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Processing of the intron-encoded U16 and U18 snoRNAs: the conserved C and D boxes control both the processing reaction and the stability of the mature snoRNA

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A novel class of small nucleolar RNAs (snoRNAs), encoded in introns of protein coding genes and originating from processing of their precursor molecules, has recently been described. The L1 ribosomal protein (r-protein) gene of *Xenopus laevis* and its human homologue contain two snoRNAs, U16 and U18. It has been shown that these snoRNAs are excised from their intron precursors by endonucleolytic cleavage and that their processing is alternative to splicing. Two sequences, internal to the snoRNA coding region, have been identified as indispensable for processing the conserved boxes C and D. Competition experiments have shown that these sequences interact with diffusible factors which can bind both the pre-mRNA and the mature U16 snoRNA. Fibrillarin, which is known to associate with complexes formed on C and D boxes of other snoRNAs, is found in association with mature U16 RNA, as well as with its precursor molecules. This fact suggests that the complex formed on the pre-mRNA remains bound to U16 throughout all the processing steps. We also show that the complex formed on the C and D boxes is necessary to stabilize mature snoRNA.

Keywords: fibrillarin/RNA processing/RNA splicing/snoRNA/*Xenopus* oocytes

Introduction

It has recently been discovered that a class of small nucleolar RNAs (snoRNA) has a novel type of gene organization and biosynthesis. Unlike the well-known U3, U8 and U13 snoRNAs, which are transcribed as independent units by polymerase II and possess 5′-trimethylguanosine caps, this new class of snoRNAs are encoded in introns of protein coding genes, have a 5′-monophosphate end and are released from their precursor RNAs by a processing event (Leverette *et al.*, 1992; Fragapane *et al.*, 1993; Kiss and Filipowicz, 1993; Prislei *et al.*, 1993; Tycowski *et al.*, 1993; Cecconi *et al.*, 1994; Nicoloso *et al.*, 1994; Pellizzoni *et al.*, 1994; Qu *et al.*, 1995). This discovery has opened up many new issues relating mainly to their function and biosynthesis. Since they have a nucleolar localization and display portions of sequence complementarity to specific rRNA sequences, they have been assumed to participate in rRNA maturation, similarly to what has been shown for U3, U8 and U14 snoRNAs in different organisms (Kass *et al.*, 1990; Li *et al.*, 1990; Savino and Gerbi, 1990; Hughes and Ares, 1991; Peculis and Steitz, 1993; Liang and Fournier, 1995). Indeed, in the case of U22, encoded in an intron of the UHG gene, a specific effect on accumulation of 18S rRNA was observed in experiments on snoRNA depletion in *Xenopus laevis* oocytes (Tycowski *et al.*, 1994). In consideration of the increasing number of snoRNAs (Maxwell and Fournier, 1995), it seems reasonable to assume that they are also involved in additional functions related to ribosome biogenesis, such as folding, rRNA/r-protein assembly and transport.

The second interesting aspect, specifically related to this peculiar gene organization, concerns the mechanism of their biosynthesis. In the case of U15 and U17 snoRNAs the *in vitro* systems utilized show that production of mature RNA is obtained by trimming of linear precursors (Kiss and Filipowicz, 1993; Tycowski *et al.*, 1993). More recently analysis in transfected cells has demonstrated that U17 snoRNA must be present in a spliceable intron for production of the mature snoRNA (Kiss and Filipowicz, 1995), suggesting that processing is the result of exonucleolytic trimming of the debranched lariat.

A different situation is found for frog and human U16 and U18 snoRNAs, both encoded in the L1 and its human homologue L4 ribosomal protein genes. Their processing has been studied in *X.laevis* oocytes (Fragapane *et al.*, 1993; Prislei *et al.*, 1993) and in germinal vesicle extracts (Caffarelli *et al.*, 1994; Santoro *et al.*, 1994). The *in vivo* and *in vitro* experiments have demonstrated that processing of snoRNAs from their precursor molecules is a two-step reaction. The first step consists of endonucleolytic cleavages upstream and downstream of the snoRNA sequence: one cleavage releases intermediate molecules, while two cleavages on the same precursor produce pre-snoRNA molecules. All these products have 5′ and 3′ trailer sequences. In the second step of the reaction the trailer sequences are removed by trimming and pre-snoRNA molecules are converted to the mature form (see schematic representation in Figure 1).

For U16- and U18-containing precursors it has been demonstrated that processing is alternative to splicing, which means that the L1 precursor RNA either undergoes splicing with the production of mature L1 mRNA or processing with the release of snoRNAs (Fragapane *et al.*, 1993; Prislei *et al.*, 1993; this paper). In this system the commitment to either pathway is of primary importance in determining the relative amount of the different final products. The indication that splicing of L1 pre-mRNA is critical for modulating expression of L1 protein has already been shown in a previous work on *X.laevis* oocytes and embryos (Bozzoni *et al.*, 1984; Pierandrei-Amaldi *et al.*, 1987, 1988).

Many snoRNAs share two conserved motifs, boxes C

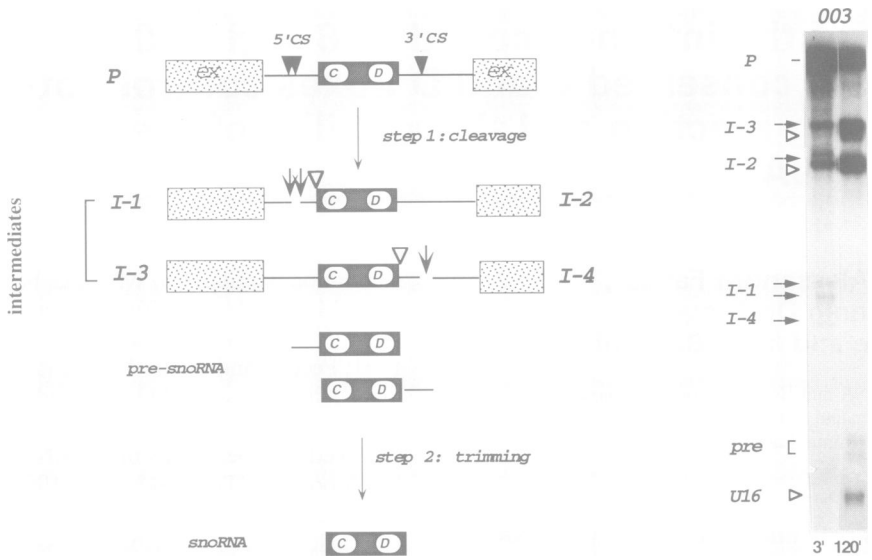

Fig. 1. Processing of the intron-encoded U16 snoRNA. ^{32}P-Labelled 003 RNA was injected into nuclei of *X.laevis* oocytes and incubated for 3 or 120 min. Nuclear RNA was recovered and analysed on a 10% acrylamide–urea gel. The different products are schematically represented at the side. The exons are shown as stippled boxes, the intron as a continuous line and the snoRNA coding region as a shadowed box. C and D represent the boxes conserved in many different snoRNAs. 5′cs and 3′cs indicate the upstream and downstream cleavage sites respectively. All the indicated intermediates have also been identified *in vitro* and for U18 snoRNA (Prislei *et al.*, 1993; Caffarelli *et al.*, 1994). The products of primary cleavage are indicated by arrows, the trimmed products by open triangles.

