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Analysis of a sea urchin gene cluster coding for the small nuclear U7 RNA, a rare RNA species implicated in the 3′ editing of histone precursor mRNAs

(RNA processing/regulatory sequences/gene structure/initiation and termination of transcription)

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ABSTRACT **A genomic 9.3-kilobase DNA fragment of the sea urchin *Psammechinus miliaris*, containing a cluster of five U7-RNA genes (or pseudogenes), has been isolated and analyzed by partial DNA sequencing. The U7-RNA coding sequences differ from one another by one or two nucleotides, one of the five gene sequences being identical to those of the cDNA U73 clone prepared earlier [Strub, K., Galli, G., Busslinger, M. & Birnstiel, M. L. (1984) *EMBO J.* 3, 2801–2807]. The spacer sequences separating the genes have, on the whole, a low degree of homology; hence, the five genes must have arisen by an ancient duplication event. The sequences preceding the coding portion contain three highly conserved sequence motifs but no "TATA box." The 3′ flanking sequences include a highly conserved AAAGNNAGA sequence that is held in common with other U-RNA genes from both sea urchins and vertebrates. Our findings confirm our classification of the U7 RNA as a genuine, if sparsely represented, member of the U-RNA family.**

There has been an increasing interest in the characterization of the family of U RNAs and their genes in eukaryotes because of the known or proposed role of small nuclear ribonucleoproteins (snRNPs), which contain these U RNAs, in a variety of RNA processing steps. The U1 and U2 snRNPs are clearly involved in RNA splicing (1, 2). U3 snRNPs may be important for processing of RNA precursors (3). U4 snRNPs may have a role in the cleavage/polyadenylylation reaction of most pre-mRNAs (4).

The snRNP containing U7 RNA is part of the machinery required for the generation of the 3′ ends of histone pre-mRNAs by RNA processing (5). The evidence for this derives from two lines. First, processing of the sea urchin histone H3 pre-mRNA is possible in the heterologous frog oocyte only when sea urchin U7 RNA is coinjected with the precursor RNA. Second, U7 RNA has sequence complementarities to the histone pre-mRNA sequences flanking the mature 3′ end, and this suggests the way in which U7 RNA might act. Thus, it has been proposed (6) that the U7 RNA base-pairs with both the highly conserved terminal stem–loop structure of the histone mRNA and the downstream CAAGAAAGA spacer transcript, forming a "blister" of unmatched nucleotides, where the mature 3′ end will appear (reviewed in ref. 7).

Although the function of other U RNAs, such as the U3, U4/U6, and U5 RNAs, are still a matter of conjecture, some of their genes have been isolated. U6-RNA genes have been cloned and characterized from mouse (8) and human (9); U3-RNA genes, from rat (10); and U1- and U2-RNA genes, from a large variety of species (11–19) ranging from sea urchin (20, 21) to *Drosophila* (22–24) and human (25–30).

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The U1–U6 RNAs are quite abundant, representing as much as 0.5%, by mass, of the total cellular RNA (31). Conversely, U7 RNA is an extremely rare RNA species present in the sea urchin at only 1/30th to 1/50th the molar concentration of U1 RNA (ref. 6; unpublished results). Detection of the rarer U-RNA species, such as the U8 (32) and the U9 and U10 RNAs (33), requires biochemical techniques of high sensitivity or, alternatively, complementing bioassay systems such as was used for the identification of U7 RNA (5).

Sea urchin U7 RNA can be precipitated with mouse monoclonal antibodies (K. Strub and M.L.B., unpublished results). Hence, U7 RNA is complexed, either directly or indirectly, with the Sm antigen typical of many U-family snRNPs. Deproteinized U7 RNA can be quantitatively precipitated with antiserum against the trimethyl cap structure (K. Strub and M.L.B., unpublished results). These are all indications that U7 RNA is a genuine member of the family of snRNPs. As we show in this paper, this classification is further supported by our finding that both 5′ and 3′ flanking sequences of the U7-RNA genes share features in common with other U-RNA genes.

MATERIALS AND METHODS

Gene Cloning. Restriction enzymes were purchased from Boehringer Mannheim. *Escherichia coli* HB101 competent cells prepared by the method of Hanahan (34) were obtained from Bethesda Research Laboratories (Basel). Cloning and nucleic acid purification were carried out according to Schleif and Wensink (35).

