agarose gel. The DNA was transferred to nitrocellulose filters and analyzed by RNaseH mapping. The templates for the pre-RNA containing the second introns are hereafter described. L1a gene copy (002a): HinfI digestion of plasmid 00234 (14) and transcription with SP6 polymerase produce an RNA with 20 bases of the upstream polylinker, 100 nucleotides of exon 2, 210 nucleotides of intron and 20 nucleotides of exon 3. L1b gene copy (002b): XhoI digestion of the corresponding plasmid and transcription with T7 polymerase produce an RNA with 67 bases of the T7 upstream polylinker, 112 nucleotides of exon 2, 199 nucleotides of intron and 50 nucleotides of exon 3. L1t gene copy (002t): XhoI digestion of the corresponding plasmid and transcription with T7 polymerase produce an RNA with 67 bases of the T7 upstream polylinker, 112 nucleotides of exon 2, 187 nucleotides of intron and 50 nucleotides of exon 3. L4h gene copy (002h): EcoRI digestion of the corresponding plasmid and transcription with T3 polymerase produce an RNA with 35 bases of the T3 upstream polylinker, 84 nucleotides of exon 2, 193 nucleotides of intron 2 and 54 nucleotides of exon 3.

Cloning of oocyte endogenous U18 RNA and 5' mapping

Total RNA extracted from ovariess was fractionated on 10—30% sucrose gradient. 45 μg of the 4—8S fraction were run on a 6% acrylamide—urea gel and the RNA 50-80 nucleotide long was eluted. The RNA was 3' elongated with poly(A) polymerase and reverse transcribed in the presence of an oligo(dT) primer. The cDNA was then PCR amplified utilizing as forward primer the oligo A1 containing the first 17 nucleotides of the conserved intron region plus a XhoI site (5'-GCTCTAGATGATGAGTTCGTT-CCACTTC-3') and as backward primer an oligo(dT) containing an XhoI site (5'-GCGCGAGTTTTTTTTTTTTTTTTT- TT-3'). Each amplification cycle consisted of 1 min. denaturation at 94°C, 90 sec. annealing at 48°C and 2 min. extension at 72°C. Twenty five cycles were performed. The XhoI and XbaI sites of the primers allow the cloning of the amplified DNA in the corresponding sites of Bluescript vector (Stratagene). The recombinant plasmids were sequenced according to the Sanger method.

To characterize the 5' end of the U18 RNA, reverse transcriptase elongation was performed on 10 μg of 4—8S RNA from sucrose gradient (see above) utilizing as labelled primer the A2 oligonucleotide (5'-CCAAGCTTAAACGAAACTTC-CAC-3') which includes 15 nucleotides of the 3' portion of the conserved intron region (underlined nucleotides). The reaction was performed as described by Fragapane et al. (2), with the only modification of decreasing the annealing temperature to 48°C. The products of the reaction were run on 6% denaturing polyacrylamide gel in parallel with a Sanger sequence (13) performed with the same primer on 004 plasmid, which includes the intron 4 of the X.laevis L1a gene (11).

Circularization of U18 RNA

Gel purified U18 RNA, originating from oocyte injection of 002b labelled RNA, was circularized with T4 RNA ligase. The reaction was performed in 20 μl of 50 mM Tris—HCl pH 7.5, 10 mM MgCl2, 1 mM ATP, 20 mM DTT, 0.1 mg/ml BSA, in the presence of 18 units of T4 RNA ligase (Pharmacia) and 20 units of RNasin (Boehringer). The sample was incubated at 37°C for 2 hrs, phenol extracted, ethanol precipitated and analyzed on 8% acrylamide—urea gel.

RESULTS

Phylogenetical conservation of sequences present in introns of the L1 r-protein gene

Previous work described that a highly conserved region of approximately 70 nucleotides in length is present in the second, fourth, seventh and eighth intron of the X.laevis L1a gene (11). The same region is conserved also in the seventh and eighth intron of the X.tropicalis L1 r-protein gene (18). In addition to the already published sequences, we also isolated the second intron from the second copy of the X.laevis L1 gene (Lib) and from the unique copies of X.tropicalis (L1t) and H.sapiens (L4h) corresponding genes (10, 12). All these different introns contain the same conserved region where sequences of perfect homology are intermingled with variable nucleotides (Fig. 1). The positions and the type of substitutions of these variable bases, together with the comparison of flanking intron sequences (18 and not shown) indicate that there is higher homology among members of the same intron rather than among different introns, suggesting that this gene organization must have been present in the ancestor L1 gene. Among these introns, the best characterized is the second one for which we obtained the complete set of sequences: both copies of the L1 X.laevis gene (002a and 002b) and the unique copies of X.tropicalis (002c) and H.sapiens (002h).

