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Processing of the Precursors to Small Nucleolar RNAs and rRNAs Requires Common Components

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The genes encoding the small nucleolar RNA (snoRNA) species snR190 and U14 are located close together in the genome of *Saccharomyces cerevisiae*. Here we report that these two snoRNAs are synthesized by processing of a larger common transcript. In strains mutant for two 5′→3′ exonucleases, Xrn1p and Rat1p, families of 5′-extended forms of snR190 and U14 accumulate; these have 5′ extensions of up to 42 and 55 nucleotides, respectively. We conclude that the 5′ ends of both snR190 and U14 are generated by exonuclease digestion from upstream processing sites. In contrast to snR190 and U14, the snoRNAs U18 and U24 are excised from the introns of pre-mRNAs which encode proteins in their exonic sequences. Analysis of RNA extracted from a *dbr1*-Δ strain, which lacks intron lariat-debranching activity, shows that U24 can be synthesized only from the debranched lariat. In contrast, a substantial level of U18 can be synthesized in the absence of debranching activity. The 5′ ends of these snoRNAs are also generated by Xrn1p and Rat1p. The same exonucleases are responsible for the degradation of several excised fragments of the pre-rRNA spacer regions, in addition to generating the 5′ end of the 5.8S rRNA. Processing of the pre-rRNA and both intronic and polycistronic snoRNAs therefore involves common components.

Eukaryotic cells contain a large number of small nucleolar RNA (snoRNA) species that play major roles in the processing and modification of the pre-rRNAs (reviewed in references 29 and 41). The mode of synthesis of many of the snoRNA species differs from that of other small RNAs. Rather than being expressed from simple genes, most of the known snoRNAs are encoded in the intronic sequences of genes which also encode mRNAs in the exonic sequences. This was first observed for the mammalian U14 snoRNA (27) and has subsequently been demonstrated for several other species (for reviews, see references 29, 31, and 37). In most cases there is some relationship between the protein product of the host gene and ribosome synthesis or function. Several snoRNAs are located in the introns of genes encoding ribosomal proteins (r-proteins), nucleolar proteins, or translation factors (reviewed in reference 29). It has been suggested that this provides a mechanism for the coregulation of the synthesis of the snoRNAs and other components involved in ribosome synthesis. No small RNA species other than the snoRNAs are known to follow this pathway of biosynthesis.

The mechanism of synthesis of vertebrate snoRNAs has been the subject of considerable interest. The faithful synthesis of snoRNAs in *Xenopus* oocytes and in vitro has been reported. The best-studied example is the U16 snoRNA, which is encoded within intron III of the L1 r-protein gene (7, 9, 13, 34). Synthesis of U16 is mutually exclusive with pre-mRNA splicing and involves endonucleolytic cleavage within intron III, followed by trimming to generate the 5′ and 3′ ends of the mature snoRNA. A similar pathway of processing has been reported for U18, which is also encoded in introns of the L1 r-protein gene (34). Processing of other snoRNAs, i.e., U15 (44), U17 (10, 21, 22), and U19 (20), appears to be slightly different; for these snoRNAs, no upstream endonuclease cleavage site was identified, and both 5′ and 3′ processing appear to be purely by exonuclease digestion of either the pre-mRNA or the excised intron. The exonucleases that carry out these processing reactions have not been identified.

Two homologous proteins with in vitro 5′→3′ exoribonuclease activity have been purified from yeast (19, 24, 38). These are Xrn1p (also known as Rar5p, Kem1p, Dst2p, and Sep1p) (reviewed in reference 18), which functions in the cytoplasm (17), and Rat1p (also known as Tap1p and Hke1p) (1, 2, 12, 19), which functions in the nucleus (17). The genes encoding each of these proteins have been cloned by a number of different groups by using selection techniques which are not obviously related, although in several cases they potentially involve RNA metabolism. We have previously reported that strains which have the *XRN1* gene deleted and carry a temperature-sensitive lethal mutation in *RAT1* are impaired in the formation of the 5′ end of the 5.8S rRNA (14). In addition, *xrn1* mutant strains accumulate high levels of an excised pre-rRNA spacer fragment containing the 5′ region of internal transcribed spacer 1 (ITS1) between sites D and A_2 (Fig. 1B) (39). Other studies have shown that *xrn1* mutants are impaired in 5′→3′ degradation of mRNA (15, 32, 33). Homologs of both Rat1p and Xrn1p have been identified in mice (5, 36), and mouse Xrn1p can functionally replace the yeast protein, showing that their functions have been highly conserved in evolution.

The yeast U18 and U24 snoRNAs are intron encoded (23, 29, 35), like their vertebrate homologs (8, 35). Vertebrate U14 is encoded within introns of the *hsc70* gene (27, 29), but the genomic arrangement is different in *Saccharomyces cerevisiae*. The yeast *SNR128* gene, which encodes U14, lacks consensus promoter elements and is located just 67 bp 3′ to the end of the gene encoding another snoRNA, snR190 (46). The U14 and snR190 snoRNAs lack the 5′ cap structure characteristic of primary transcripts of RNA polymerase II, and both species show 5′ heterogeneity, consistent with synthesis by processing of larger transcripts (reference 3 and this work). This suggested that these snoRNAs are transcribed from a polycistronic

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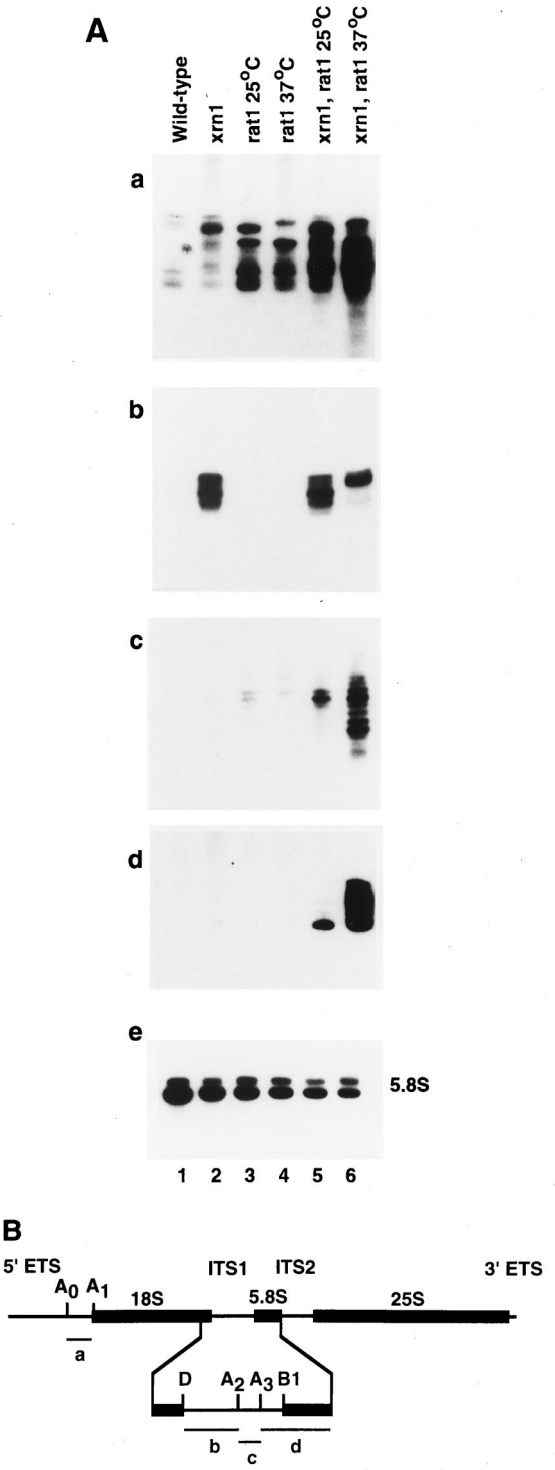


