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Intronic U14 snoRNAs of *Xenopus laevis* are located in two different parent genes and can be processed from their introns during early oogenesis

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ABSTRACT

U14 is a member of the rapidly growing family of intronic small nucleolar RNAs (snoRNAs) that are involved in pre-rRNA processing and ribosome biogenesis. These snoRNA species are encoded within introns of eukaryotic protein coding genes and are synthesized via an intron processing pathway. Characterization of *Xenopus laevis* U14 snoRNA genes has revealed that in addition to the anticipated location of U14 within introns of the amphibian hsc70 gene (introns 4, 5 and 7), additional intronic U14 snoRNAs are also found in the ribosomal protein S13 gene (introns 3 and 4). U14 is thus far a unique intronic snoRNA in that it is encoded within two different parent genes of a single organism. Northern blot analysis revealed that U14 snoRNAs accumulate during early oocyte development and are rapidly expressed after the mid-blastula transition of developing embryos. Microinjection of hsc70 pre-mRNAs into developing oocytes demonstrated that oocytes as early as stages II and III are capable of processing U14 snoRNA from the pre-mRNA precursor. The ability of immature oocytes to process intronic snoRNAs is consistent with the observed accumulation of U14 during oocyte maturation and the developmentally regulated synthesis of rRNA during oogenesis.

INTRODUCTION

Eukaryotic rRNA is synthesized in the nucleolus as a large precursor transcript that subsequently undergoes processing to produce mature 18S, 5.8S and 25S/28S rRNAs (1–3). Biochemical analysis has revealed an ever increasing number of RNA species or small nucleolar RNAs (snoRNAs) contained within the nucleolus (4–7). The snoRNAs are believed to participate in ribosome biogenesis, playing potential roles in pre-rRNA folding, rRNA processing, ribosome assembly and subunit transport to the cytoplasm. Indeed, specific snoRNAs in both unicellular and metazoan organisms have been shown to be important for pre-rRNA processing. Gene deletion experiments in *Saccharomyces cerevisiae* have shown that loss of yeast snoRNAs U3, U14, snR10 or snR30 disrupts pre-rRNA processing patterns and affects accumulation of mature rRNA species (8–11). Similarly, RNase H-directed knock-out experiments in mouse and *Xenopus laevis* have demonstrated that U3, U8 and U22 snoRNAs are required for processing of higher eukaryotic pre-rRNAs (12–15).

Examination of snoRNA genes in metazoans has revealed an interesting dichotomy of genomic organization. Several snoRNAs species have a classical gene organization, with the snoRNA produced by direct transcription of the gene from a snoRNA-specific promoter (16). However, the majority of metazoan snoRNA genes are positioned within introns of protein coding genes and biosynthesis of the snoRNA involves processing of the intronic species from the respective intron of the parent pre-mRNA transcript (4,5,17–24). Many intronic snoRNAs possess conserved nucleotide boxes C and D and are immunoprecipitable with antibodies directed against the nucleolar protein fibrillarin (4,5,25).

U14 is an essential intronic snoRNA required for rRNA processing and 18S rRNA accumulation (9). The mammalian U14 genes (mouse, rat, hamster and human) are positioned within introns 5, 6 and 8 of the parent cognate hsc70 heat shock gene (17). Sequencing of the trout cognate hsc70 gene has demonstrated the intronic location of six U14 genes, suggesting an evolutionary conservation of parental gene positioning for intronic U14 genes in vertebrates (26). U14 belongs to the class of intronic snoRNAs which possess boxes C and D and are immunoprecipitable with fibrillarin antibodies (4,27). Work in our laboratory has recently indicated the importance of the box C and D elements in intronic U14 snoRNA processing (Watkins, Leverette, Andrews and Maxwell, manuscript submitted).

In this work we have characterized the U14 snoRNA genes from *X.laevis*. In addition to the anticipated intronic U14 snoRNAs of the cognate hsc70 gene, additional species were found within introns of the ribosomal protein S13 gene. Developmental profiles of U14 expression and the ability of immature oocytes to process intronic U14 snoRNAs are consistent with the requirement of U14 snoRNA for *de novo* rRNA

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synthesis during the early stages of oocyte maturation and the production of new ribosomes in developing embryos after the mid-blastula transition.