Oocyte microinjections

The splicing phenotype of the different 002 transcripts was analyzed by oocyte microinjection of in vitro made transcripts (fig.2). The second intron-containing precursors are good splicing substrates as shown by the accumulation of large amounts of lariats and mature RNAs. Besides splicing they undergo site specific cleavages, with the production of truncated molecules (C, and D) and the release of an RNA corresponding to the internal conserved region (F-molecules). The different cleavage products were eluted from the gel and analyzed by RNaseH digestion with an oligo internal to the conserved region (not shown). They result analogous to the molecules previously characterized in the processing reaction of the third intron (2,8,19): D-molecules originate from cleavage in the 5' portion...
of the intron, while C-molecules derive from cleavage in the 3' portion. When both cleavages occur on the same precursor, F-type of molecules are produced (see schematic representation of fig.2). In vitro experiments have shown that the primary cleavages are localized some 20 nucleotides upstream and downstream of the conserved intron region and trimming occurs subsequently removing the 5' and 3' trailer sequences (Caffarelli et al., manuscript in preparation). The efficient trimming activity of the oocyte does not allow the accumulation neither of the primary cleavage products nor of the complementary cut-off molecules. Instead, large amounts of the trimmed C, D and F-molecules are visualized. The stability of these RNAs, and in particular of the uncapped D and F-molecules, suggests that some factor or secondary structure must protect them from degradative trimming.

All four substrates analyzed show efficient production of F-molecules corresponding in size to the length of the conserved intron sequence (68-70 nucleotides); in addition, some molecules few nucleotides longer than the mature F-RNA can also be detected (F' bands). RNase H analysis has demonstrated that these RNAs have a short trailer sequence in the 3' portion (not shown). This is more pronounced in 002b and 002h substrates that show the presence of F'-molecules still after three hours of incubation. This is more pronounced in 002b and 002h substrates that show the presence of F'-molecules still after three hours of incubation.

The internal conserved region hybridizes to a snRNA

We utilized an oligonucleotide complementary to the central part of the coding strand of the conserved intron region as probe (U18-oligo) in Northern blots in order to check whether it recognizes any specific class of molecules in the endogenous RNA pool of the oocyte. Fig. 4 shows that this probe hybridizes to a discrete nuclear RNA species. Subcellular fractionations and RNP selective immunoprecipitations have allowed to define that this RNA is predominantly localized in the nucleolus (lane No), is associated with the fibrillarin antigen (lane fibr) and does not possess a trimethylguanosine cap at its 5' end (lane m3G). The figure shows control hybridizations with other well characterized snRNAs, such as U1 and U3. These controls allow to verify the subcellular and antigen-association preparation of the RNA samples (2). The nucleolar localization and fibrillarin association of this RNA is also supported by the presence in the conserved intron sequence of the canonical C and D boxes (2,21,22) contained in all the fibrillarin-associated snoRNAs identified so far (see fig.1).
Figure 2. In vivo splicing analysis of 002a, 002b, 002t and 002h RNAs. Upper panels: electrophoretic analysis of 32P-labelled transcripts injected into the germinal vesicles of X.laevis oocytes and incubated for 0 min (lanes 1), 30 min (lanes 2), 1 hr (lanes 3) and 3 hrs (lanes 4); nuclei were manually dissected and RNA was extracted and loaded on 10% denaturing polyacrylamide gels. M: Mspl-digested pBR322 plasmid.

