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The *Xenopus* intron-encoded U17 snoRNA is produced by exonucleolytic processing of its precursor in oocytes

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ABSTRACT

U17 is a small nucleolar RNA encoded in the introns of the *Xenopus laevis* gene for ribosomal protein S7 (formerly S8, see Note). To study the mechanisms involved in its *in vivo* processing from S7 transcripts, various *in vitro* synthesized RNAs embedding a U17 sequence have been microinjected into the germinal vesicle of *Xenopus* oocytes and their processing analysed. In particular, the *Xenopus U17* gene copies a and f and a *U17* gene copy from the pufferfish *Fugu rubripes* have been used. Information about the nature of the processing activities involved in U17 RNA maturation have been sought by injecting transcripts protected from exonucleolytic attack at their 5′-end by capping and/or lengthened at their 3′-end by polyadenylation. The results obtained indicate that U17 RNA processing is a splicing-independent event and that it is mostly or entirely due to exonucleolytic degradation at both the 5′- and 3′-ends of the precursor molecules. Moreover, it is concluded that the enzymes involved are of the processive type. It is suggested that the apparatus for U17 RNA processing is that responsible for the degradation of all excised and debranched introns. Protection from exonucleolytic attack, due to the tight structure and/or to the binding of specific proteins, would be the mechanism by which U17 RNA is produced.

INTRODUCTION

Xenopus U17 RNA is a 220 nt small RNA (1; where it was called $U17^{XS8}$ RNA) encoded by sequence repeats (copies a–f) present in each of the six introns of one of the two gene copies coding for ribosomal protein S7 (formerly S8, see Note). This peculiar genome organization has been described for several other small nucleolar RNAs (snoRNAs) that in vertebrates are encoded in the introns of ribosomal protein (r-protein) genes or of genes for other ribosome- or nucleolus-associated proteins (for references see 2–4). Occasionally during evolution the snoRNA coding sequences can jump from one 'host gene' to another. This is exemplified by U17 RNA (5), which is encoded in the introns of the S7 r-protein gene in *Xenopus* (1) and in two introns of the human gene for RCC1 (6), which is a chromatin-bound protein involved in several nuclear processes (7).

The specific function of U17 RNA, as for other snoRNAs, is not yet understood, although their roles in rRNA processing and/or ribosome assembly can be hypothesized. For some snoRNAs, regardless of whether they are encoded in introns or not, it has been shown that their depletion affects the rRNA processing pattern (8, and references therein). By DNA and RNA microinjection into *Xenopus* oocytes we have previously shown that U17 RNA, or at least its f copy, is not the product of independent transcription units, but is produced by processing of intron sequences of the r-protein S7 transcript (1), as also occurs for the other intron-encoded snoRNAs.

In the present paper we report analysis of the processing pathway leading to production of U17 RNA, carried out by microinjection into *Xenopus* oocytes of various *in vitro* synthesized transcripts. For this study we have used the *U17* gene copies a and f, located respectively in the first and sixth introns of the *Xenopus* r-protein *S7* gene, and a *U17* gene copy located in an intron of the homologous r-protein gene of *Fugu rubripes*, a fish recently adopted as a model for its small and compact genome (9).

MATERIALS AND METHODS

Plasmid constructions

Plasmids were constructed and prepared by standard procedures (10). Plasmids pi6 and pU17f′, containing the U17f sequence, have been previously described (1). Plasmid pi1, containing the U17a sequence, was obtained by cloning into the *Sma*I site of the Bluescript KS(+) vector a DNA fragment encompassing a portion of the first intron, including the U17a sequence, and part of the second exon of the *Xenopus S7* gene. This fragment was generated by PCR amplification of the region between nt 120 and 1314 of the *S7* gene (11).

Cloning and structural analysis of the *F.rubripes* homologous r-protein gene will be described elsewhere. Plasmids pF-S7.11 and pF-S7.15 were obtained by cloning PCR-generated DNA fragments