and D. They were originally described in U3, U8, U13 and U14 snoRNAs (Tyc and Steitz, 1989; Liu and Maxwell, 1990). *In vivo* and *in vitro* studies demonstrated that these conserved sequences are required for formation of specific complexes and for accumulation of stable snoRNAs (Baserga *et al.*, 1991; Huang *et al.*, 1992; Lubben *et al.*, 1993; Peculis and Steitz, 1994). Subsequently the same C and D motifs were found in many intron-encoded snoRNAs: U15 (Tycowski *et al.*, 1993), U16 (Fragapane *et al.*, 1993), U18 (Prislei *et al.*, 1993), U20 (Nicoloso *et al.*, 1994), U21 (Qu *et al.*, 1994), U22 (Tycowski *et al.*, 1994) and U24 (Qu *et al.*, 1995). The role played by the conserved boxes in the processing of intron-encoded snoRNAs has not been investigated until now. In the present paper we show that mutations in boxes C and D prevent processing of U16 and U18 snoRNAs, in addition to affecting the stability of the mature snoRNA. The same effect of processing inhibition can be obtained in competition experiments with wild-type substrates, indicating that the interaction of boxes C and D with specific *trans*-acting factors determines commitment to the processing pathway. Fibrillarin, which is a protein found in many snoRNP complexes, is found associated with mature U16 snoRNA, as well as with the precursor molecules, including the pre-mRNA. The presence of fibrillarin-containing snoRNP complexes early in the processing reaction suggests that the complex present on the mature snoRNA is the same as that assembled on the U16 coding region in the pre-mRNA and that the presence of this complex mediates commitment of the pre-mRNA to the processing pathway.

Results

Recognition of boxes C and D commits the U16- and U18-containing precursors to the processing pathway

A typical pattern of U16 snoRNA excision from the third intron of the L1 gene is shown in Figure 1, together with its schematic representation. At short incubation times (3 min), before significant trimming has begun, the primary cleavage products can be visualized (arrows). These products have very short half-lives *in vivo*, because the unprotected ends produced after cleavage are rapidly attacked by exonucleases. In particular, I-1 and I-4 are rapidly degraded, whereas I-2 and I-3 are trimmed up to the 5′- and 3′-ends respectively of U16 (open triangles), suggesting the presence of some protecting activity (Caffarelli *et al.*, 1994). A second cleavage produces pre-snoRNA from the I-2 and I-3 intermediate molecules, which is then trimmed to the mature form.

In order to understand whether boxes C and D participate in the biosynthesis of intron-encoded snoRNAs we tested the effect of base substitution mutations on the processing of U16 from its precursor molecule (003 RNA). *In vitro*-transcribed ^{32}P-labelled 003 RNA was injected into oocyte nuclei together with ^{32}P-labelled U6 RNA as an internal control (Terns and Dahlberg, 1994) and the products of the reactions analysed at different times. The co-injection of U6, which is maintained inside the nucleus, allows normalization of the different bands, which were quantitated by densitometric scanning. In this experiment U16 is hidden by co-migration with U6 RNA, which is present in excess. The U16 signal is, anyway, poor, even at 40 min incubation (not shown). Figure 2a demonstrates that two and three base substitutions in boxes C and D respectively (mutants bC and bD) of 003 RNA totally inhibit cleavage. In fact, no cleaved molecules are detected with the mutant substrates, even at short incubation times (3 min, lanes 1), at which point wild-type substrates produce primary cleavage products. In fact, at 3 min some I-2 and I-3 molecules are still present in the untrimmed form (arrows) and I-1 molecules are still visible; I-4 products, which are always less represented than I-1, are only detected in longer exposures (not shown). In addition, accumulation of uncleaved pre-mRNA at longer incubation times is

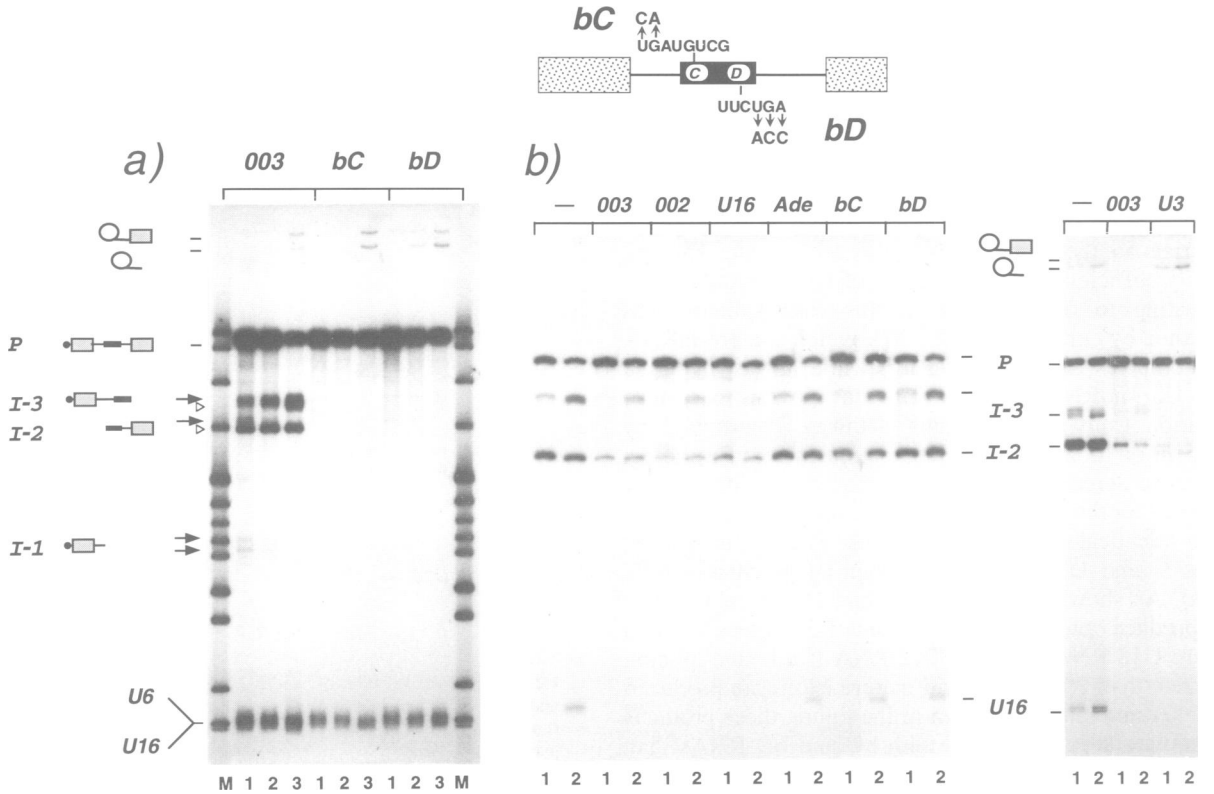

Fig. 2. Gel electrophoretic analysis of the injection products of 003 RNA and of its mutant derivatives bC and bD. The bC and bD mutations are indicated in the upper panel, where the consensus sequence of the two boxes is reported. (**a**) ^{32}P-Labelled 003, bC and bD RNAs were co-injected together with ^{32}P-labelled U6 RNA into oocytes and incubation was allowed to proceed for 3 (lanes 1), 20 (lanes 2) or 40 min (lanes 3). U6 co-migrates with mature U16 (112 and 106 nt respectively); due to the large excess of U6 counts the contribution by U16 is irrelevant. The products of primary cleavage are indicated by arrows, the trimmed products by open triangles. (**b**) *In vivo* competition experiment. 003 RNA ^{32}P-labelled at high specific activity (see Materials and methods) was injected alone (lanes –) or together with a 10-fold molar excess of different cold competitor RNAs: U16 snoRNA-containing precursor (lanes 003), U18 snoRNA-containing precursor (lanes 002), U16 snoRNA (lanes U16), adenovirus major late transcript (lanes Ade), mutant bC (lanes bC), mutant bD (lanes bD) and U3 RNA (lanes U3). The oocytes were incubated for 20 (lanes 1) or 60 min (lanes 2). Nuclear RNA was analysed on 10% acrylamide–urea gels. The processing and splicing products are indicated at the side; the dot represents the cap structure. The experiments in (b) were performed in two different batches of oocytes. The different migration of I-3 molecules in (b) has been frequently observed in 10% acrylamide gels. Lanes M, molecular weight markers (pBR322 plasmid DNA, *Msp*I digested).