MATERIALS AND METHODS

Cloning and characterization of *X.laevis* U14 snoRNA genes

A λ phage Charon 4A genomic library of *X.laevis* DNA fragments generated by limited digestion with *Mbo*I restriction endonuclease was kindly provided by Igor Dawid. This library was screened with a DNA oligonucleotide complementary to the last 39 nt of mouse U14.5 snoRNA as previously described (28). Restriction mapping of six positive clones revealed two independent genomic organizations, the subgroups being represented by clones CSX191 and CSX141 respectively. Fragments of clones CSX191 and CSX141 were subcloned into plasmid pT3T7 (Promega) and sequenced. Analysis of determined primary sequences was carried out using the PC/Gene Package of the Intelligenetics Corporation.

Preparation and analysis of *X.laevis* RNA

Xenopus laevis oocytes were surgically removed from female frogs and prepared for microinjection or RNA extraction as previously described (18). Oocytes were developmentally staged according to Dumont (29) and RNA prepared from either whole oocytes or manually dissected germinal vesicles (18). For developmental studies embryos of the same sibling group were prepared by fertilizing extruded eggs as previously described by Tashiro *et al.* (30). At designated times of embryo development total RNA was prepared by phenol extraction (31). Prepared RNA was resolved on 10% polyacrylamide–7 M urea gels, electroblotted onto nylon membranes and hybridized with DNA oligonucleotides radiolabeled at the 5′-terminus with [γ-^{32}P]ATP (32). Oligonucleotide probes used for hybridization included the 39mer complementary to the conserved 3′-terminal region of U14 snoRNA (29), a 22mer complementary to nt 4–25 of human U1 snRNA (5′-GGTATC-TCCCCTGCCAGGTAAG-3′) and a 23mer complementary to nt 9–31 of human U3 snoRNA (5′-ACTTTCAGGGATCATTTC-TATAG-3′). U14 snoRNA processing was studied by microinjecting ^{32}P-radiolabeled hsc70 pre-mRNA transcripts into *Xenopus* oocytes of various developmental stages as previously described (18). Oligonucleotide primers for the *Xenopus* (exon 5 primer, 5′-CCCGAATTCCAGAGGTACCTTGAC-3′, *Eco*RI cloning site underlined; exon 6 primer, 5′-CCCGACGT-CATCTCCACATTGTAG-3′, *Pst*I cloning site underlined) and mouse (exon 5 primer, 5′-GCGCGGTACCCTGCAAGACT-TCTTCAATGG-3′, *Kpn*I cloning site underlined; exon 6 primer, 5′-GCGCGAGCTCGAGGAGTGACATCCAAGAGC-3′, *Sac*I cloning site underlined) constructs were used to generate PCR fragments. Inserts of *Xenopus* or mouse U14.5 snoRNAs containing portions of upstream exon 5 and downstream exon 6 were subsequently cloned into the appropriate plasmid pT3T7 (Promega) or Bluescript 2SK+ (Stratagene) restriction sites respectively. These constructs, when transcribed *in vitro* with T7 RNA polymerase (18), generated *Xenopus* and mouse hsc70 pre-mRNA transcripts of 491 and 391 nt respectively. *Xenopus* and mouse hsc70 pre-mRNA transcripts were injected into selected oocytes as previously described (18). After overnight incubation (12–18 h) total RNA was prepared by phenol extraction and processed RNAs analyzed on 10% polyacrylamide–7 M urea gels.

RESULTS

Characterization of *X.laevis* U14 snoRNA genes

A *X.laevis* genomic library was screened with a U14-specific oligonucleotide probe complementary to the highly conserved 3′-terminal region of U14 snoRNA. Six positive λ phage recombinants were isolated and preliminary analysis was carried out by restriction mapping. All six exhibited one of two restriction patterns, indicating two different and independent genomic locations for U14 genes in the *Xenopus* genome. An individual clone from each subset of recombinant constructs was selected, designated CXS191 and CSX141.

