Mouse U14 snRNA is encoded in an intron of the mouse cognate hsc70 heat shock gene

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ABSTRACT

Mouse U14 snRNA (previously designated mouse 4.5S hybRNA) is an evolutionarily conserved eukaryotic low molecular weight RNA capable of intermolecular hybridization with both homologous and heterologous 18S rRNA (1). A single genomic fragment of mouse DNA containing the U14 snRNA gene(s) has been isolated from a Charon 4A lambda phage mouse genomic library and sequenced. Results have surprisingly revealed the presence of three U14 snRNA-homologous regions positioned within introns 5, 6, and 8 of the mouse cognate hsc70 heat shock gene. Comparative analysis with the previously reported rat and human cognate hsc70 genes revealed a similar positioning of U14 snRNA-homologous sequences within introns 5, 6 and 8 of the respective rat and human genes. The U14 sequences contained in all three introns of all three organisms are highly homologous to each other and well conserved with respect to the diverging intron sequences flanking each U14-homologous sequence. Comparison of the mouse U14 snRNA sequence with the U14 DNA sequences contained in the three mouse hsc70 introns indicates that intron 5 is utilized for U14 snRNA synthesis in normally growing mouse ascites cells. Analysis of the determined mouse, rat, and human U14-homologous sequences and the upstream and downstream flanking regions did not reveal the presence of any previously defined RNA polymerase I, II, or III binding sites. This suggests that either higher eukaryotic U14 snRNA is transcribed from a unique transcriptional promoter sequence, or alternatively, is generated by intron processing of the hsc70 pre-mRNA transcript.

INTRODUCTION

We have previously reported the characterization of mouse U14 snRNA (originally designated 4.5S hybridizing or 4.5S hybRNA), a low molecular weight (lmw) RNA species of 87 nucleotides capable of intermolecularly base-pairing with mRNA and 18S rRNA sequences (2). Subsequent work in our laboratory

has demonstrated the evolutionary conservation of U14 snRNA in eukaryotes (1). Southern blot analysis indicated the presence of single or low copy number U14 snRNA-homologous genes in human, rat, hamster, *Xenopus laevis*, and yeast (*Saccharomyces cerevisiae*) as well as mouse. The demonstrated ability of mouse, *Xenopus laevis*, and yeast U14 snRNAs to base-pair with 18S rRNA suggested a role for this evolutionarily conserved intermolecular RNA:RNA interaction in U14 snRNA function (1).

More recently, the Fournier laboratory has cloned and sequenced the yeast U14 snRNA gene (originally designated snR128) (3). Their experiments have shown that it is a capped lmw RNA species of 128 nucleotides transcribed from its own independent, upstream promoter. Gene deletion experiments have also shown that yeast U14 is an essential snRNA in *Saccharomyces cerevisiae*, required for cell growth. Localization of yeast U14 snRNA in the nucleolus suggested a role in ribosomal RNA synthesis and/or processing, consistent with our observations demonstrating a conserved ability of mammalian, amphibian, and fungal U14 snRNAs to base-pair with 18S rRNA (1). Most recently, gene deletion experiments have shown that loss of U14 snRNA in yeast results in the underaccumulation of 18S rRNA and the alteration of rRNA precursor patterns (4).

To learn more about U14 snRNA in higher eukaryotes, we have examined the organization of the U14 snRNA gene in the mouse genome. Sequencing the cloned mouse U14 snRNA gene(s) has unexpectedly revealed that three U14-homologous sequences are positioned within introns 5, 6, and 8 of the constitutively-expressed mouse cognate hsc70 heat shock gene. Sequence analysis of the previously published rat (5) and human (6) hsc70 genes has revealed U14 snRNA-homologous sequences similarly positioned in introns 5, 6, and 8 of these genes as well. This suggests a common genomic organization for this snRNA gene in higher eukaryotes. Present sequence evidence indicates that the U14 coding region contained in intron 5 of the mouse hsc70 gene is used in normally growing mouse Taper ascites cells for the synthesis of mouse U14 snRNA. The positioning of multiple U14 snRNA-homologous sequences/genes within multiple introns of a single eukaryotic gene may have important implications for the organization and evolution of gene structure.

