U1 snRNA is cleaved by RNase III and processed through an Sm site-dependent pathway

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ABSTRACT

Core snRNP proteins bind snRNA through the conserved Sm site, PuA(U)≥3GpU. While yeast U1 snRNA has three matches to the Sm consensus, the U1 3′-terminal Sm site was found to be both necessary and sufficient for U1 function. Mutation of this site inhibited pre-mRNA splicing, blocked cell division and resulted in the accumulation of two 3′-extended forms of the U1 snRNA. Cells which harbor the Sm site mutation lack mature U1 RNA (U1α) but have a minor polyadenylated species, U1γ, and a prominent, non-polyadenylated species, U1β. Metabolic depletion of the essential Sm core protein, Smd1p, also resulted in the increased accumulation of U1β and U1γ. In vitro, synthetic U1 precursors were cleaved by Rnt1p (RNase III) very near the U1β 3′-end observed in vivo. We propose that U1β is an Rnt1p-cleaved intermediate and that U1 maturation to the U1α form occurs through an Sm-sensitive step. Interestingly, both U1α and a second, much longer RNA, U1ε, were produced in an rnt1 mutant strain. These results suggest that yeast U1 snRNA processing may progress through Rnt1p-dependent and Rnt1p-independent pathways, both of which require a functional Sm site for final snRNA maturation.

INTRODUCTION

Soon after synthesis, the spliceosomal snRNAs are exported to the cytoplasm where they bind the phylogenetically conserved set of common snRNP proteins: B, B′, D1, D2, D3, E, F and G (1,2). The common snRNP proteins are bound through an RNA element, the Sm site, which contains the sequence PuA(U)≥3GpU (3,4). The Sm site–common protein interaction stabilizes the snRNA (5–8) and is required for, or assists in, cap hypermethylation (9) and subsequent nuclear import through the β-importin/RAN GTPase-dependent pathway (10–13). The role of common snRNP proteins in splicing is unknown although there is experimental evidence suggesting that the common proteins help recruit the snRNP-specific proteins required for splicing (14,15). ATP-independent interactions between the U1 snRNP particle and the pre-mRNA 5′ splice site and branch point regions nucleate the assembly of the spliceosome (reviewed in 16). In metazoan, the U1 snRNP consists of the common snRNP proteins and a set of three snRNP-specific proteins, U1-A, U1-C and U1-70K (reviewed in 17,18). The highly unusual Saccharomyces cerevisiae U1 snRNP possesses equivalents of the metazoan common and snRNP-specific proteins (19 and references cited therein) and five proteins not observed in vertebrates, Prp39p (20), Prp40p (21), Prp42p (22), Smu56p/Mud10p and Smu71p, as well as a possible sixth protein, Npl3p (19). Some of these additional proteins likely interact with the yeast-specific snRNA core, an insertion which expands yeast U1 snRNA to 3.5 times the length of its vertebrate counterparts (23,24). All of the known yeast-specific proteins (except Np13p) are essential and likely contribute directly to U1 snRNP assembly or function.

Embedded within the extraordinarily large yeast U1 snRNA are three matches to the Sm consensus element (23,24). In the present investigation we used a mutagenesis approach to learn whether any or all of these putative Sm sites support biological function. Our results show that a single 3′-terminal Sm site is both necessary and sufficient for U1 snRNP function in splicing. In addition, this study reveals that U1 snRNA 3′-end formation is strictly Sm site-dependent. Perturbation of the Sm RNP structure by Sm site mutation or the removal of a core protein causes increased intracellular accumulation of 3′-extended U1 snRNA forms. Two RNase III cleavage sites were identified directly 3′ of the U1 snRNA gene body. Curiously, unlike what has been observed for U2 (25) and USL (26), mutation of the yeast RNase III gene, RNT1, does not reduce the level of mature U1 snRNA but, rather, causes the accumulation of a third form of lengthened U1 snRNA. This study supports a model in which yeast U1 snRNA is processed along two parallel or convergent pathways, both of which pass through an Sm-dependent step.