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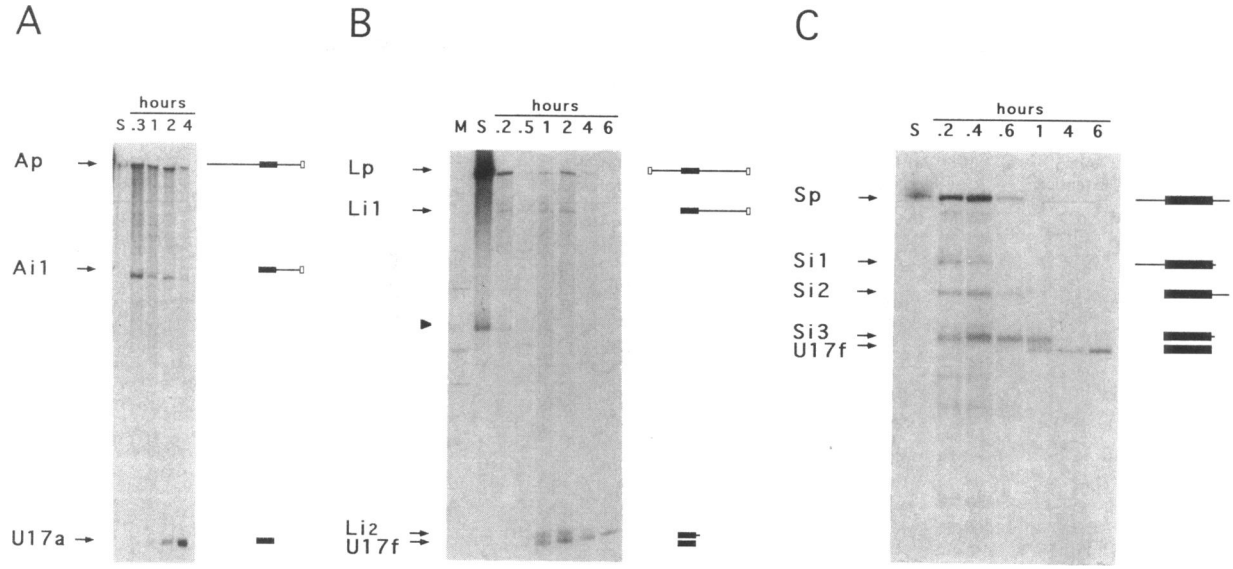

Figure 1. U17 RNA processing from intron transcripts injected into *Xenopus* oocytes. *In vitro* synthesized radioactive RNA precursors Ap, Lp and Sp were injected into the nuclei of *Xenopus* oocytes. After incubation nuclei were manually prepared from oocytes, the RNA extracted and analysed by denaturing polyacrylamide gel electrophoresis and autoradiography. Results relative to injection of transcripts Ap (**A**), Lp (**B**) and Sp (**C**). On the left of each panel the positions of the precursor substrates, of the intermediate products and of mature U17 RNA are indicated, while on the right schematic representations of the corresponding RNA molecules are shown. Lanes S, injected RNA substrates; lane M in (B), RNA size marker run in parallel. An arrowhead in (B) indicates the position of a short product, due to a premature transcription stop or to degradation, present in the transcript before injection.

into the *Sma*I site of the Bluescript KS(+) vector: the two cloned fragments, both bearing the U17 homologue sequence, cover the putative third intron and part of the flanking third and fourth exons of the *S7* gene homologue (pF-S8.11) respectively and only a part of the putative third intron and a portion of the 3′ flanking fourth exon (pF-S7.15).

In vitro synthesis of radioactive transcripts

To obtain the transcripts to be used as processing substrates the plasmids (1 μg) were cut with the appropriate enzymes and transcribed with T7 RNA polymerase (pi6, pi1, pF-S7.11 and pF-S8.15) or with SP6 RNA polymerase (pU17f′) in the presence of 50 μCi [α-^{32}P]CTP as described (12). The *Xenopus* 1260 (Ap), 1268 (Lp) and 345 nt (Sp) transcripts were obtained respectively from the pi1, pi6 and pU17f′ plasmids. The *Fugu* 592 (FLp) and 516 nt (FSp) transcripts were obtained from plasmids pF-S7.11 and pF-S7.15 respectively. After transcription and DNase digestion the RNAs were purified by phenol/chloroform/isoamyl alcohol (50:50:1) extraction and ethanol precipitation and resuspended in H_2O for microinjection.

The 5′ capped RNAs were produced by *in vitro* transcription in the presence of the dinucleotide m^{7}G(5′)ppp(5′)G (Boheringer) in a 10-fold molar excess over GTP (13). To control capping efficiency *Eco*RI-linearized Bluescript KS(+) plasmid (originating a 64 nt transcript) was added as an internal control in the T7 RNA polymerase reactions; the 1 nt shift in the size of this control transcript was checked by gel electrophoresis. The 3′ polyadenylated RNAs were produced by post-transcriptional polyadenylation of previously synthesized RNAs with *Escherichia coli* poly(A) polymerase as already described (14).

RNA microinjection into *Xenopus* oocytes

Isolation of stage V–VI oocytes and microinjection of RNA into the germinal vesicle were carried out essentially as previously described (15). Oocytes were injected with 40 nl H_2O containing ~80 000 c.p.m. (corresponding to 10–40 ng) of the *in vitro* transcribed RNAs and incubated for increasing times at 22°C. After incubation nuclei from pools of 10 oocytes were manually prepared (15) and then lysed in 300 μl 100 mM Tris, pH 7.5, 300 mM NaCl, 10 mM EDTA, 2% SDS, containing 1 mg/ml proteinase K (16). RNA was extracted and analysed by 6 or 8% polyacrylamide–8 M urea gel electrophoresis according to standard procedures. RNA extracted from the cytoplasm of enucleated oocytes never yielded detectable radioactive bands.