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METHODS

Materials

[α - 32 P]-ATP was purchased from New England Nuclear Corp. Restriction endonucleases and Klenow fragment were obtained from Promega. DNA oligonucleotides were synthesized on an Applied Biosystems Inc. synthesizer and purified on polyacrylamide gels before use.

Mouse U14 snRNA Gene Isolation and Sequencing

A mouse genomic library, prepared by limited digestion of mouse genomic DNA with the restriction endonuclease Mbo I and subsequent insertion into a Charon 4A cloning vector (7), was generously provided by Steven Weaver. Phage plaques were screened as previously detailed (8) using a 5' terminally-labeled 39mer DNA oligonucleotide complementary to the 3' end of mouse U14 snRNA (1). Filter washes were carried out at 55°C in 1×SSC, 0.1% SDS buffer. A single U14-positive plaque (JLM 2772) was isolated from the approximately 200,000 screened plaques after 3 rounds of hybridization and plaque isolation/purification. A 1.5 kb Bam H1-Pst 1 restriction fragment containing the U14-homologous sequences was cut from the cloned mouse DNA and cloned/subcloned into M13mp18 and M13mp19 sequencing vectors (Bethesda Research Laboratories). DNA sequencing was performed by the dideoxy method (9) initially using DNA oligonucleotide primers synthesized from the previously determined mouse U14 sequence (2) and labeled at the 5' end with 32 P-ATP (1). Additional DNA oligonucleotide primers were synthesized based upon the rat (5)/human (6) exons 6, 7, and 8 sequences bordering introns 5, 6 and 8. Sequence analysis was accomplished using the Wisconsin Program of Gene Bank.

RESULTS

For the isolation of the mouse U14 snRNA gene(s), a mouse genomic library in Charon 4A lambda phage was screened with a synthetic DNA oligonucleotide 39mer (1) complementary to the 3' terminal region of mouse U14 snRNA. A single, U14 snRNA-positive construct (JLM 2772) was isolated from the approximately 200,000 plaques screened. Isolation of a single U14-positive clone was consistent with earlier Southern blot analysis indicating a single copy number for this snRNA gene in the mouse genome (1). A 1.5 kb Bam H1-Pst 1 mouse restriction fragment from this construct was then cloned/subcloned into M13mp18/mp19 sequencing vectors for analysis. The complete sequencing strategy for this mouse DNA fragment is outlined in Figure 1.

Initial sequence determination of the 5' end of the subcloned 1.5 kb mouse DNA fragment revealed the presence of a U14

Figure 1. Sequencing strategy of the isolated 1.5 kb Bam H1-Pst 1 DNA fragment of the mouse cognate hsc70 heat shock gene containing U14 snRNA-homologous sequences. The solid, boxed regions represent exons and the thin solid lines represent introns. Exons and introns are numbered appropriately.

snRNA-homologous coding region (Figure 2). This U14-homologous sequence matched the previously published (2) mouse U14 snRNA sequence (previously designated mouse 4.5S hybRNA). [Based upon this determined DNA sequence and those discussed below, we have corrected a previously ambiguous nucleotide at position 35 in the U14 snRNA sequence, changing the originally reported guanosine (2) to a uridine residue.] Subsequent analysis of upstream and downstream regions flanking the U14 coding sequence surprisingly revealed that this U14 coding sequence is positioned within intron 5 of the constitutively-expressed mouse cognate hsc70 heat shock gene (Figure 2). Protein coding regions contained in exons 5 and 6 are highly homologous to the corresponding exon coding regions found in the heat-induced hsp70 proteins of *Drosophila* (10), chicken (11), *Xenopus laevis* (12), rainbow trout (13), and yeast (14) (data not shown), as well as exons 5 and 6 of the non-heat-induced and constitutively expressed cognate rat (5) and human (6) hsc70 genes (Figure 2). [Typical of higher eukaryotic cognate hsc70 genes and in contrast to the stress-induced hsp70 genes, the mouse, rat, and human cognate hsc70 gene possess introns (10).] Further comparative analysis of the previously published rat (5) and human (6) hsc70 genes also demonstrated the similar positioning of U14 snRNA-homologous sequences in intron 5 of these hsc70 genes as well (Figure 2). The U14 coding sequences contained in intron 5 of all three hsc70 genes are highly homologous and well conserved with respect to the divergent intron 5 flanking regions.