observed in bC and bD mutants. These results allow exclusion of the possibility that the absence of truncated products is due to their poor stability and demonstrate instead that both mutations affect the cleavage step. The mutations produced are not expected to alter the overall structure of U16 RNA and, in particular, the short conserved 5′–3′ terminal stem, which is another conserved motif shared with many snoRNAs (Tycowski *et al.*, 1993). This experiment also shows that the splicing efficiency of the mutated substrates is improved, as witnessed by increased accumulation of the lariat forms. This result supports the notion that processing and splicing are alternatives and compete with each other. In this respect it is important to remember that 003 RNA is an inefficient splicing substrate, because of the presence of a suboptimal 5′ splice site (Caffarelli *et al.*, 1992; Fragapane *et al.*, 1992).

Of the other mutations tested in the U16 coding region only one abolished processing (Prislei *et al.*, 1992), affecting a stem structure present in the central region of U16 which is conserved in *Xenopus tropicalis* and human homologous sequences. Because mutations affecting the stability of the stem can be suppressed by compensatory changes in the opposite strand, we concluded that this region must have an important structural function (Prislei *et al.*, 1992). Study of this mutant has so far not been pursued further.

In order to confirm that the no-cleavage phenotype of mutants bC and bD is not due to structural alterations in the pre-mRNA, but rather to lack of interactions with specific *trans*-acting factors, we performed *in vivo* competition experiments. ^{32}P-Labelled 003 RNA was co-injected together with a 10-fold molar excess of different cold competitor RNAs. Figure 2b shows that cleavage is inhibited by homologous competition (003 lanes) and by the U18 snoRNA precursor (002 lanes), which contains the same C and D boxes. The same competition effect is obtained with mature U16 RNA (U16 lanes). Conversely, adenovirus major late precursor (Ade lanes) does not affect cleavage. Interestingly, mutants bC and bD do not affect cleavage either (bC and bD lanes). A different RNA containing boxes C and D, such as U3, was also used in a different co-injection experiment; lanes U3 show that this RNA acts as a very strong competitor preventing cleavage of the control 003 RNA. Inhibition of cleavage is revealed by the increase in precursor accumulation paralleled by a decrease in intermediates and U16 RNA. Densitometric analysis of the pre-mRNA signal at 60 min

with respect to time 0 (not included in the figure) has shown that wild-type 003 converts 64% of the pre-mRNA into truncated products; inactive competitors allow conversion of 50–70% (Ade, bC and bD) of the pre-mRNA, whereas active competitors reduce this value to 30–40% (002, 003 and U16). In the experiments shown two different batches of oocytes were utilized: the first had very poor splicing activity, as shown by the absence of lariats in the control lanes; the second had splicing activity sufficient for visualization of lariat products. It is interesting to observe that in this case splicing was abolished by competition with 003, which is a pre-mRNA and competes for splicing factors,while it was increased with U3 snoRNA as the competitor. In this case also deviation from the processing pathway, determined by competition for C and D binding factors, increases splicing. This is in agreement with the increased splicing activity observed for the bC and bD mutants observed in Figure 2a.

An identical mutational analysis was performed on boxes C and D of the U18-containing precursors (002 RNA). As shown in Figure 3, C and D box mutants do not produce intermediates (I-2 and I-3 molecules) and mature U18 RNA. Because 002 RNA is a better splicing substrate than 003, lariats and mature exons are produced in higher amounts. As shown in the figure, these products accumulate at higher levels with bC and bD RNAs than with wild-type 002 RNA. Densitometric analysis has indicated that the increased accumulation of spliced products in mutants bC and bD accounts for the decrease in their pre-mRNA levels. In each experiment the amount of RNA in the different lanes is comparable, because the RNA loaded was extracted from single nuclei injected with the same number of counts. Incubation times were prolonged to 6 h because U18 is released less efficiently than U16; at short incubation times, which allow visualization of primary cleavage products in U16 precursors, almost no cleavage is visible with these substrates (not shown).

These results indicate that boxes C and D must interact with specific diffusible factors in order to enable the U16- and U18-containing pre-mRNAs to undergo cleavage. In addition, they show that these factors are the same as those interacting with boxes C and D of U3 snoRNA. The fact that mature U16 snoRNA is able to compete for processing indicates that the factors interacting with the boxes on the pre-mRNA are the same as those present on mature snoRNA.

Box C and D mutants can be cleaved in vitro

The sites utilized *in vivo* and *in vitro* for cleavage correspond to short U stretches, localized some 10–20 nucleotides (nt) upstream and downstream of the mature ends of U16. In order to test whether recognition of the C and D boxes is essential for commitment of the pre-mRNA to the processing pathway or for the cleavage reaction itself we compared the *in vivo* activity of 003-bC and 003-bD RNAs with *in vitro* activity. We previously described the setting up of an *in vitro* system competent for processing U16 snoRNAs from their precursor molecules (Caffarelli *et al.*, 1994). This extract, from *X.laevis* nuclei (ONE), is able to direct efficient cleavage at the same sites as those utilized *in vivo*. At extract concentrations allowing only the first step of the reaction (cleavage

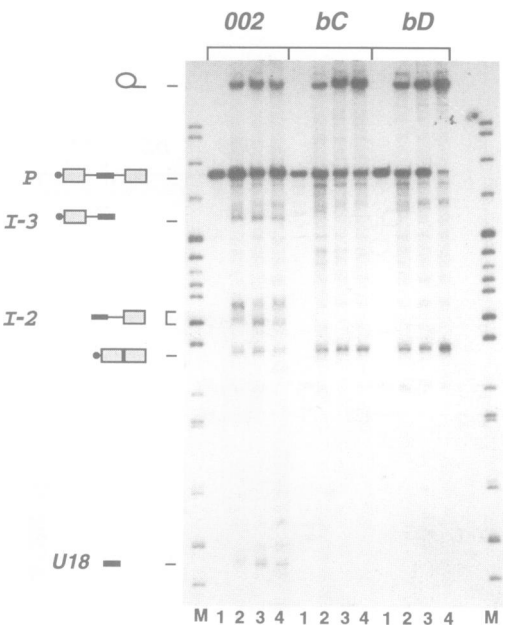

Fig. 3. ^{32}P-Labelled 002, bC and bD RNAs were injected into oocytes and incubation was allowed to proceed for 0 (lanes 1), 1 (lanes 2), 3 (lanes 3) or 6 h (lanes 4). The products were analysed on a 10% acrylamide–urea gel. The different products of splicing and processing are schematically represented at the side; the dot represents the cap structure. Lanes M, molecular weight markers (pBR322 plasmid DNA, *Msp*I digested).