Analysis of recombinant clone CSX191 revealed the previously uncharacterized *X.laevis* cognate hsc70 heat shock gene (Fig. 1A). U14 snoRNA coding regions are located within introns 4, 5 and 7 of the *Xenopus* hsc70 gene. This differs from the arrangement of U14 genes found in introns 5, 6 and 8 of mammals (17). Analysis of the second recombinant clone, CSX141, revealed the ribosomal S13 protein gene (Fig. 1B). Two U14 genes are positioned within introns 3 and 4 of the S13 gene. Both the *Xenopus* hsc70 and *Xenopus* S13 gene coding regions exhibited high homology with the corresponding genes characterized in other eukaryotic organisms (Table 1). The presence of U14 coding regions within the hsc70 gene was anticipated, since previous analyses of five other vertebrate organisms has revealed an identical parent gene for this intronic snoRNA species (17,26). However, the positioning of additional U14 genes within a second, distinctly different parent gene, the ribosomal S13 protein gene, was not expected. U14 snoRNA thus represents the first example of an intronic snoRNA species encoded within two different parent genes of a single organism.

Sequence alignment of all five *Xenopus* U14 genes revealed a high degree of homology among themselves (Fig. 2) and with other U14 snoRNA species previously characterized, indicating that these are *bona fide* U14 genes. Highly conserved regions included the terminal base paired ends, flanking nucleotide boxes C and D, and regions A and B, which exhibit complementarity to 18S rRNA. The middle third of the *Xenopus* U14 snoRNAs exhibited the least sequence conservation, consistent with the variability seen in this region of the molecule for other U14 species characterized from mammals, fish, fungi and plants (17,26,44).

Careful comparison of all five *Xenopus* U14 variants revealed specific sequence homologies and corresponding differences in specific sites in the molecule for those species encoded within the two different parent genes. Nucleotides 3, 5, 13, 27, 34, 41, 48, 49, 54, 61, 64 and 94 were conserved among all U14 variants found in the hsc70 parent gene, but were distinctly different from the nucleotides in those positions found for both U14 variants encoded in the ribosomal S13 gene. The variations in the terminal stem (nt 3 and 94) still preserved the terminal stem structure of U14. Those occuring in rRNA complementary region A (nt 27 and 34) also maintained complementarity with the corresponding bases in 18S rRNA to preserve the proposed intermolecular helix between these two RNAs (27,28). The variability of intron location for U14 genes in the hsc70 gene between mammals and amphibia, as well as the sequence differences between U14 variants of the two parent genes, may provide some clues as to the evolutionary origin of U14 intronic snoRNAs (see Discussion).