MATERIALS AND METHODS

Oligonucleotides

U1-2, 5′-CCC GAA TTC ATA CTT ACC TTA AGA TAT CA-3′; U1-3, 5′-GGG AAG CTT AAA ATA AA TTA CAA-3′; U1-9, 5′-ATC AGT TGT GTG TGA CCA AG-3′; U1-10, 5′-AAC AAG GGA ATG GAA ACG TC-3′; U1-16, 5′-GAT CAG TAG GAC TTC TTG ATC-3′; U1-16, 5′-GGG TGG TGA ATC TTA CAA-3′; U1-23, 5′-GGT CTT ATT TAT TTT CAA AAG TAC-3′;

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Yeast strains

PSU1∆192-507, MATa Δrtp1 Δhis3 ura3-52 lys2-801 snr19::LYS2, pSE358(BRP1 snr19∆192-507); XLT10 p-XL46, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1); (27); XLT10 pXl46T, MATa ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1); RSY1, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), YEplac112(BRP1 snr19∆192-507Sm); RSY2, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), YEplac112(BRP1 snr19Sm); BZY0-14, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), YEplac112(BRP1 snr19Sm); BYZ0-14, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), Yplac128(LEU2 SMD1HA); RSY3, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), Yplac128(LEU2 SMD1HA); YEplac112(BRP1 snr19Sm); PAY4-192, MATa trp1-289 ura3-52 leu2-3,112 arg4(RV) ade2 snr19∆::ADE2, pXL46(URA3 GAL::U1), PRP39HA(integrated) YEplac112(BRP1 snr19∆192-507); BYZ0-16, MATa trp1 ura3-52 leu2-3,112 smd1::LEU2 snr19::LYS2, pBM150(URA3 GAL::SMD1HA) (5); BZY2-51a, MATa trp1 ura3-52 smd1::LEU2 snr19::LYS2, pBM150(URA3 GAL::SMD1HA), pSE358(BRP1 snr19∆192-507); MA52, MATa leu2-3,112 his3-d200 lys2A trp1 pep4-3 prb1 prc1 rnt1::HIS3 (28); MA6, MATa leu2-3,112 ura3-52 his3-d200 lys2A trp1 pep4-3 prb1 prc1 rnt1::HIS3, pRS316-RNT1 (28); TS192, MATα trp1-289 ura3-52 leu2-3,112 his3 cyth5 prp38-1 (29).

Plasmid construction and analysis

The full-length SNR19 (U1) gene was subcloned as a 2.3 kb AccI-HindIII fragment (23) into the TRP1-marked Escherichia coli yeast shuttle vector YEplac112 (US Biological; 30). Construction of the U1∆192–507 functional deletion mutant was previously described (31). To be compatible with the yeast strains used, U1∆192–507 was transferred as a 900 bp EcoRI fragment from pSE358 (31) into vector YEplac112. Sm site mutations were previously described (31). To be compatible with the yeast strains used, the diploid progeny selected on galactose-containing medium that lacked uracil and tryptophan. The diploid strain was sporulated and Trp-positive, galactose-dependent haploid progeny identified. Northern analysis with a U1-specific probe was then used to identify yeast that expressed U1∆192–507 as the sole source of U1 snRNA.

RNA analysis

Total cellular RNA was extracted from logarithmically growing cultures by glass bead disruption followed by phenol extraction and ethanol precipitation (34). To metabolically deplete U1 snRNA or Smd1/Hap, the appropriate yeast strain (XLT10-pXL46, RSY1, RSY2, BZY0-14, RSY3 or BZY2-51a) was initially cultured in 1% yeast extract, 2% Bactopeptone, 2% galactose broth (YPgal) (35) to mid-log phase at 30°C. The cells were then collected by centrifugation at 7000 g and resuspended in the same medium with 2% glucose substituted as the sugar (YPgfu). All cultures were incubated with periodic dilution to maintain cell densities below 1 x 10⁷ cells/ml. RNA samples isolated from these cultures were fractionated on denaturing 1% agarose-0.22 M formaldehyde gels or on 7 M urea–5% polyacrylamide gels (29). Northern transfers were hybridized with random prime-labeled PCR fragments corresponding to RPS51A intron plus exon sequences (29), mature U1 snRNA (primers U1-2 and U1-3) and U1 downstream sequences (primers U1-25 and pTZ-80). RNA from yeast that express GAL::U1, U1∆192–507 (strain PAY4-192), GAL::U1, U1∆192–507Sm (strain RSY1), U1∆192–507 (strain PSU1∆192-507), GAL::U1 (strain XLT10-pXL46), GAL::U1, U1Sm (strain RSY2), GAL::SMD1HA, U1∆192–507 (strain BZY2-51a), mnt1::HIS3 (strain MA52) and RNT1 (strain MA6) were analyzed by this method.

Oligonucleotide-directed RNase H cleavage was done essentially as described (36). Ten micrograms of RNA was denatured at 70°C for 10 min in 19 µl of 1 mM EDTA, pH 7.4. Next, 1 µg of oligonucleotide U1-10, SPP381-8 or oligo(dT)20 was added and the hybridization mixture incubated at 26°C for 15 min. One microliter of 4 M KCl was added and the incubation continued for 15 min at 26°C. To effect cleavage, 20 µl of TM buffer (40 mM Tris–HCl, pH 7.6, 60 mM MgCl₂) and 1 U of E.coli RNase H (Gibco BRL) were added for 30 min at 37°C. The RNAs were then phenol extracted, concentrated by ethanol precipitation and used for northern analysis. RNA from yeast that express GAL::SMD1HA, U1∆192–507 (strain BZY2-51a) and GAL::U1, U1∆192–507Sm (strain RSY1) were analyzed by this method.