FIG. 1. Accumulation of excised pre-rRNA fragments in exonuclease mutants. (A) Northern hybridization of RNAs extracted from strains of the indicated genotypes following growth at 25°C or 2 h after transfer to 37°C. Panels: a, Riboprobe specific for the A_0-A_1 region; b, probe specific for the D-A_2 region (oligonucleotide 002); c, riboprobe specific for the A_2-A_3 region; d, 5′-extended 5.8S detected with the probe for the A_3-B_1 region (oligonucleotide 003); e, mature 5.8S rRNA (oligonucleotide 017). RNA was separated on a gel containing 8% polyacrylamide. (B) Structure of the pre-rRNA, showing the locations of processing sites above the pre-rRNA. Lines below the pre-rRNA indicate the species detected in panels a to d of panel A. The effects of the mutations on the accumulation of the fragments referred to as a, b, c, and d are shown in panels a, b, c, and d, respectively, of panel A.

snoRNA precursor, as is also the case for maize U14 (25, 26). Here we analyze the in vivo processing of both intron-encoded and polycistronic snoRNAs in yeast and report that formation of their 5′ ends involves the 5′→3′ exonucleases Rat1p and Xrn1p. We also show that these enzymes process a variety of pre-rRNA spacer fragments, in addition to forming the 5′ end of the 5.8S rRNA.

MATERIALS AND METHODS

Strains and media. The growth and handling of *S. cerevisiae* were by standard techniques. Cultures were grown in SD minimal medium containing 2% glucose, 0.67% yeast nitrogen base (Difco), and appropriate supplements. The strains used were as follows: *XRN1* strain, *MAT***a** *trp1-1 ura3-52 his3-11,15 ade2-1* (strain W303-1A; kindly provided by S. Kearsey); *xrn1*-Δ strain, same as the *XRN1* strain except for *xrn1::URA3* (strain R934; kindly provided by S. Kearsey) (in the *xrn1::URA3* mutation the *URA3* gene is inserted at a point corresponding to 97 amino acids from the amino terminus of the protein encoded by the gene; no Xrn1p can be detected on Western blots prepared from this strain [17a]); *rat1-1* strain, *MAT***a** *rat1-1 leu2-Δ1 ura3-52 his3-Δ200* (DAH18; kindly provided by C. Cole) (2); *rat1 xrn1* strain, *MAT***a** *rar5::URA3 rat1-1* (strain 966-1C; kindly provided by S. Kearsey); *DBR1* strain *MAT***a** *trp1-Δ1 his3-Δ200 leu2-Δ1 ura3-167* (strain YH8; kindly provided by J. Boeke) (11); and *dbr1*-Δ strain, same as the *DBR1* strain but with Δ*dbr1::HIS3* (strain KC99) (11).

RNA extraction, Northern hybridization, and primer extension. RNA extraction (43) Northern hybridization (40), and primer extension (42) were performed as previously described. For Northern hybridization and primer extension, total RNA equivalent to that of cells at an optical density at 600 nm of 0.1 (approximately 2×10^6 cells; equivalent to 4 μg of RNA for wild-type cells) was used for each sample. Prior to RNA extraction, the *rat1-1* and *tap1-1* strains were pregrown at 25°C to mid-log phase (optical density at 600 nm = 0.3) and either harvested or transferred to 37°C for 2 or 6 h prior to being harvested.

Hybridization probes. (i) rRNA probes. For the A_0-A_1 and A_2-A_3 fragments, riboprobes overlapping the entire regions were used (28, 45). The hybridization probe for the D-A_2 fragment was oligonucleotide 002 (GCTCTTTGCTCTTGC C), the oligonucleotide probe for the A_2-A_3 region was oligonucleotide 003 (TGTTACCTCTGGGCCC), the probe for the 5′-extended 5.8S rRNA was oligonucleotide 001 (CCAGTTACGAAAATTCTTG), the probe for the 5.8S rRNA was oligonucleotide 017 (GCGTTGTTCATCGATGC), the probe for the 5S rRNA was oligonucleotide 041 (CTACTCGGTCAGGCTC), the probe for the 5′ region of ITS2 was oligonucleotide 013 (GGCCAGCAATTTCAAGTT A), the probe for the 3′ region of ITS2 was oligonucleotide 006 (AGATTAGC CGCAGTTGG), and the probe for the external transcribed spacer (ETS) 5′ to site A_0 was oligonucleotide 024 (TCGGGTCTCTCTGCTGC).

(ii) snoRNA probes. The oligonucleotide complementary to the 5′ flanking sequence of snR190 was CAATCAATTCTTCTTTTCTG (αsnR190+1), the internal snR190 oligonucleotide was CGTCATGGTCGAATCGG (αsnR190), the oligonucleotide complementary to the 5′ flanking sequence of U14 was ATAT ATTATCTGTCTCCTC (αU14-9), the internal U14 oligonucleotide was TGC GAATGTTAAGGAACC (αU14), the internal U24 oligonucleotide was TCAG AGATCTTGGTGATAAT (αU24), the U24 3′ flanking oligonucleotide was AAACCATTCATCAGAG (U24-3′fl), the U18 internal oligonucleotide was GTCAGATACTGTGATAGTC (αU18), and the U18 3′ flanking oligonucleotide was GCTCTGTGCTATCGTC (U18-3′fl).

RESULTS

Excised pre-rRNA spacer fragments accumulate in 5′→3′ exonuclease mutants. RNA was extracted from a strain carrying an *xrn1::URA3* gene disruption and from an otherwise isogenic wild-type strain following growth at 25°C and from the *rat1-1* mutant strain and the *rat1-1 xrn1*-Δ double-mutant strain following growth at 25°C and after transfer to 37°C for 2 or 6 h. All data shown are for the 2-h time point; the 6-h time point gave similar results. Growth of *rat1-1* strains essentially ceased within 2 h of transfer to 37°C (2).