Detection of U7-RNA Genes with U7 cDNA Transcripts. As the hybridization target for each U7-RNA gene is only 58 nucleotides long, detection of these sequences requires a hybridization probe of high specific radioactivity. Further, because of the short length of the hybrid, the stringency of the hybridization conditions has to be lowered. As a consequence, we were faced with the inherent problem of obtaining false positives from hybridization of the colonies or Southern blots with radioactive probes, unless these probes were free of extraneous sequences. This precluded nick-translation of the cDNA clones as hybridization probes and called for highly specific probes such as provided by SP6 transcripts of U7 cDNA clones. However, when cDNA U77 (6) was placed downstream of the SP6 promoter (36, 37), efficient transcription was hindered by the poly(dA)·poly(dT) tails (which had been donated to the U7 RNA during cloning and flanked the cDNA segments in the cloned DNA). Once these were removed by a BAL-31 resection, transcription led to a utilization of up to 60% of the radioactive nucleotide rGTP or rUTP. This was true even for rUTP (3000 Ci/mmol; 1 Ci =

Abbreviations: snRNP, small nuclear ribonucleoprotein; kb, kilobase(s).

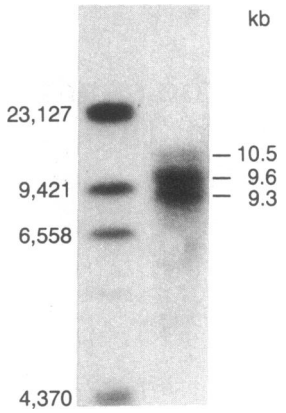

FIG. 1. Southern blot analysis of *Hin*dIII-restricted DNA from a single sea urchin (right lane). The hybridization probe was an SP6 transcript of cloned U7 cDNA (see text). Left lane: *Hin*dIII digest of bacteriophage λ DNA, run as size markers (lengths given in base pairs at left).

37 GBq) at 3–6 μM concentration. SP6 plasmids to be transcribed were cut consecutively, downstream of the cDNA insert, by at least two restriction enzymes to prevent any transcription into the downstream plasmid sequence. Hybridization was at 65°C in 450 mM NaCl/3 mM EDTA/45 mM Tris·HCl, pH 7.5/10% (wt/vol) polyethylene glycol 6000/0.1% sodium pyrophosphate/0.2% $NaDodSO_4$ containing rRNA at 200 μg/ml. Washing was at 50°C in 15 mM NaCl/0.1 mM EDTA/1.5 mM Tris·HCl, pH 7.5/0.5% $NaDodSO_4$. Some filters were also treated with RNase to reduce background. Hybridization signals became apparent after an overnight exposure of the autoradiograms.

RESULTS

Cloning of a 9.3-Kilobase (kb) Cluster of U7-RNA Genes. Sperm DNA isolated from individual sea urchins was digested with a variety of restriction enzymes, and the DNA was transferred onto nitrocellulose filters and hybridized with highly labeled transcripts of U7-RNA cDNA (see *Materials and Methods*). Most restriction enzymes gave relatively complex hybridization patterns based on restriction fragments that coincided in their electrophoretic mobility with those of the bulk of the genomic DNA. This was not true for the three hybridizing *Hin*dIII restriction fragments. These had electrophoretic mobilities commensurate with lengths of 9.3–10.5 kb (Fig. 1). Two of them (9.3 and 9.6 kb) hybridized strongly, and a third (10.5 kb) only weakly, with the radioactive probe.

Recovery of the DNA from this area of the gel represented a 20-fold purification of the DNA hybridizing to U7 RNA. The DNA was cloned in the vector pSP64, using *E. coli* HB101 competent cells prepared by the method of Hanahan (34). Gene libraries were screened by the standard colony-hybridization technique (38). False positive colonies were eliminated by Southern hybridization experiments. Clone pgU7 (genomic U7-RNA genes), which survived this test, was used for further analysis. A restriction map of the pgU7 insert was constructed (39) and is shown in Fig. 2.

