Purification, cloning and characterization of XendoU, a novel endoribonuclease involved in processing of intron-encoded small nucleolar RNAs in Xenopus laevis

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Running title: XendoU, a novel RNase involved in snoRNA biosynthesis

Summary

Here we report the purification, from Xenopus laevis oocyte nuclear extracts, of a new endoribonuclease, XendoU, that is involved in the processing of the intron-encoded box C/D
U16 small nucleolar RNA (snoRNA) from its host pre-mRNA. Such an activity has never been reported before and has several uncommon features that make it quite a novel enzyme: it is poly-U specific, it requires Mn$^{2+}$ ions and it produces molecules with 2’-3’ cyclic phosphate termini. Even if XendoU cleaves U-stretches, it displays some preferential cleavage on snoRNA precursor molecules.

XendoU also participates in the biosynthesis of another intron-encoded snoRNA, U86, that is contained in the NOP56 gene of *X.laevis*. A common feature of these snoRNAs is that their production is alternative to that of the mRNA, suggesting an important regulatory role for all the factors involved in the processing reaction.

**Introduction**

Endoribonucleases play essential roles in RNA metabolism participating both in “degradative” pathways, such as mRNA decay, and in “maturative” pathways, to generate functional RNA molecules (1, 2). Despite the plethora of functions played by processing enzymes in RNA metabolism, in eukaryotes only a few endoribonucleases have been isolated to date. Most of these activities are involved in the biosynthesis of translation components. In particular, RNase P and RNase MRP are ribonucleoprotein enzymes, functioning as site-
specific endoribonucleases (3, 4). Other well characterized endonucleolytic activities, such as
the 3’-tRNase, the tRNA splicing endonuclease and members of the RNase III-like family
are protein-only enzymes (5-7). While the majority of these activities participate in the
biosynthesis of a specific class of RNA molecules, RNase III was shown to be required for a
large number of different maturative pathways. *S.cerevisiae* RNase III (Rnt1p) was shown to
be involved in pre-rRNA, snRNA and snoRNA processing (8-12). Recently, Rnt1p was also
shown to participate in processing the intron-encoded snoRNAs U18 and snR38 from their
host pre-mRNA (13). Furthermore, a new member of the metazoan RNase III family has
been identified to be involved in the RNA interference process (14).

Another process in which the participation of endoribonucleases was expected to play
an important role is the biosynthesis of snoRNAs. These RNAs are part of a complex class of
molecules which are localized in the nucleolus where participate, as small ribonucleoprotein
particles (snoRNPs), in different rRNA maturative events: processing and nucleotide
modifications (15, 16). Most snoRNAs in vertebrates are encoded in introns of protein-
coding genes and are released from the host primary transcript either by debranching and
exo-trimming of the spliced lariat (splicing-dependent pathway) or by endonucleolytic
cleavage of the pre-mRNA (splicing-independent pathway) (15, 16). There are only a few
cases of intron-encoded snoRNAs in vertebrates which are released through the intervention
of endoribonucleases (17, 18), but so far these activities have not been purified and
characterized. We previously showed, by microinjection experiments in *X.laevis* oocytes, that
precursors containing U16 and U86 snoRNAs undergo very little splicing, while they
efficiently produce snoRNAs through a processing pathway, involving specific
endonucleolytic cleavages inside the intron (17, 18). The common feature of U16 and U86
snoRNAs is their localization in introns which are poor splicing substrates, due to the
presence of non-canonical consensus sequences.

We previously reported the identification in oocyte nuclear extracts (ONE) of an endoribonucleolytic activity, named XendoU (19), that produced the release of U16 snoRNA from its host intron, by cleaving at the same sites identified in vivo (17). The same activity was described to operate also for the processing of U86 snoRNA (18 and this paper).

From massive preparation of *X.laevis* ONE, we purified to homogeneity the XendoU endoribonuclease and characterized its activity. Partial protein sequencing enabled us to clone a XendoU cDNA, to express it and to perform a functional characterization of this enzyme. Several aspects of XendoU make this protein a novel enzyme, different from all known endoribonucleases characterized so far: i) it is poly-U specific, ii) its activity depends on Mn\(^{2+}\) ions and iii) it releases cleavage products with 2’-3’ cyclic phosphate termini.