Cloning of the U18 snoRNA

In order to prove that the snoRNA identified by hybridization indeed corresponds to the intron conserved sequence, we proceeded to the cloning of this snoRNA. RNA from a 4-8S sucrose gradient fraction was separated on a 6% polyacrylamide gel and the RNA corresponding to the size of 50-80 nucleotides was eluted and utilized as substrate for poly-A tailing with poly-A polymerase and reverse transcription in the presence of oligo-dT primers. The cDNA was PCR amplified with a forward primer corresponding to the first 20 nucleotides of the second intron conserved region and with a backward primer complementary to the synthetic poly-A tails (see materials and methods). The amplified DNA was cloned into the XbaI-XhoI sites of the Bluescript vector and the clones were screened for hybridization to the conserved intron region (U18-oligo). Several clones were found having sequences perfectly matching that of the second intron conserved region (fig.5a). This previously uncharacterized snoRNA was named U18. In order to screen more rapidly for the presence of snoRNAs deriving from the other introns besides the second, we cloned the product of RT-PCR performed on the same size-selected RNA, utilizing as primers two oligonucleotides complementary to the terminal portions of the conserved intron region. Sequencing of several independent clones has demonstrated the existence of U18 RNAs originating from the fourth and eighth intron of the L1a gene copy. In addition, we found also two U18 RNAs not corresponding to any

Figure 4. (Left): Northern analysis of RNAs from: 10 nuclei (Nu), 30 nuclei immunoprepared with anti-trimethylguanosine cap (m3G) and anti-fibrillarin (fibr) antibodies. Right panel: Northern analysis of RNAs from: nucleolar (No) and nucleoplasm (Super) preparations from 30 nuclei. The same filters were utilized for subsequent hybridizations with the different snoRNA probes indicated beside.
Figure 5. Panel a) Top: the nucleotide sequence of the U18 cDNA is shown aligned with that of the 002a intron. The perfect sequence homology demonstrates the 002a origin of the characterized U18 RNA. The comparison allows the identification of the 3' end at the T indicated by the arrow. The underlined regions correspond to the C and D boxes. Lower panel: identification of the 5' end of U18 RNA. The products of the reverse transcriptase reaction (RT) were run in parallel with the sequence (G, A, T and C) performed with the same oligo on 004 plasmid DNA that contains the fourth intron of the L1a gene (9). On the side the deduced complementary nucleotides to the shown sequence are reported. Panel b): Southern blot analysis of total genomic DNA digested with EcoRI and BamHI. The probes utilized are indicated above: 002 contains the second intron (see materials and methods). The L1 cDNA contains all the L1 coding region but the first exon. The size of the hybridization bands are indicated on the side.

sequenced intron. The most possible explanation is that they represent the U18 RNAs deriving from the L1b introns not yet cloned. From these data it is possible to assume that very likely all the introns containing the conserved region contribute to the endogenous pool of U18 RNA, generating a family of U18 related sequences.

The 3' terminal nucleotide of U18 was identified, by the cDNA sequence, in the last nucleotide before the poly-A tail; the possibility of an A being the 3' end, was ruled out by the comparison with the 002a intron sequence that does not show any A at the corresponding position (see fig.5a). In order to characterize the 5' end of U18 RNA, we performed reverse transcriptase analysis on 10 μg of the same size-selected RNA utilized for cloning with the A2 primer which is complementary to the 15 terminal nucleotides of U18. The products of primer extension were run in parallel with the nucleotide sequence performed on the fourth intron containing plasmid (004). Four different products are identified (fig.5a), the longest extending up to the G of the 004 sequence which is 6 nucleotides upstream of the C box (see fig.1). Since the primer extension was performed on a mixed RNA population and since the different U18 coding sequences differ in length up to 4 nucleotides (see fig.1), it is possible that each band contains the extended products of a single class of U18 RNA. In this case the different intron-encoded U18 RNA would all start 6 nucleotides before the conserved UGAUGA sequence of the C-box (see fig.1).

Genomic organization of U18 coding sequences

X.laevis genomic DNA was digested with EcoRI and BamHI and analyzed by Southern blot with a second intron probe (002a, see materials and methods), in order to check whether the only coding sequences reside in the L1 gene. Figure 6 shows that four bands of hybridization are visualized. The interpretation of these bands derives from sequencing and restriction analysis data of the cloned genes; the bands at 2 and 1.7 Kb correspond to the genomic fragments containing the second and fourth introns of the L1a and L1b gene copies respectively. The band at 7 Kb represents the genomic fragment containing the seventh and eighth intron of the L1a gene copy, while the band at 5 kb, although not available as a cloned fragment, very likely corresponds to the same gene portion of the L1b copy. All these bands are visualized also with the L1 cDNA probe showing that the U18 RNA coding sequences are only present inside the L1 gene.