U7-RNA Coding Sequences Form a Small Gene Family in the Sea Urchin. Dot hybridization experiments with genomic sea urchin DNA, in conjunction with reconstruction experiments in which plasmid DNA was mixed with known amounts of U7-RNA cDNA (6), indicate (results not shown) that the haploid genome of the sea urchin contains about five U7 RNA-like coding sequences (for simplicity, we shall call them U7-RNA genes). All of the U7 cDNA clones prepared previously (6) include an *Xba* I restriction site within the U7 coding sequence. The genomic pgU7 DNA clone has five such sites (see restriction map of Fig. 2). Hence, it seemed possible that the genomic clone included five U7-RNA genes.

DNA sequencing from these *Xba* I restriction sites shows that all of them lie within U7-RNA genes, gene U71 being of opposite transcriptional polarity. Southern hybridization with U7 RNA transcripts, after restriction of the pgU7 DNA with a variety of restriction enzymes, suggests that pgU7 DNA contains five, and no more than five, U7-RNA genes (results not shown). By comparison to the total number of genes detected by dot hybridization and to Southern blots of total genomic DNA, it would appear that the clone pgU7 includes most, if not all, U7-RNA genes within the sea urchin, although one or two U7-RNA genes may lie outside the cloned chromosomal fragment (for instance, in the weakly hybridizing 10.5-kb DNA fragment). If this conclusion is correct, then the two strongly hybridizing *Hin*dIII genomic fragments (Fig. 1) are likely to be allelic, with the assignment of the third, weakly hybridizing *Hin*dIII fragment (10.5 kb) uncertain.

U7-RNA Genes Are Polymorphic. The five gene sequences of the clone pgU7, together with the cDNA sequences obtained previously (6), are shown in Fig. 3. All cDNAs obtained by retrotranscription of (capped) sea urchin U7 RNA have a 5′-terminal uridine. We prefer the preceding adenosine as the initial nucleotide of the U7 genes because the consensus 5′ terminal sequence for most U-series RNAs is m_3GAU, with the m_3G added posttranscriptionally. Most cDNA sequences tailed with an adenosine homopolymer terminate with an AAC, one with an AACU sequence. It has not been determined whether the two adenosines following the AAC in the genomic DNA belong to the U7-RNA coding sequence (see also ref. 6).

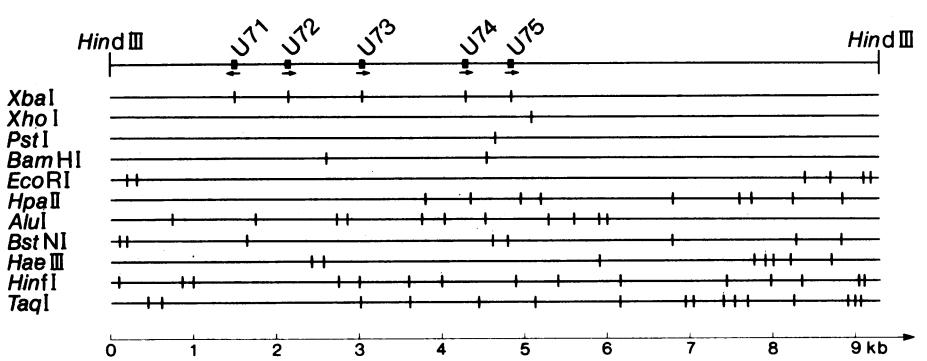

FIG. 2. Restriction map of the 9.3-kb *Hin*dIII fragment containing the U7-RNA gene cluster of *P. miliaris*. U7-RNA genes U71–U75 and their proposed directions of transcription are indicated.