**Experimental Procedures**

**Purification of XendoU activity.** The *X.laevis* oocyte nuclear extracts were prepared as already described (17). The pellet obtained after two sequential ammonium sulphate precipitations (45% and 70% saturation) was dissolved in buffer A (25 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.5) and applied onto an hydroxyapatite column (CHT-II Econocolumn, Biorad). Elution was carried out with 100 mM Na-phosphate, pH 7, in buffer A; the active fractions were diluted with 3 volumes of buffer A and applied on a Blue Sepharose column (Blue Sepharose Fast Flow Pharmacia). Elution was performed with 0.2 M NaCl in buffer A and fractions containing XendoU activity were pooled and dialyzed against buffer A. The protein mixture was subjected to a second
fractionation on hydroxyapatite column. The elution was performed with 10 column volumes of a linear gradient 0-100 mM Na-phosphate, pH 7, in buffer A. Selected fractions were then applied on a gel-filtration column (Pharmacia) previously equilibrated in buffer A.

By this procedure, starting from 15 ml of ONE (7 mg/ml) we obtained sufficient amount for protein sequencing analysis (approximately 70 µg).

**Preparation and isolation of tryptic peptides.** The protein band from SDS-PAGE (5 µg) stained with Coomassie Blue R250 was excised, reduced with DTT and carboxamidomethylated. The gel piece was equilibrated in 25 mM NH₄HC0₃, pH 8, and finally digested *in situ* with trypsin at 37 °C for 18 h. Peptides were extracted by sonication with 100 µl of 25 mM NH₄HC0₃/acetonitrile 1:1 v/v, pH 8 (twice). Peptide mixture was fractionated by reverse-phase HPLC on a Vydac C₁₈ column 218TP52 (250 x 1 mm), 5 µm, 300 Å pore size (The Separation Group, USA) by using a linear gradient from 5% to 60% of acetonitrile in 0.1% TFA over 60 min, at flow rate of 90 µl/min. Individual components were manually collected and lyophilized.

**Peptide Sequencing and mass spectrometry analysis.** Sequence analysis was performed using a Procise 491 protein sequencer (Applied Biosystems, USA) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems, USA) for the automated identification of PTH-amino acids, as already described (20).

Matrix assisted laser desorption ionization mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, USA), as previously reported (20); a mixture of analyte solution, α-cyano-4-hydroxy-cinnamic acid was applied to the sample
plate and dried. Mass calibration was performed using the molecular ions from peptides produced by trypsin auto-proteolysis and the matrix as internal standards.

**Plasmids and templates for RNA transcription.** U16 and U86-containing precursors were already described (17, 18). The following U16-containing mutant derivatives were obtained by inverse PCR on plasmid 003 (17) with the oligonucleotides indicated in parentheses: “pre-open stem” (open stem fw and open stem rev), “pre-Ms1” (Ms1a and Ms1b) and “pre-ΔU16 (003 int fw and 003 int rev). "pre-ΔC/bD mutant was instead derived by inverse PCR on plasmid 003 bD (21) with oligos ΔC/bd fw and Ms1 ΔC rev.

**Oligonucleotides.** The following oligonucleotides were used for obtaining the templates for *in vitro* transcription:

open stem fw (GTAATTTGCCTCCTACTCTAC);
open stem rev (GACATCATATTTTGTAAAAAAAGCAC);
Ms1a (ATTACGACATCATAGCAAGTA);
Ms1b (TATCGCGTTCTGAGCAAAAA);
003 int fw (CTTGGATAAGTTTAGAATATATTAATA);
003 int rev (GTAAAAAAAGCACAAATCTAAATC);
ΔC/bd fw (CGTAATTTGCGTCCTACTCTAT);
MS1 ΔC rev (AGCAAGTAAAAAAAGCAC).

**In vitro processing reactions of wild type and mutant derivative RNAs.** U16 and U86-containing precursors (17, 18) were *in vitro* transcribed in the presence of [α-32P]UTP and
pre-mRNAs were injected into nuclei of stage VI oocytes as already described (22).