Endonucleolytic cleavage of the pre-rRNA releases a number of discrete fragments from the transcribed spacer regions (see Fig. 1B for the structure of the pre-rRNA). The levels of these excised fragments were assessed by Northern hybridization (Fig. 1A). Strong accumulation of a number of spacer fragments was observed in the exonuclease mutants. Hybridization with a probe specific for the region from site A_0, in the 5′ ETS, to site A_1, the 5′ end of the 18S rRNA, showed that this fragment (fragment a in Fig. 1B) accumulated significantly

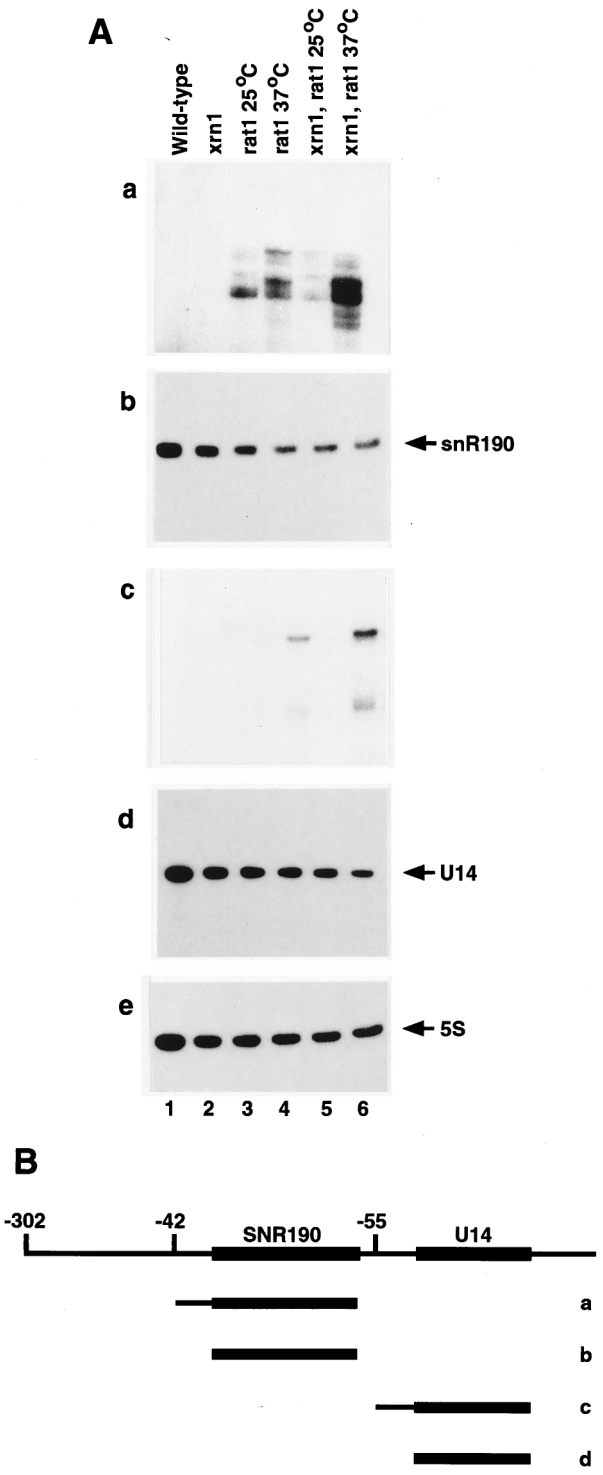


FIG. 2. Northern analysis of the synthesis of U14 and snR190. (A) Northern hybridization of RNAs extracted from strains of the indicated genotypes following growth at 25°C or 2 h after transfer to 37°C. Panels: a, 5′-extended forms of snR190 (oligonucleotide αsnR190+1); b, mature snR190 (oligonucleotide αsnR190); c, 5′-extended forms of U14 (oligonucleotide αU14-9); d, mature U14 (oligonucleotide αU14); e, mature 5S rRNA (oligonucleotide 041). RNA was separated on a gel containing 8% polyacrylamide. (B) Predicted structure of the precursor to snR190 and U14. Lines below the pre-snoRNA indicate the species detected in panels a to d of panel A.

in either the *xrn1*-Δ or *rat1-1* single-mutant strain. The accumulation was, however, much stronger in the double mutant at 37°C, the nonpermissive temperature for the *rat1-1* strain (Fig. 1A, panel a). This indicates that both exonucleases normally play roles in the degradation of this pre-rRNA region. The largest band visible in the wild-type strain probably corresponds to the full-length A_0-A_1 fragment (91 nucleotides [nt]) (45), indicating that most of the accumulated spacer fragments in the mutants have undergone some digestion.

Cleavage at sites A_0 and A_1 is inhibited in strains with the U3 snoRNA depleted (16); to confirm the identification of the hybridizing RNA, the A_0-A_1 probe was used on a Northern blot of RNA from a strain genetically depleted of U3 by growth of a *GAL::U3* strain (16) on glucose medium. As expected, the A_0-A_1 fragment was lost during U3 depletion (data not shown).

Interestingly, cleavage at site A_0 can be detected by primer extension in wild-type strains by using a primer which hybridizes 3′ to site A_1, (6, 16, 45), but no effects of the exonuclease mutations were observed with this primer (data not shown). We conclude that while degradation of the excised A_0-A_1 fragment from A_0 very rapidly follows processing at A_1, the 5′ exonuclease digestion occurs only after cleavage of A_1.

Strains carrying mutations of *XRN1* have previously been reported to accumulate the pre-rRNA fragment from site D, the 3′ end of the 18S rRNA, to site A_2 in ITS1 (fragment b in Fig. 1B). As expected, the *xrn1*-Δ strain strongly accumulated this fragment (Fig. 1A, panel b, lane 2). The fragment which accumulated in the *rat1-1 xrn1*-Δ double-mutant strain at the nonpermissive temperature (Fig. 1A, panel b, lane 6) was, however, significantly longer than that observed in the *xrn1*-Δ single mutant (Fig. 1A, panel b, lane 2) or in the double mutant grown at the permissive temperature for the *rat1-1* strain (Fig. 1A, panel b, lane 5). We conclude that while the degradation of fragment b is largely due to Xrn1p, Rat1p also plays a role in this activity, at least in *xrn1* mutants.

A hybridization probe specific for the region between the A_2 and A_3 cleavage sites in ITS1 (fragment c in Fig. 1B) showed that this fragment was accumulated in the *rat1-1* single-mutant strain (Fig. 1A, panel c), with much stronger accumulation in the double-mutant strain, particularly after growth at the nonpermissive temperature for the *rat1-1* strain (Fig. 1A, panel c, lane 6). Clear accumulation was not seen in the *xrn1*-Δ single-mutant strain. The hybridization probe used for Fig. 1A, panel c, is an antisense riboprobe to the A_2-A_3 region. Hybridization with an oligonucleotide complementary to the sequence immediately 5′ to site A_3 gave the same result (data not shown), suggesting that the accumulated fragments have undergone partial digestion from the 5′ end.