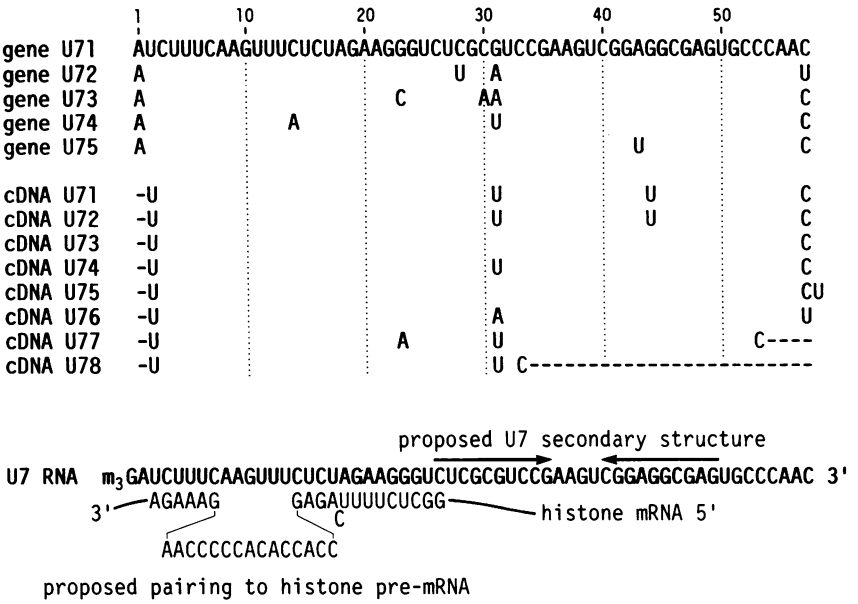

FIG. 3. U7-RNA gene sequences and hypothetical base-pairing scheme for histone pre-mRNA and U7 RNA. (*Upper*) Gene sequences determined by the method of Maxam and Gilbert (40) and cDNA sequences reported earlier (6). (*Lower*) U7 RNA sequence of gene U71 in conjunction with the 3'-terminal sequences of histone H3 pre-mRNA. For detailed discussion of the model, see refs. 6 and 7.

The five gene sequences show minor sequence variations, as do the individual isolates of the U7 cDNA clones (6). As reported previously, position 31 (Fig. 3) is a mutational hotspot in that guanosine, adenosine, and uridine are found at this site, as is the terminal nucleotide where uridine can replace cytidine. Gene U71 and cDNA U73 have the exact same sequence. Conversely, the adenosine in position 14 of gene U74 and the cytidine at position 23 of gene U73 are unusual in that such nucleotide arrangements would disturb the proposed hybrid structure between U7 RNA and histone mRNA during 3' processing (but see *Discussion*).

5' Flanking Sequences Contain a TGCA Motif but No "TATA Box." Sequence analysis of far upstream and downstream sequences flanking the U7-RNA genes is as yet incomplete, but, from partial sequencing (unpublished results) and the restriction map (Fig. 2), it can be deduced that the pGU7 gene cluster is not composed of five repeating, closely similar units. If the gene cluster arose by a saltatory event (followed by an inversion of gene U71), it must have occurred during ancient times.

The sequences of gene U75 shown in Fig. 4 were compared to the flanking sequences of other genes coding for snRNAs. The U75 gene does not contain a TATAAA box at position −30. The consensus sequence detected near position −50 in vertebrate U1- and U2-RNA genes (41) is not present, nor are there extensive homologies to the U1-RNA gene of the sea