Alternatively, 3 X 10^4 cpm of 32P-labeled pre-mRNAs were incubated with 1 µg of ONE (17) or with 1 ng of purified XendoU, in 5 mM MnCl₂, 50 mM NaCl, 25 mM Hepes, pH 7.5, 1 mM DTT, 10 µg of E.Coli tRNA, 20 U of RNAse inhibitor (Promega). The products of the reactions were then analyzed on 6% polyacrylamide-7 M urea gels.

Oligoribonucleotides P1 (5’-GGAAACGUAUCCUUUGGGAG–3’), P2 (5’-GGAAACGUAUCCUUUGGGAGG–3’) and P3 (5’-GGAAACGUAUCCUCUGGGAG–3’) were 5’ end-labeled and incubated in the same conditions as above. Double stranded P2 (dsP2) was obtained by incubating labeled P2 oligo with its reverse complementary oligo, in a molar ratio1:2, for 1h at 37°C in 100 mM KOAc, pH 7.5, 30 mM Hepes KOH, pH 7.4, and 2 mM Mg(OAc)₂. Double stranded formation was controlled on a native gel. The RNA ladder was obtained by incubation of P1 oligo (200,000 cpm) in 500 mM NaHCO₃, at 90 °C, for 20 min.

**Analysis of 3’ termini of cleavage products.** This analysis was performed by two different approaches. In the first one, 32P-labeled gel-purified I-1b molecules, generated by incubation of U16-containing precursor with ONE, with purified XendoU or *in vivo*, were treated with 10 µl of 10 mM HCl, at 25 °C, for 2 hr, to hydrolyse the cyclic phosphate moiety as described by Forster (23). The phosphate was then removed by incubation of the RNA in 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5, in the presence of 1 U of calf intestine alkaline phosphatase at 50 °C, for 60 min. RNA was subjected to two subsequent purification steps with phenol:chloroform, ethanol precipitated and analyzed on 10% polyacrylamide-7 M urea.
gel. In the second approach, the I-1b molecule, obtained by incubation with purified XendoU, was gel-eluted and redissolved in 30 mM Tris, pH 8.0, 15 mM MgCl₂ and 1.5 units/µl T4 polynucleotide Kinase, and the mixture was incubated for 45 min at 37 °C to remove the 2-3 cyclic phosphate (24). RNA was then extracted and labeled using [5-³²P]pCp and T4 RNA ligase for 5 h at 16°C. RNA was then analyzed on 6% polyacrylamide-7 M urea gel.

**Isolation of XendoU cDNA and its expression in reticulocyte lysate and in bacteria.** A *X.laevis* stage 28 embryo cDNA library, constructed in λZAP II vector, was screened using a specific probe obtained by PCR amplification on *X.laevis* cDNA with degenerate oligonucleotides (MAHs 5’-ATGGCICAYGAYTAYYTIGT–3’ and IGTa 5’-ACIGGRTAIGCIGTICCIAT–3’) designed on tryptic peptides of purified XendoU. The XendoU ORF was cloned into Blue Script vector and [³⁵S] methionine-labeled proteins were produced by *in vitro* transcription and translation using the TnT-coupled Reticulocyte Lysate System Kit (Promega). Translational products were analyzed on 10% SDS-PAGE. The XendoU coding sequence was also cloned downstream to the 6His coding region of the pQE30 vector (Qiagen). The fusion protein (6His-XendoU) was induced in the *E.Coli* M15 strain.

**Primer extension analysis.** The I-2 and I-4 products, derived from U16 processing, were gel purified and reverse transcribed with 5’-end labeled oligonucleotides B3 (5’-TACGTCCACCACGACACAT-3’) and γ (5-TTTTCCTCAGAAACGCAAT-3) respectively. For the I-4 products derived from U86 processing, oligonucleotide U*Hind*III (5-
AAGCTTCTTCATGGCGGCTCGGCCAAT-3) was utilized.