RNA analysis

RNA analyses were performed essentially according to standard laboratory manuals (10). Probes for RNase mapping were prepared from 1 μg linearized plasmid template (pi6, pU17f′ or pi1) as described (1,12) using NTPs at a total concentration of 0.5 mM. RNase mapping experiments were performed according to Zinn *et al.* (17), using gel-purified bands as substrates. Reaction products were treated for 15 min with 0.1 mg/ml proteinase K, purified by phenol/chloroform/isoamyl alcohol (50:50:1) extraction and ethanol precipitation and analysed on a sequencing gel together with DNA and RNA size markers. Primer extension analysis was performed as follows. A synthetic oligonucleotide complementary to the U17a sequence from nt 22 to 38 (1) was 5′-end-labelled with T4 polynucleotide kinase in the presence of 30 μCi [γ-^{32}P]ATP. The labelled primer was hybridized to gel-purified fragments, extended with reverse transcriptase and the products analysed as above (10).

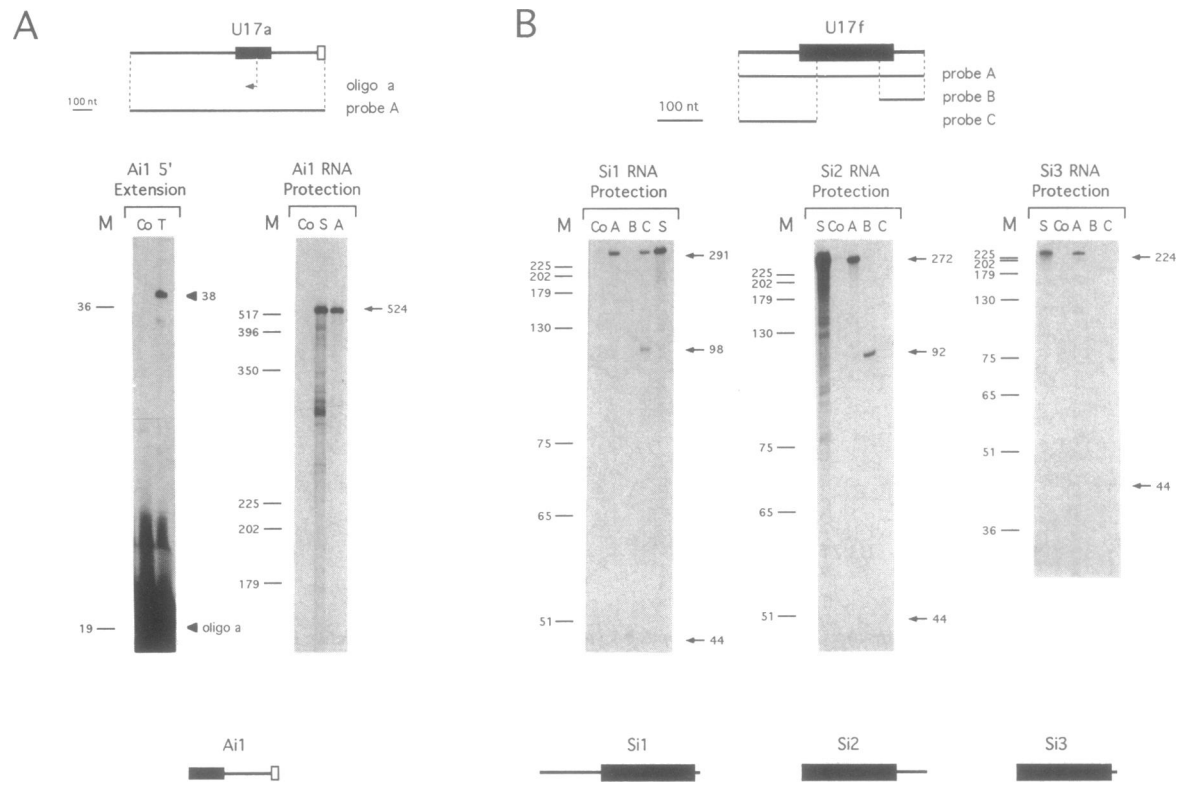

Figure 2. Analysis of the termini of the U17 RNA processing intermediates. At the top are given schematic representations of the probes for RNase protection and the primer for the extension experiment. At the bottom are shown the determined structures of intermediates. (**A**) The Ai1 intermediate (see Fig. 1) has been analysed by primer extension (left panel) and RNase protection (right panel). (Left) Lanes Co and T, extended products without and with RNA template respectively. (Right) Lane Co, control without antisense RNA; lane S, RNA substrate (gel eluted band); lane A, protection by probe A. (**B**) The Si1, Si2 and Si3 intermediates (see Fig. 1) have been analysed by RNase protection experiments. Lanes Co, controls without antisense RNA; lanes S, RNA substrates (gel eluted bands); lanes A–C, protection by probes A, B and C respectively. Filled arrowheads in (**A**) (left) indicate the primer (19 nt) used for extension and the major extended product (38 nt). Arrows in (**A**) (right) and (**B**) point to the protected bands whose deduced sizes (nt) are indicated. Size marker positions are indicated (nt) on the left of each panel.