We also show the level of the mature 5.8S rRNA (Fig. 1A, panel e) and the accumulation of the 5′-extended form of the 5.8S rRNA (fragment d in Fig. 1B) by using a hybridization probe to the 3′ region of ITS1 (Fig. 1A, panel d). Here, too, accumulation was strongest in the double-mutant strain grown at the nonpermissive temperature (Fig. 1A, panel d, lane 6). As previously reported (14), the major product has undergone partial digestion from the 5′ end to the base of a stable stem structure located between site A_3 and the 5′ end of the 5.8S rRNA.

Probes to the 5′ or 3′ region of ITS2 or further upstream in the 5′ ETS did not detect accumulation of pre-rRNA fragments in the mutant strains (data not shown; see Materials and Methods for details of the hybridization probes used). From these data we conclude that both Xrn1p and Rat1p play roles in the turnover of several excised fragments of the pre-rRNA. In each case, the steady-state level of the pre-rRNA fragment

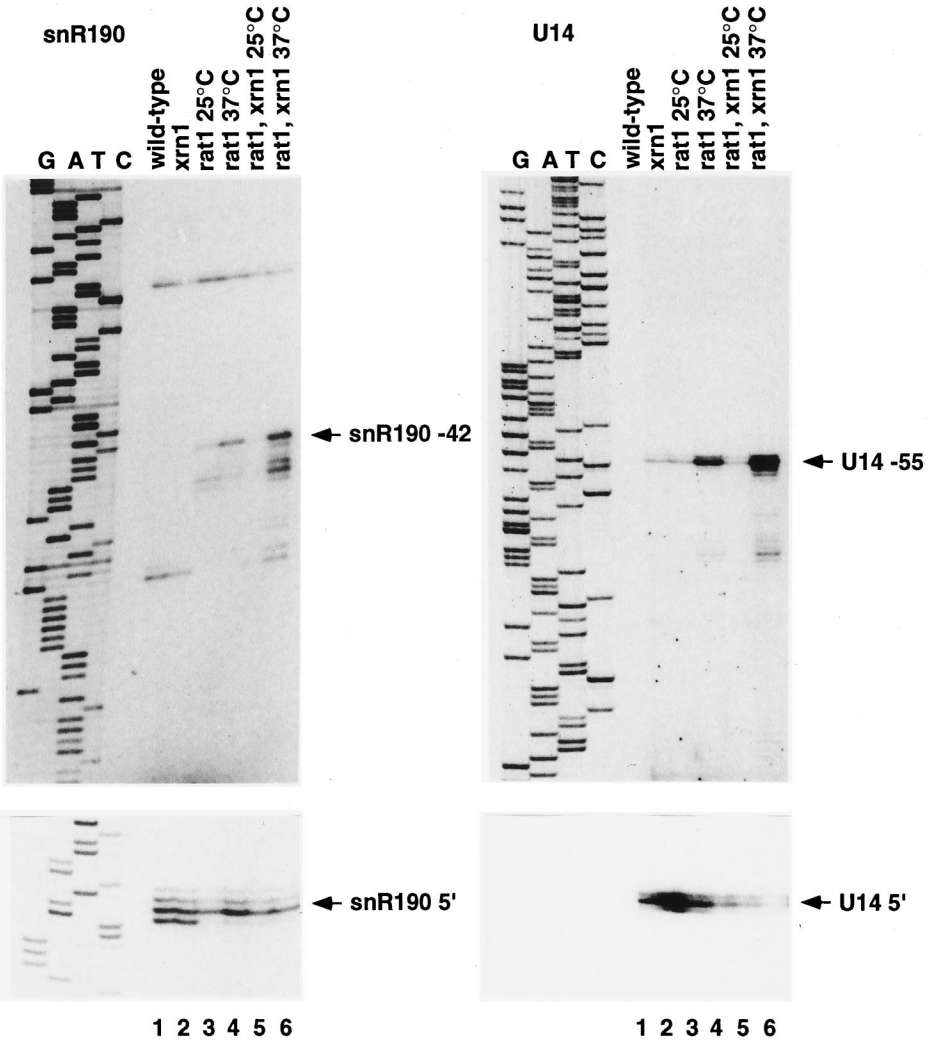


FIG. 3. Primer extension analysis of the synthesis of U14 and snR190. 5′-extended forms of snR190 and U14 are detected in 5′→3′ exonuclease mutants. Primer extension was performed with the αU14 or αsnR190 oligonucleotide on RNA extracted from strains of the indicated genotypes following growth at 25°C or 2 h after transfer to 37°C. The lower panels show shorter exposures of the same primer extensions as the corresponding upper panels.

was very low in wild-type cells, indicating that degradation very rapidly follows generation of the excised pre-rRNA fragments.

5′-extended forms of snR190 and U14 accumulate in 5′→3′ exonuclease mutants. Northern hybridization with probes to the mature snR190 (Fig. 2A, panel b) and U14 (Fig. 2A, panel d) sequences revealed a reduction in the levels of the mature snoRNAs compared to 5S rRNA (Fig. 2A, panel e), particularly in the *xrn1*-Δ *rat1-1* double mutant at 37°C. The mature snoRNAs are expected to be stable, so the reduction observed 2 h after transfer to nonpermissive conditions indicates that the synthesis of new snR190 and U14 was strongly inhibited. The *rat1-1* strain essentially ceases growth after 2 h at 37°C (reference 2 and data not shown), and further reduction was not observed at later time points (data not shown).

Probes to the 5′ flanking sequences of snR190 (Fig. 2A, panel a) and U14 (Fig. 2A, panel c) revealed the accumulation of 5′-extended forms of the snoRNAs in the exonuclease mutants. For both snR190 and U14 the 5′-extended RNA was most clearly seen in the double mutant at the nonpermissive temperature (Fig. 2A, panels a and c, lanes 6). A lower accumulation of 5′-extended snR190 was seen in the *rat1-1* single-mutant strains. In addition, longer RNAs were weakly detected; RNAs with the same gel mobility were detected by both snR190 and U14 internal and 5′ flanking probes (data not shown), indicating that these represent common transcripts of snR190 and U14 pre-snoRNAs. The 5′-extended snoRNA species accumulated to much lower levels than the mature snoRNAs (ca. 10%), suggesting that other pathways for their turnover exist.