Results

Purification of XendoU from X. laevis oocyte nuclear extracts. We previously developed an in vitro system able to reproduce the in vivo processing of U16 snoRNA from its host intron (17). When $^{32}$P-labeled U16-containing precursor was incubated with X. laevis oocyte nuclear extracts (ONE), specific endonucleolytic products were obtained (Fig. 1B): the I-1 and I-2 molecules derive from cleavage upstream to the U16 coding region, while the I-3 and I-4 molecules are produced by cleavage downstream to U16. When double cleavage occurs on the same precursor molecule, pre-U16 products accumulate; these intermediates are eventually converted by exo-trimming to the mature snoRNA (see Fig.1A). The cleavage sites were previously mapped in correspondence of short U-stretches: four of them are clustered upstream to U16 and one is located downstream. This cleaving activity was named XendoU (19).

In this work we carried out the biochemical purification of XendoU (Fig. 1D). The enzymatic activity was followed, throughout the different steps, by incubating $^{32}$P-labeled U16-containing precursor with aliquots of the different fractions and by analyzing the cleavage products on polyacrylamide gels. Since we previously observed the dependence of XendoU activity on Mn$^{2+}$ ions (19), this cofactor was always added to the reaction mixture. The protein content of the active fractions is shown in Fig. 1E. After several chromatographic steps, a single component of 37 kDa was identified in those fractions displaying specific
activity (Fig. 1E, lane 6). The elution profile on the gel filtration chromatography was consistent with XendoU being a monomeric protein (not shown). Fig. 1B shows the comparison of processing activity of 1 µg of ONE (lanes ONE) with that of 1 ng of the purified 37 kDa polypeptide (lanes XendoU). In both cases, the same primary cleavage products (I-2 and I-3), their complementary cut-off molecules (I-1 and I-4) and pre-U16 molecules were generated.

**Characterization of XendoU cleavage.** In order to analyze the specificity of cleavage of the purified enzyme, primer extension analysis was performed on gel-purified I-2 and I-4 products (Fig. 1C). The results indicate that, at short incubation times, XendoU cleaves intronic sequences at the same U-rich regions previously identified *in vivo* and in extracts (17): four I-2 molecules are generated by cleavages at the a, b, c and d sites, while two I-4 molecules are produced by cleavage at two adjacent U residues, 14 nucleotides downstream to U16 (see representation of Fig. 1A). From the reverse transcriptase experiment it appears that the a and b sites are preferentially utilized in the upstream cleavage. As a consequence two major I-1 molecules (a and b) are identified (see gels of Fig. 1 and 4).

Efficient cleavage with the purified enzyme was obtained only when Mn$^{2+}$ ions were present in the reaction (Fig. 1B, lane 7). It is remarkable that the addition of Mn$^{2+}$ ions in the *in vitro* assay is required for the purified protein and for all the fractions obtained after the blue-sepharose step: this finding, along with the requirement of Mn$^{2+}$ supply when "aged" oocyte extracts are utilized (not shown), is consistent with a loosely bound metal ion in the protein moiety. The purified enzyme is poorly activated by Mg$^{2+}$, while it is inactive in the presence
of Cd$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Pb$^{2+}$ (not shown).

The time course of Fig. 1B shows that the purified enzyme cleaves preferentially at specific sites. Nevertheless, at longer incubations also the primary cleavage products become substrates for further digestion, producing small sized RNAs only visible on short run gels (not shown). The substrate specificity of XendoU was then tested by incubating the purified enzyme with a synthetic oligoribonucleotide (P1), containing the upstream distal XendoU cleavage site (site d, Fig. 1A), and with mutated derivatives thereof (P2 and P3). The results indicate that the sequence specificity of XendoU is limited to U-stretches and that only two U residues are sufficient for cleavage (Fig. 2A). Incubation of XendoU with the P2 oligo in a double stranded configuration, did not produce any cleavage (lanes dsP2), indicating that the enzyme is unable to cleave U-stretches present in double stranded structures.

