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Functional Characterization of XendoU, the Endoribonuclease Involved in Small Nucleolar RNA Biosynthesis*[S]

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XendoU is the endoribonuclease involved in the biosynthesis of a specific subclass of *Xenopus laevis* intron-encoded small nucleolar RNAs. XendoU has no homology to any known cellular RNase, although it has sequence similarity with proteins tentatively annotated as serine proteases. It has been recently shown that XendoU represents the cellular counterpart of a nidovirus replicative endoribonuclease (NendoU), which plays a critical role in viral replication and transcription. In this paper, we combined prediction and experimental data to define the amino acid residues directly involved in XendoU catalysis. Specifically, we find that XendoU residues Glu-161, Glu-167, His-162, His-178, and Lys-224 are essential for RNA cleavage, which occurs in the presence of manganese ions. Furthermore, we identified the RNA sequence required for XendoU binding and showed that the formation of XendoU-RNA complex is Mn^{2+}-independent.

XendoU is an RNA processing enzyme that participates in the production of small nucleolar RNAs (snoRNA(s)),[1] a large family of non-coding RNAs with essential roles in ribosome biogenesis (reviewed in Ref. 1). There are two major families of such molecules, namely box H/ACA and box C/D snoRNAs. The components of the first class participate in pseudouridylation, whereas the members of the second class are involved in site-specific 2′-*O*-methylation of rRNA and spliceosomal small nuclear RNAs (2). In a previous study, we showed that the box C/D U16 snoRNA, encoded by an intron of the L4 ribosomal protein gene of *Xenopus laevis*, originated from site-specific processing of the intron within the pre-mRNA (3). Subsequently, we studied the *in vitro* processing of U16 and demonstrated that an extract prepared from *X. laevis* germinal vesicles was able to reproduce the cleavage pathway observed *in vivo* (4). In particular, we showed that double cleavage upstream and downstream of intronic U16-coding region produced pre-U16 molecules that are converted, by exonuclease trimming, to the mature snoRNA (see schematic representation in Fig. 1*A*). More recently, we purified, from *X. laevis* nuclear extracts, the endoribonucleolytic activity responsible for the release of U16 and U86 snoRNAs from their host primary transcripts. Characterization of this enzyme, named XendoU, showed that it is a Mn^{2+}-dependent nuclease that cleaves U stretches on the RNA substrate and releases 2′-3′-cyclic phosphodiester products (5).

Depending on the production of 5′- or 3′-phosphate extremities, the RNases can be subdivided into two groups (6). The main characteristic of the enzymes belonging to the first group, which includes many intracellular endo- and exoribonucleases, is that they all possess a metal ion in their active site. The second class includes the families of barnase, RNase A, RNase T1, and RNase T2. The components of this class release reaction products carrying cyclic 2′-3′-phosphodiester termini, and their catalytic activity is ensured by two side chains, one acting as a general base, the other as a general acid. In addition, in all known cases the general acid catalyst is always a histidine, whereas the general base catalyst can be a histidine or a glutamate (7–10). So far, no RNase has been described that requires manganese ions and releases products with 2′-3′-cyclic phosphodiester termini, suggesting the existence of another class of enzymes that employs different reaction mechanism.

A data base search for XendoU homologs with the whole protein sequence did not show any homology with cellular RNases, yet identified significant homology (38% identity and 55% similarity) with pp11, a putative serine protease (11) from organisms as distant as *Arabidopsis thaliana*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens.* However, data base searches with nidovirus proteins led to the recent identification of XendoU as the cellular homolog of the nidovirus NendoU (12). NendoU is one of the replicative components conserved in coronaviruses and in all their distant nidovirus relatives, and its activity is required for virus replication and transcription (13).

In this study, we analyzed the contribution of active site residues to the function of XendoU and the requirements for enzyme-RNA interaction. We started with phylogenetic comparisons of XendoU and its eukaryotic orthologs and used the information about sequence conservation as a guide for site-directed mutagenesis experiments. Several mutants were designed, and the roles of mutated residues were investigated by characterizing their catalytic activity in parallel with their binding capability. The results of such analysis indicate the critical role of five amino acid residues; their chemical nature suggests an interesting hypothesis about the role of manganese in RNA cleavage.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[1] The abbreviation used is: snoRNA, small nucleolar RNA; ONE, oocyte nuclear extracts.

EXPERIMENTAL PROCEDURES

Sequence Analysis—The non-redundant protein data base was searched with PSI-BLAST (14) for 10 iterations or until convergence; typical inclusion threshold *E*-value was 0.001, although we experimented also with $E = 0.01$. The data base was searched separately for three iterations, and the checkpoints were saved for secondary structure predictions (15). Multiple alignment figures were generated using CHROMA (16). Profile-profile comparison and alignments of protein families were done using COACH (17).