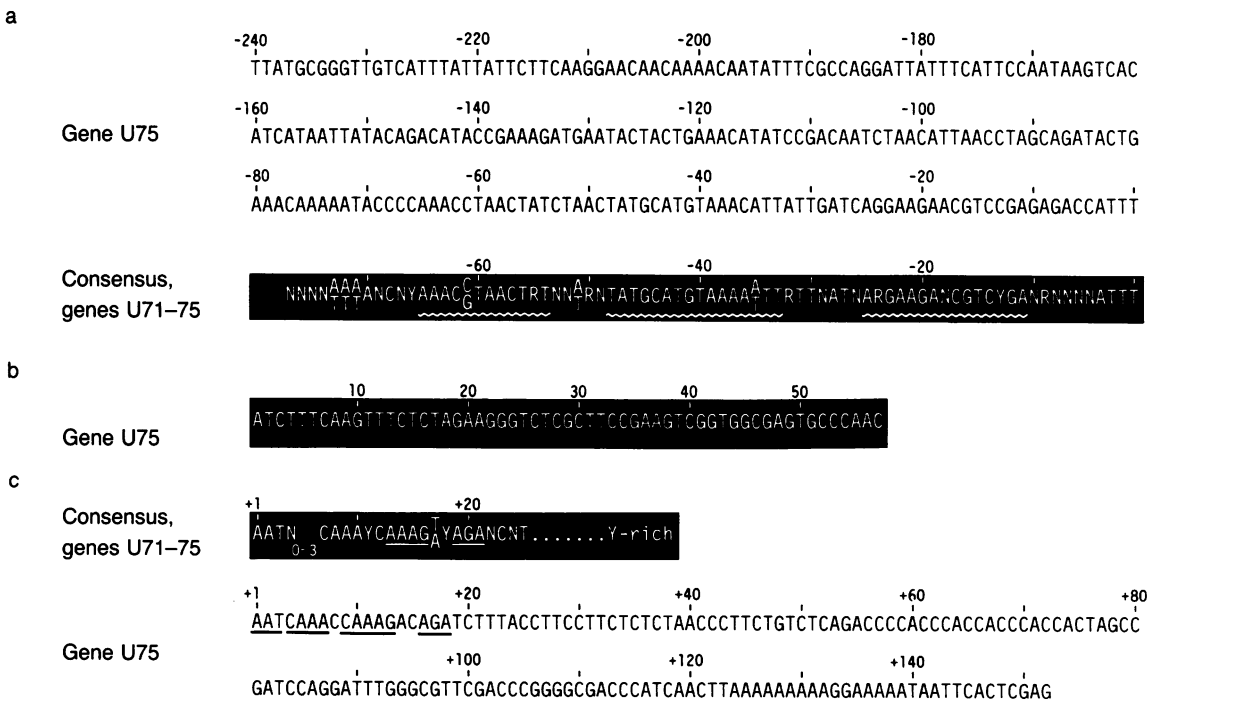

FIG. 4. Coding (*b*) and 5' (*a*) and 3' (*c*) flanking sequences of gene U75, derived from DNA clone pgU7. The consensus sequences common to all known U7-RNA genes of the sea urchin are shown white-on-black. The spacer nucleotides +1 and +2 may in fact be part of the U7-RNA coding sequence (see text).

urchin *Lytechinus* (21). However, a TATGCATGTAAAA with the degenerate TGCA/TGTA repeat is reminiscent of the far upstream enhancer-like element of vertebrate U-RNA genes (42–45) and is found in the 5′ flanking sequence of gene U75 near position −40 (see *Discussion*). The sequence containing alternating purines and pyrimidines has a Z-configuration potential. All five U7-RNA genes of clone pgU7 contain a consensus sequence of this type (M.DeL. and M.L.B., unpublished results). It is flanked by the consensus sequence YAAACNTAACTRT (Y = C or T; R = A or G) near −60 and by the sequence ARGAAGANCGTCYGA near −20. The sequence ATTT immediately 5′ of the "cap" site is also conserved in all U-RNA genes (Fig. 4).

3′ Flanking Sequences Contain an AAAGNNAGA Sequence in Common with Most U-RNA Genes. Sequences essential for correct 3′ end formation of vertebrate pre-U1 and pre-U2 RNAs have been found (46, 47) to consist essentially of the sequence $GTTTN_{0-3}$AAARNYAGA (conserved core sequences underlined), located 9–19 nucleotides downstream of the mature 3′ end. The downstream sequence of gene U75 (Fig. 4) and of all other U7-RNA genes of clone pGU7 (M.DeL. and M.L.B., unpublished results) contain an AAARNYAGA sequence at an appropriate distance downstream of the mature U7 RNA end. The homology of the U7-RNA genes of *P. miliaris* to the U1-RNA gene of *Lytechinus variegatus* (21), where the sequence reads ATTCAAN_5CAAAGAAAGA, is even more extensive. Hence, the $GTTTN_{0-3}$ of vertebrates appears to be replaced by an $AATN_{0-3}$CAAAYC sequence in the sea urchin U7-RNA genes. As pointed out by Hernandez (46) and Yuo *et al.* (47), the AAARNYAGA sequence is reminiscent of the conserved spacer sequence CAAGAAAGA located just downstream of the 3′ termini of the sea urchin histone mRNAs (48, 49).

DISCUSSION

Putative Regulatory Sequences of Sea Urchin U7-RNA Genes. Many investigators (30, 41–45) have found it convenient to describe the promoter of U-RNA genes as being composed essentially of two functional elements: (*i*) a proximal unit acting as a selector of the transcription initiation site and (*ii*) a distal element representing an enhancer-like activator. In all U-RNA genes, including U7-RNA genes of the sea urchin, there is a singular lack of conserved TATA box at −30.