From these data it appears that, despite the presence of several U-stretches in the U16-host intron upstream and downstream to the snoRNA, specific sites are preferentially cleaved by XendoU. This is similar to what described for the bacterial RNase E endonuclease which, even if has a low primary sequence specificity, cleaves the substrate only at a limited number of sites. In this case, it was shown that the overall secondary structure of the substrate modulated cleavage activity and that the role of stem-loop structures was to limit rather than promote RNase E cleavages (25). Since snoRNAs are folded in specific secondary structures characterized by the conserved “terminal core motif” (see wild type RNA in lower part of Fig.2B) (16, 26) and, in many cases, by an apical stem-loop region (27, 28), we asked whether these structural elements could influence XendoU activity. Mutants affecting either structures were raised on precursor molecules and tested for XendoU cleavage. The “terminal core motif” was destroyed in the “pre-open stem” and “pre-ΔC/bD mutants, while the apical
stem-loop structure was deleted in the pre-Ms1 derivative (see schematic representation of Fig. 2B). The pattern of XendoU cleavage on such mutants (Fig. 2B), was not altered suggesting that these structures do not represent entry sites or positioning elements for XendoU. This conclusion was definitely confirmed by the analysis of pre-ΔU16, a mutant completely lacking the snoRNA sequence: XendoU cleaves this RNA at the same sites as the wild type RNA substrate, as indicated by the size of the cleavage products (refer to Fig.1A for schematic representation).

**Characterization of the reaction products.** The chemistry of XendoU cleavage was assessed by determining the chemical nature of the termini in the cleaved products. The ends of 32p-labelled I-1b molecules produced with ONE or with the purified XendoU were analyzed; these molecules were gel purified and treated either with HCl or alkaline phosphatase, or with both. Fig. 2C shows that a slight decrease in migration, due to the loss of a negative charge, is obtained only when the alkaline phosphatase follows the HCl treatment (lanes 3). These data allowed to conclude that the 3’ end of the cleavage products, obtained with ONE (lanes ONE) and with the purified XendoU (lanes XendoU), carry a 2’-3’ cyclic phosphate (23, 29). In fact, only after the acid treatment the phosphate group can be removed by phosphatase such as to confer slight decrease in gel mobility. As previously reported (21), the products of primary cleavage such as I-1 molecules, are quite unstable *in vivo* because, after cleavage, they are rapidly trimmed out. Nevertheless, at very short incubation times, we were able to purify little amounts of I-1b molecules and to subject them to the same treatment described above. Fig. 2C (lanes *in vivo*) shows that a slight reduction in migration was obtained, demonstrating that also the products of the *in vivo* reaction have 2’-3’ cyclic phosphate ends.
The nature of the 3’ ends was also tested by a different approach (24): the I-1b molecule, generated by XendoU cleavage, was ligated to [5’-32P]pCp directly or after kinase treatment, which removes the 2’-3’ cyclic phosphate. The appearance of radioactive band only after kinase treatment (lane 3 of Fig. 2D) confirms that this molecule has 2’-3’ cyclic phosphate.

Isolation of a XendoU cDNA. After elution from the gel, the 37 kDa polypeptide was reduced, alkylated and digested with trypsin as reported in the experimental procedures. The resulting peptide mixture was resolved by reversed-phase HPLC and selected peptide fractions were submitted to automated Edman degradation. Three tryptic peptides (indicated as #1, #2 and #3 in Fig. 3) were utilized to derive degenerate oligonucleotides. These were employed, in different combinations, in PCR amplification reactions on cDNA from polyA+ RNA, extracted from X.laevis oocytes. Only the reaction performed with sequence #1 (forward) and sequence #3 (reverse) gave a specific amplification product of 500 nucleotides. Sequencing of this product indicated the presence of an Open Reading Frame containing peptide #2. This cDNA probe was then utilized for screening a X.laevis stage 28 embryo cDNA library, allowing the isolation of a full-length cDNA (Fig. 3). 65% of the amino acid sequence determined was confirmed by MALDI-MS spectra of the tryptic peptides.

Activity of in vitro translated and recombinant XendoU. The XendoU ORF, 876 bp long, was cloned into the Blue Script vector and the protein was produced by in vitro transcription and translation, using a reticulocyte lysate. The translation product was analyzed on SDS-PAGE and resulted in a 37 kDa protein (Fig. 4A). In order to assess the activity of this polypeptide,
the reticulocyte lysate expressing the XendoU ORF was incubated with $^{32}$P-labeled U16-containing precursor. Figure 4B shows that the cleavage pattern produced by the in vitro translated 37 kDa protein (lane 2) matches that obtained with the extracts (lane 1).