Oligonucleotides—The following DNA oligonucleotides, purchased from MWG Biotech, were used for cloning the different constructs: ATG, 5′-GGGGATCCATGGCGAGTAACAGGG-3′, STOP, 5′-GGAAGCTTTC-AGTACAGATCCGGGTTATT-3′, A, 5′-CACAAACACGTGCTGAAAGC-CGC-3′, B, 5′-GGACAATCGAAGCGAGGGCAGGAG-3′, C, 5′-GGAGA-ATCGAAGCGAGGGCAGGAG-3′, D, 5′-CACAAACACGTGCTCAAAG-CCGC-3′, E, 5′-GGAGAATCGAAGCGAGGGCAGGAG-3′, F, 5′-CACA-AACACGGCCTCAAAGCCGCAC-3′, G, 5′-ACAACCTGGGCCACCCC-CGTG-3′, H, 5′-GACTCCGGAGGAAATCGCTGAAAAC-3′, I, 5′-GATG-CGTGCGGCTTTGAGCACGTGTTTGTGGG-3′, J, 5′-GGGTCTGCTCC-CTGGGGCCCG-3′, K, 5′-GCCGTCAGCCCGGAATTCGAATTCGCCC-3′, L, 5′-AATGAAGCTGCTGCCGACGGG-3′, M, 5′-GGCGTCGCCCC-GGAATTCGAATTCGCCC-3′, N, 5′-GGCGTCAGCGCGGAATTCGAA-TTCGCCC-3′, O, 5′-GAGATGATGGGGCTTGCCAACTGGG-3′, P, 5′-C-TGCCCTCGCTTCGATTCTCCCAC-3′, Q, 5′-AAGGAGATGGTGGCAC-CCGTCGGC-3′, R, 5′-CCAATTGAACTGCAGGTTCAACACC-3′, S, 5′-GGCGTCAGCCCGCAATTCGAATTCGCCC-3′, mini 003fw, 5′-CT-GGTAATACGACTCACTATAGGGGTATCCTTTTGGGATTTA-3′, mini 003rev, 5′-AAGCTTCCCGAAGAGAAATTTTTG-3′, B3, 5′-TACGTCC-ACCACGACACAT-3′, and γ, 5′-TTTTCCTCAGAACGCAAT-3′. For competition experiments, we purchased the following oligoribonucleotides P1 (5′-GGAACGUAUCCUUUGGGAG-3′) and P3 (5′-GGAACGU-AUCCUCUGGGAG-3′) from RNA-TEC.

Plasmids and Templates for in Vitro RNA Transcription—The 003 construct, encoding U16-containing precursor (003 RNA), was described previously (18). The mini-003 construct, encoding for a shorter variant of 003 RNA, was obtained by PCR-based methods. The 003 template was amplified using oligonucleotides mini 003fw and mini 003rev and cloned in Bluescript KS plasmid (Stratagene). The mini-003 RNA is 207 nucleotides long and includes the upstream (a, b, c, and d) and downstream (e and f) XendoU cleavage sites, flanking the U16-coding region (see schematic representation in Fig. 3*A*).

Cloning of Hexahistidine-tagged XendoU and of Its Mutant Derivatives—All fusion proteins were expressed as N-terminally His_6-tagged proteins by using the expression vector pQE30 (Qiagen). The open reading frame for the wild type XendoU was PCR-amplified using *X. laevis* oocyte cDNA, with the primers ATG and STOP (pQE30-XendoU). The constructs expressing XendoU mutant derivatives were obtained by inverse PCR on pQE30-XendoU plasmid with the oligonucleotides indicated in parentheses: E161Q/E167Q (A, B), E161Q (A, C), E167Q (B, D), H162A (E, F), E92Q (G, H), S157A (I, J), G232A (K, L), S234A (L, M), P235A (L, N), H178A (O, P), K224A (Q, R), and E236Q (L, S).

Expression and Purification of Recombinant Proteins—Recombinant XendoU (His-XendoU) and its mutant derivatives were expressed in M15 [pREP4] strain and purified to greater than 90% homogeneity by affinity chromatography with nickel (II) nitrilotriacetic acid-agarose, according to Qiaexpress protocol (Qiagen). Protein preparations were dialyzed against storage buffer (10 mM Hepes, pH 7.5, 75 mM NaCl and 20% glycerol) and stored at −80 °C.

In Vitro Transcription and Processing Reaction—U16-containing precursors (003 and mini-003 RNAs) were synthesized *in vitro* (19) in the presence of 10 μCi of [α-^{32}P]UTP and 15 μM UTP. After transcription, the RNAs were gel-purified, phenol-extracted, and precipitated with ethanol. In a typical processing reaction, 3×10^4 cpm (corresponding to ~2 fmol of ^{32}P-labeled RNA precursors) were incubated with recombinant proteins or oocyte nuclear extracts as described (5). The reaction products were then analyzed by denaturing PAGE.

Analysis of 3′ Terminus—Gel-purified 5′-terminal cleavage product (molecule I-1 derived by cleavage at the b site) was treated as described (5) After extraction and labeling in the presence of 5′-[^{32}P]cytidine 3′,5′-bisphosphate, the RNA was analyzed by denaturing PAGE and autoradiography.

Primer Extension Analysis—Unlabeled I-2 and I-4 molecules produced by XendoU cleavage on U16-containing precursor (003 RNA) were gel-extracted and annealed with γ-^{32}P-labeled γ and B3 oligonucleotides, respectively. Resulting molecules were reverse-transcribed using SuperScript II System (Invitrogen), and reaction products were analyzed by denaturing PAGE.