Mammalian U1- and U2-RNA genes have a consensus sequence mapping around position −60 to −40. U7-RNA gene sequences are also conserved at this position, although with a different nucleotide arrangement. In fact, the TATGCATGTAAAA at positions −48 to −36 resembles most the far upstream activator sequence of the U-RNA genes, which in *Xenopus* U1B genes takes the form of $(TGCA)_4AA$ (44). Removal of the TGCA from the human U2-RNA gene activator sequence GGGCATGCAAAT abolishes all stimulatory transcriptional effect (42); hence this motif is an essential feature of the upstream activator. Curiously, in several species a similar TGCA sequence occurs at about −80 in the specific conserved sequence block of the H2B histone gene (50), as well as at positions −72 to −110 in the proximal region of the promoters of the immunoglobulin light and heavy chain genes (51). If indeed there is a functional connection between the TGCA sequence in all of these genes, then the U7-RNA consensus sequence is unusual in that the TGCA sequence is close to the putative transcription initiation site.

The interpretation of the 3′ conserved sequences is probably more straightforward. The CAAAYCAAAGWYAGA (W = A or T) consensus sequences of the U7-RNA genes is almost identical to that of the U1-RNA genes of *L. variegatus* (21) and is closely similar to the vertebrate sequence (46, 47), the U-RNA genes of all species known to date sharing the core consensus sequence AAARNNAGA. Surrogate genetics experiments have shown that the conserved 3′ sequences are essential for the generation of 3′ ends of the U1 RNAs (46, 47), most likely by inducing transcriptional termination near this site (52). If the assignments of both initiation and termination sites are correct, the U7-RNA genes would represent one of the smallest eukaryotic transcription units ever recorded.

U7-RNA Gene Sequences. It can easily be calculated that, because of the sparsity of the U7 RNA in the sea urchin (see the Introduction), one or two U7-RNA genes per haploid genome would suffice to produce all U7 RNA in dividing cells, if the gene were donated with a strong promoter and if the turnover of this RNA were limited. This raises the question whether or not all of the U7 RNA-like coding sequences in the clone pgU7 are genuine U7-RNA genes. The sequence of gene U71 corresponds exactly to that obtained from cDNA clone U73 prepared by tailing and retrotranscription of sea urchin egg U7 RNA (6). Gene U71 is therefore a good candidate for a productive gene, but the definitive assignment awaits functional tests of this and other U7-RNA genes.

Why is there otherwise no coincidence between cDNA and gene sequences? One cause (that we consider to be less likely) could be errors introduced by the retrotranscription of U7 RNA. However, it should also be taken into account that the cDNA clones were prepared from RNA from pooled eggs of many sea urchins. This opens up the possibility that the sequence variations seen in the cloned cDNAs reflect a population polymorphism of a single active gene (per haploid genome) with nucleotide changes in positions where they would be most easily tolerated. It is indeed noteworthy that none of the 10 cDNA clones sequenced to date (ref. 6; unpublished results) contain nucleotide alterations in positions 1–27, which would interfere with the proposed base-pairing between histone mRNA precursors and U7 RNA. This is obviously not true for those genomic U7 RNA sequences that contain an adenosine at position 14 or a cytidine at position 23 (genes U73 and U74), base changes that would clearly interfere with the proposed hybrid structure (see Fig. 3). Since these types of sequences are not represented in the cDNA library, we have at the present level of sensitivity of detection no evidence that genes U73 and U74 (or, for that matter, U72 and U75) are ever expressed during oogenesis or in the sea urchin egg.

The U7-RNA gene sequences allow us to resolve whether or not the interior loop near positions 31–44, predicted by sequencing of cDNA clones, is a genuine feature of the U7 RNA. Mismatched nucleotides are indeed predicted at this site from all five gene sequences. In some cases the interior loop appears to be accentuated by two mismatched base pairs rather than one, as seen in genes U73 and U75.

With the U7-RNA genes now in hand, it should be possible to coexpress cloned histone genes and their 3′ mutants (48, 49) with suitably modified U7-RNA genes and, in this way, to establish essential contact sites between the histone pre-mRNA and the U7 RNA.

Note Added in Proof. Coexpression of sea urchin U7 genes (driven by a *Xenopus* U2 promoter) and of H3 histone genes in the frog oocyte complements faulty 3′ maturation of histone pre-mRNA. This shows that the sea urchin U7 RNA is indeed an essential component of the 3′-processing machinery (K. Strub and M.L.B., unpublished observations).

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