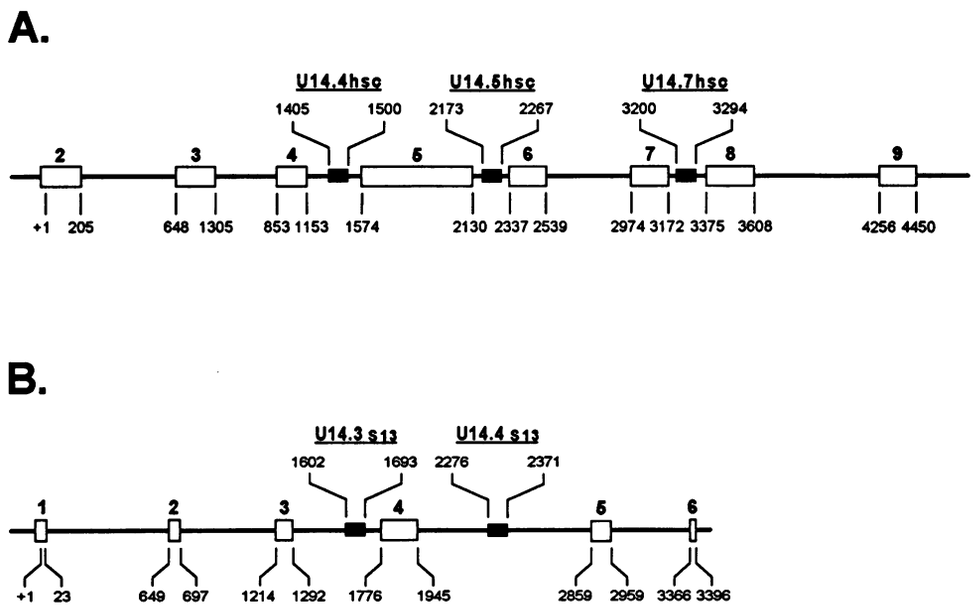

Figure 1. Intron-encoded U14 snoRNAs of the *X.laevis* cognate hsc70 heat shock and ribosomal S13 protein genes. Schematic presentation of the cloned and sequenced *Xenopus laevis* cognate hsc70 heat shock (**A**) and ribosomal S13 (**B**) protein genes. Intron-encoded U14 snoRNAs are indicated by solid black boxes. Exon coding regions and intronic U14 snoRNAs are numbered with respect to the first nucleotide of methionine initiator codons (designated +1). Complete nucleotide sequences are entered in the EMBL Gene Bank under accession nos Z49896 and Z49897 for the hsc70 and S13 genes respectively.

Table 1. Amino acid sequence homology

Species	Homology (%)	Reference
A. Hsc70 proteins		
Xenopus laevis	100	
Human	96.6	34
Rat	96.6	35
Mouse	96.6	36
Hamster	96.3	37
Bovine	96.6	38
Trout	94.5	26
Drosophila	84.9	39
B. S13 proteins		
Xenopus laevis	100	
Human	99.3	40
Rat	99.3	S.McNabb and M.Ashburner, unpublished, S31453
Drosophila	82.8	41
Nematode	82.1	42
Pea	76.8	U.Bertsch, S.Clausen-Krueper and J.Soll, unpublished, Q05761
Schizosaccharomyces pombe	74.8	43
Maize	72.8	44

Developmental expression of *Xenopus* U14 snoRNA

The expression of U14 snoRNA during *X.laevis* oogenesis and embryogenesis was assessed using Northern blot analysis. Nuclear RNA was prepared from germinal vesicles of stage I–VI oocytes. From the same sibling frogs, eggs were prepared, fertilized and total RNA extracted from developing embryos at various stages of embryogenesis. Figure 3 shows the developmental profile of *Xenopus* U14 snoRNAs. Small amounts of U14 were detected in stage I oocytes and increased in amount through stage V, appearing to reach maximal levels in stage V and VI oocytes. Similar expression patterns were seen for the U1 snRNA and U3 snoRNA controls. U14 was observed in all stages of developing embryos, but a great increase in U14 accumulation was seen after the mid-blastula transition (stage 8), when transcription is activated in developing embryos. Due to the high degree of sequence homology, the accumulation of individual U14 variants was not examined. However, high resolution gels clearly demonstrated no difference in expression profiles for those U14 species capable of being resolved upon the basis of different U14 sequence lengths (data not shown). Interestingly, a differential expression of U3 variants was observed at the mid-blastula transition of developing embryos, suggesting the possible developmental regulation of U3 snoRNA expression during embryogenesis.

Ability of developing oocytes to process intronic U14 snoRNA

The ability of developing oocytes to process U14 snoRNA was assessed by microinjecting uncapped hsc70 pre-mRNA transcripts containing intronic U14 snoRNA into oocytes of different developmental stages. Both the mouse and *Xenopus* hsc70 pre-mRNAs contained the U14 snoRNA encoded within intron 5 and included portions of flanking exons 5 and 6 (see Materials and Methods). Both stage II and III oocytes produced mature U14.5 snoRNAs at low levels (more easily seen at longer exposure times), while stage IV–VI oocytes produced significantly larger quantities of processed U14.5 (Fig. 4). The heterologous mouse transcripts consistently produced larger amounts of U14 in comparison with the *Xenopus* species, raising