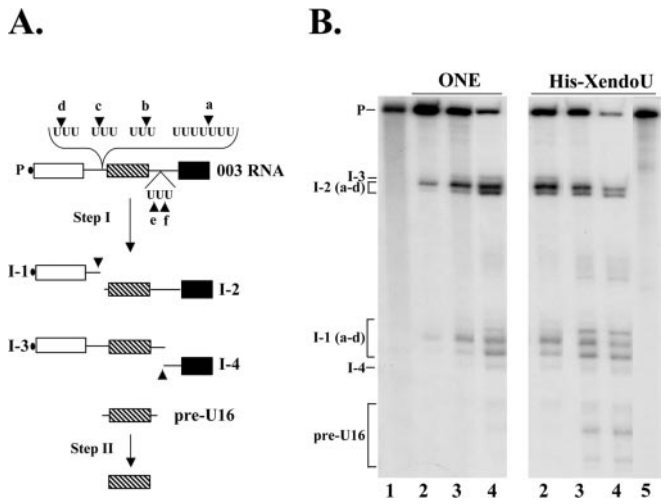

FIG. 1. **Analysis of recombinant XendoU (His-XendoU) cleavage activity.** *A*, schematic representation of U16 snoRNA processing. The U16-containing precursor (003 RNA) is indicated by *P*. The Cap structure is represented as a *black dot*, exon III as a *white box*, exon IV as a *black box*, the intron as a *continuous line* and U16 snoRNA coding region as a *striped box*. Cleavages at sites upstream to U16 (*a*, *b*, *c*, and *d*) generate I-1(a–d) and I-2(a–d) molecules. Although the four I-1 molecules are well resolved on the gel, the complementary I-2 molecules are not well separated; however, their production is demonstrated by primer extension analysis (see Fig. 2*B*). Double cleavages (*e* and *f*) at the site downstream to U16, produce I-3 and I-4 molecules. *B*, *in vitro* processing assay. ^{32}P-Labeled 003 RNA was incubated with 1 μg of unfractionated oocyte nuclear extracts (*lanes ONE*) or with 10 ng of recombinant His-XendoU (*lanes His-XendoU*), under standard conditions, for 0 (*lane 1*), 5 (*lanes 2*), 10 (*lanes 3*), and 15 min (*lanes 4*) or without the addition of Mn^{2+} (*lane 5*). The specific cleavage products are indicated on the *side*.

Binding Assays—Binding assays were performed by incubating 2 fmol of [α-^{32}P]UTP *in vitro* transcribed mini-003 RNA with increasing amounts of recombinant proteins in a final volume of 10 μl of binding buffer (10 mM Hepes, pH 7.5, 75 mM NaCl, 20 mM EGTA, 1 mM dithiothreitol, and 20% glycerol). After 30 min of incubation at 24 °C, the RNA-protein complexes were fractionated on 8% polyacrylamide (29:1 acrylamide:bis) native gels containing 4% glycerol. In competition experiments, binding assays were carried out by incubating [α-^{32}P]UTP *in vitro* transcribed mini-003 RNA with His-XendoU, in the presence of increasing amounts of different unlabeled RNAs as competitors.

RESULTS

Characterization of Recombinant XendoU Activity—The first step toward the characterization of the XendoU residues involved in the catalysis was the production of the recombinant enzyme. N-terminal hexahistidine-tagged XendoU (His-XendoU) was expressed from bacteria, and the affinity-purified protein was tested for its ability to correctly process the U16 snoRNA from its host precursor (003 RNA). 003 RNA mimics the natural substrate of XendoU and includes the U16-containing intron and portions of the flanking exons (18).

A typical processing assay was carried out by incubating His-XendoU with ^{32}P-labeled 003 RNA in the presence of Mn^{2+} (5). Fig. 1*B* shows the comparison of processing activity of 1 μg of Oocyte Nuclear Extract (*lanes ONE*) with that of 10 ng of recombinant XendoU (*lanes His-XendoU*). The results confirm that the enzyme releases the same products previously identified *in vivo* and in extracts (4) (see schematic representation in Fig. 1*A*). Furthermore, the lack of cleavage when Mn^{2+} was not added to the reaction (*lane 5*) confirms its requirement for His-XendoU activity.

His-XendoU cleavage activity was further analyzed by characterizing the 3′ termini of the reaction products (Fig. 2*A*) and by primer extension experiments on gel-purified I-2 and I-4 products (Fig. 2*B*). The results indicated that His-XendoU pro-

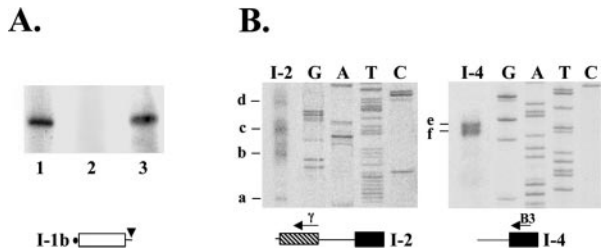

FIG. 2. **Characterization of His-XendoU properties.** *A*, analysis of 3′ termini of His-XendoU products. I-1b molecule (schematically represented *below*), obtained after incubation of unlabeled 003 RNA with His-XendoU, was 5′-[^{32}P]pCp-labeled directly (*lane 2*) or after kinase treatment (*lane 3*). In *lane 1*, a labeled marker I-1b molecule is run. *B*, mapping of His-XendoU cleavage sites. Unlabeled I-2 and I-4 molecules, generated after 10 min of incubation with His-XendoU, were gel-eluted and incubated with oligos γ and B3, respectively, for primer extension analysis. The products of the reaction were run in parallel with the sequencing reaction (*lanes G*, *A*, *T*, and *C*) carried out with the same oligonucleotides on 003 template. *Letters* on the left indicate His-XendoU cleavage sites defined by the extended products.

duces molecules carrying 2′-3′-cyclic phosphate ends and that it cleaves intronic sequences at the same U-rich sites recognized by the native enzyme (5). We concluded that the recombinant enzyme could be used for mutagenic analysis.