For the RNase protection assays (36), the U1 snRNA genes described above were subcloned as HindIII–EcoRI fragments into the in vitro transcription vector pGEM-4Z (Promega). 32P-labeled RNA complementary to U1 was prepared by T7 RNA polymerase transcription of Сell-labeled DNA with [α-32P]UTP. Aliquots of 5–10 µg of total yeast RNA were hybridized with 100 000–500 000 c.p.m. of uniformly labeled, gel-purified RNA probe for 1 h at 65°C in 20 µl of solution R1 (0.9 M NaCl, 7.5 mM EDTA, pH 7.4). Next, 105 µl of solution R2 (5 mM EDTA, pH 7.4, 0.5 U RNase Oneµg; Promega) was added and the sample digested at room temperature for 1 h. After phenol extraction, the protected RNA fragments were resolved on a denaturing 7 M urea–6% polyacrylamide gel together with RNA molecular weight markers. The markers used included in vitro transcripts of EcorI-cleaved pTZ19U (61 nt), PsuII-cleaved pTZ19U (149 nt), HindIII-cleaved U1 pGEM-3Z (769 nt), HindIII-cleaved U1∆192–507 (462 nt) and BamHI-cut 25S rRNA (228 nt; S.Abou Elela and M.Ares, personal communication). One nucleotide has been added to the size of the markers to account for the inclusion of a CAP analog, diguanosine triphosphate (Pharmacia), in the reaction mixture. To more precisely map the RNase digestion products, the RNAs were also resolved adjacent to an RNA sequence (37) of 3′-end-labeled U1∆192–507 RNA.
RNA from yeast that express GAL::U1 (strain XLT10-pXL46), GAL::U1, U1Sm (strain RSY2), U1ΔI92–507 (strain PSU1Δ192–507), GAL::U1, U1ΔI92–507Sm (strain RSY1) and U1 (strain TS192) were analyzed by this method.

The 5′-ends of the various U1 transcripts were mapped by primer extension with 5′-end-labeled oligonucleotide U1-16, complementary to nucleotides +26 to +45 of U1. For each reaction, 10 ng of total yeast RNA and 3 U of avian myeloblastosis virus reverse transcriptase were used (Life Sciences Inc., St Petersburg, FL) under conditions previously described (38). A DNA sequence generated with the same primer and the RNA from yeast that express virus reverse transcriptase were used (Life Sciences Inc., St Petersburg, FL) for 5 min at 37°C. The reactions were stopped by the addition of 100 µl of PK buffer (100 mM Tris–HCl, pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% SDS, 2 mg/ml proteinase K; Gibco BRL) for 5 min at 37°C. The RNAs were recovered by phenol extraction and ethanol precipitation and then resolved by denaturing gel electrophoresis with the untreated substrate and the molecular weight standards described above. The RNAs used for RNase III digestion included the T7 RNA polymerase transcribed BamHI-cut 25S rDNA control substrate and the SP6 RNA polymerase transcribed HindIII-cut U1 pGEM-4Z derivatives. In some experiments 5′-end-labeled RNA (40) or 3′-end-labeled RNA (38) was substituted for the uniformly labeled RNA.

Purified GST–Rnt1p, a kind gift of M. Ares and S. Abou-Elela (26), was also used to map RNase III cleavage sites in the synthetic U1 snRNA precursors. Here, 0.5 µl of Rnt1p was added to a 10 µl reaction composed of the RNA substrate and R3 buffer (30 mM Tris–HCl, pH 7.5, 10 mM MgCl2, 5 mM spermidine, 30 mM KCl, 0.1 mg/ml wheat germ RNA) (26). After 1–10 min the reactions were terminated as described for the yeast extract cleavages and the samples analyzed by denaturing gel electrophoresis.

RESULTS

The 3′-terminal U1 Sm site is essential in vivo

The yeast U1 snRNA has three sequence elements that match the Sm site consensus. Two of these three sites are found within a double-stranded region of the internal yeast-specific domain (positions 224–230 and 241–247) and can be deleted without obvious loss of U1 function (31,41). The third resides at the more characteristic Sm site location near the 3′-end of the transcript (position 553–559). To test whether the 3′-terminal site was essential for U1 snRNA activity, we replaced the natural Sm sequence, AUUUUGA, with a different sequence of equivalent length, AUUCACAC. This mutation was introduced into a plasmid-borne copy of the natural full-length SNR19 gene and into a functional deletion derivative that lacked the first two Sm sites (U1ΔI92–507; 31). The shorter derivative, U1ΔI92–507Sm, allowed for clean resolution of the mutant RNA from a co-expressed wild-type U1 RNA. In addition, the parallel use of U1Sm and U1ΔI92–507Sm mutants permitted assessment of the 3′-terminal Sm site activity in the presence and absence of the potentially compensatory upstream sites.