RESULTS

The production of U17a and U17f RNAs is splicing independent

The time course of the processing of three r-protein S7 gene transcripts containing the U17 sequences a and f has been studied by microinjection into *Xenopus* oocytes. The following radioactive RNAs, synthesized *in vitro* as described in Materials and Methods, have been used: (i) a 1260 nt transcript (Ap) containing almost the entire first intron of the *S7* gene, including the U17a RNA sequence, and part of the second exon; (ii) a 1268 nt transcript (Lp) containing the entire sixth intron, including the U17f RNA sequence, and part of the flanking sixth and seventh exons; (iii) a 345 nt transcript (Sp) corresponding to a small portion of the sixth intron that contains the U17f RNA sequence. After incubation of the injected oocytes total RNA was extracted and analysed by gel electrophoresis and autoradiography. The results obtained in these experiments are shown in Figure 1. On the right of each panel we have schematically represented the structures of the injected transcripts (Ap, Lp and Sp), of the mature U17 RNA and of the intermediate molecules (Ai1, etc.), deduced by RNase mapping and/or primer extension analysis of the relevant RNA bands recovered from gel slices (most relevant 5′- and 3′-end mappings are shown in Fig. 2).

Figure 1A shows that the injected transcript Ap is processed to produce mature U17a RNA. An intermediate product (band Ai1) is also visible after short incubations, which then disappears in parallel with accumulation of the mature product. RNase mapping and primer extension analyses have shown that the Ai1 intermediate has a 5′-end corresponding to the 5′-end of mature U17a RNA and the still intact 3′-end of the precursor transcript (Fig. 2A). Figure 1B and C shows, respectively, that the long (Lp) and the short (Sp) transcripts of the sixth intron, containing the f copy of U17 RNA, are also processed to produce the mature molecule. In both cases intermediate products are visible during the time course. The Li1 intermediate of the Lp transcript has the same 5′-end as mature U17f RNA and the 3′-end of the precursor transcript. The situation is somewhat different in the case of the Sp transcript, characterized by short sequences flanking the U17f RNA, 73 and 54 nt at the 5′- and 3′-ends respectively. In fact, the Si1 intermediate has a 5′-end corresponding to that of the injected transcript (Fig. 2B), while the 3′-end is almost identical to that of mature U17 RNA (with a few extra nucleotides). In contrast, Si2 has a 5′-end corresponding to that of mature U17 RNA, but still bears the intact 3′-end of the injected transcript (Fig. 2B).

It must be mentioned that, while the processing patterns of injected transcripts are qualitatively reproducible when using oocytes from different *Xenopus* individuals, sometimes they are quantitatively not perfectly reproducible, the relative abundance