Analysis of XendoU-RNA Interaction—To analyze the binding of His-XendoU to its RNA substrate, we investigated the ability of the protein to bind RNA without cleaving it. For this purpose, the RNA-protein interaction was tested in the absence of Mn^{2+}, and the assembled complex was visualized by electrophoretic mobility shift assay. For this assay, a suitable RNA substrate was constructed (mini 003). Mini 003 RNA is 207 nucleotides long. It includes the upstream and downstream cleavage sites flanking the U16 snoRNA coding region and lacks the exon sequences (see schematic representation in Fig. 3*A*).

Fig. 3*A* shows that when incubated with His-XendoU this substrate produces the same cleavage profile (*lanes His-XendoU*) as that obtained with the oocyte nuclear extracts (*lanes ONE*). A typical gel-shift assay is shown in Fig. 3*B*. ^{32}P-Labeled mini 003 RNA was incubated with XendoU, at concentrations of 0.1 (*lane 2*) or 0.6 μM (*lane 3*) (see "Experimental Procedures"); the assembled complexes were then fractionated on native gels and visualized by autoradiography. Densitometric analysis showed that His-XendoU, at a concentration of 0.1 μM, is able to shift ~10–15% of the input RNA. Based on these data, we concluded that manganese ions are not required for RNA-protein interaction.

Association of XendoU to Its Substrate Is Specific and Requires Oligo(U)—To verify the specificity of the RNA-protein interaction, competition experiments were performed. A gel mobility shift assay was carried out by incubating ^{32}P-labeled mini 003 RNA with His-XendoU alone, at a protein concentration (0.6 μM) that shifts 90% of the RNA input or in the presence of different concentrations of cold competitors. The RNAs used as competitors were mini 003 RNA, *Escherichia coli* tRNA (Fig. 3*C*) and the two oligoribonucleotides, P1 and P3 (Fig. 3*D*). P1 oligo contains the distal cleavage site upstream to U16 (site d), whereas P3 is its mutant derivative in which the cleaved sequence UUU is replaced by the sequence UCU. This analysis revealed that the RNA containing the XendoU cleavage sites (mini 003 competitor) was able to compete out the binding of the protein already at a concentration of 4 nM, whereas tRNA molecules did not compete even at higher concentrations. The use of smaller 20-nucleotide long oligoribonucleotides P1 and P3 provided additional information about the sequence requirements for RNA-protein interaction. Three U residues present in P1 oligo are sufficient to make this RNA an active competitor, whereas the substitution of three U residues with the sequence UCU makes the competitor P3 completely inactive. These data are consistent with functional data, which showed that P1 is a substrate for XendoU cleavage, whereas P3 is not (5). These results support the conclusion that the binding of His-XendoU to its substrate is highly specific and revealed a strict requirement for oligo(U) sequences.

Prediction of XendoU catalytic residues—A data base search with the whole XendoU sequence revealed homology to a number of proteins from animals (both vertebrates and invertebrates) and plants, including a few surprising hits to cyanobacteria. The only group of identified proteins with established annotations were putative serine proteases, with homologs found in human, mouse, fruit fly, and nematodes. Given that XendoU and its homologs did not contain any known protein domain, and in particular those with a known RNase activity, we generated a multiple alignment to identify the most conserved residues (Fig. 4). Knowing that XendoU needs Mn^{2+} for its activity (5), we looked in particular for the conserved residues that may bind metal ions or those that are typically involved in metal-based cleavage by nucleases. Divalent metal ions are commonly bound by carboxylate groups of acidic residues (Asp and Glu) and are thought to stabilize the transition state in cleavage reactions. Often found in these enzymes are Asp-His conserved pairs that are used to deprotonate a water molecule for nucleophilic attack on the phosphate (20, 21). It also has been shown that catalytic His residues can be directly involved by forming hydrogen bonds to one of the oxygens in of the target phosphate (22). The most conserved part of the alignment is between XendoU residues 157–167, with a consensus sequence SSsFEHVF*x*sE (where capital letters represent individual amino acids, while "s" and "x" are for small and any amino acid residues, respectively). We considered this region a prime candidate for the active site of XendoU and decided to mutate potential catalytic residues identified by red triangles in Fig. 4. In addition, we singled out for mutagenesis other conserved residues that were outside of this putative active site (Glu-92, His-178, and Lys-224 of XendoU, marked by *green triangles* in Fig. 4).

Because many conserved residues were identified (Fig. 4, *yellow shade*), we reasoned that not all of them are involved in catalysis, in particular, because XendoU can bind RNA. To test whether RNA binding and catalytic functions are separated within the protein, we decided to individually mutate residues 232–236 in yet another conserved patch of XendoU (Fig. 4, *blue triangles*).

While this work was in preparation, it was reported that nidoviruses contain a homolog of XendoU named NendoU (12, 13). This group of viral proteins never appeared as significant hits in our PSI-BLAST searches, even when we raised the significance threshold. However, in reciprocal searches starting with viral proteins, the homologs of XendoU appeared just below the significance threshold. Because XendoU and NendoU proteins do not align over their whole lengths, we used profile-profile alignments (17) to define the region they share. This common region encompasses our predicted active site, and the multiple alignment of XendoU and NendoU homologs is shown in supplemental Fig. 1. Red triangles in that figure mark XendoU residues mutated in this study, whereas blue triangles denote residues mutated by Ivanov *et al.* (12).