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                                  Box C                    Region A
Xenopus   U14.4hsc   TCGCTGTGATGAATGTGATTCCAAAGCCATTCGTAGTTTCCACCAGATG
Xenopus   U14.5hsc   .T...AA.......AC.............................
Xenopus   U14.7hsc   ............CT..-.............................
Xenopus   U14.3s13   ..A.A.......C..-.T......--T......C......T......AA
Xenopus   U14.4s13   ..A.A.......C..-.T......--T......C......T......AA

  Mouse   U14.5hsc   ............-..-.........A.....................A.
                     *  *   *****              *****   ***** **   *


                                                 Region B  Box D
Xenopus   U14.4hsc   TCTCACGACTTATGATGGTTTATTACCTTCCTTGGATGTCTGAGCGAT    97
Xenopus   U14.5hsc   ..GT.A....C.......C.C--.....................A    95
Xenopus   U14.7hsc   C.GA.A.G..G.......CC..GC....................C    96
Xenopus   U14.3s13   G..TT-.C-.GG..T...CC.G.A...................T..G    92
Xenopus   U14.4s13   G..TT-.C..GG..T...C.AG.A...................T..G    93

  Mouse   U14.5hsc   .------G..G-..T...C.AG..-...................A    87
                              * * *       ******* ****** **** **
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Figure 2. Sequence alignment of *X.laevis* U14 snoRNAs. Coding regions of all five intronic U14 snoRNAs are aligned and compared with the mouse U14.5 snoRNA sequence (17). Asterisks below the mouse sequence indicate those nucleotides conserved in all U14 snoRNAs thus far characterized from mammals, amphibians, fish and yeast. Conserved snoRNA nucleotide boxes C and D are underlined and conserved 18S rRNA-complementary regions A and B are overlined.

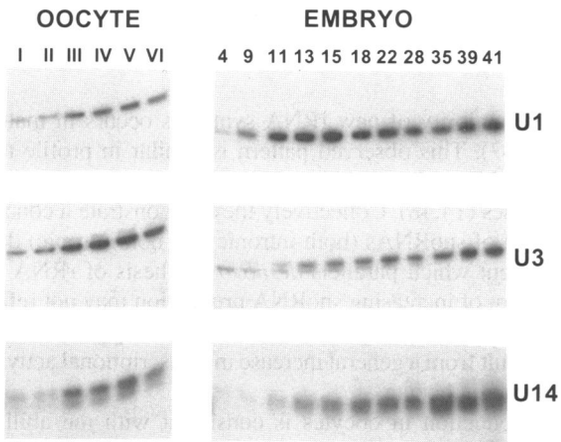

Figure 3. Expression of U14 snoRNAs during *X.laevis* oogenesis and embryogenesis. RNA was isolated from developing oocytes and embryos, resolved on polyacrylamide gels, electroblotted onto nylon membranes and probed with U14-, U1- and U3-specific probes as detailed in Materials and Methods. Specific oocyte and embryo stages are indicated above the appropriate gel lanes with probes for the specific RNAs designated at the side.

the question of possible sequences (regulatory?) within the homologous *Xenopus* U14 snoRNA that affect U14 snoRNA processing. It is possible that *Xenopus* U14 binds a *Xenopus*-specific protein to unique sequences found within the variable region of the molecule, thus affecting snoRNA processing efficiency.

Interestingly, the increase in processed U14 seen in more mature oocytes paralleled the apparent increase in splicing, as noted by the large increase in the lariat form of hsc70 intron 5. The lariat form of intron 5 appears to be quite stable and we believe that the large accumulation of this splicing product is due to very low levels of debranching activity. Large amounts of spliced exons were typically not observed in these experiments, since the absence of a cap on the 5′-end of exon 5 led to destabilization of the final spliced product. However, injection of capped hsc70 pre-mRNAs led to increased accumulation of spliced exons, which paralleled the observed levels of the lariat form (data not shown). Larger amounts of unprocessed hsc70 precursor in conjunction with lower levels of the intron lariat and processed U14 in stage II and III oocytes suggests the potential linkage of U14 snoRNA production with parent pre-mRNA splicing.