without trimming; see Figure 1) primary cleavage products accumulate. For the second step (removal of the 5′ and 3′ trailer sequences and conversion of the pre-snoRNA into mature and stable U16) trimming activity must be provided either by increasing the concentration of ONE or by adding HeLa nuclear extract at the concentration normally utilized for splicing. This two-step reaction was previously shown for the wild-type U16-containing precursor and is reported here as a control in Figure 4 (lanes 003). When 003-bC and 003-bD RNAs are tested *in vitro* under conditions that allow cleavage, truncated products originating from upstream and downstream cleavage are produced (Figure 4, lanes ONE), as in the case of the wild-type precursor. These results indicate that cleavage of bC and bD can occur. They also show that the oocyte nuclear extract is unable to reproduce the regulated processing observed *in vivo*. The fact that mutant subtrates can be cleaved *in vitro* at the correct sites indicates that the *in vivo* pre-mRNA commitment to processing is not a default process dependent solely on the presence of the cleavage sites, but is a regulated event based on specific interactions occurring at the level of boxes C and D.

If the second step of the reaction is allowed to proceed (trimming activity is provided by adding HeLa nuclear extract after 20 min pre-incubation with ONE) no accumulation of intermediates and mature U16 RNA is observed in the bD and bC mutants (Figure 4, lanes ONE+HeLa). These data suggest that boxes C and D must also play a role in stabilization of the snoRNA and of the intermediates from which it originates.

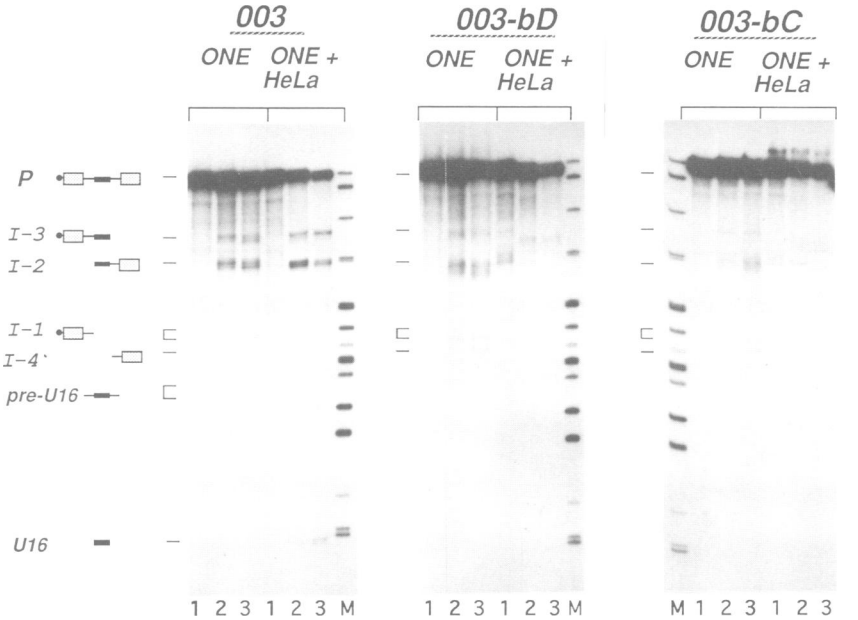

Fig. 4. *In vitro* processing analysis of 003 RNA and mutant derivatives 003-bD and 003-bC. ^{32}P-Labelled RNAs were incubated in 10 μg ONE (lanes ONE) or 10 μg ONE followed by the addition, after 20 min, of 300 μg HeLa cell nuclear extract (lanes ONE + HeLa). The reactions were allowed to proceed for 0 (lanes 1), 60 (lanes 2) or 120 min (lanes 3). In the double extract incubations 0 min corresponds to addition of the second extract. The products of the reactions were analysed on 10% acrylamide–urea gels. The different products are represented at the side; the dot represents the cap structure. Lanes M, molecular weight markers (pBR 322 plasmid DNA, *Msp*I digested).

Role of boxes C and D in the stabilization of U16 snoRNA

In order to analyse the role played by boxes C and D in stabilization of the snoRNA we set up a stability assay *in vivo*: U16 snoRNA or mutant derivatives (U16-bC and U16-bD; Figure 5, upper panel) were microinjected into nuclei of *X.laevis* oocytes and their maturation and stability analysed at different times. A 4 nt 3′ trailer sequence was added in order to verify the occurrence of trimming followed by stabilization of the mature form. The base substitutions utilized in these mutants are the same as those indicated in Figure 2. Figure 5a shows that 40% of the input U16 RNA undergoes trimming of the 3′ trailer sequence and is stably accumulated as the mature form (106 nt) after 4 h incubation. Conversely, almost no accumulation of mutated substrates is visualized at corresponding incubation times (lanes U16-bC and U16-bD). The stability assay was performed *in vitro* as well. The same constructs, except for a longer 3′ trailer sequence (19 nt), were incubated for 20 min in ONE and then incubated for 1 or 2 h more in the presence of HeLa extract. At the extract concentrations used ONE provides the U16-specific binding factors, while HeLa cells essentially provide trimming activity. Wild-type U16 precursor (Figure 5b, lanes U16) is converted after 2 h incubation into the mature form (band 106) and into a product with a 2 nt 3′ extension (band 108). Conversely, U16 RNAs with box C (lanes U16-bC) or box D (lanes U16-bD) mutations do not accumulate any stable mature forms. In order to prove that stability is determined by specific RNA–protein interactions on the C and D boxes and not solely by the structure of the substrate we performed the same experiment in the presence of excess amounts of different competitor RNAs. Figure 5c shows that only the substrate with intact boxes C and D is able to compete for stabilization of U16 RNA (lanes U16). In fact, competition with both the bC (lanes U16-bC) and bD (lanes U16-bD) mutants does not affect accumulation of stable U16 RNA. The same results were obtained if HeLa cell extracts were utilized as the source of binding activity, suggesting that C and D binding factors must be conserved in evolution (not shown).

Both *in vivo* and *in vitro* experiments demonstrate that boxes C and D affect stability of the mature U16 snoRNA and that the effect is mediated by diffusible factors. A comparison between the percentage protection of input RNA obtained *in vivo* (40%) and *in vitro* (5%) demonstrates that the oocyte nuclear extract is poor in C and D binding factors. This could explain why this extract is unable to regulate cleavage as well as the *in vivo* system.