DISCUSSION

In the last year there has been a continuous increase in the number of different snoRNAs identified in introns of protein coding genes and that originate by processing of the pre-mRNA (for a review
see 23 and 24). A series of new questions related to the function of these snoRNAs, to the mechanism of processing and to the evolutionary and functional implications of their gene organization are now opened. The availability of a large number of different examples will help in elucidating some of these points. The L1 ribosomal protein gene of *X. laevis* represents a well suited case to study because one of its intron (the third one) was shown to undergo splicing regulation (25–27) and to encode for a snoRNA (U16) produced in alternative to the splicing process (2). For this reason the modulation of splicing of this intron can influence both the amount of the L1 protein and the production of the snoRNA. In this paper we describe that a second snoRNA (U18) is encoded in other four introns of the same gene. U18 RNA has features similar to U16: it is localized in the nucleolus associated with fibrillarin and it is processed from the pre-mRNA by the same mechanism. In addition, both sequence and gene organization of this snoRNA are conserved in evolution: a sequence 75% homologous to the *X. laevis* U18 is found in the second intron of the human L1-like r-protein gene (L4, ref. 12).

The second intron is a more efficient splicing substrate with respect to the third one and allows a better analysis of the relationship between splicing and processing. In injected oocytes, a large proportion of the precursor is converted almost equally into products of splicing and processing. Densitometric analysis, normalized for the U-content of the molecules, indicated that lariat and spliced exons are accumulated in equimolar amounts, showing that lariat is not quantitatively converted into U18 RNA. In addition, experiments of microinjection of purified lariat into oocyte nuclei have proven that the lariat is maintained in circular form without any conversion into the snoRNA. These data suggest that in oocytes, also in the case of a good splicing substrate such as the second intron, only the pre-mRNA is substrate for snoRNA production. It cannot be excluded that in somatic cells, which have efficient debranching activity, the lariat could be converted to the snoRNA by an alternative pathway, based on trimming of its linearized form (3, 4).

Several evidences allow to conclude that, similarly to U16 RNA, also U18 RNA originates by processing of the pre-mRNA and not by independent transcription: i) U18 RNA is uncapped; ii) it has the same sequence of the intron conserved region (F-molecule) that becomes released from the pre-mRNA in experiments of RNA microinjection into oocyte nuclei have proven that the lariat is maintained in circular form without any conversion into the snoRNA. These data suggest that in oocytes, also in the case of a good splicing substrate such as the second intron, only the pre-mRNA is substrate for snoRNA production. It cannot be excluded that in somatic cells, which have efficient debranching activity, the lariat could be converted to the snoRNA by an alternative pathway, based on trimming of its linearized form (3, 4).

*X. laevis* contains 8 potential coding regions for U18 RNA per haploid genome: four from each of the two L1 gene copies (L1a and L1b, see ref. 9–11). The cloned intron sequences available comprise the four L1a copies and only one (the second intron) of the L1b gene. Among the cDNA clones analyzed, we found represented five different classes of U18 RNA: three corresponding to the known sequences of the second, fourth and eighth intron of the L1a copy, and two not represented among the available clones. The possibility exists that they originate from some of the L1b intron sequences not yet characterized. Additional cDNA and genomic clones should be analyzed in order to definitely prove that all the U18-containing introns are substrates for the production of the corresponding snoRNA. Up to now we cannot draw any conclusion on the stoichiometry of the U18 RNA synthesis from the different introns and with respect to U16 RNA. It will be very interesting to find out how the synthesis of these components is regulated and whether their relative abundance has any implication in their function.

In conclusion, the L1 r-protein gene appears to have a mosaic structure in which sequences encoding for a specific r-protein are intermingled with sequences coding for two different snoRNAs (see fig.6). The common feature of all these components is the localization in the nucleolus, where very likely they play some important role in ribosome biosynthesis. While the function of the r-proteins seems clear, the same is not true for the snoRNAs. Some of them have been shown to be involved in rRNA processing (28–31) but additional functions can be envisaged such as assembly and transport. For several snoRNAs association with RNA was described (23); a prerequisite for such interactions is given by the presence of sequences complementary to specific portions of the rRNA (30, 32). In the case of U18 RNA, a 13 nucleotide long region, shows perfect complementarity with the 28S rRNA and is 100% conserved from amphibian to human (see fig.1). The corresponding sequence on the 28S rRNA, is contained in an expansion segment which is particularly conserved in all the eukaryotic major rRNA species (33). Is then possible that U18 could interact via base pairing with the rRNA in order to performe its function.

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