DISCUSSION

Analysis of the *X.laevis* genome revealed five intronic U14 snoRNAs located within two different parent protein coding genes. The pseudotetraploid nature of the *Xenopus* genome (45,46) makes it difficult to determine the total number of U14 snoRNA genes in this amphibian. However, the observation that all six U14-positive clones were members of the two characterized genomic organizations could suggest that the total component of *Xenopus* U14 genes has been characterized. Clearly, Northern blot and primer extension analysis (data not shown) have revealed that all the U14 snoRNA species observed in both oocytes and embryos exhibit lengths consistent with the intronic U14 snoRNAs characterized in these two parent genes.

The finding that U14 snoRNA coding regions are found within introns of the cognate hsc70 heat shock gene was anticipated, since previous characterization of mammalian (mouse, rat, human and hamster) and fish (trout) U14 snoRNA genes has demonstrated a location in this common parent gene (17,26). The characterization of additional U14 genes within the ribosomal protein S13 gene was unexpected and represents the first example of an intronic snoRNA located within introns of two different parent genes of a single organism. The occurence of U14 snoRNA genes in the ribosomal protein S13 gene of the mouse has not been directly assessed. However, Southern blot analysis demonstrating that only a single mouse genomic fragment contains U14 snoRNA sequences (28) strongly indicates that U14 sequences are absent from the introns of the S13 gene. The absence of U14 sequences in the ribosomal protein S13 gene in mammals suggests the potential mobility of these intronic snoRNA elements during evolution. Similarly, the variation in intronic location even within a common parent gene from organism to organism (mammals, hsc70 introns 5, 6 and 8; fish, hsc70 introns 2 and 4–8; amphibian, hsc70 introns 4, 5 and 7) is consistent with

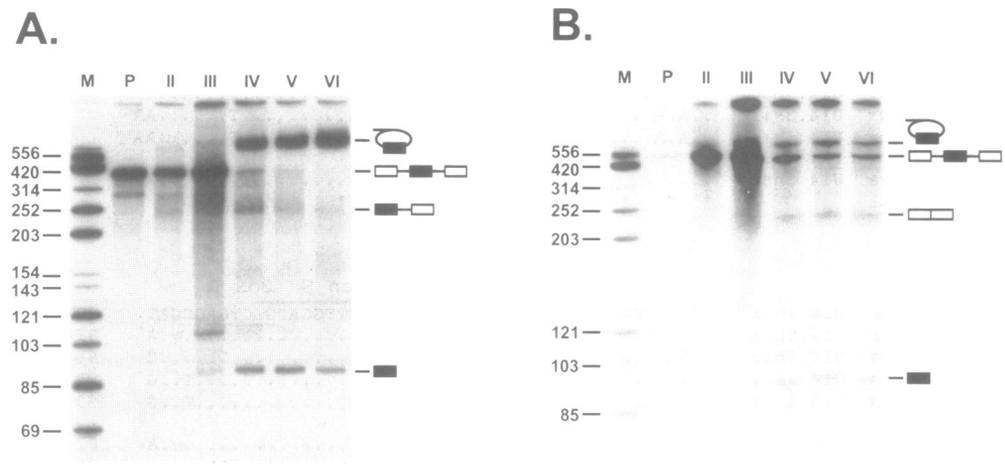

Figure 4. Processing capability of *X.laevis* oocytes. Radiolabeled mouse (**A**) or *X.laevis* (**B**) hsc70 pre-mRNA transcripts containing intron-encoded mouse U14.5 or *Xenopus* U14.5 were injected into stage II–VI oocytes. After 12–18 h incubation total RNA was prepared and U14 snoRNA processing assessed by resolution of the radiolabeled transcripts on polyacrylamide gels as detailed in Materials and Methods. DNA molecular weight marker sizes and RNA processing products are designated at the side. Lane M, molecular weight markers; lane P, injected hsc70 pre-mRNA precursor containing intronic U14 snoRNA; lanes II–VI, processed hsc70 pre-mRNA transcripts incubated in stage II–VI *Xenopus* oocytes. The designated U14 snoRNA processing intermediate containing the flanking 3′ intron is inferred from its migration position and earlier work examining U14 snoRNA processing intermediates (50).