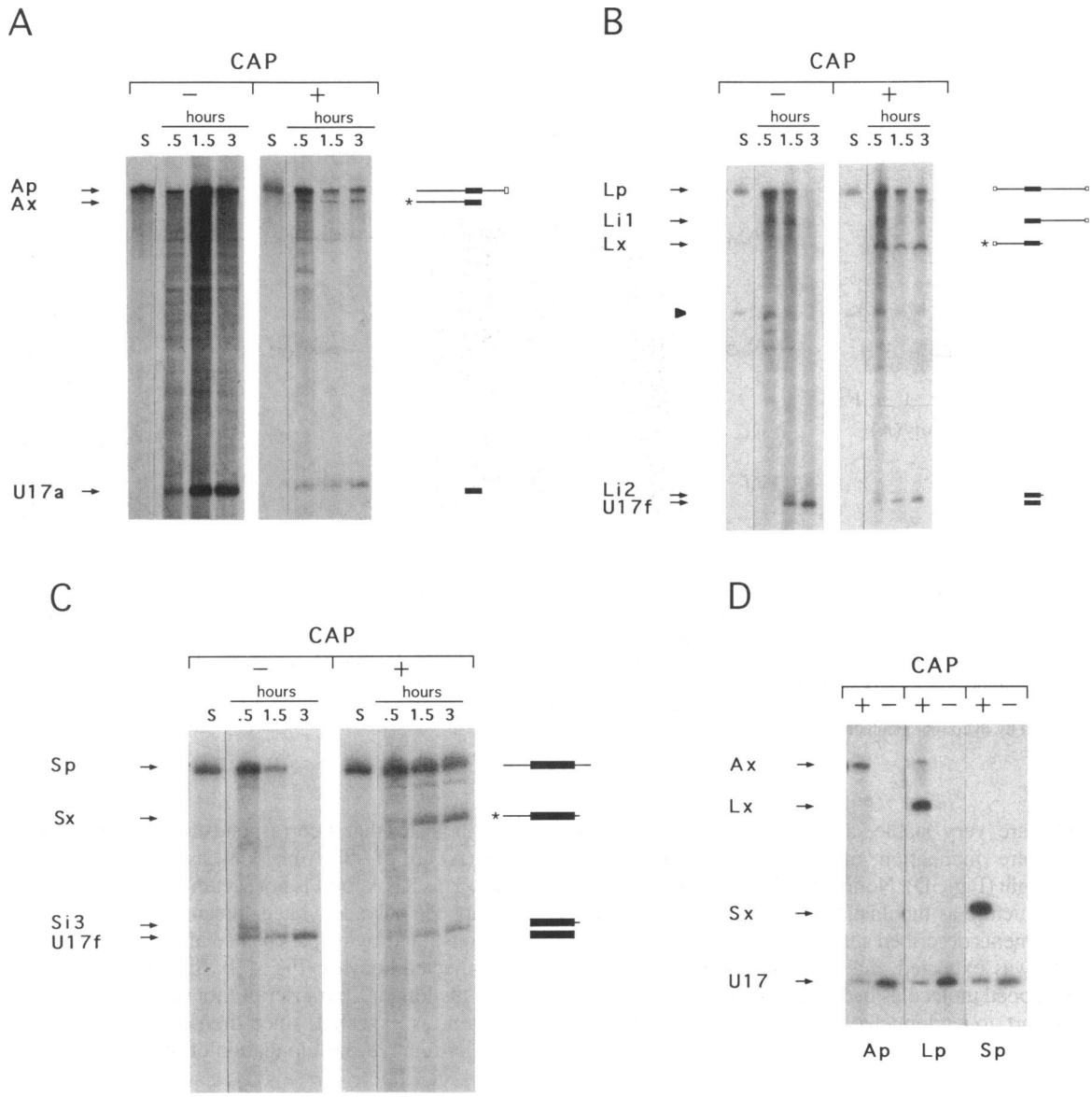

Figure 3. U17 RNA processing from capped transcripts injected into *Xenopus* oocytes. Results obtained in injection experiments performed respectively with uncapped and capped transcripts Ap (**A**), Lp (**B**) and Sp (**C**). After incubation the RNA was extracted from manually dissected nuclei and analysed. Lanes S, injected RNA substrates. On the left of each panel the positions of the precursor substrates, intermediate products and mature U17 RNA are indicated, while on the right schematic representations of the corresponding RNA molecules are shown. The stars indicate capped 5′-ends. (**D**) Results obtained on injection of the three capped and uncapped precursors followed by overnight incubation.

of the intermediates being somewhat variable. Although in this paper we only show some example experiments, our conclusions are derived from results obtained in several duplicates (not shown).

5′-End processing of U17 RNA precursors is inhibited by 5′ capping

To investigate the catalytic nature of the processing enzymes involved in production of U17 RNA we injected *Xenopus* oocytes with radioactive RNA precursors (Ap, Lp and Sp) capped at their 5′-end by addition of the dinucleotide $m^7G(5')ppp(5')G$ to the *in vitro* transcription reaction. In order to evaluate capping efficiency *Eco*RI-linearized Bluescript KS(+) plasmid was included in the reaction mixture as an internal standard; this originates a 64 nt transcript whose capped and uncapped forms can be distinguished by gel electrophoresis. This internal control RNA was in general capped with very good efficiency (not shown), thus we assumed that the co-transcribed RNA precursors were also mostly capped. Figure 3A–C shows the results obtained in injection experiments with capped and uncapped Ap, Lp and Sp transcripts respectively. It appears that in all three cases the production of mature U17 RNA from injected capped transcripts, compared with uncapped, was substantially reduced. In fact, a new band accumulates during processing of each of the three capped transcripts (bands Ax, Lx and Sx respectively), which is not observed during processing of the uncapped counterparts. These RNA forms, whose sizes indicate processed 3′-ends and