Fibrillarin is associated early in the processing reaction

We previously showed that U16 snoRNA is associated *in vivo* with complexes containing fibrillarin (Fragapane *et al.*, 1993). Even though fibrillarin does not directly interact with RNA (Lubben *et al.*, 1993), it represents the only marker available for analysing when snoRNP particles are formed. Fibrillarin immunoprecipitations were performed in order to identify at which step during U16 snoRNA processing these particles are assembled. In order to be able to analyse the possible interaction of fibrillarin with splicing products a mutant derivative of 003 RNA (F4) that splices efficiently, thanks to a mutation in the 5′ splice site (AC′GUAUG→AG′GUAAG), was utilized as substrate. Figure 6a shows the time course of *in vitro* processing. It appears that at short incubation times only the pre-mRNA is reactive (lane 4), while at longer incubation times the intermediates and mature U16 snoRNA are precipitated (lane 6). A similar timing of

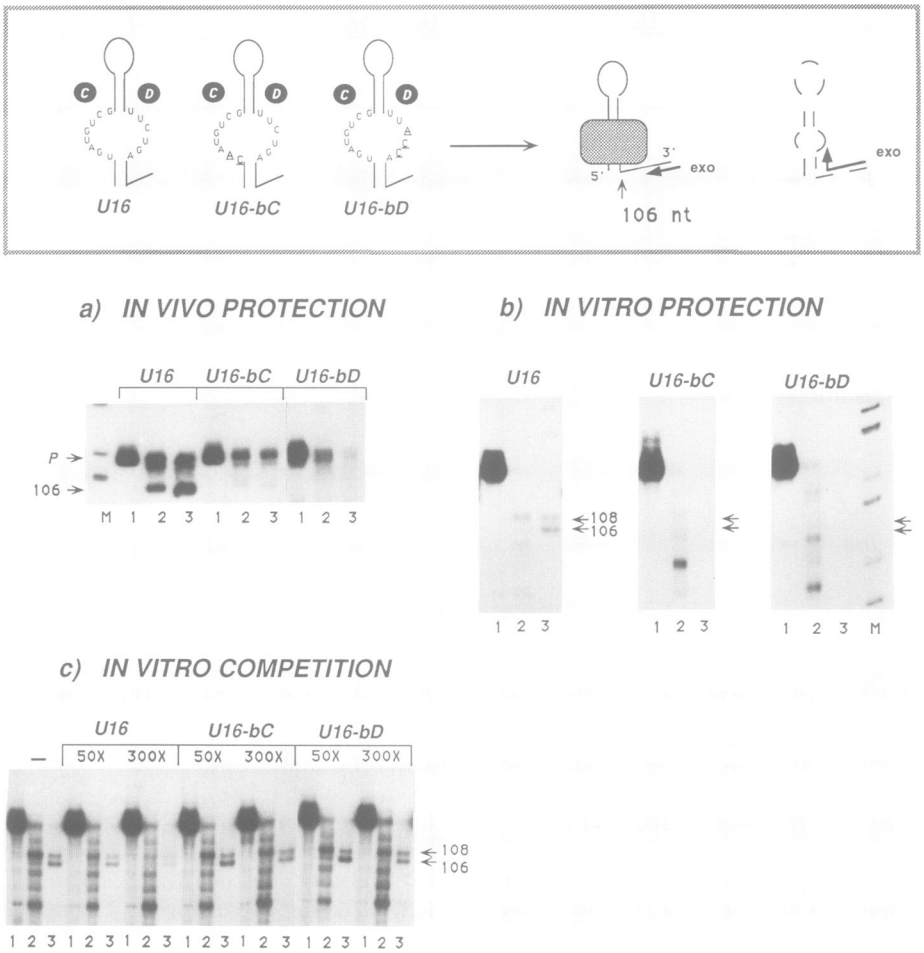

Fig. 5. Assay of *in vivo* and *in vitro* stability of wild-type and mutant U16 snoRNAs. The upper panel shows a schematic representation of the constructs and the rationale of the assay. The substrate corresponds to the mature U16 snoRNA with a 3′ trailer sequence; the 106 nt species represents the mature form obtained after trimming. (**a**) *In vivo* protection experiment. ^{32}P-Labelled U16 snoRNA with a 4 nt 3′ extension (lanes U16) or its bC and bD derivatives (lanes U16-bC and U16-bD) were injected into nuclei of *X.laevis* oocytes and incubated for 0 (lanes 1), 90 (lanes 2) or 240 min (lanes 3). Nuclear RNA was extracted and analysed on a 6% acrylamide–urea gel. The arrows indicate the injected RNA (P) and the mature U16 snoRNA (106). (**b**) *In vitro* protection experiment. ^{32}P-Labelled U16 snoRNA with a 19 nt 3′ extension (lanes U16) or its bC and bD derivatives (lanes U16-bC and U16-bD) were pre-incubated in ONE for 20 min, HeLa cell nuclear extract was then added and the reaction was allowed to proceed for 0 (lanes 1), 60 (lanes 2) or 120 min (lanes 3). RNA was analysed on 6% acrylamide–urea gels. The arrows indicate mature U16 snoRNA (106) and a 2 nt longer species (108). (**c**) *In vitro* competition experiment. ^{32}P-Labelled U16 snoRNA with a 19 nt 3′ extension (lanes U16) was pre-incubated in ONE alone (lane –) or in the presence of a 50- or 300-fold molar excess of cold competitors as indicated above. After 20 min HeLa cell nuclear extract was added and the reaction was allowed to proceed for 0 (lanes 1), 60 (lanes 2) or 120 min (lanes 3). The arrows indicate mature U16 (106) and a 2 nt longer species (108). All the transcripts utilized in these experiments are uncapped. Lane M, molecular weight markers (pBR 322 plasmid DNA, *Msp*I digested).

fibrillarin interaction is found *in vivo* (Figure 6b, lanes 4–6). These data demonstrate that fibrillarin-containing complexes initially assemble on pre-mRNA and are subsequently chased into mature products. Figure 6b also shows that fibrillarin associates very poorly with the lariat at short incubation times (lane 4), while increased reactivity is observed on prolonged incubation and exclusively with the mature form (lanes 5 and 6). The absence of fibrillarin interaction with the lariat intermediate, which is instead efficiently precipitated by Sm antisera (upper panel), shows that fibrillarin cannot co-exist with snRNPs during the splicing reaction. The poor precipitation of the mature lariat with Sm antibodies (lanes 4–6 of the upper panel) suggests that in the oocyte the lariat, accumulated after splicing, rapidly becomes devoid of snRNPs; very likely fibrillarin associates with the lariat only after splicing factors have dissociated. In order to analyse whether the interference between fibrillarin-containing complexes and splicing factors could be due to the small size of the intron a precursor RNA (007) containing a 521 nt intron was utilized. This pre-mRNA encodes for U18 snoRNA, which is also released by processing (Prislei *et al.*, 1993). Figure 6c shows that the timing of fibrillarin interaction is the same as in F4 RNA. Moreover, fibrillarin-containing complexes are found associated with the lariat only on prolonged incubation (lane 6), whereas Sm antigens are detected on the lariat only after short incubation times (upper panel, lane 4; the size of this intron does not allow a distinction between intermediate and mature lariats). This indicates that association of fibrillarin-containing complexes with the lariat is an alternative to that of splicing factors. In oocytes the lariat does not undergo debranching and no production of intron-encoded snoRNA can be obtained by this pathway (Caffarelli *et al.*, 1993;