Furthermore, the lack of cleavage when Mn$^{2+}$ ions were not added to the reaction mixture (lane 3) confirms the specific ion requirement of XendoU and suggests that the binding to this cofactor is reversible. As a negative control the activity assay was carried out by incubating the RNA substrate with an uncommitted reticulocyte lysate in the presence of Mn$^{2+}$ ions (lane 4). Fig. 4 also shows that a 6His-XendoU recombinant protein expressed in bacteria is able to reproduce the cleavage pattern of the purified enzyme (lane 5).

**XendoU also participates in the biosynthesis of another intron-encoded snoRNA.** We previously identified a novel box C/D snoRNA, named U86, which is encoded by an intron of the NOP56 gene of *X.laevis* (18). Similarly to U16 snoRNA, also U86 is contained in a poorly spliceable intron and its biosynthesis appears to be alternative to that of the co-transcribed mRNA. Injection of $^{32}$P-labeled U86-containing precursor into *X.laevis* oocytes generates the truncated products I-2 and I-3 and their 5’ and 3’ cut-off molecules, I-1 and I-4 (Fig. 5A, lanes *in vivo*).

Processing of U86-containing precursor with purified XendoU (Fig. 5A, lanes XendoU) or with the reticulocyte lysate expressing XendoU ORF (Fig. 5C, lane 2) demonstrates that the enzyme is responsible for the cleavage occurring downstream to the U86 coding region. The activity responsible for the cleavage upstream to U86, that produces I-2 and I-1 molecules, is still unidentified and it is not reproduced also in oocyte nuclear
extracts (Fig. 5A, lanes ONE). The XendoU cleavage sites, downstream to U86, were mapped by primer extension on I-4 molecules and found to localize in correspondence of three U-rich sequences (Fig. 5B).

**DISCUSSION**

In this paper we report the purification to homogeneity of XendoU, an activity previously shown to be involved in the release of the intron-encoded U16 and U86 snoRNAs from their host primary transcripts in *X.laevis* oocytes.

XendoU is a novel endoribonuclease in that it requires Mn$^{2+}$ ions and produces 2'-3' cyclic phosphate termini. Such ends have been previously associated solely with metal-independent (30, 2) or Mg$^{2+}$-dependent endoribonucleases (31, 32, 33). On the contrary, Mn$^{2+}$-requiring endonucleases usually produce 5'-P and 3'-OH ends (34, 35). The chemistry of cleavage of XendoU strongly resembles that of ribozymes, where the metal, positioned near the attacking 2' oxygen, increases its nucleophilicity and allows the transesterification reaction with the production of cyclic 3' ends (36). Until structural data are available, it will not be possible to assess the role of Mn$^{2+}$ in the catalytic activity of XendoU. A clear example that metal ions can have a direct role in phosphoryl-transfer reactions in the context of metallo-proteins was derived from the crystal structure of the DNA polymerase I, 3'-5'-exonuclease domain, complexed with single stranded DNA. In this case, two metal ions form complexes with the scissible phosphate and water, facilitating formation of the attacking hydroxide ion and stabilizing the transition state (37, 38). The role of the protein component would be exclusively to correctly orient the metal ions, the substrate
and the attacking water molecule. By analogy, it could be possible that in the case of XendoU, Mn$^{2+}$ might participate directly in the catalytic step, while the protein component may assist the reaction by orienting the ions to specific sites on the substrate. The availability of the active recombinant protein will allow us to answer this question in the near future.

Characterization of XendoU activity indicated that it does not have a stringent sequence specificity in that only two U-residues are sufficient for cleavage; nevertheless, preferential cleavages occur at specific U-stretches localized upstream and downstream to U16 snoRNA. At prolonged incubations, also the other U-rich regions are cleaved, converting the primary products into small sized RNA species. Since snoRNAs, and in particular U16, have been described to be folded in specific secondary structures characterized by the conserved “terminal core motif” (16, 26, 39) and, in many cases, by an apical stem-loop region (27, 28), we asked whether these elements could provide some structural information for directing the preferential activity of the enzyme. Instead, we have observed that these structural motifs of U16 are not required for positioning XendoU cleavage. This is analogous to the case of RNase E in which McDowall et al. (25) have described that stem-loops do not serve as entry sites for the enzyme, but instead they limit cleavage at potentially susceptible sites, more accessible than others to the nuclease.