this hypothesis. The positioning of U14 within the hsc70 and S13 parent genes is also consistent with the recurring theme that intronic snoRNAs are typically located in parent genes whose protein products are involved in nucleolar function, ribosome biogenesis or mRNA translation (4,5). If these intronic snoRNA coding regions are or have been mobile in evolution, the homing mechanism which targets them to this group of parent genes remains unknown. Interestingly, the U14 sequences found in each *Xenopus* parent gene exhibit sequence homologies between themselves which are distinctly different from the U14 snoRNAs of the other parent gene. This would suggest that duplication and divergence of the U14 sequences occurred after insertion into the different parent genes.

While only the *Xenopus* U14.5hsc species has been directly tested for processing in oocytes, we believe that all five U14 sequences are *bona fide* genes. All five are highly homologous to the more than 25 available U14 sequences; all five possess base paired 5′- and 3′-ends, conserved nucleotide boxes C and D flanking this terminal stem and regions A and B that are complementary to 18S rRNA and separated by a variable region. Recent work in this laboratory has demonstrated that the U14 terminal structural motif, which consists of the terminal stem and flanking boxes C and D, is sufficient for processing of U14 from the intron (Watkins, Leverette, Andrews and Maxwell, manuscript submitted). All five *Xenopus* intronic U14 snoRNAs possess this structural element, which strongly suggests that all can be processed from their respective introns. Indeed, the U14 sequence within hsc70 intron 5 varies the most from this consensus structure (variation in box C), yet this species is clearly processed from its parent intron, as demonstrated in these experiments.

Analysis of U14 expression in developing oocytes and embryos revealed an increasing accumulation of U14 snoRNAs during oogenesis and significant increases in U14 production after the mid-blastula transition of embryo development. U14 snoRNA accumulates early in oogenesis and is present in stages II–IV, when the majority of new rRNA synthesis occurs in maturing oocytes (47). This observed pattern is similar in profile to U3 control in this study and U3 and U8 expression profiles seen in other studies (14,48). Collectively they demonstrate a concerted expression of snoRNAs (both intronic and non-intronic) during development which parallels *de novo* synthesis of rRNA (47). This pattern of increasing snoRNA production may not reflect a regulatory mechanism specific for ribosome biogenesis, but may simply result from a general increase in transcriptional activity of the developing oocyte.

U14 production in oocytes is consistent with the ability of immature oocytes to process this intronic snoRNA, albeit at apparently lower levels. Analysis of various ribosomal protein mRNAs has revealed that they are expressed early in oogenesis and increase in amount with maturation (49). This pattern would be expected for mRNA species that contain intronic snoRNAs (S13-U14, S1-U15, L1-U16, L1-U18 and S8-U17). The nucleolar protein fibrillarin, which is associated with snoRNAs possessing nucleotide boxes C and D, also exhibits a similar expression profile (48), indicating coordinated expression of snoRNA binding proteins with snoRNA biosynthesis early in oogenesis.

Finally, the relationship between hsc70/S13 pre-mRNA splicing and intronic U14 snoRNA processing remains to be defined. mRNA synthesis for several ribosomal proteins and several intronic snoRNAs exhibit similar accumulation profiles. Unfortunately, production of both mature message and intronic snoRNAs from the same pol II RNA transcript cannot be inferred from these data. Interestingly, the accumulation of both U14 and the lariat form of both mouse and *Xenopus* hsc70 intron 5 exhibit similar patterns of accumulation. Co-production of U14 with splicing intermediates and spliced exons (when hsc70 pre-mRNA transcripts are 5′ capped) may suggest that intronic U14 snoRNA synthesis and hsc70 mRNA splicing are coupled and produced by co-existent pathways from the same pre-mRNA transcript.

Indeed, in this scenario the lariat form of the intron could serve as the direct precursor for U14 snoRNA production.

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