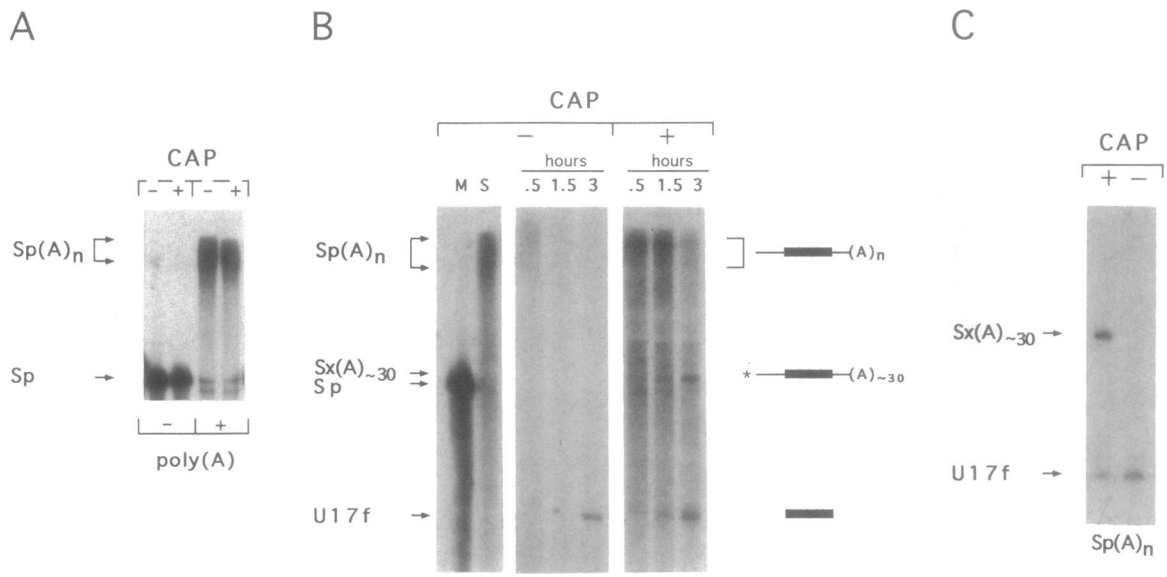

Figure 4. U17 RNA processing from polyadenylated Sp transcript injected into *Xenopus* oocytes. (**A**) Control of polyadenylation efficiency. Capped and uncapped Sp transcripts have been polyadenylated and electrophoresed in parallel with the corresponding non-polyadenylated transcripts. Arrows point to substrate transcript and polyadenylated products. (**B**) Results obtained on injection into oocytes of the capped and uncapped polyadenylated Sp transcript. After 0.5, 1.5 and 3 h incubation the RNA was extracted from manually dissected nuclei and analysed. Lane S, injected RNA substrate; lane M, precursor transcript before polyadenylation. On the left of the panel the positions of the precursor substrates, intermediate products and mature U17 RNA are indicated, while on the right schematic representations of the corresponding RNA molecules are shown. The star indicates the capped 5′-end. (**C**) Results obtained on injection of the same capped and uncapped polyadenylated precursors followed by overnight incubation of the injected oocytes.

intact 5′-ends, are very stable, as demonstrated in a similar experiment where incubation of the injected oocytes was extended overnight (Fig. 3D). Notice that the stable RNA form Sx has the same structure as the unstable Si1 except for the 5′ cap. In all the experiments described some mature U17 RNA was still produced; this was expected, due in part to the presence of a fraction of uncapped molecules in the injected transcript preparations and in part to endonucleolytic degradation that would expose uncapped 5′-ends.

3′-End processing of the U17 RNA precursors is slowed by 3′ polyadenylation

Another set of injection experiments has been carried out with Lp and Sp transcripts modified at their 3′-end by polyadenylation. In order to control the *in vitro* polyadenylation efficiency the addition of the poly(A) tail to the capped and uncapped transcripts was monitored by gel electrophoresis. The results obtained with the short transcript Sp are shown in Figure 4. Both uncapped and capped Sp transcripts have acquired long poly(A) tails, ranging from 500 to 600 A residues (Fig. 4A). These capped and uncapped polyadenylated transcripts were microinjected into the oocyte nuclei. Figure 4B shows that the polyadenylated Sp transcript is also processed to produce mature U17f RNA, but this processing requires a longer time compared with the non-polyadenylated transcript shown in Figure 3C. This comparison is possible since the experiments shown in Figures 3 and 4 were performed in parallel on the same oocyte batch. Moreover, during processing of the capped polyadenylated Sp transcript another band [Sx(A)$_{\sim 30}$] accumulated, besides that of mature U17f RNA. The 3′-end of this RNA form has not been mapped; however, its size indicates that it corresponds to the Sp transcript plus a short poly(A) tail of ~30 A residues, as also occurrs in other systems (25). This RNA form is not accumulated during processing of the uncapped polyadenylated precursor and is very stable, as demonstrated by its persistence after an overnight incubation of the injected oocytes (Fig. 4C). Similar experiments carried out with the longer Lp transcript (not shown) are consistent, but less evident, as expected, since the poly(A) tail represents a much smaller relative size elongation of the transcript.