Construction of XendoU Mutant Derivatives—A collection of XendoU mutant derivatives was produced by site-directed mutagenesis. Two Glu residues of XendoU, Glu-161 and Glu-167, were substituted both simultaneously and individually by Gln, giving rise to the double mutant E161Q/E167Q and to the single mutants E161Q and E167Q. Mutants E92Q and E236Q

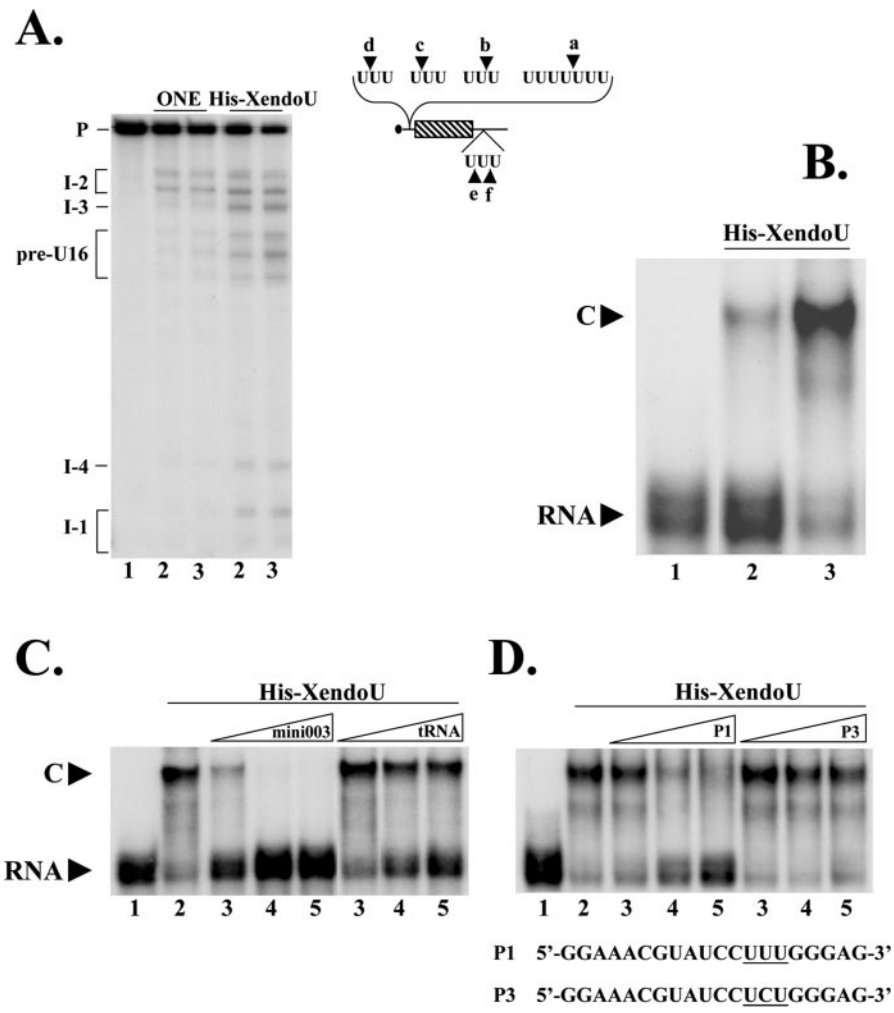

FIG. 3. **Processing of mini 003 RNA substrate and gel mobility shift assays.** *A*, *in vitro* processing assay. ^{32}P-Labeled mini 003 RNA was incubated with 5 μg of unfractionated oocyte nuclear extracts (*lanes ONE*) or with 40 ng of recombinant His-XendoU (*lanes His-XendoU*), under standard conditions, for 0 (*lane 1*), 5 (*lanes 2*), or 10 min (*lanes 3*). The RNA precursor (*P*) and the specific cleavage products are indicated on the *side*. *B*, gel mobility shift assay of His-XendoU. 2 fmol of ^{32}P-labeled mini 003 RNA were incubated with XendoU at a concentration of 0.1 (*lane 2*) or 0.6 μM (*lane 3*). As control non-incubated RNA was loaded in *lane 1*. The RNA-protein complexes were fractionated on 8% polyacrylamide native gel and visualized by autoradiography. *C*, competition experiment with mini 003 RNA and *E. coli* tRNA as competitors. The same gel mobility shift assay as in *B* was carried out by incubating 2 fmol of ^{32}P-labeled mini 003 RNA with His-XendoU alone, at a concentration of 0.6 μM (*lane 2*), or in the presence of 4 (*lanes 3*), 40 (*lanes 4*), or 100 nM (*lanes 5*) of cold competitors. As control non-incubated RNA was loaded in *lane 1*. *D*, competition experiment with the oligoribonucleotides P1 and P3 as competitors. The sequences of the two oligoribonucleotides are shown *below*. P1 contains the distal cleavage site upstream to U16 (site d), P3 is its mutant derivative in which the cleaved sequence UUU is replaced by the sequence UCU. 2 fmol of ^{32}P-labeled mini 003 RNA were incubated with His-XendoU alone, at a concentration of 0.6 μM (*lane 2*) or in the presence of 40 (*lanes 3*), 400 nM (*lanes 4*), and 4 μM (*lanes 5*) of cold competitors. In *lane 1* non-incubated RNA was loaded. In *B*, *C*, and *D* panels the *arrows* point to free RNA (*RNA*) and to the RNA-protein complex (*C*).