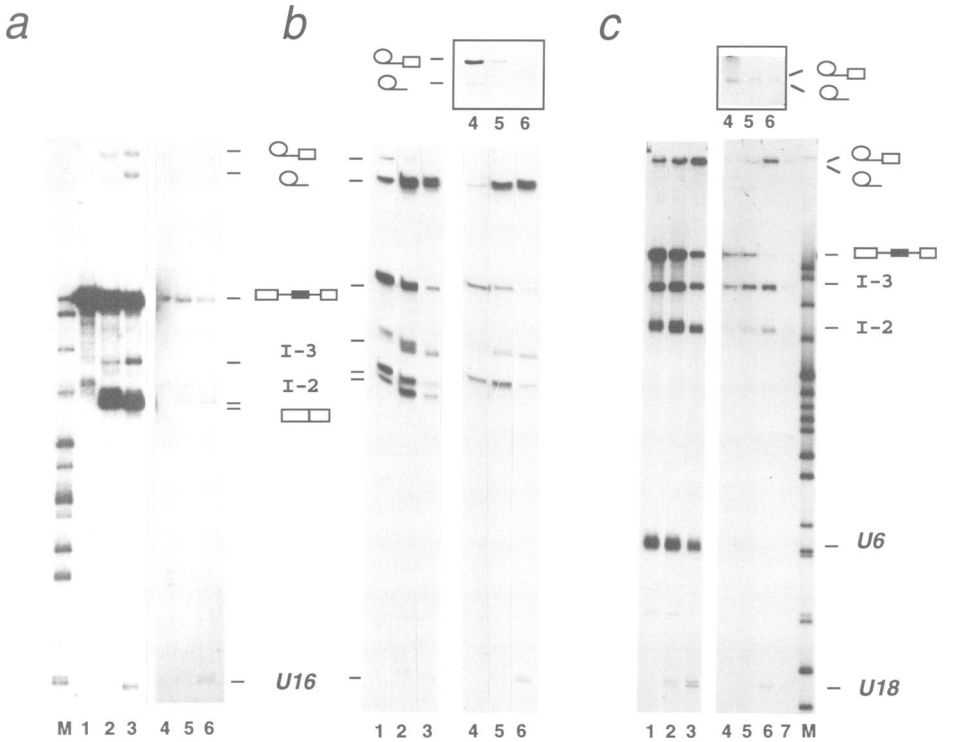

Fig. 6. Analysis of fibrillarin-containing complexes. (**a**) ^{32}P-Labelled F4 RNA was incubated with oocyte nuclear extract for 20 min (lanes 1 and 4), HeLa cell nuclear extract was then added and incubation was allowed to proceed for an additional 60 (lanes 2 and 5) or 120 min (lanes 3 and 6). Half of each sample was directly extracted (lanes 1–3), while the remaining half was immunoprecipitated with fibrillarin antibodies (lanes 4–6). (**b**) ^{32}P-Labelled F4 RNA was injected into germinal vesicles of *X.laevis* oocytes and incubation was allowed to proceed for 20 (lanes 1 and 4), 60 (lanes 2 and 5) or 120 min (lanes 3 and 6), after which nuclei were manually isolated. One third of each sample was directly extracted (lanes 1–3), one third was immunoprecipitated with fibrillarin antibodies (lanes 4–6) and one third was immunoprecipitated with Sm antibodies (lanes 4–6, upper panel). (**c**) As (b) except that ^{32}P-labelled 007 RNA was injected together with control ^{32}P-labelled U6 snRNA. Anti-Sm immunoprecipitation of the lariat at the corresponding times is presented in the upper panel. On this type of gel and for the size of this intron it is impossible to resolve the intermediate lariat from the mature form. Specificity of fibrillarin immunoprecipitation is demonstrated by the lack of reaction with U6 snRNA and by immunoprecipitation with pre-immune antisera, which is shown in lane 7. The different products are indicated at the side. All the RNA samples were run on 10% acrylamide–urea gels. Lane M, molecular weight markers (pBR322 plasmid DNA, *Msp*I digested).

Prislei *et al.*, 1993). Endonucleolytic cleavage cannot be responsible for snoRNA release from the lariat, as demonstrated by the fact that purified lariat reinjected into oocytes stably accumulates without any conversion (Prislei *et al.*, 1993). In addition, wild-type substrate (003 RNA; see Figures 1 and 2) produces only minimal amounts of lariat, which cannot account for the observed accumulation of U16 snoRNA. In conclusion, in oocytes splicing-independent processing is the only way of producing U16 snoRNA.

In these experiments antibody specificity is shown by the absence of interaction with spliced exons in F4 and with control U6 snRNA in 007 injections. In addition, pre-immune antisera do not display any specific reactivity (Figure 6c, lane 7)

Two snoRNAs can be independently released from the same intron

Further evidence that endonucleolytic activity is responsible for U16 and U18 snoRNA processing is presented in Figure 7, where a pre-mRNA containing both snoRNAs in the same intron (U16/U18) was tested by oocyte microinjection. If exonucleolytic processing was responsible for production of mature snoRNA no monomers should be found, as already described in the case of U17 snoRNA (Kiss and Filipowicz, 1995). The experiment instead shows that mature U16 and U18 snoRNAs accumulate independently (lanes 2–4), as in the control injection of 00234 RNA (lane 6), which covers the genomic region containing the second (U18) and third (U16) introns of the L1 gene. The intermediate products that accumulate, although not characterized in detail, correspond to I-2- and I-3-like molecules (see schematic representation to the side).

Discussion

The *X.laevis* and *X.tropicalis* L1 ribosomal protein genes and the corresponding human L4 gene have a composite structure in which protein coding exons are intermingled with introns encoding two different snoRNAs. U16 snoRNA is encoded in intron 3 (Fragapane *et al.*, 1993), whereas U18 snoRNA is encoded by introns 2, 4, 7 and 8 (Prislei *et al.*, 1993).

A combination of *in vivo* and *in vitro* studies has allowed a detailed analysis of the processing reaction responsible for biogenesis of such RNAs (Fragapane *et al.*, 1993; Prislei *et al.*, 1993; Caffarelli *et al.*, 1994; Santoro *et al.*, 1994). U16 and U18 snoRNAs are released from their precursor molecules in a two-step process: endonucleolytic cleavages upstream and downstream of the snoRNA coding regions release a precursor snoRNA with

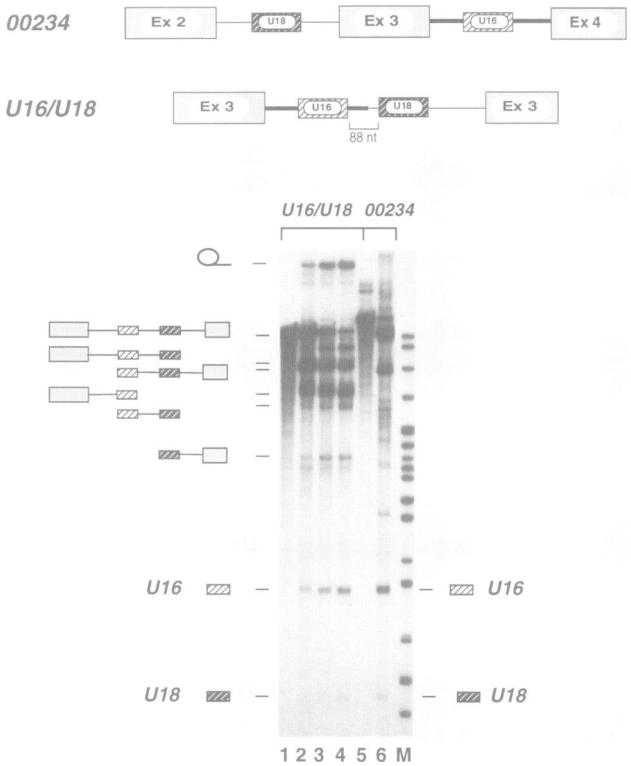

Fig. 7. Excision of U16 and U18 from the same intron. A schematic representation of the constructs utilized (00234 and U16/U18; exons represented by boxes, the third intron by a thick line and the second intron by a thin line) is shown above. ^{32}P-Labelled U16/U18 RNA (lane 1) and 00234 RNA (lane 5) were injected into germinal vesicles of *X.laevis* oocytes and incubations were allowed to proceed for 1 (lane 2), 3 (lane 3) or 6 h (lanes 4 and 6), after which nuclei were manually isolated. RNA was run on a 10% acrylamide–urea gel. The different products are indicated on the side. Lane M, molecular weight markers (pBR322 plasmid DNA, *Msp*I digested).