More detailed analysis of the published rat (5) and human (6) hsc70 genes revealed the presence of additional U14-homologous sequences contained within introns 6 and 8 of these hsc70 genes (Figures 3 and 4). The nucleotide sequences of mouse hsc70 introns 6 and 8 were therefore subsequently determined by sequencing the appropriate regions of the cloned 1.5 kb fragment of the mouse hsc70 gene. DNA oligonucleotides complementary to flanking regions of exons 6, 7, and 8 [deduced from the rat hsc70 sequence (5)], in conjunction with the universal M13 oligonucleotide, were used as sequencing primers. Shown in Figures 3 and 4 are the determined mouse hsc70 introns 6 and 8 sequences with U14-homologous regions positioned within each intron, compared with the corresponding regions of the rat and human hsc70 genes. As seen with the U14 snRNA sequence of intron 5, the U14-homologous sequences within hsc70 introns 6 and 8 are highly conserved with respect to each other and with respect to the diverging sequences of the flanking intron regions.

Analysis of all intron-encoded U14 sequences found in the mouse, rat and human hsc70 introns 5, 6 and 8 as well as the yeast U14 snRNA sequence (previously designated snR128) clearly revealed several regions of high homology (Figure 5). Most notable are regions encompassing nucleotides 1–12, 18–46, 53–62, and 65–86. Two of these regions contain the previously defined Box C and D sequences (15–18) conserved in those snRNAs which are found in the nucleolus and are believed to play a role in rRNA biogenesis (see Discussion).

Comparison of the sequenced mouse U14 snRNA transcript (2) with the U14 sequences contained in mouse hsc70 introns 5, 6, and 8 indicates that the U14 coding sequence found in intron 5 is responsible for the synthesis of U14 snRNA in normally-growing mouse Taper ascites cells. Analysis of the U14 coding sequence in mouse hsc70 intron 6, however, reveals only a single nucleotide difference at position 47 (an A in place of the determined G). Careful review of the original RNA sequencing gels (both chemical and primer extension sequencing) has

MOUSE ATGATATTGTCCTGGTGGGTGGTTCTACCAAGAATCCCCAAGATTTCAGAACTTCTCGAAGACTTCTTCAATCGAAAAAGAGCTGAAACAAGAGCATTAAAC
 RATC.....C.....T.....
 HUMANC...T.....TC.T.....G...C.....A...T.....C...
 MOUSE AspIleValLeuValGlyGlySerThrArgIleProLysIleGlnLysLeuLeuGlnAspPhePheAsnGlyLysGluLeuAsnLysSerIleAsn
 RAT
 HUMAN

EXON 5⁻⁵⁴ INTRON 5⁺¹
 (M) CCGATGAAGCTGTTGCCATATGGTGCAGgtaaatacatacagocctttatagctctttataatctca—gcaagtcctgtgaagattogctgtgatgatgg
 (R)t—.....C.ta..c.g..g....acaa.gg..g.....t.
 (H) ..T.....T.....ca.tggatct.aa.ta.cc.—...agg..g—...g.c..aaggt..c.....gt.
 (M) ProAspGluAlaValAlaTyrGlyAlaA
 (R)
 (H)

(M) attcAAAAaccattogtagtttccaccagaagtgcgtg-----tggtggctagttccttctctggatgctgagogaatgaaacatacatatgtttgagta
 (R)at.....a.....
 (H) ..gt-t..a.....a...-..tggtctaa..a....a.ca-.....g...t.gt..a.aca.qa.ctat

	INTRON 5	+159	EXON 6	+189	HOMOLOGY (Coding Region)
(M)	gattgcagttacagtgogttgtttctaactttttttttctt—tttagctgTGCAGGCAGCATTCTATCTGGAGACA				—
(R)	...a.a.....a.a...C.....a...a....tgctc...g.....				98%
(H)	.tactgg...ttct.ttaact.c.t.t.aog..aac....tg.—.gc.....CT.G.....				90%
(M)	laValGlnAlaAlaIleIeuSerGlyAsp				—
(R)					100%
(H)					100%

Figure 2. A U14 snRNA coding sequence is contained in intron 5 of the mouse hsc70 heat shock gene. The determined sequence of mouse hsc70 intron 5 containing the U14 snRNA coding sequence, as well as the flanking regions of hsc70 exons 5 and 6, are compared with the exon 5-intron 5-exon 6 region of the rat (5) and human (6) hsc70 cognate heat shock genes. Conserved nucleotides and amino acids are indicated by dots (·) and nucleotide insertions/deletions are indicated by dashes (–). The mouse U14 snRNA sequence is indicated by the solid line over that region of intron 5. The 5' terminal nucleotide of U14, determined by primer extension (unpublished data), is designated as +1.