It is possible to suggest that in vivo specific RNA/protein interactions should control the accessibility of the nuclease only to the specific sites on the pre-mRNA. hnRNP C was previously shown to interact in vivo with the U-stretches which are XendoU substrates and to interfere with cleavage (40). It is possible to imagine that the hnRNP C, deposited on the nascent RNA during transcription, can be displaced at specific locations and under specific circumstances. An attractive hypothesis is that the assembly of snoRNP factors on the nascent
snoRNA could displace hnRNP C from the flanking regions and help the recruitment of
XendoU, allowing site-specific cleavage and release of the snoRNA. This would be similar
to what demonstrated in the yeast system where the endonucleolytic release of U18 snoRNA
from its host intron depends on assembly of snoRNPs which in turn recruit the Rnt1p
endoribonuclease (13).

The antagonistic effect of hnRNP C and XendoU can represent the basis for the post-
transcriptional regulation of the U16 snoRNA processing.

In analogy with Rnt1p, which is involved in the maturation of different RNA molecules
(pre-rRNA, snRNAs and snoRNAs) it is possible that XendoU is required for many other
processes of RNA maturation or turnover. So far, we do not know whether XendoU is also
present in the cytoplasm and whether it plays any role in this compartment.

Database search for XendoU homologs identified significant homology (38%
identity and 55% similarity) only with a human putative serine protease (41),
which, in turn, has homologs in *C.elegans, A.thaliana* and *M.musculus*.

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**Footnotes**

1 The abbreviations used are: snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein particle; ONE, oocyte nuclear extracts; ORF, Open Reading Frame.

The nucleotide sequence for XendoU cDNA has been deposited in the GenBank database under GenBank Accession Number AJ507315

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Figure legends

Fig. 1. Purification and activity assay of XendoU. (A) Schematic representation of U16 snoRNA processing. The U16-containing precursor is indicated by P. Cleavage upstream to U16 produces the I-1 and I-2 molecules, while cleavage downstream generates the I-3 and I-4 products. Double cleavage produces pre-U16 molecules. a, b, c and d, indicate the cleavage sites upstream to U16; the major sites of cleavage are represented by large arrows. e and f point to the cleavage sites downstream to U16. The Cap structure is shown as a black dot, exons as boxes, the intron as a continuous line and U16 snoRNA coding region as a thicker line. γ and B3 indicate the oligonucleotides utilized in primer extension experiments.

(B) 32P-labeled U16-containing precursor was incubated with 1 µg of unfractionated oocyte nuclear extracts (lanes ONE) or 1 ng of purified XendoU (lanes XendoU) under standard conditions, for 0 min (lanes 1), 5 min (lanes 2), 15 min (lanes 3), 30 min (lanes 4), 60 min (lanes 5) and 120 min (lanes 6) or without the addition of Mn2+ for 30 min (lane 7). The specific cleavage products are indicated on the side. (C) Unlabeled I-2 and I-4 molecules, obtained after 5 min of incubation with purified XendoU were gel eluted and incubated with oligos γ and B3 respectively (see panel A). The products of primer extension were run in parallel with the sequencing reaction (lanes G, A, T, and C) performed with the same oligonucleotides on U16-containing precursor template; the arrows point to the extended products and letters on the left side indicate XendoU cleavage sites. (D) XendoU purification
scheme. (E) Proteins from the active fractions of the different fractionation steps of panel D were separated on SDS-PAGE and visualized by Blue Coomassie staining. The arrow points to the purified enzyme with an apparent molecular mass of 37 kDa.