5′ and 3′ trailer sequences; subsequently these tails are removed by trimming and mature molecules are produced. The cleavage sites for U16 snoRNA processing map at the level of U stretches and coincide exactly in the *in vivo* and *in vitro* systems (Caffarelli *et al.*, 1994). The cleavage activity has been characterized as a uridine-specific endonucleolytic activity (manuscript in preparation). Because several U stretches can be found along the precursor molecule, specific RNP complexes must be formed in order to control the appropriate choice of cleavage regions. Several experiments show that splicing and processing of U16 and U18 pre-mRNAs are alternatives: (i) reinjection of purified lariats into oocytes or incubation in extracts does not give rise to snoRNA (Prislei *et al.*, 1993; unpublished data); (ii) 5′ splice site mutants (Santoro *et al.*, 1994) and 3′ splice site deletions (unpublished results) do not affect U16 snoRNA processing; (iii) if processing is inhibited, splicing is increased (this paper). For these reasons specific regulation must control the amount of pre-mRNA committed to the splicing or processing pathways. Therefore, the study of interactions of the L1 pre-mRNA with protein factors is of primary importance in understanding how the production of the different components (L1 mRNA, U16 and U18 snoRNAs) is controlled.

Mutagenesis throughout the U16-containing precursor allowed the study of the elements involved in control of the processing reaction. Two specific mutants were found to be very interesting, because they map inside the snoRNA coding region and because they were found to completely abolish cleavage *in vivo*. These mutants are cleaved *in vitro*, showing that the cleavage reaction *per se* is not affected. Therefore, the two regions must play some important role in regulating the level of processing *in vivo*. Specific interactions must be lost in the *in vitro* extract which are important for modulating cleavage *in vivo*. It is well known that oocyte nuclear extracts do not always reproduce the *in vivo* stoichiometry of factors, as for instance is shown by their poor splicing activity.

The above mutants map at the level of boxes C and D, which are conserved in the majority of snoRNAs characterized so far (Maxwell and Fournier, 1995). We have analysed two and three base substitution mutants in the best conserved nucleotides of boxes C and D respectively (Tyc and Steitz, 1989; Fragapane *et al.*, 1993). These substitutions were designed so as not to alter the overall structure of the snoRNA and in particular so as not to affect the conserved terminal stem (Tycowski *et al.*, 1993). The demonstration that boxes C and D play a role by binding to specifc factors was given by *in vivo* competition experiments. Only competitor substrates containing functional C and D boxes inhibit cleavage of a wild-type precursor; among these the U18 snoRNA-containing precursor, the mature U16 snoRNA and U3 snoRNA are able to compete. These data show that the same C and D binding factors are present on mature U16, on its precursor molecule, on U18 and on U3 snoRNAs. The interaction of boxes C and D with these factors is then necessary to commit the pre-mRNA to the cleavage pathway and subsequent snoRNA release. The competition with U3 snoRNA opens the interesting question as to the structural correlation between these snoRNAs encoded in introns and originating by processing and the previously well-characterized snoRNAs that are independently transcribed. The biochemical characterization of these snoRNPs will help in elucidating these correlations. Mutations in boxes C and D of the U18-containing precursor were also tested by oocyte microinjection; the results very clearly indicate that no cleavage of the pre-mRNA and no accumulation of U18 snoRNA occur in this case. It is interesting to note that when processing of both U16 and U18 snoRNAs is inhibited, either by mutation or by competition, splicing is increased. This is in line with other experiments showing that processing is an alternative to splicing.

The conserved C and D boxes have been shown to control the stability of yeast U14 snoRNA (Huang *et al.*, 1992) and of mammalian U8 snoRNA (Peculis and Steitz, 1994). In line with these results, we set up *in vivo* and *in vitro* experiments in order to analyse the effect of mutants bC and bD on the stability of mature U16 snoRNA. Synthetic U16 snoRNA containing 3′ trailer sequences can be converted by trimming to the mature form both in extracts and *in vivo* and their stability can be analysed. Both approaches demonstrate that the wild-type substrate can be trimmed to the mature form and stably accumulates. In contrast, bC and bD mutant pre-snoRNAs are degraded. In this case it can also be shown that the mutations affect the binding of specific factors, in that the stability of wild-type U16 snoRNA is abolished

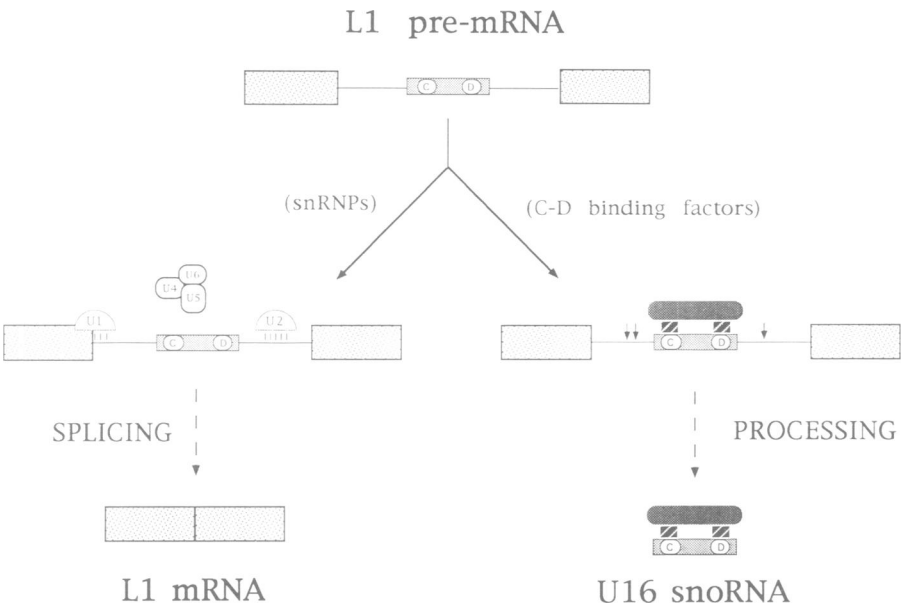

Fig. 8. Model of the alternative choice between splicing and processing.

if co-incubated with a molar excess of homologous competitor sequences, while is unaffected in the presence of bC and bD competitor substrates.