The U1 derivatives were transformed into a yeast strain that contained a chromosomal null allele of SNR19 (27) and a plasmid-borne U1 gene allele under the transcriptional control of the GAL10 promoter (GAL::U1; 42). While natural promoters are required for proper U snRNA processing in vertebrates (43–45), GAL10-driven yeast snRNAs are functional and appear to be processed normally (6,46–49; below). Neither the full-length mutant, U1Sm, nor the deletion derivative, U1ΔI92–507Sm, produced a dominant negative phenotype when co-expressed with GAL::U1 (Fig. 1A, tryptophan + galactose). To test for function, the transformants were streaked on medium that repressed GAL::U1 transcription and selected against the physically linked URA3 gene (Fig. 1A, +5-FOA, + galactose). As expected, the U1ΔI92–507 allele compensated for the loss of GAL::U1 expression and colonies formed on the 5-FOA plates. In contrast, the U1Sm and U1ΔI92–507Sm mutants did not form colonies and appeared, therefore, to be defective in U1 function. Consistent with a primary defect in pre-mRNA splicing, transcriptional repression of GAL::U1 caused cellular mRNA to decrease and pre-mRNA to accumulate in yeast transformed with U1Sm or U1ΔI92–507Sm (Fig. 1B and data not shown). Thus, the terminal Sm site of the U1 snRNA gene is both necessary and sufficient to support in vivo pre-mRNA splicing.

Interference with Sm site–protein interactions causes the accumulation of lengthened forms of U1 snRNA

GAL::U1 yeast co-transformed with U1ΔI92–507 accumulated RNAs corresponding to the predicted lengths of the fully processed 568 nt U1 and 258 nt U1Δγ, showing that these alternative snRNA forms were not transcribed from the GAL10 fusion construct. Equivalent results were obtained when U1Sm was introduced as a mutant construct, although a prominent U1 form was not observed (Fig. 2B). Thus, for both U1 and U1ΔI92–507, mutation of the terminal Sm site was lethal and caused the accumulation of extended U1 snRNA derivatives.

The Sm sequence might contribute directly to the processing of the U1 snRNA or act indirectly through the association of the Sm-bound common snRNP polypeptides. To address this question, we asked whether an alternative means to perturb the Sm RNP structure produced a similar pattern of U1 snRNA forms. Yeast were assayed for U1 snRNA length both before and after transcriptional repression of the nutritionally regulated GAL::SMD1HA fusion gene which encodes the essential Smd1p common snRNP protein tagged with a hemagglutinin epitope (5,8; Fig. 2C). Although less complete than what was observed
with the Sm site change, U1Δα levels decreased and U1Δβ and U1Δγ levels increased with depletion of the Sm-associated Smd1HAp protein (Fig. 2C). Thus, the RNP structure of the Sm site plays an important role in U1 snRNA 3′-end formation.

Extended forms of U1 snRNA have additional 3′-sequence

It is of interest that while lower in abundance, the U1Δβ and U1Δγ RNAs are present in yeast transformed with the functional U1ΔJ92–507 construct (Fig. 3, lanes 1 and 3). Oligonucleotide-directed RNase H digestion was used to confirm that these RNAs were truly derived from the U1 gene. An oligo complementary to the U1Δα, U1Δβ and U1Δγ RNAs (lane 2). Each U1 band was also cleaved with oligonucleotides directed against U1 nt 26–45 and 130–148 but not with the control oligonucleotide, 381–1 (lane 1 and data not shown). Equivalent results were obtained with the U1-specific oligonucleotides when U1ΔJ92–507Sm-derived RNA was used as an enriched source of the U1Δα, U1Δβ and U1Δγ RNAs (data not shown). When oligo(ΔT)20 was used to direct RNase H cleavage, the U1Δγ band was selectively lost (compare lanes 3 and 4). U1Δγ was also selectively enriched by oligo(ΔT) chromatography (data not shown). Since no extended poly(A) tract flank the U1 gene (23,24) the U1γ oligo(ΔT)20 sensitivity is most likely due to post-transcriptional modification. RNase H treatment of the U1ΔJ92–507Sm-derived RNA with oligo(ΔT)20 likely shifts U1Δγ to the U1Δβ position since no novel product was generated by this treatment (Fig. 3B). Taken together, the RNase H digestion results indicate that U1Δβ and U1Δγ are transcribed from the U1ΔJ92–507 allele and that U1Δγ is a polyadenylated U1 derivative of U1Δβ.