**Fig. 2** Analysis of XendoU cleavage. (A) $^{32}$P-labeled synthetic oligoriboligonucleotide P1, containing the distal cleavage site upstream to U16 (site d), its mutant derivatives (P2 and P3) and a double stranded P2 derivative substrate (dsP2), were incubated under standard conditions, for 30 min, with: buffer (lanes 1), unfractionated extracts (lanes 2) or purified XendoU (lanes 3). Lane M reports the ladder generated by alkaline digestion of P1. On the side, the sequences of the oligoribonucleotides are reported; arrows indicate the cleavage sites. (B) XendoU processing of U16-containing precursor and of its mutant derivatives “pre-open stem”, pre-$\Delta$C/bD, pre-Ms1 and pre-$\Delta$U16 (schematised in the lower part of the panel). $^{32}$P-labeled RNAs were incubated with XendoU, under standard conditions, for 0 min (lanes 1), 10 min (lanes 2), 20 min (lanes 3), 40 min (lanes 4) and 60 min (lanes 5). The processing products are indicated on the sides (refer to Fig.1A for schematic representation).

(C) $^{32}$P-labeled I-1b molecules, schematically represented on the left side, obtained with oocyte nuclear extracts (lanes ONE), with purified XendoU (lanes XendoU) or after injection in oocytes (lanes *in vivo*), were gel purified and incubated with 1 unit of alkaline phosphatase (lanes 1) or with 10 mM HCl (lanes 2) or with alkaline phosphatase after acid treatment (lanes 3). Untreated molecules were run as control in lanes 4. (D) I-1b molecule obtained by incubation of unlabeled U16-containing precursor with purified XendoU was gel purified and subjected to $[5^{\prime}-^{32}$P]pCp labeling directly (lane 2) or after Kinase treatment (lane 3).
lane 1, a labeled marker I-1b molecule is run.

**Fig. 3.** cDNA and deduced amino acid sequence of XendoU. 5’ and 3’ untranslated regions are shown in lower case letters while the ORF is in capital letters. Above each codon the deduced amino acid is shown. The peptides determined by automated Edman degradation (see Experimental Procedures) that were utilized for deriving the degenerated oligonucleotides are indicated by #1, #2 and #3. The stop codon is identified by an asterisk. The amino acid sequences covered by MALDI-mapping experiments are underlined.

**Fig. 4.** Functional analysis of *in vitro* translated and recombinant XendoU. (A) SDS-PAGE analysis of [35S] methionine-labeled *in vitro* transcription and translation products of XendoU cDNA (lane 2) and of control luciferase (lane 1). The arrow points to 37 kDa product. (B) The U16-containing precursor (P) was incubated for 45 min, in the presence of Mn$^{2+}$ ions, with ONE (lane 1), with the *in vitro* translated XendoU (lane 2), or with an uncommitted reticulocyte lysate (lane 4). The RNA substrate was also incubated with *in vitro* translated XendoU, in the absence of Mn$^{2+}$ ions (lane 3). In lane 5 the cleavage pattern obtained with recombinant His-XendoU is shown.

**Fig. 5.** XendoU is involved in U86 snoRNA biosynthesis. (A) $^{32}$P-labeled U86-containing precursor (P) was injected in *X.laevis* oocytes (lanes *in vivo*), or incubated *in vitro* with ONE (lanes ONE), or with purified XendoU (lanes XendoU). Incubations were allowed to proceed for: 0 min (lane1), 10 min (lanes 2), 45 min (lanes 3), 3 hours (lanes 4), 16 hours (lanes 5).
The processing products are schematically represented on the side. Arrows indicate the cleavage sites. (B) $^{32}$P-labeled UHindIII primer was reacted with unlabeled, gel purified, I-4 molecules obtained after 10 min incubation in oocytes (lane *in vivo*), 45 min with ONE (lane ONE), or 45 min with purified XendoU (lane XendoU). The products of primer extension were run in parallel with the sequencing reaction (lanes G, A, T, and C) performed with the same oligonucleotide on U86-containing precursor template. The cleavage sites are indicated on the corresponding sequence. (C) U86-containing precursor (P) was incubated for 45 min, in the presence of Mn$^{2+}$ ions, with ONE (lane 1), with *in vitro* translated XendoU (lane 2), or with an uncommitted reticulocyte lysate (lane 4). As a control, pre-mRNA was incubated with *in vitro* translated XendoU in the absence of Mn$^{2+}$ ions (lane 3).
Purification, cloning and characterization of XendoU, a novel endoribonuclease involved in processing of intron-encoded small nucleolar RNAs in Xenopus laevis
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