were generated by substitution of Glu-92 and Glu-236 by Gln. Acidic residues were replaced with their amides, because these mutations remove the carboxyl group needed for metal coordination while negligibly affecting the whole structure of the protein (23). Substitution of His-162 and His-178 by Ala produced the mutant H162A and H178A. Finally, Ser-157, Lys-224, Gly-232, Ser-234, and Pro-235 were individually replaced by Ala producing mutants S157A, K224A, G232A, S234A, and P235A. XendoU mutant derivatives, expressed as recombinant proteins in bacterial cells, were first tested for their ability to interact with the RNA substrate in a mobility shift assay (Fig. 5). ^{32}P-Labeled mini 003 RNA was incubated with increasing concentrations of wild type XendoU and its mutant derivatives. Using Packard Instant Imager, we calculated, for each mutant, the protein concentrations giving 10–15% shift of the RNA substrate. For proper comparison of binding activities for different mutants, we determined the amount of active protein in each preparation by reverse titration experiments with constant amounts of protein and increasing amounts of the hot probe (data not shown). The results of such analyses indicated that all mutated proteins were able to bind mini 003 RNA. Some of them (H178A, K224A, G232A, P235A, S234A, and S237A) bind the RNA substrate as efficiently as the wild type protein, whereas other mutants (E161Q/E167Q, H162A, and E92Q) interact less efficiently. E236Q is the only protein that shows higher affinity for the RNA substrate (see Fig. 6*A*).

Characterization of Processing Activity of XendoU Mutant Derivatives—We first established the amounts of wild type and of mutated proteins needed to bind 10–15% of the RNA substrate, to standardize subsequent studies of the processing activity. The predetermined amounts of proteins were incubated with a constant amount of ^{32}P-labeled RNA substrate, and the efficiency and specificity of processing activity were evaluated. For those mutants that did not display any detectable cleavage activity (H162A, E161Q/E167Q, H178A, and K224A), we tested higher concentration of proteins (up to 4 μM) in the activity assay (Fig. 6*B*). Such analysis allowed us to identify five residues (Glu-161, Glu-167, His-162, His-178 and Lys-224) that are indispensable for cleavage. Interestingly, the single mutants E161Q and E167Q were still able to specifically process the transcript (data not shown), indicating that individually these two residues have functions that are not strictly required for the catalytic activity. Somewhat surprisingly, G232A, P235A, and E236Q proteins at the concentration giving 15% of RNA shift did not produce an efficient cleavage pattern. However, at higher concentrations (2 μM for G232A and P235A and 0.4 μM for E236Q), they were able to process the RNA substrate specifically.

DISCUSSION

In this paper, we report functional characterization of XendoU, the *X. laevis* endoribonuclease responsible for processing several intron-encoded snoRNAs. Our results shed new light on the association between XendoU and its substrate, including the identification of XendoU residues that participate in catalysis.

The chemical requirements and specificity of RNA-protein interaction were investigated by *in vitro* binding assays. Such analysis revealed that the interaction of XendoU to its RNA