Primer extension was used to better characterize the 5′-extended forms of snR190 and U14 seen in the exonuclease mutant strains. The *rat1-1* mutant strains accumulated a ladder of 5′-extended forms of snR190 which extend to a position 42 nt longer than mature snR190, but not beyond. This accumulation was evident in the *rat1-1* single mutant (Fig. 3, lanes 3 and 4) but was much stronger in the double-mutant strain at the nonpermissive temperature (Fig. 3, lane 6). Consistent with the results of Northern hybridization, the level of mature U14 fell substantially in the *rat1-1* strains, particularly in the *rat1-1* *xrn1*-Δ strain at 37°C (Fig. 3, lane 6). A ladder of 5′-extended forms of U14, which extended to a position 55 nt longer than mature U14 but not beyond, was also observed in the *rat1-1*

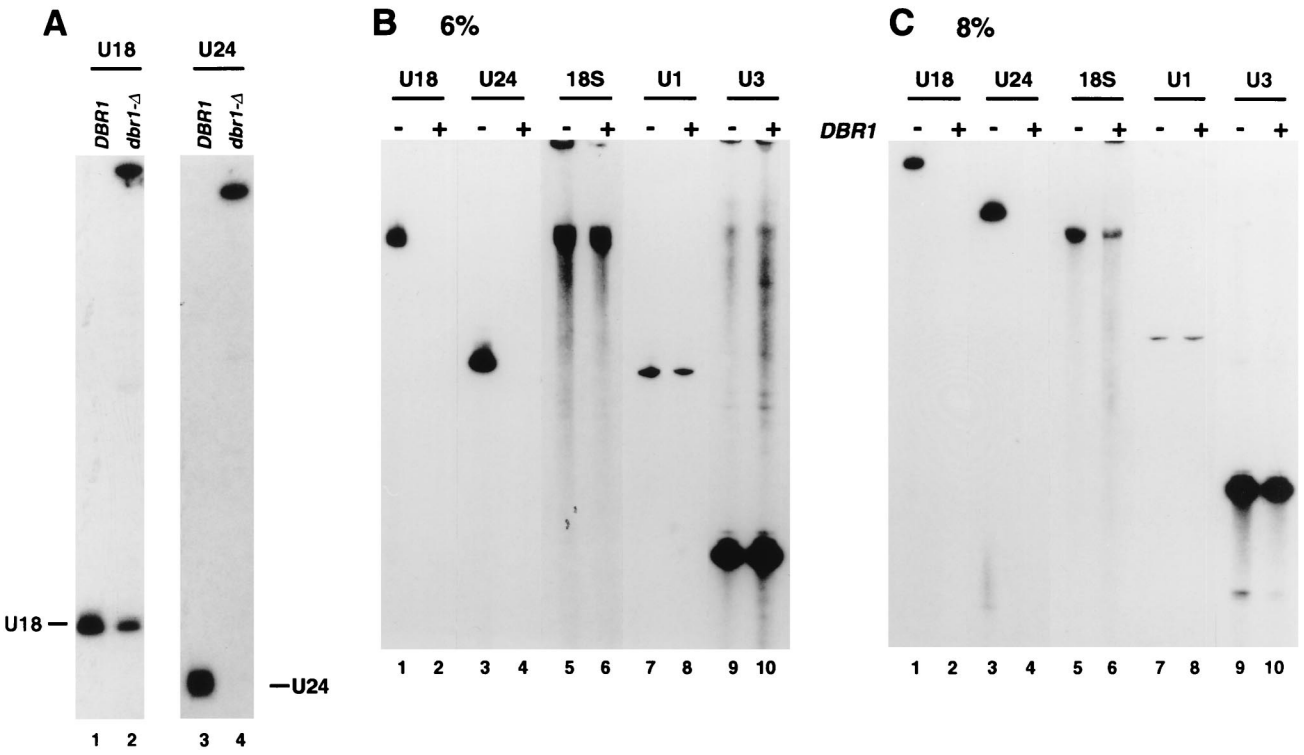


FIG. 4. Lariat forms of U18 and U24 accumulate in an intron-debranching mutant. (A) In RNA from the *dbr1*-Δ strain, mature U18 and U24 are underaccumulated and longer forms are detected. (B and C) RNAs extracted from the *dbr1*-Δ strain and an otherwise isogenic *DBR1* strain were separated by long migration on gels containing either 6% (B) or 8% (C) polyacrylamide. Lanes + and −, *DBR1* and *dbr1*-Δ strains, respectively. The slow-migrating forms of U18 and U24 differ in their relative migrations on the gels compared to the linear RNA species U3 (311 nt), U1 (568 nt), and 18S rRNA (1,860 nt). Note that mature U18 and U24 are substantially smaller than the linear RNA species shown and have been lost from the gels in panels B and C.

strain at 37°C (Fig. 3, lane 4) and was more strongly accumulated in the *xrn1*-Δ *rat1-1* double-mutant strain (Fig. 3, lane 6).

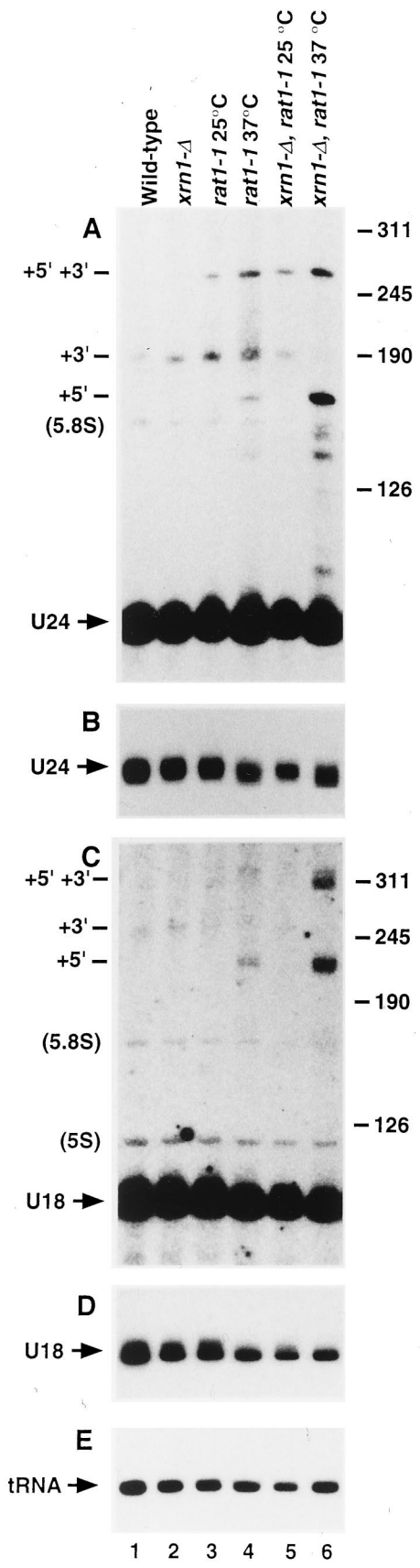
As with pre-rRNA processing (14), the accumulation of the pre-snoRNAs was similar but not identical in strains carrying the *tap1-1* mutation (data not shown), which is allelic with *rat1-1* (1). From these data we conclude that Rat1p has the major activity in the processing of mature snR190 and U14 from 5′-extended pre-snoRNAs, with Xrn1p also playing a role, at least in *rat1-1* mutant strains.