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      -53 |
      EXON 6      INTRON 6
Mouse  GTGTACTCATTCAGTgtatgtgtccctagaattttgtagc—ttgtatagaagacc—aggggaagt——tcc
Rat    .....C.....g.....gtagt.....a..a..—.....t.
Human  ...G..T.....tctgtacttctc...ttggc..actg.t..cagataa.....cttgactga.
Mouse  ValLeuIleIleIln
Rat    . . . .
Human  . . . .

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+1
 (M) togctgtgatgatggattocaaaaaccattogtagtttccaccagaaatgc-tgtgttggctagtttootttoottggat
 (R)C.....g.a.-.....
 (H)a.....gt.t.a.....c.....

+87

(M) gtctgagogaacttatcttcccc-aaatttgagcttaagocacagagcagtagtttctaaaatgga—tgtctta

(R)C.g.....C.....-.....tat.t....t.....—..t.....

(H) ..t.....c.a-.....ttagc.ggaacctagc.ctgt...aga.ctggag.c.attgtacta.tt...t...

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                                +170
                        INTRON 6      EXON 7
(M)   attttattgtctcttct--agGTGATGAAGGTGAAAGGGCCATGACCAAGGCAC
(R)   ....C.c.....t.ct.aa.....
(H)   -...cctacc-----....T.....C..GC.T.....A....T
(M)                                   ValTyrGluGlyGluArgAlaMetThrLysAsp
(R)                                   . . . . .
(H)                                   . . . . .

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Figure 3. A U14 snRNA-homologous sequence is contained in intron 6 of the mouse hsc70 heat shock gene. Exon-specific primers deduced from the rat hsc70 gene sequence (5) were used to sequence intron 6 of the mouse hsc70 gene contained in the cloned 1.5 kb mouse DNA fragment. The determined mouse intron 6 sequence is compared with the corresponding rat (5) and human (6) hsc70 heat shock gene sequences. Conserved nucleotides and amino acids are indicated by dots (·) and nucleotide insertions/deletions designated by dashes (—). The U14-homologous sequence of intron 6 is indicated by the solid line over that region of the intron. The corresponding 5' terminal nucleotide (with respect to the intron 5/U14 snRNA sequence) is designated as +1.

Figure 4. A U14 snRNA-homologous sequence is contained in intron 8 of the mouse hsc70 heat shock gene. An exon 8-specific primer, deduced from the rat hsc70 gene sequence (5), and the M13 universal primer were used to sequence intron 8 of the mouse hsc70 gene contained in the cloned 1.5 kb mouse DNA fragment. The determined mouse intron 8 sequence is compared with the corresponding rat (5) and human (6) hsc70 heat shock gene sequences. Conserved nucleotides and amino acids are indicated by dots (·) and nucleotide insertions/deletions designated by dashes (-). The U14-homologous sequence of intron 8 is indicated by the solid line over that region of the intron. The corresponding 5' terminal nucleotide (with respect to the intron 5/U14 snRNA sequence) is designated as +1.

Figure 5. Evolutionary conservation of U14 snRNA genes/homologous sequences. The mouse, rat, human, and yeast U14 snRNA coding sequences are aligned. Dots (·) indicate conserved nucleotides and dashes (-) indicate nucleotide insertions/deletions. The mouse U14 snRNA sequence (at the top in capitals) has been determined by RNA sequencing (2). The remaining sequences have been determined by DNA sequencing. The intron 5, 6, and 8 sequences of the mouse are from this work, and the corresponding rat and human sequences are from the previously published rat (5) and human (6) hsc70 genes, respectively. The yeast U14 snRNA sequence is the published U14 (snR128) gene sequence (3). The underlined mouse U14 snRNA sequences encompassing nucleotides 7-12 and 77-82, correspond to Box C and Box D sequences, respectively. These sequences are characteristic of nucleolar snRNAs (18).