While not definitive, the lengths of the RNase H products relative to the oligonucleotide binding sites suggested that U1Δα, U1Δβ and U1Δγ RNAs shared a common 5′-end. Primer extension analysis confirmed that GAL::U1, U1ΔJ92–507 and U1ΔJ92–507Sm all initiated transcription at the previously reported 5′ adenosine (Fig. 4A, lanes 1–4; 23,24). In the case of U1ΔJ92–507Sm, the RNA was assayed both in the presence of the wild-type U1 (lane 3) and after transcriptional repression depleted U1α to nearly undetectable levels (lane 4). Since the cDNA produced was the same length, U1Δβ must differ from U1α due to heterogeneity 3′ of the primer binding site.

An RNase protection assay (36) was used to map the 3′-end of the U1 derivatives (Fig. 5B). Cellular RNA was hybridized to a uniformly labeled probe that extended from U1 snRNA nt 508 to a position 191 nt downstream of the natural U1 3′-end (23,24). The cellular U1ΔJ92–507 RNA protected probe fragments of between 61 and 69 nt (Fig. 4B, lane 4, and C, lane 3); a 66 nt product corresponds to the published 3′-end of U1α snRNA (23,24). The high A+U content of the 3′-end likely contributes to the minor heterogeneity of the RNase digestion products. GAL::U1 and wild-type U1 produced equivalent fragments, foreshortened by 6 nt due to a linker inserted in the 3′-end of the U1ΔJ92–507 probe (31; Fig. 4B, lane 2, and C, lanes 2 and 4; Materials and Methods).

GAL::U1-containing yeast that simultaneously expressed U1Sm or
precursor (Fig. 5 B). Of the six RNA forms observed, the 35 nt F positions 75 and 110 upstream of the 3′-end products. The 3′-processing, bands D and E were present in the final reaction Δ suggesting that it is an intermediate in the processing reaction. (Fig. 5 B). Band B was present only in the earliest time points respectively, downstream of the U1 α A–C were visible. Band A is the uncut precursor while bands B cleaved to produce five additional bands (Fig. 5 A, labeled B–F).

When the pre-U1 GAL :: Δ U1 downstream sequence, respectively. Both pre-U1 α and U1β 64–78 nt longer than the mature U1 snRNA, termed U1Δβ (lanes 5 and 6) and U1β (lanes 2 and 3). Together, the RNase mapping data show that unlike the vertebrate system (43–45,50) β (lanes 5 and 6) and U1 α in galactose medium (lane 4), U1Δβ was not significantly influenced by the large internal deletion Δ as a 5′- or 3′-end-labeled species) and was derived from a double cleavage at positions 75 and 110 upstream of the 3′-end. Pre-U1 snRNA was processed at precisely the same nucleotides as the pre-U1Δβ–507 derived probe, showing that the processing reaction was not significantly influenced by the large internal deletion (compare lanes 5 and 11).

The electrophoretic pattern of 5′- and 3′-labeled products was consistent with endonucleolytic cleavages of the synthetic U1 pre-snRNA. Recently, the yeast RNT1 gene product, Rnt1p (RNase III), was shown to cleave the 3′-ends of synthetic U2 and U5 snRNA precursors (25,26). Two experiments were performed to determine whether RNase III was responsible for the U1 snRNA cleavages. First, processing in the wild-type extract was compared with that observed in an extract prepared from an rnt1::HIS3 mutant strain (Fig. 6, RNT1 and rnt1::HIS3, respectively). The RNT1 extract efficiently cleaved a 228 nt 25S rRNA control

**Figure 3.** Oligonucleotide-directed RNase H cleavage of extended U1 snRNAs. (A) A northern blot of U1 snRNA isolated from galactose grown U1Δβ 192–507 transformed yeast. The RNA was hybridized with a non-specific oligonucleotide (381–8, lane 1), a U1-specific oligonucleotide complementary to U1 at nt 170–189 from the 5′-end (U1-10, lane 2) or oligo(dT) (lane 4) and then digested with RNase H. Undigested RNA is shown in lane 3. (B) RNase H analysis of U1 snRNA isolated from cells that express GAL::U1 and U1Δβ 192–507Sm. RNA was hybridized with oligo(dT) (lane 1) or a non-specific oligo (381–8, lane 2) and digested with RNase H.