In primer extension analysis, the internal snR190 oligonucleotide also gave a strong stop at a position 302 nt 5′ to the end of the mature snR190 (data not shown). A primer extension stop at a similar position was detected with an internal U14 oligonucleotide, but this product is too long for its end to be accurately identified. In addition, the internal U14 primer gave a stop at the position corresponding to the 5′ end of the mature snR190; as expected, this stop was reduced in the *xrn1*-Δ *rat1-1* double-mutant strain (data not shown). These data are consistent with processing of both U14 and snR190 from a common pre-snoRNA transcript.

Processing of intron-encoded snoRNAs. In contrast to snR190 and U14, the snoRNAs U18 (4) and U24 (35) are encoded in introns of the genes *EFB1* (encoding EF-1 beta) and *BEL1*(encoding a G-beta-like protein of unknown function), respectively. To determine whether intron debranching is required for their synthesis, RNA was extracted from a strain with *DBR1*, the gene encoding the intron lariat debranching enzyme (11), deleted. Strains lacking this activity are viable and accumulate the excised intron lariats to high levels (11). Since these are circular molecules, synthesis of intron-encoded snoRNA species that are processed exclusively by exonucleases is expected to be severely inhibited in the *dbr1*-Δ strain. In contrast, synthesis of snoRNAs that are generated by pathways involving endonuclease cleavage is predicted to be little affected.

Northern hybridization (Fig. 4A) showed that formation of mature U24 was almost entirely abolished in the *dbr1*-Δ mutant. This strongly indicates that U24 is synthesized exclusively by exonuclease digestion. Synthesis of U18 was also reduced, but only to ~30% of the wild-type level. Slow-migrating RNA species were detected with both the U18 and U24 probes in the *dbr1*-Δ strain. To determine whether these represent the intron lariats, their mobilities on gels containing either 6% (Fig. 4B) or 8% (Fig. 4C) polyacrylamide were compared. The relative mobility of circular RNA species compared to linear RNA is expected to be more retarded in the 8% gel than the 6% gel, and this was observed. Strains carrying *dbr1*-Δ are reported to accumulate intron lariats which lack the sequence 3′ to the intron branch site (11). This has not been tested for the lariats containing the U24 and U18 sequences but is likely to be the case.

The effects of 5′→3′ exonuclease mutations on the synthesis of U18 and U24 were also assessed. Northern hybridization (Fig. 5) showed some reduction in the levels of mature U24 (Fig. 5B) and U18 (Fig. 5D) relative to $tRNA_3^{Leu}$ (Fig. 5E) 2 h after transfer to 37°C, indicating an inhibition of their synthesis. Interestingly, the mobility of U24 was reproducibly shifted downwards, i.e., to slightly shorter forms. Since the location of the 5′ end of U24 is unaltered on primer extension (see Fig. 6), this presumably represents the formation of 3′-shortened spe-



cies. Larger forms of U18 and U24 were detected in the *rat1-1* mutant strain at 37°C (Fig. 5, lane 4) and were more abundant in the *xrn1*-Δ *rat1-1* double mutant (Fig. 5, lane 6). For both snoRNAs two major extended species were detected. The gel mobility of the larger U24 species (+5′ +3′ in Fig. 5A) is appropriate for the entire intron (273 nt). From its mobility, the smaller species (+3′ in Fig. 5A) is predicted to be 3′ matured but 5′ extended to the end of the intron (169 nt). Consistent with this, the U24 +5 +3 band hybridized to the U24-3′ flanking oligonucleotide probe, while the U24 +5′ band did not (data not shown); as the probe lies across the 3′ end of the mature U24, the U24 +5′ species is very likely to be fully 3′ matured. The accumulation of the U24 intron in the exonuclease mutant suggests that some inhibition of 3′ processing also occurs. In other systems, e.g., pre-tRNA processing, 3′ processing is inhibited in the absence of prior 5′ processing. In the wild-type strain an RNA species of ~190 nt (+5′ in Fig. 5A) is detected with the U24 probe. This is in agreement with the size of a pre-snoRNA which is 5′ mature but 3′ extended to the end of the intron (192 nt). Consistent with this, the 190-nt species is depleted in the double-mutant strain (Fig. 5, lane 6) and is detected with the U24-3′ probe in the same samples (data not shown). From the primer extension data (see below), this species would not, however, be expected in the *rat1-1* mutant strain, and we assume that the band at this position in Fig. 5, lane 4, represents an aberrant processing intermediate.

In the case of U18, the mobility of the U18 +5′ band in Fig. 5C, lane 6, is consistent with a species which is 3′ mature but 5′ extended to the end of the intron (220 nt). The mobility of the larger U18 +5′ +3′ species in Fig. 5C, lane 6, is, however, greater than that predicted for the intact intron (366 nt); the faint band above this species may represent the intact intron. The U18 +5′ +3′ species may therefore be a form of the intron which has undergone partial digestion. A faint band present in the wild-type but not in the double-mutant strain (+3′ in Fig. 5C) shows a gel mobility consistent with U18 that is 5′ mature but 3′ extended to the end of the intron (253 nt)

The synthesis of U24 and U18 was also examined by primer extension. In the *dbr1*-Δ strain, the mature 5′ end of U24 is lost and a strong primer extension stop at the position corresponding to the 5′ end of the mRNA intron was observed (labeled 5′ IVS in Fig. 6) with either an internal U24 oligonucleotide or an oligonucleotide complementary to the 3′ flanking sequence. With the internal U24 oligonucleotide, an RNA species extended to the 5′ end of the intron was detected in the *rat1-1* strains at 37°C (Fig. 6); a reduction in the mature 5′ end of U24 was also observed, particularly in the *xrn1*-Δ *rat1-1* double-mutant strain at 37°C. A striking reduction in the stop corresponding to the mature 5′ end of U24 was observed in the *rat1-1* strains at 37°C by using the 3′ flanking oligonucleotide, which detects the 3′-immature pre-snoRNA species. This is consistent with the inhibition of 5′ processing of newly synthesized U24 in the mutant. Similar results were obtained for U18 (data not shown), with the interesting difference that the ma-

FIG. 5. Northern analysis of the synthesis of U18 and U24. Longer forms of U18 and U24 are detected in 5′→3′ exonuclease mutants. Northern hybridization of RNAs extracted from strains of the indicated genotypes is shown. For U18, RNA was separated on a gel containing 6% polyacrylamide; for U24, which is smaller, a gel containing 8% polyacrylamide is shown. The positions of migration of U14 (126 nt), snR190 (190 nt), snR10 (245 nt) and U3 (311 nt) determined by subsequent Northern hybridization of the same filters are indicated, as are the positions of migration of mature 5S and 5.8S rRNAs. Species predicted to be 5′- and 3′-extended forms of U24 and U18 are indicated (+5′, +3′, and +5′ +3′).