Figure 6. The conserved 5', 3' extended helix structure of U14 snRNA may serve as an RNA processing site. Secondary structural analysis of all eukaryotic U14 snRNA sequences has indicated a conserved 5'–3' terminal helix structure, such as those shown in this figure. For those U14 snRNA sequences encoded within introns 5 and 8 of the mouse, rat, and human hsc70 genes, this helix may be extended beyond the 5' and 3' terminal nucleotides of each U14 snRNA sequence into the flanking intron sequences as shown. Arrows indicate nucleotide changes which conserve, extend, or strengthen this proposed helix structure.

indicated that a guanosine nucleotide is indeed found in this position of the U14 snRNA transcript. While we cannot rule out the presence of U14 transcripts synthesized from the intron 6 sequence at this time, we are confident that they could represent no more than several percent of the total U14 snRNA synthesized in normally-growing mouse Taper ascites cells. The U14 snRNA-homologous sequence found in intron 8 differs in length and sequence from the intron 5-and 6-encoded U14 sequences as well as the determined U14 snRNA sequence. Under ordinary growth/culture conditions, the intron 8 U14-coding region is apparently not utilized.

DISCUSSION

U14 snRNA is an evolutionarily conserved, eukaryotic small nuclear RNA that appears to play a role in rRNA processing. The strongest evidence to support this hypothesis comes from work examining the yeast U14 snRNA gene. Genetic analysis has shown that it is an essential gene in the yeast *Saccharomyces cerevisiae* (3) and that deletion of the U14 coding region results in an underaccumulation of 18S rRNA and an alteration in the rRNA precursor patterns (4). Consistent with this biological role, mouse U14 snRNA is expressed in all mouse tissues (1) and is localized in the nucleolus complexed with fibrillarin (Maxwell

and Liu, unpublished results). U14 snRNA also possesses conserved nucleotide Boxes C (UGAUGA) and D (GUCUGA) sequences characteristic of those snRNAs which appear to play a role in rRNA biogenesis (15–18). Finally, the demonstration that fungal, amphibian, and mammalian U14 snRNAs are capable of intermolecular hybridization with both homologous and heterologous 18S rRNAs (1) suggests a possible role for this observed intermolecular RNA:RNA interaction in U14 snRNA function.

In this work examining mouse U14 snRNA gene structure, we have demonstrated the positioning of the mouse U14 snRNA gene(s) within an intron of the constitutively-expressed mouse cognate hsc70 heat shock gene. This appears to be the sole location of U14 snRNA-homologous sequences in the mouse genome since digestion of mouse genomic DNA with various restriction endonucleases has repeatedly resulted in the recognition of a single DNA fragment by the U14 snRNA probe (1). Indeed, digestion of mouse DNA with Pst 1 yields a single 3 kb fragment of containing the U14 snRNA gene(s). This fragment, trimmed to 1.5 kb with Bam H1, is the DNA fragment which has been inserted into the M13 vector for sequencing. The finding of U14-homologous sequences conserved within introns 5, 6, and 8 of the rat and human hsc70 genes supports the idea that these are the bona fide U14 snRNA coding regions.

We believe that the U14 snRNA transcript isolated from normally growing mouse Taper ascites cells is synthesized from the U14 coding sequence positioned in intron 5 since the sequenced DNA matches the sequenced U14 snRNA. However, we also feel that it is quite possible, and even likely, that the U14-homologous sequences found within hsc70 introns 6 and 8 are functional U14 genes as well. First, the high degree of sequence conservation for all three intron sequences in all three organisms suggests sequence conservation for functional purposes. Second, all three intron-contained U14 coding sequences possess the conserved Box C and D regions characteristic of nucleolar snRNAs. Third, careful comparison of all U14-homologous sequences contained in one intron with respect to those of other introns reveal variations in U14 sequence specific for one group of intron sequences versus another. For example, specific and conserved nucleotide changes at positions 3, 6, 32–33 and 84 of all intron 8 U14 sequences suggest a possible functional/structural importance for these nucleotide differences in a biologically significant U14 coding region. Finally, the expression of two different sized Xenopus U14 snRNA transcripts during different stages of oogenesis (Maxwell and Andrews, unpublished results), suggests the possible utilization of more than one U14 snRNA coding region.