Precursor transcripts of *Fugu* U17 snoRNA are processed in the *Xenopus* oocyte nucleus

The availability of a genomic fragment from *F.rubripes* containing a U17 sequence allowed us to question whether the processing mechanism for production of U17 RNA is evolutionarily conserved. The following radioactive precursors of *Fugu* U17 RNA have been microinjected in the germinal vesicles of *Xenopus* oocytes: (i) a 592 nt transcript (FLp) containing the entire third intron, including the U17 RNA sequence, and part of the flanking third and fourth exons; (ii) a 516 nt transcript (FSp) containing almost the entire third intron, including the U17 RNA sequence, and part of the fourth exon. Figure 5 shows that the injected uncapped transcripts FSp and FLp are both correctly processed to produce mature U17 RNA (FU17); in both cases this is already visible after 30 min incubation, together with some intermediate forms which then decrease with time. Experiments have also been performed with capped and with polyadenylated FSp and FLp transcripts. The results obtained (not shown) are in line with those obtained with the capped and polyadenylated *Xenopus* transcripts (see above).

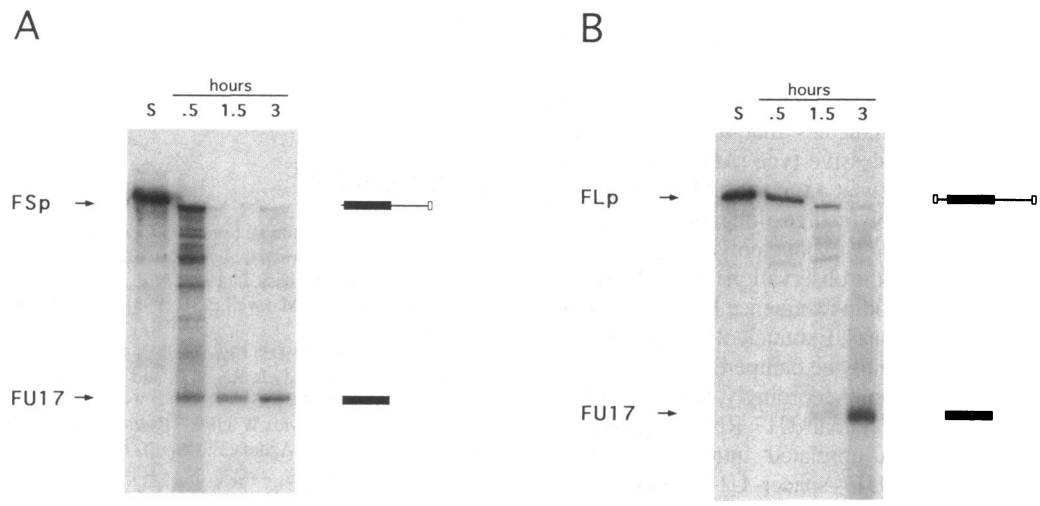

Figure 5. *Fugu* U17 RNA is correctly processed from transcripts injected into *Xenopus* oocytes. *In vitro* synthesized radioactive RNA precursors FSp and FLp have been injected in the nuclei of *Xenopus* oocytes. After incubation the RNA was extracted from manually dissected nuclei and analysed by denaturing gel electrophoresis. Results relative to injection of transcripts FSp (**A**) and FLp (**B**). Lanes S, injected RNA substrates. On the left of each panel the positions of the precursor substrates and of mature U17 RNA (FU17) are indicated, while on the right schematic representations of the corresponding RNA molecules are shown.

DISCUSSION

It has been shown that several snoRNAs are encoded in the introns of protein-encoding genes and that they are not produced by independent transcription, but by the processing of the host gene intron sequences (1,6,8,18–24). In the present work we have investigated the mechanisms involved in processing of one of these snoRNAs, U17 RNA, encoded in the six introns of the *Xenopus* gene for r-protein S7 (1) and in two of the introns of the human *RCC1* gene (6). We have previously shown that mature U17 RNA is produced by processing of a transcript of an *S7* gene intron injected into *Xenopus* oocytes (1). In the present paper we have used transcripts corresponding to two different introns (the first and sixth) of the *Xenopus S7* gene containing the sequences coding for U17a and U17f RNAs respectively. These two *U17* gene copies are slightly divergent (96% homology), as also are the other four copies present in the remaining introns of the same gene. A relevant point is that the three transcripts used in this work differ in their structure: one (Lp) contains the entire intron and portions of the two flanking exons; the other (Ap) contains most of the intron and part of the downstream exon, but lacks the 5′ donor splicing site; the third (Sp) is a small intron fragment containing the U17 sequences and a few tens of nucleotides upstream and downstream, lacking both donor and acceptor splice sites. The observation that all three transcripts are correctly and efficiently processed after microinjection into oocytes indicates that U17 RNA processing is a splicing-independent event, i.e. it does not require that the transcript goes through the splicing pathway.