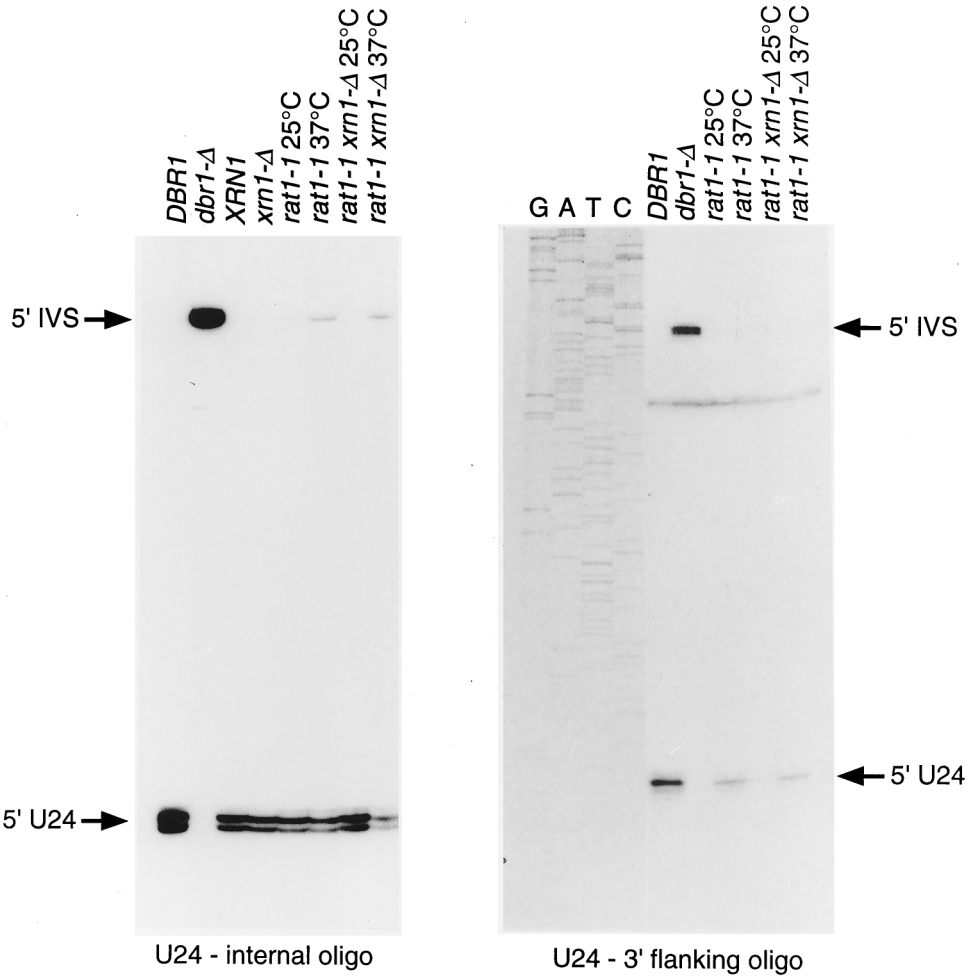


FIG. 6. Primer extension analysis of the synthesis of U24. (Left) Primer extension with a probe to mature U24 (αU24). (Right) Primer extension with an oligonucleotide (oligo) spanning the 3′ end of U24 (U24-3′fl). The positions of the mature 5′ end of U24 and the intron 5′ splice site (5′ IVS) are indicated on the DNA sequence ladder.

ture 5′ end of U18 was only weakly detected with the 3′ flanking oligonucleotide even in the wild-type strain. This suggests that 3′ processing of U18 normally precedes 5′ processing.

We conclude that the 5′ ends of U18 and U24 are synthesized by exonuclease digestion requiring Rat1p, with Xrn1p also playing a role, at least in the *rat1-1* strains. U24 can be synthesized only from the debranched intron lariat, while U18 can be generated with moderate efficiency in the absence of intron debranching.

DISCUSSION

Results from higher eukaryotic systems indicate that the 5′ ends of many snoRNA species are generated by 5′→3′ exonuclease activities (7, 13, 20, 22, 34, 44). Two 5′→3′ exonucleases, Xrn1p and Rat1p, have been identified in *S. cerevisiae*, and we therefore tested whether mutants defective in these activities are also defective in the 5′ processing of pre-snoRNA species. The data presented here establish that Xrn1p and Rat1p play roles in the formation of the 5′ ends of the yeast snoRNAs, snR190, U14, U18, and U24. In each case, the *rat1-1* mutation led to some accumulation of 5′-extended species at 37°C, but this was stronger in the *xrn1*-Δ *rat1-1* double-mutant strain, whereas the *xrn1*-Δ single mutation alone had little effect. After 2 h at the nonpermissive temperature, depletion of the mature snoRNAs was observed in the *rat1-1 xrn1*-Δ strains, indicating that the major biosynthetic pathway was inhibited. As *rat1-1* strains rapidly cease growth at 37°C (2), stronger depletion of the mature snoRNAs was not observed at later time points. The pre-rRNA spacer fragments and the 5′-extended snoRNA and 5.8S rRNA species were, however, present at relatively low levels compared to the mature rRNA and snoRNAs. The *xrn1*-Δ allele is a gene disruption construct (14), whereas the *rat1-1* allele is a temperature-sensitive point mutation (2). It is not clear whether Rat1p retains some residual processing activity in the *rat1-1* strain or whether the accumulated RNAs were degraded by another pathway. In the *rat1-1* mutant strain, the nuclear poly(A) signal is lost after prolonged incubation at the nonpermissive temperature (2), suggesting that there is some residual activity in the mutant. A complex of 3′→5′ exonucleases processes the 3′ end of the 5.8S rRNA (30) and degrades the excised pre-rRNA spacer 5′ to site A_0 (11a). This complex may also contribute to turnover of the RNAs that accumulate in the 5′→3′ exonuclease mutants.

Northern and primer extension data indicate that snR190 and U14 are synthesized from a common, dicistronic transcript. In the *rat1-1* and *xrn1*-Δ *rat1-1* strains, ladders of 5′-extended snoRNAs that extend to positions −42 for snR190