U1Δβ 192–507Sm also produced the U1α RNA fragment (45 nt, *) but this band was much reduced after glucose repression of GAL::U1 (compare lane 2 with 3 and 5 with 6). In addition, yeast with Sm mutant genes produced a large amount of RNA that is 64–78 nt longer than the mature U1 snRNA, termed U1Δβ (lanes 5 and 6) and U1β (lanes 2 and 3). Together, the RNase mapping data show that unlike the vertebrate system (43–45,50) a major step in yeast U1 snRNA 3′-end formation requires the Sm site and is not promoter dependent.

**RNase III cleaves U1 snRNA precursors**

Synthetic U1 snRNAs were produced to investigate whether extended U1 snRNA could be processed by yeast splicing extract. These uniformly radiolabeled RNAs consisted of the U1Δβ 192–507 or U1 mature RNA sequences followed by 190 and 188 nt of downstream sequence, respectively. Both pre-U1Δβ 192–507 (Fig. 5A, lanes 2–10) and pre-U1 (lane 11) RNAs were efficiently cleaved to produce five additional bands (Fig. 5A, labeled B–F). When the pre-U1Δβ 192–507 RNA was 5′-end-labeled only bands A–C were visible. Band A is the uncut precursor while bands B and C correspond to stable RNA products that end 80 and 115 nt, respectively, downstream of the U1α 3′-end observed in vivo (Fig. 5B). Band B was present only in the earliest time points suggesting that it is an intermediate in the processing reaction. When the pre-U1Δβ 192–507 snRNA was 3′-end-labeled before processing, bands D and E were present in the final reaction products. The 3′-end-labeled D and E RNAs were cleaved at positions 75 and 110 upstream of the 3′-end of the synthetic precursor (Fig. 5B). Of the six RNA forms observed, the 35 nt F band was the only internally derived fragment (i.e. was not found

**Figure 4.** Mapping the ends of lengthened U1 transcripts. (A) Primer extension analysis of U1 snRNA 5′-ends from yeast that express GAL::U1 (lane 1), U1Δβ 192–507 (lane 2), GAL::U1 and U1Δβ 192–507Sm (lane 3) and GAL::U1, U1Δβ 192–507Sm after GAL::U1 repression. The end-labeled cDNA products were fractionated alongside a U1 gene sequencing reaction done with the same oligonucleotide (lanes 5–8). (B) RNase protection of the U1 snRNA 3′-ends. Uniformly labeled RNA probes complementary from U1 nt 508 to the HindIII site located 190 nt downstream of the natural U1 3′-end were used for hybridization. A U1Δβ 192–507-derived probe was used in reactions shown in lanes 2, 5 and 8. A U1Δβ 192–507Sm-derived probe, which contains an additional 24 nt at its 5′-end, was used in reactions 2, 4, 6, 7 and 9. The RNA sources were GAL::U1 (lane 2), GAL::U1, U1Sm in galactose medium (lane 3) and after 4 h in glucose medium (lane 4), U1Δβ 192–507 (lane 5) and GAL::U1, U1Δβ 192–507Sm in galactose medium (lane 6) and after 4 h in glucose medium (lane 7). Lanes 8 and 9 contain rRNA controls for probe self- annealing. RNA markers are shown in lane 1 with sizes noted on the right. The relevant protected products are indicated by arrows at the left as U1Δα, U1β, U1Δβ and U1α, respectively. A 45 nt GAL::U1-derived fragment protected by the Sm probe is noted with an asterisk. RNA fragment lengths were determined by fractionating the protected fragments on high resolution sequencing gels adjacent to an RNA sequencing reaction (data not shown). (C) Comparison of the 3′-end RNA protection of endogenous U1 snRNA (strain TS192, lane 4) and GAL::U1 (lane 2). Lane 3 contains RNA from the shortened functional U1 gene, U1Δβ 192–507. The 61 nt RNA marker band is also shown (lane 1).
substrate at the expected positions, 15 and 50 nt from the 3′ end (lanes 2, 4 and 6–14; S.Abou Elela and M.Ares, personal communication; 28). The synthetic U1 snRNA was processed by the Rnt1p cleavage patterns adds support for endogenous Rnt1p, GST–Rnt1p, was incubated directly with U1 snRNA since the levels of U1 ε rnc RNA, E.coli met-orf15A-nusA RNA, E.coli sib3 RNA, the bacteriophage \( \lambda \) N-leader RNA and T7 bacteriophage RNA (reviewed in 54).

As a second test of Rnt1p activity in U1 processing, recombinant Rnt1p, GST–Rnt1p, was incubated directly with U1 snRNA (lanes 12–14). The pattern of cleavage was identical to that observed with the yeast extract except that band D, a possible intermediate in processing, was not found. The concordance of the RNA cleavage patterns adds support for endogenous Rnt1p, GST–Rnt1p, was incubated directly with U1 snRNA since the levels of U1 ε rnc RNA, E.coli met-orf15A-nusA RNA, E.coli sib3 RNA, the bacteriophage \( \lambda \) N-leader RNA and T7 bacteriophage RNA (reviewed in 54).