During processing of the three transcripts a smear with many faint bands is visible in the gel, indicating a heterogeneous RNA population of decreasing size and a few well-defined intermediates. An intermediate consisting of RNA molecules precisely processed at their 5′-end and with a still intact 3′-end is always present. Only the very small precursor Sp also produces an appreciable amount of an intermediate with a still intact 5′-end and a processed 3′-end (except for the short tail). Experiments carried out with transcripts protected at their 5′-ends by capping and/or at their 3′-ends by polyadenylation led us to the conclusion that the production of intermediates and of mature U17 RNA is attained mainly by exonucleolytic activity. In fact, both modifications strongly reduce the efficiency of mature U17 RNA production. In the case of protection of the 5′-end by capping a large part of the precursor remains as a very stable form with an intact 5′-end. The remaining fraction, still processed with kinetics similar to those obtained for the control uncapped precursor, is due to incomplete capping and, possibly, to some endonucleolytic degradation exposing uncapped 5′-ends. In the case of 3′-end polyadenylation the kinetics of appearance of mature U17 RNA are much slower with respect to the non-polyadenylated transcript, as much longer 3′-ends must be removed. The appearance of a RNA form with ~30 A residues at the 3′-end is probably due to its interaction with poly(A) binding protein (PABP), which protects the RNA from exonucleolytic digestion (for references see 25). The finding that this occurs with the capped but not with the uncapped transcript is not surprising, as an interaction between the 5′ cap and 3′ poly(A) has been described (26, and references therein). We have also confirmed the previous observation (1,6) that U17 RNA with a few extra nucleotides at the 3′-end is produced first and then trimmed to give the final mature molecule.

Thus the results presented in this paper indicate that *S7* gene intron transcripts containing a U17 sequence are processed to give mature U17 RNA independently of the occurrence of *S7* gene transcript splicing and mostly or entirely by exonucleolytic activity at both 5′- and 3′-ends. Experiments performed with human U17 RNA, encoded in two *RCC1* gene introns, led to the conclusion that the mammalian counterpart is also produced by exonucleolytic activity (27). A somewhat different situation has been described for U16 RNA, encoded in a very small intron of the gene for the *Xenopus* r-protein L4 (formerly L1), where *in vitro* processing analysis has shown the occurrence of endonucleolytic cleavage followed by exonucleolytic trimming (28).

As to the type of exonucleases involved, it is relevant to notice that we have not observed a progressive change in electrophoretic

migration of the bands. On the contrary, we have observed a decrease in intensity of the precursor bands with a parallel increase in intensity of the intermediate and then of the mature products. A smear is always present in between the bands. This pattern of depolymerization indicates that the exonucleolytic activity involved is of the processive type. After injection of a large number of transcripts a few at a time would be taken up by the enzyme and their exonucleolytic digestion rapidly completed.

The production of snoRNAs by exonucleolytic degradation of intron sequences could explain the observation that in all the cases described only one snoRNA coding sequence has been found in each intron, even in very long ones. Exonucleolytic processing of snoRNA sequences would actually be hampered by the presence two or more in the same intron. Accordingly, the experimental insertion of two copies of the human U17 RNA sequence in a tandem arrangement into an unrelated intron leads to the accumulation of a continuous U17–spacer–U17 product (27).

Several cases of involvement of 5′→3′ and/or 3′→5′ exonucleases in RNA degradation processes are known, as for instance in the control of mRNA decay in the cytoplasm (26) and in 5.8S rRNA processing in the nucleolus (29). It is now shown here that 5′→3′ and 3′→5′ exonucleolytic activities are involved in the processing of U17 RNA. In our opinion these exonucleolytic degradations might represent a general mechanism for depolymerization of introns after these have been excised from primary transcripts and debranched. The presence in an intron of a highly structured RNA region complexed with specific proteins would create a nuclease-resistant core which remains stable as an 'intron-encoded RNA'. Thus its structure and its protein binding capacity would be sufficient for proper snoRNA processing, the surrounding intron sequences being uninfluential. This conclusion is also supported by the fact that no sequence homology can be detected among the six introns of the *Xenopus* gene for r-protein S7, except for the U17 RNA coding region, and by the finding that *Fugu* U17 RNA is correctly and efficiently processed in the *Xenopus* oocyte, in spite of the fact that, in this case also, intron homology between the two species is limited to the U17 sequence.

Note

In our previous papers we have used the ribosomal protein numbering system introduced in our first study of *Xenopus* r-proteins (30). The large amount of sequencing data now accumulated allows us to adopt, as a unified nomenclature, the rat system (31). Thus the r-protein that we previously designated S8 is now identified as XS7, meaning that it is the *Xenopus* r-protein homologous to the rat r-protein S7, or simply S7 when the specific origin is not relevant.

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