While the positioning of the U14 snRNA gene(s) within an intron of a heat shock gene could suggest a potential relationship of U14 function with the stress response, at present we have no evidence to support such a possibility. Preliminary experiments have indicated no obvious association of mouse U14 snRNA with the mouse hsp70/hsc70 proteins (Maxwell and Welch, unpublished results). Not unexpectedly, the expression of mouse U14 snRNA is not greatly increased during heat shock and no obvious alteration in nucleolar distribution is observed under these stressed culture conditions (Liu and Maxwell, unpublished results).

The positioning of the mouse U14 snRNA gene(s) within an intron is an unexpected location for a eukaryotic snRNA gene. The finding of such an intron-encoded gene is not unique however. Specific introns of some mitochondrial genes encode

proteins required for mRNA splicing (19, 20). The gene for the pupal cuticle protein of *Drosophila melanogaster* is located with an intron of the purine pathway gene (21). In each of these examples however, the intron-contained gene coding sequence is located on the non-coding strand of the gene in which it is nested. Strikingly, the mouse U14 snRNA gene(s) is unique in that it is found on the coding strand of the hsc70 gene. Perhaps more striking, U14-homologous sequences are found in three separate introns of the same gene. It is possible that the U14 sequence has been positioned in 3 different introns of the hsc70 gene by some type of transposition mechanism. The recent examples of mobile introns (22–25) may be analogous to what has occurred for the U14 gene sometime earlier in evolution. The recent finding of a U14-homologous sequence in intron 5 of the *Xenopus laevis* hsc70 gene (Sage and Maxwell, unpublished results) suggests a common and conserved organization for this snRNA gene at least since the divergence of amphibians and reptiles/mammals some 350 million years ago (26). It should be noted that the genomic organization of the yeast U14 snRNA gene is very different from higher eukaryotic U14 genes in that it possesses its own upstream promoter site and is not positioned within an intron sequence.

The biosynthetic pathway utilized for mouse U14 snRNA biogenesis is not yet clear. Examination of the U14-homologous sequences with accompanying upstream and downstream flanking regions has not revealed the conserved presence of any obvious RNA polymerase I, II, or III binding sites. Such sites included in this analysis were the mouse Pol I rRNA promoter sequence (27–29), the eukaryotic Pol II TATA (–35) sequence and upstream CAATT (–70) sequence (30), the internal Pol III binding site with the 3' termination oligo U signal site characteristic of small RNAs transcribed by Pol III (31), and the newly defined upstream (–25) Pol III promoter site (32, 33). Perhaps more significantly, the proximal sequence element or PSE characteristic of small nuclear RNA transcription, which is conserved in mouse (34), human (35), and *Xenopus laevis* (36), is not found 5' (–60) to the intron-encoded U14 snRNA-homologous sequences nor is an upstream enhancer element at –200 (34). The snRNA termination signal typical of Pol II transcription (35–38) is also not found downstream from any of the intron-encoded U14 sequences. This suggests that the U14 snRNA gene(s) may possess a unique and as yet undefined RNA polymerase promoter site.

Alternatively, mouse U14 snRNA could be produced via RNA processing of the U14 snRNA sequence from the intron 5 region of the hsc70 pre-mRNA transcript. Clearly, the transcription of the hsc70 gene results in the production of 3 intron-contained U14 snRNA transcripts whose ultimate fate is presently unknown. Interestingly, structural analysis has indicated that the 5' and 3' terminal regions of all U14 snRNA sequences could base-pair to form an evolutionarily conserved helix structure, such as those illustrated in Figure 6. For the U14 sequences encoded in introns 5 and 8 of the mouse, rat, and human hsc70 genes, this conserved helix structure can be extended a few nucleotides beyond the 5' and 3' termini of the U14 sequences to include upstream and downstream flanking nucleotides in each of these introns (Figure 6). The existence of such an evolutionarily conserved structure may be indicative of an RNA processing site, similar to that previously described for *E. coli* 5S rRNA processing (39). The utilization of such a U14 'intron transcript' for rRNA processing may suggest an earlier, more widespread utilization of intron transcripts in the 'RNA World' (40). Experiments to determine

if mouse U14 snRNA is produced by intron processing or by a more conventional transcription pathway are presently underway.

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