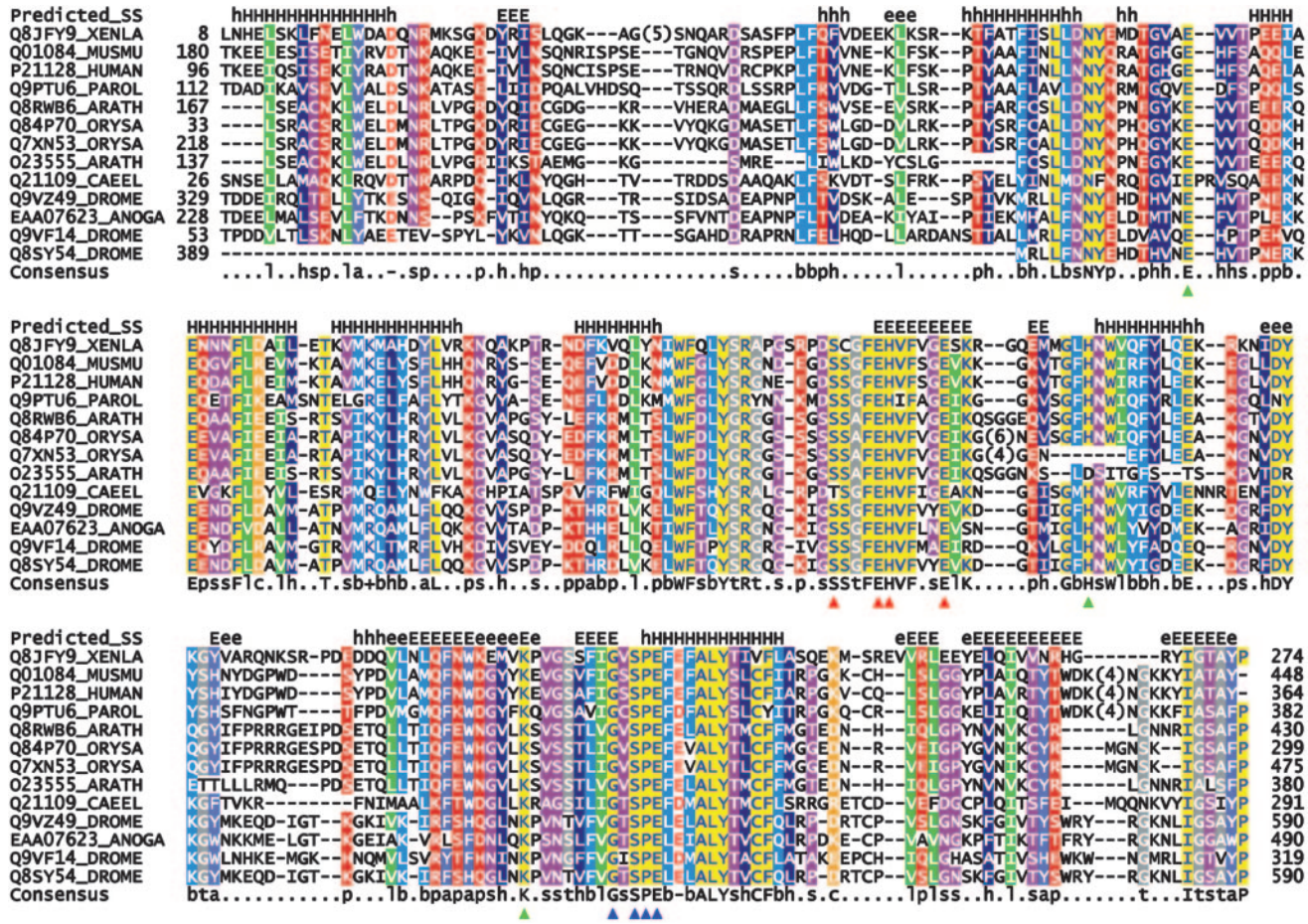

FIG. 4. **Sequence alignment of XendoU and its eukaryotic homologs.** The alignment includes secondary structure prediction (15) above the sequences (*H* for helices, *E* for strands, *C* for coils, shown in *lowercase letters* when the reliability of prediction is less than 70%). The residues are colored according to physicochemical properties (*b*, big; *s*, small; *t*, tiny; *h*, hydrophobic; *a*, aromatic; *l*, aliphatic; *p*, polar; +, positively charged; −, negatively charged) or shown in *yellow* when a single amino acid is found in at least 80% of all sequences. Aligned sequences are denoted by their SwissProt numbers and standard species abbreviations. Mutated residues are indicated by *red*, *green*, and *blue triangles* beneath the alignments (see text for details).

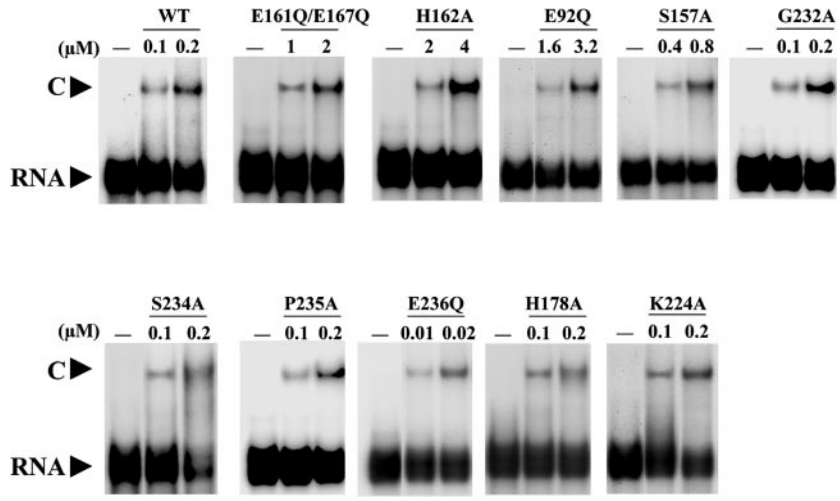

FIG. 5. **Characterization of binding activity of XendoU mutant derivatives.** Band shift assays were performed by incubating 2 fmol of ^{32}P-labeled mini 003 RNA with increasing concentrations (expressed in μM above each lane) of wild type (*WT*) XendoU and its mutant derivatives. The *arrows* point to the RNA-protein complex (*C*) or to the non-incubated RNA (*RNA*).

substrate does not require metal ions. Because manganese ions are essential for cleavage (5), we concluded that RNA binding and processing activities can be functionally separated.

The specificity of the interaction was verified by competition experiments. We showed that oligo(U) sequences are directly involved in binding and recognition of the enzyme; in fact, the UUU → UCU mutation is sufficient to prevent binding. This indicates that U stretches represent not only the target for cleavage activity (5) but also the critical element for protein recognition. The low XendoU sequence specificity observed *in vitro* contrasts with the enzyme selectivity *in vivo* (4) suggesting that the enzymatic activity in the cellular context is finely controlled. Therefore, it seems likely that specific factors and/or secondary structures assist the function of the enzyme *in vivo* by providing substrate selectivity. A similar conclusion has already been reached for other enzymes such as the endoribonuclease Rnt1p, which participates in the biosynthesis of monocistronic and polycistronic snoRNA transcripts in yeast

A.

Mutants	Protein (μM)
Wild type	0.1
E161Q/E167Q	1
H162A	2
E92Q	1.6
S157A	0.4
G232A	0.1
S234	0.1
P235A	0.1
E236Q	0.01
H178A	0.1
K224A	0.1

B.

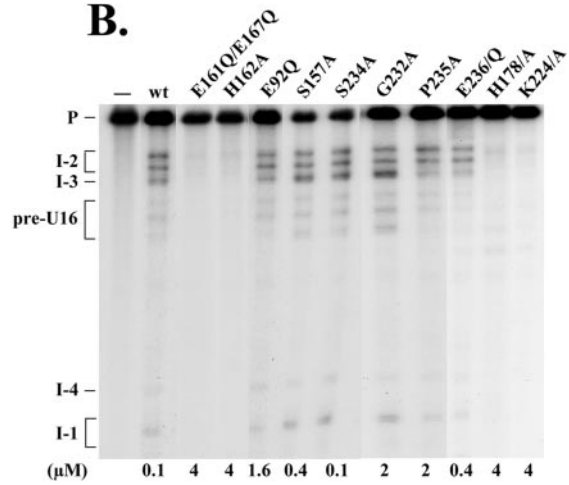

FIG. 6. **Analysis of activity of XendoU mutants.** *A*, binding activity calculated as the protein concentrations getting 10–15% of RNA shift. These values were calculated by Instant Imager quantification on band shift gels shown in Fig. 5. *B*, catalytic activity of different XendoU mutant derivatives analyzed by processing assays. The different proteins at the specific concentrations indicated below, were incubated with 2 fmol of ^{32}P-labeled mini 003 RNA for 20 min under the standard conditions. The specific cleavage products are indicated on the *side*.