Little is known about the protein factors present in snoRNPs. A detailed analysis of these proteins has been performed only for U3 snoRNPs in CHO cells and for snR30 particles in yeast (Lubben *et al.*, 1993, 1995). Fibrillarin is the only common factor that has been characterized until now; it is weakly bound and very likely it associates by protein–protein interaction (Lubben *et al.*, 1993, 1995). Immunoprecipitations with fibrillarin antibodies were performed in order to determine at which step of the processing reaction assembly of snoRNPs occurs. This analysis has shown that fibrillarin-containing complexes are found on pre-mRNA molecules, on processing intermediates and on mature U16 snoRNA. This indicates that snoRNPs form on the precursor and remain associated throughout all the processing steps. Fibrillarin is also found associated with the mature lariat, nevertheless, data indicate that this interaction is not correlated with the splicing reaction: (i) fibrillarin is never found on the lariat intermediate, which, instead, is associated with snRNPs; (ii) fibrillarin interaction with the mature lariat shows kinetics opposite to those of snRNPs. These data suggest that fibrillarin-containing particles cannot co-exist with splicing factors and become associated with the lariat when this is devoid of snRNPs. Similar results have been obtained with a pre-mRNA containing a longer intron (007 RNA), suggesting that the mutual exclusion of fibrillarin and splicing factors is not simply due to the small size of the intron. In spite of the presence of fibrillarin-containing snoRNPs, the lariat does not appear to be a substrate for U16 snoRNA release: oocytes show poor debranching activity and lariats stably accumulate even after reinjection (Prislei *et al.*, 1993). Endonucleolytic cleavage of pre-mRNA is the process required for U16 and U18 snoRNA release in oocytes, as further demonstrated by their independent accumulation as mature molecules when they are present together in the same intron. It cannot be excluded that in somatic cells, where debranching is active, a second default pathway could be operating; experiments in embryos are in progress in order to answer this question.

Altogether these results suggest that commitment of pre-mRNA to the processing pathway is determined by the assembly of specific snoRNP complexes on the pre-mRNA; only molecules devoid of complexes on boxes C and D would be able to assemble into functional spliceosomes. These considerations could explain the molecular basis of why, in this system, processing and splicing are alternatives. This alternative choice would be favoured in those cases where splicing is inefficient, such as the U16-containing intron. A schematic representation of the proposed model is shown in Figure 8. This model has important regulatory implications. U16 synthesis would not occur constitutively at each splicing event, as suggested for other intron-encoded snoRNAs, such as U17, which depend uniquely on splicing (Kiss and Filipowicz, 1995). Instead, it would depend on the formation of U16-specific snoRNPs on the pre-mRNA. This matches very well with our previous data on the tight control of L1 gene expression exerted at the level of splicing (Caffarelli *et al.*, 1987; Pierandrei-Amaldi *et al.*, 1987, 1988; Fragapane *et al.*, 1992): the availability and assembly of U16-specific snoRNPs would become the key element for regulated expression of L1 ribosomal protein and of the snoRNAs encoded in its introns.

Materials and methods

RNA transcripts

The templates used for 003, F4 and Ade RNA transcription have been described previously (Caffarelli *et al.*, 1987; Fragapane *et al.*, 1992). Mutants 003-bC and 003-bD were constructed by inverse PCR (Inouye and Inouye, 1987) on plasmid 003. Mutants 002-bC and 002-bD were constructed by inverse PCR on plasmid 00234 (Caffarelli *et al.*, 1987). The bC mutants contain a UG→CA substitution in box C, while bD mutants contain a UGA→ACC substitution in box D (see Figure 2). The template for U16, with the 19 nt 3′ extension (125 nt long, used for *in vitro* experiments) was constructed by PCR using oligonucleotides B5 (5′-TAATACGACTCACTATAGGGCTTGCTATGATGTCGTAA-3′) and G1a (TTCTATACTTATCCAAGTATT). The underlined sequence

represents the T7 promoter sequence (Milligan *et al.*, 1987). The bC derivative was constructed with oligonucleotides B7 (TAATACGACTCACTATAGGGCTTGCTA**CA**ATGTCGTAA) and G1a and the bD derivative with oligonucleotides B5 and G1a on mutant 003bD. The template for U16, with the 4 nt 3′ extension (110 nt long, used for *in vivo* experiments), was constructed using oligonucleotides B5 and γwt (AGTATTTTTTGCTCAGAA). Its bC derivative was constructed by PCR with oligonucleotides B7 and gwt and the bD derivative with oligonucleotides B5 and γd (AGTATTTTTTGCT**GGT**AACGCGATAT). The mutated nucleotides are indicated by bold type. The template used for 007 RNA transcription was constructed by PCR on a genomic clone of rpL1 (Bozzoni *et al.*, 1984) using oligonucleotides 7a (CCGAATTCAGCCAAGCTGAA) and 9b (CCAAGCTTGAGCTCCCTTC). The 599 nt DNA molecule was cloned between the *Eco*RI and *Eco*RV sites of the Bluescript plasmid. The *Hin*dIII-linearized transcript contains 67 nt of upstream T7 polylinker fused to 26 nt of exon 7, 521 nt of intron 7 and 52 nt of the downstream exon. The template containing U16 and U18 in the same intron (U16/U18) was obtained by cloning a 223 nt region, extending from 23 nt upstream of U18 to nt 50 of exon 3, 65 nt downstream of U16 in the 003 plasmid. The *Xho*I-linearized transcript contains 53 nt of upstream T7 polylinker fused to 89 nt of upstream exon, 384 nt of the chimeric intron and 50 nt of the downstream exon. The control plasmid 00234 extends from the second to the fourth exon; linearized with *Hha*I this gives a 757 nt transcript. The template used for T7 transcription of U6 snRNA was obtained by PCR amplification of the *X.laevis* U6 gene (Reddy and Bush, 1988), kindly provided by E.Lund. U3 snoRNA was obtained by SP6 transcription of a PCR template kindly provided by M.P.Terns (Terns and Dahlberg, 1994).

In vitro transcription

RNA was synthesized *in vitro* (Melton *et al.*, 1984) in the presence of 10 μCi [α-^{32}P]UTP (800 Ci/mmol) and 60 μM UTP or, when necessary at higher specific activity, with 40 μCi [α-^{32}P]UTP and 12.5 μM UTP. CAP nucleotide (0.165 mM; G5′ppp5′G; Boehringer) was also included in the reaction except for the experiment shown in Figure 2, where the RNA was hypercapped in the presence of 1 mM CAP nucleotide and 500 μM GTP. After transcription the RNA was gel purified, phenol extracted and precipitated with ethanol.

Oocyte microinjections

In vitro-transcribed RNA was dissolved in bidistilled water at a concentration of 0.8–1.9 pmol/μl RNA and 20 nl were injected into germinal vesicles of stage VI oocytes as already described (Caffarelli *et al.*, 1987). Oocytes were incubated at 19°C for the times indicated and total RNA was extracted from manually isolated nuclei (Birckenmeir *et al.*, 1978) and analysed on 6 or 10% acrylamide–7 M urea gels.

In vitro protection and competition experiments

In vitro-transcribed wild-type or mutated U16 RNAs were pre-incubated at 24°C in ONE (10 μg) in the presence of the reaction mixture described by Caffarelli *et al.* (1994). After 20 min 300 μg HeLa cell nuclear extract were added and the reaction was allowed to proceed at 30°C for the indicated times. Reactions were then stopped and processed as described by Caffarelli *et al.* (1992). In the competition experiment wild-type U16 was pre-incubated in ONE under the same experimental conditions as described above except for the presence of a 50× or 300× molar excess of cold competitors. After 20 min 300 μg HeLa cell extract were added and the reaction was allowed to proceed at 30°C for the indicated times. Reactions were then stopped as previously described (Caffarelli *et al.*, 1992).

Immunoprecipitation

Immunoprecipitations with antibodies against fibrillarin (IgG 72139) were performed in NET-2 (40 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40; Baserga *et al.*, 1991). Washes were carried out in the same buffer. Immunoprecipitations with Sm antibodies were performed in IPP-500 (40 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Nonidet P-40; Hamm *et al.*, 1987). Washes were in the same buffer. The RNA recovered from pellets was analysed on 10% denaturing acrylamide gels.

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