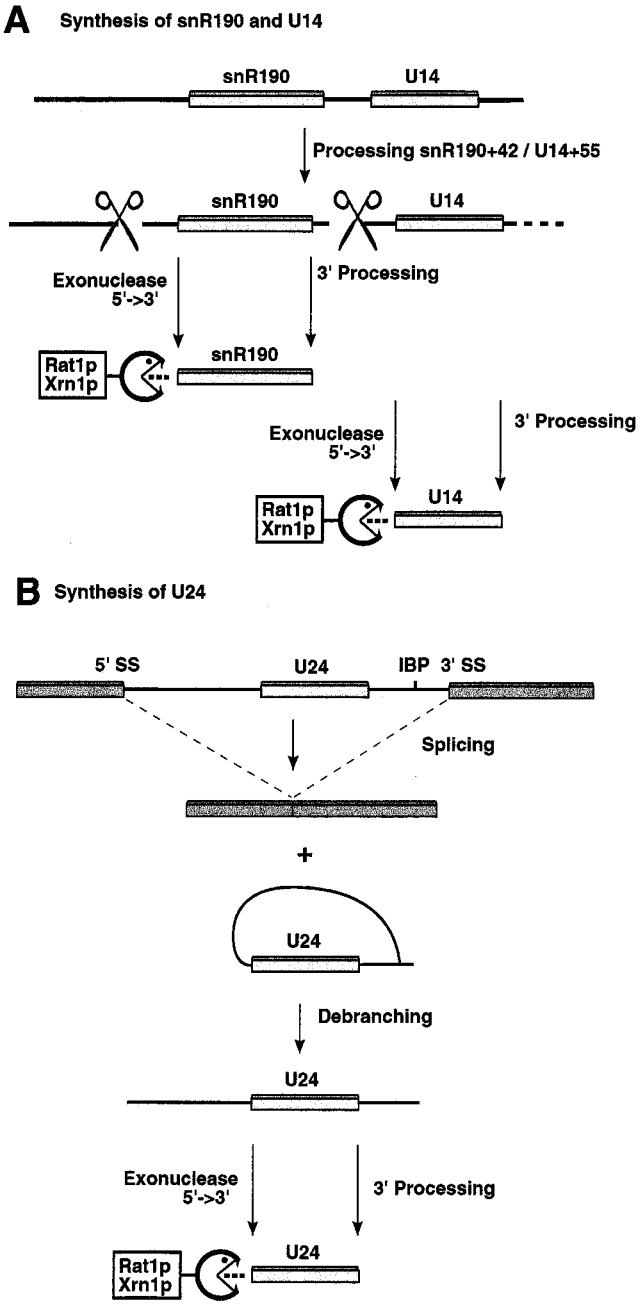


FIG. 7. (A) Model for the processing of pre-snR190 and pre-U14. The coding sequences of snR190 and U14 lie in the same orientation in the genome and are separated by only 67 nt (46). We propose that they are synthesized from a common precursor which extends from 302 nt 5′ to snR190 to beyond the 3′ end of U14. Cleavage of the pre-snoRNA at snR190 position −42 and U14 position −55 is envisaged to be followed by exonuclease digestion by Rat1p to the 5′ ends of the snoRNAs and 3′ trimming. (B) Model for the processing of pre-U24. U24 is encoded in the intron of the *BEL1* gene (23, 35) and is generated from the excised intron lariat. Following intron debranching, processing is envisaged to consist of exonuclease digestion by Rat1p to the 5′ end of the snoRNA and 3′ trimming. IBP, intron branch point.

and −55 for U14 were detected. These sites do not have any obvious homology to each other or to consensus snoRNA promoter sequences. Moreover, U14 position −55 lies only 12 nt 3′ to the mature snR190 region, making it unlikely to be a transcription start site. We propose that snR190 position −42 and U14 position −55 represent intermediate sites in the processing of larger pre-snoRNA species. These could be the products of either endonuclease or 5′→3′ exonuclease digestion. However, exonuclease digestion would presumably have to involve an exonuclease(s) other than Xrn1p and Rat1p, and other 5′→3′ exonuclease have not been identified in yeast extracts (19, 24, 38). Moreover, the 5′ cap structure would be expected to confer protection against exonucleases, and an endonuclease activity is more probable. The furthest-upstream primer extension stop that we detected lies 302 nt 5′ to snR190. It has not been established whether this represents the transcription start site or is a further upstream processing site; however, the 5′ end of the next open reading frame lies only 190 nt upstream of this site, making this likely to be the start site.

U18 and U24 are synthesized from the introns of host genes that also encode mRNAs. U24 can be synthesized only from the debranched intron lariat; the snoRNA was found almost entirely in circular form in a mutant which lacks intron-debranching activity. This strongly indicates that both 5′ and 3′ processing of the pre-snoRNA are exclusively exonucleolytic. Moreover, little if any processing of U24 can occur on the unspliced pre-mRNA. In the case of U18, synthesis of the mature snoRNA was reduced to ~30% of the wild-type level in the debranching mutant, indicating that the major processing pathway is also via exonuclease digestion. Residual processing might be due to endonuclease cleavage of the intron lariat or exonuclease digestion of the unspliced pre-mRNA. For both U18 and U24, pre-snoRNAs that were 5′ extended to the intron 5′ splice site in the *rat1-1* and *xrn1*-Δ *rat1-1* strains accumulated, indicating that these are the 5′→3′ exonucleases responsible for processing the pre-snoRNAs. Primer extension specifically on pre-U24, using a 3′-flanking oligonucleotide, failed to detect the mature 5′ end of the snoRNA in the *rat1-1* strains at 37°C, demonstrating the inhibition of 5′ processing.

In each case the accumulation of 5′-extended snoRNA species was much stronger in the *rat1-1* strain than in the *xrn1*-Δ strain, indicating that Rat1p is the major pre-snoRNA-processing activity in wild-type cells. Since Rat1p functions in the nucleus, the presumed site of pre-snoRNA processing, while Xrn1p functions in the cytoplasm (17), it may be that the processing activity normally resides only in Rat1p, with Xrn1p functioning to process the accumulated pre-snoRNAs in the *rat1-1* mutant strains.

Together, the data suggest the models shown in Fig. 7. We envisage that snR190 and U14 are synthesized from a dicistronic pre-snoRNA species which extends from a position 302 nt 5′ to snR190 to beyond the 3′ end of U14 (Fig. 7A). This is processed, probably endonucleolytically, at positions 42 nt 5′ to snR190 and 55 nt 5′ to U14, within the intergenic spacer region. These processing reactions are followed by 5′ and 3′ trimming reactions which generate the mature snoRNAs. In contrast, U24 (Fig. 7B) is processed from the excised pre-mRNA intron. In wild-type cells processing is probably exonucleolytic from the debranched intron lariat.

In general, the host genes for vertebrate snoRNAs encode protein products that have some relationship to ribosome synthesis or function. This coexpression may facilitate the coordinated synthesis of the protein and snoRNA products. The data reported here extend the interaction between the synthesis of the snoRNAs and the function of the nucleolus by demonstrating that the snoRNAs and pre-rRNAs are processed by common components. It is possible that as the snoRNAs developed, they simply made use of whatever processing machinery was available. Alternatively, the use of common components might have been selected because of the obvious possibilities that it offered for coregulation of the synthesis of the rRNAs

and snoRNAs. Such coregulation might indeed be the reason that so many snoRNAs, but no other known small RNA species, are synthesized by such excision mechanisms.

All studies on the in vitro processing of vertebrate snoRNAs have implicated 5′→3′ exonuclease activities in formation of the 5′ ends of the snoRNAs (7, 13, 22, 34, 44; reviewed in references 20 and 29). In no case have the nucleases yet been identified, but we strongly predict that, at least in some cases, these activities will involve the vertebrate homologs of Rat1p and Xrn1p (5, 36).

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