(24, 25). It has been shown that the interaction of specific snoRNP proteins with U18 snoRNA recruits Rnt1p to the specific target, thus activating cleavage (26). Another example is RNase E, where the stem-loop structures of its target RNA modulate the enzyme activity by limiting access to potentially susceptible sites (27).

The identification of the residues involved in XendoU catalysis was primarily based on prediction data. A comparison analysis showed sequence homologies between XendoU and several proteins from animals, plants, and even cyanobacteria, suggesting a very ancient origin for this protein. XendoU does not contain any known protein domain; among its homologs, the only annotated function was that of putative serine proteases. After establishing the most conserved residues by multiple alignments, we generated several XendoU mutants containing specific amino acid substitutions. They were first tested for the ability to interact with the RNA substrate and then for processing activity.

Our experiments identified several residues that are important for the catalytic function of XendoU. The first group includes five amino acids (Glu-161, Glu-167, His-162, His-178, and Lys-224) that are essential for the catalytic activity (Fig. 6). Based on their chemical nature and the known residue requirements for other nucleases (20, 28), His-162, His-178, and Lys-224 are probably directly involved in the catalysis. Because Glu-161 and Glu-167 are individually dispensable for catalysis, their role may be indirect, *e.g.* their carboxylate groups could form hydrogen bonds with imidazole rings of the catalytic histidines (20). Alternatively, glutamates and histidines could be involved in the coordination of the manganese ions, thus promoting the nucleophilic attack.

Mutants in a second group of residues (Gly-232, Pro-235, and Glu-236) display only reduced catalysis (Fig. 6). These three residues could have indirect, yet important, roles in catalysis. It has been proposed for 5-phosphatases, which share the same fold with Mg^{2+}-dependent nucleases (21), that a conserved Gly may be important, because its unusual conformation, which is not allowed for non-Gly residues, is needed to correctly position the nearby Asp (29). Gly-232 and Pro-235 are in the short loop that is sandwiched between a predicted β-strand and α-helix, raising the possibility that these conformationally flexible residues are needed for the tight turn between the two structural elements. Other conserved residues, such as Glu-92, Ser-157, and Ser-234, do not affect XendoU catalysis significantly (Fig. 6). Interestingly, among all the mutants analyzed, only the replacements of few residues (Glu-161, Glu-167, Glu-92, and His-162) significantly reduce the binding affinity of XendoU, indicating that binding and catalytic activities of XendoU are partially overlapped.

XendoU is the cellular homolog of NendoU, an endoribonuclease that is highly conserved in coronaviruses and in all their distant nidovirus relatives. Both nucleases are involved in basic RNA processing events: NendoU is required for nidovirus RNA synthesis, whereas XendoU is implicated in the biosynthesis of cellular snoRNAs. Notably, three of five catalytically essential residues (His-162, His-178, and Lys-224) coincide with those already described as essential for NendoU catalytic activity (12). The other two residues participating in the catalysis are in both cases acidic residues, glutamates for XendoU (Glu-161 and Glu-167) and aspartates for NendoU.

The fact that the homologous region shared by the two enzymes includes the putative active sites supports the conclusion that this domain carries out a conserved function. The two distantly related enzymes indeed display analogous activities. They exhibit a similar substrate specificity, the same chemistry of reaction, and the same ion requirement. However, several differences can be pointed out. NendoU, whose natural substrate is still unknown, shows a preference for cleavage of double stranded RNA substrate and displays a U specificity confined to single U residues (12).

Based on its biochemical features, XendoU cannot be included in either of the two RNase groups already described (6). We propose XendoU as the founder of a third class of RNA processing enzymes whose members require manganese ions for the catalysis and release products with 2′-3′-cyclic phosphate termini. It would be very interesting to investigate the catalytic mechanism of the members of this new RNase family. We can envision different modes of action. One possibility is that the identified histidine residues could be directly involved in the catalysis by acting as nucleophiles, with manganese ions stabilizing the transition state intermediate. Alternatively, Mn^{2+}can have a direct role in a phosphoryl-transfer reaction. In this context, the catalytically relevant glutamates and histidines could coordinate manganese ions that promote RNA cleavage by deprotonizing water molecules. This possibility is especially attractive, because we previously demonstrated that the natural substrate of XendoU, the third intron of the *X. laevis* L4 r-protein gene, is able to release the U16 snoRNA precursor by a self-cleavage reaction (30). Such autocatalytic processing requires Mn^{2+} ions and releases products containing 2′-3′-cyclic phosphate termini. Nonetheless, cleavage sites utilized in this autocatalytic process encompass but do not coincide with those recognized during enzymatic reaction (30). It is tempting to speculate that during molecular evolution a predominantly RNA-based mechanism was replaced by a pro-

tein-mediated reaction in which Mn^{2+} still induces endonucleolytic cleavage, whereas the protein moiety determines the specificity of the reaction. Future structural studies will eventually provide deeper understanding of the catalytic mechanism employed by these endoribonucleases.

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Functional Characterization of XendoU, the Endoribonuclease Involved in Small Nucleolar RNA Biosynthesis

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