While GST–Rnt1p is able to cleave synthetic U1 snRNA precursors, mutation of this gene does not appreciably decrease the intracellular abundance of fully mature U1 snRNA (Fig. 8; 26). However, another RNA species, U1 ε, was much increased in the \( rnt1::HIS3 \) mutant yeast. U1 ε migrates above U1 β as a closely spaced doublet~750 nt in length. U1 ε is a 3′-extended form as this RNA hybridized both with a probe restricted to the U1 α sequence as well as with a probe that extended from the U1 α 3′-end to the \( \text{HindIII} \) site located 192 nt downstream (Fig. 8). U1 ε may not be polyadenylated since its electrophoretic mobility is insensitive to RNase H digestion with oligo(dT)\(_{20}\) (data not shown). However, given the large size of U1 ε, a short poly(A) tail might not be detectable by this method. Since the levels of U1 ε were reduced when the \( rnt1::HIS3 \) mutant was complemented by a plasmid-borne copy of \( \text{RNT1} \) (Fig. 8, compare lanes 3 and 4), U1 ε abundance directly relates to the endogenous levels of RNase III. Based on this, we conclude that Rnt1p, while not required for U1 snRNA maturation, does influence steady-state levels of 3′-extended U1 snRNAs.

DISCUSSION

Although three sites corresponding to the Sm consensus are present in yeast U1 snRNA, only the 3′-terminal Sm site is required for splicing. This result is consistent with a proposed secondary structure of yeast U1 snRNA (25) in which only the 3′-terminal Sm element is present as a characteristic single-
stranded element (3,4). Both upstream Sm-like sites are predicted to be partially or fully base paired with other regions of the yeast-specific core (55). The location of the final site is directly downstream of a base paired segment joining the two ends of the snRNA (the closure sequence, 23,56), is within 8 nt of the 3′-end of the mature transcript and is well placed for an element that participates in 3′-end maturation. Here we demonstrate a pivotal role for the Sm site and its associated proteins in the biosynthesis of yeast U1 snRNA.

Two prominent forms of 3′-extended U1 snRNA are found in yeast, a non-polyadenylated snRNA extended 64–78 nt (U1Δε) and a related polyadenylated form (U1γ). Several lines of evidence suggest that one or both of these snRNAs may be precursors to U1 snRNA. First, 3′-extended biosynthetic precursors are the rule rather than the exception for pol II transcripts (57) and recent studies have suggested that 3′-extended precursor forms of U2 and U5 snRNAs exist in yeast (6,25,26,58). Second, the 3′-extended U1 snRNA species are observed in low abundance in wild-type yeast but increase in abundance with experimental manipulation of the Sm site or the Smd1p core protein (Fig. 2 and data not shown). This impact of Smd1p depletion is consistent with a build-up of precursors (U1Δβ and U1Δγ) resulting from a kinetic barrier to product formation (U1Δα). Inactivation of the common snRNP protein Brr1p slows conversion of the putative U2 precursor to its mature form (6). Although Brr1p has not been shown to be part of the Sm core particle, this protein interacts genetically with Smd1p and the removal of either activity produces similar defects in snRNA stability and cap modification (5,6,8). Third, like the U2 and U5 snRNAs (25,26), U1 has a pair of Rnt1p cleavage sites located downstream of the mature snRNA (Figs 5 and 6). In each case the rnt1::HIS3 mutation changes the wild-type level of the snRNA. The steady-state levels of mature U2 and USL snRNA, but not U1 snRNA, decrease in the rnt1::HIS3 background. For U2, but not U1, the rnt1::HIS3 mutation also results in a large increase in polyadenylated snRNA. Taken together, the available data support the view that the pol II spliceosomal snRNAs are matured at their 3′-ends through similar though not necessarily identical means.

*Xenopus laevis,* human and mouse U1 molecules are synthesized as nuclear precursors with 10–12 nt extensions (59–62) that are trimmed to having one or two extra nucleotides in the cytoplasm (59,60). A nuclear activity has been identified that specifically removes the remaining nucleotide of the U1 precursor (50), consistent with the observation that mature U1 snRNAs are exclusively nuclear (50,60). Since in vertebrates the Sm site is not required for nuclear export (63), the initial step of snRNA processing is Sm site-independent. In contrast, the general inhibition of nucleocytoplasmic exchange by vesicular stomatitis viral infection (64,65) or viral M protein expression (66) inhibits the processing reaction and results in the accumulation of nuclear snRNA precursors. If yeast behaves similarly, the Sm site mutation would trap U1 precursors in the cytoplasm. In this case, the 3′-extended U1Δβ RNA might be viewed as a much longer version of the vertebrate +1 cytoplasmic species. It is not clear why the 3′-extensions of the yeast Sm mutant are not processed more closely to the natural 3′-end, as in vertebrates. It is possible, however, that in yeast the Sm site mutation inhibits nuclear export and the extended RNAs are completely nuclear and thus unavailable to a cytoplasmic processing activity. Enhanced nuclear retention might also lead to promiscuous polyadenylation and, hence, the observed increased levels of U1Δγ observed with the Sm site change. However, since depletion of Smd1p also results in increased U1Δγ levels (Fig. 2C), one would need to argue that: (i) Smd1p binds prior to nuclear export (in contrast to vertebrates; 67,68); (ii) Smd1p assists in the removal of polyadenylated tails; or (iii) that U1Δγ levels may increase due to an indirect consequence of splicing inhibition that occurs with Smd1p depletion.

U1 snRNA is an efficient substrate for Rnt1p and is processed almost identically in yeast extract and with the purified enzyme. Thus, it seems almost certain that Rnt1p, not an Rnt1p-cleaved catalytic RNA, contains the observed U1 processing activity in yeast. A minor difference noted between the extract and Rnt1p processing results was that band D, generated in the yeast extract,
Figure 9. Model for U1 snRNA 3′-end formation. U1 snRNA appears to be processed along two alternative pathways. One pathway uses Rnt1p (yeast RNase III) to process the RNA to an intermediate state, U1β, which may become polyadenylated. An unknown activity, likely an exonuclease, is required to process U1β to the mature U1α form. This activity may also be able to process the putative U1 precursor, U1ε, directly to U1α. However, in both cases, U1α formation in vivo passes through an Sm-dependent step. The closed circle represents the U1 gene body, while the sequence/structure represents the RNA including and downstream of the mature U1 3′-end.

was not observed with purified Rnt1p. However, band D is a singly cleaved precursor RNA form (Fig. 6) and this difference may simply reflect a more efficient processing by the purified enzyme into the doubly cleaved E and F RNAs. U5 snRNA is also readily cleaved by Rnt1p at sites located 26–27 (pre-U5L) and 90–91 (pre-U5S) nt downstream of the mature ends (26). In this case, however, the cleaved products are then processed further by an uncharacterized nuclease to 3–4 nt downstream of the natural U5 3′-ends (26). In vitro, U1 snRNA is a much less favored substrate for the second nuclease activity since little or no U1 is processed beyond the Rnt1p cleavage sites (Figs 5 and 6 and data not shown). It remains to be determined whether cis-inhibitory elements or missing trans-acting factors account for this difference.

Model of U1 biosynthesis

The following model accounts for our experimental observations and offers a framework for future experimentation (Fig. 9). In the rnt1::HIS3 mutant we observe a U1 snRNA form extended by at least 180 nt, U1ε. We propose that U1ε is a U1 snRNA precursor, perhaps formed by transcriptional termination, and that Rnt1p cleaves U1ε at positions 80 and 115 from the mature 3′-end to form U1β. U1β may then be processed through an Sm-dependent pathway to mature U1α RNA. Alternatively, U1β may be polyadenylated to form U1γ. Since polyadenylated spliceosomal snRNAs have not been observed (23,24,69–71), U1γ may reflect promiscuous polyadenylation enhanced by kinetic impairment of the normal processing pathway. Alternatively, as speculated for an unexpected form of polyadenylated telomerase RNA (72), snRNAs may normally cycle between polyadenylated and deadenylated species.

In the absence of Rnt1p, mature U1α still forms as long as the U1 Sm site is intact. Consequently, U1ε or a related molecule must be processed directly to the Sm-dependent (U1β) form. While yeast U2 snRNA maturation is fully Rnt1p-dependent (25), an Rnt1p bypass pathway has been suggested to account for U5S formation (26). The cellular benefit of dual pathways is unknown, but not unreasonable to contemplate. Perhaps the Rnt1p-dependent (or -independent) pathway contributes to the efficiency of U1 production at times of environmental stress or at certain times of the cell cycle. Alternatively, as shown for the dicistronic snoRNAs U14 and smr190, Rnt1p cleavage of U1 precursors may contribute to the formation of an uncharacterized co-transcribed downstream RNA (53).

Clearly, the pathway for yeast U1 snRNA biosynthesis is complex and has features that distinguish it from what has been observed for vertebrate U1 and for yeast U2 and U5 snRNAs. Future experiments will be required to test the validity of our dual pathway model and to identify the Sm-dependent and -independent enzymatic activities required for U1 3′-end formation.

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REFERENCES