

Post-transcriptional Regulation of the U3 Small Nucleolar RNA*

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A high copy shuttle vector was used to express a “tagged” U3 small nucleolar RNA (snoRNA) gene in *Schizosaccharomyces pombe* to examine regulatory responses to a high gene dosage. RNA analyses utilizing reverse transcription-PCR amplification and restriction fragment length polymorphism indicated that the tagged gene was both proportionally and highly expressed and that downstream processing and/or termination were critical to U3 snoRNA stability. In contrast, direct measurements of the total cellular U3 snoRNA showed essentially normal levels of mature RNA, although measurements of precursor levels confirmed a highly expressed gene construct. Taken together, the results indicated that the steady state amounts of mature U3 snoRNA were primarily regulated at the post-transcriptional level. This regulatory mechanism prevents over-accumulation of the cellular U3 snoRNA and can efficiently degrade mutant RNA molecules. Together with past studies on other 3' extended RNA precursors, the results support post-transcriptional regulation as a quality control mechanism in which appropriate amounts of functional RNA are stabilized by protein interaction while excess or defective RNA is rapidly degraded. Precursor processing *in vitro* and mutational analyses were consistent with this model.

In the eukaryotic cell, RNAs normally are synthesized as larger precursor molecules that are cleaved and often modified to form the mature and functional RNA products. Even small RNAs, such as the 5 S rRNA (1), which are not modified, are transcribed as slightly larger precursor molecules with a short sequence extension at their 3' end; there are 12 extra nucleotides in the 5 S rRNA of *Saccharomyces cerevisiae*. Because this sequence is removed by exonuclease trimming in the course of integration of the molecule into ribosomes structure (2), the feature seems unnecessary and wasteful. Nevertheless, mutation analyses have shown that 5 S rRNA transcripts that are not normally terminated or processed are highly unstable and not incorporated into ribosomes (3). Such observations suggest that the regulation of the 5 S rRNA of yeast is not simply confined to the transcriptional level but also is subject to post-transcriptional regulation. These results also have led to the speculation that the precursor sequence and its removal during RNA maturation, at least in part, serve as a quality control

mechanism to help ensure only functional RNA is incorporated into the nascent ribosomes (2).

Unlike the 5 S rRNA, the small nucleolar U3 RNA (U3 snoRNA)² actually is not incorporated into ribosomes. Instead, it is integrated into an alternate nucleolar ribonucleoprotein complex (4), which has been shown to play a critical role in rRNA processing and ribosome biogenesis (5). The complex is recruited into the 80–90 S pre-rRNA processing complex in the dense fibrillar component (6) acting as part of the small subunit processome (7) in the initial release of the 18 S rRNA (8). Furthermore, although the U3 snoRNA of yeast cells is transcribed by RNA polymerase II rather than RNA polymerase III, like the 5 S rRNA, the U3 snoRNA is transcribed as a 3' end extended precursor (9). This raises the possibility that termination and the maturation processes may play similar roles with respect to U3 snoRNA biogenesis.

Post-transcriptional regulation generally has not been reported or even examined in respect to snoRNA biosynthesis, although the human U6 small nuclear RNA has been observed to be subject to a specific intracellular post-transcriptional regulatory mechanism (10). In an ongoing study of functional relationships and regulatory mechanisms for U3 snoRNA synthesis in *Schizosaccharomyces pombe* cells, we have been attempting to use an efficiently expressed plasmid-associated U3 snoRNA gene system to detect critical sequence and/or structural elements. As a step in the standardization of this expression system, in this study we examined and quantified the effect of a large change in gene dosage. The results indicate that, as observed previously with the 5 S rRNA, the amount of U3 snoRNA is strongly dependent on termination and RNA processing, with the excess amounts of mature RNA clearly being regulated at the post-transcriptional level.

EXPERIMENTAL PROCEDURES

Strains and Vectors—*Escherichia coli* strain C490 (*rec A*[−], *rk*[−], *mk*[−], *thr*[−], *leu*[−], *met*[−]) was used as a host for pTZ19R (11) and pFL20 (12) plasmid recombinants. *S. pombe* (*h*[−], *leu1-32*, *ura4-D18*) cells were used as a host to express the pFL20 yeast shuttle vector recombinants. Bacterial clones were grown at 37 °C in LB-ampicillin broth or LB-ampicillin agar, and yeast transformants were grown with aeration under selective conditions in minimal medium containing 0.64% yeast nitrogen base, 0.5% dextrose, 80 μg/ml leucine, 0.36 mg/ml potassium acetate, and 0.5 mg/ml potassium dihydrogen phosphate (13, 14). Growth rates were determined using the absorbency of cultures at 550 nm.

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² The abbreviations used are: snoRNA, small nucleolar RNA; RT, reverse transcription; snRNA, small nuclear RNA; contig, group of overlapping clones.

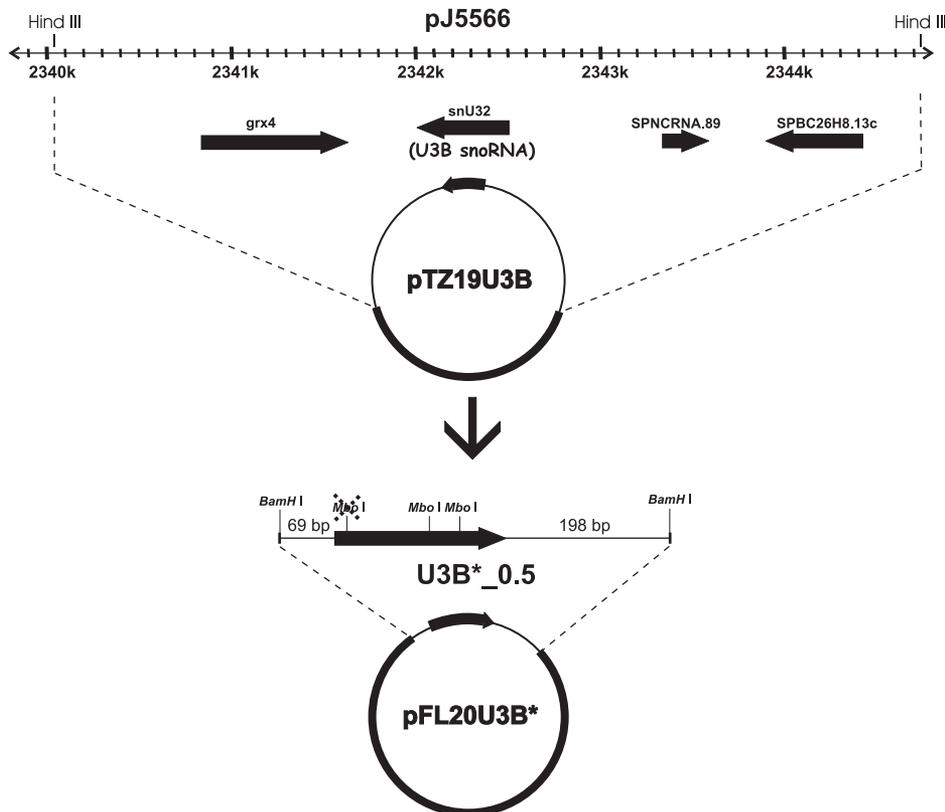


FIGURE 1. Isolation and manipulation of the U3B snoRNA gene of *S. pombe*. Primers, upstream and downstream of a HindIII fragment containing *snU32*, the gene encoding the U3B snoRNA of *S. pombe* cells, were used to amplify this region using genomic DNA prepared from *S. pombe* (h^- , *leu1-32*, *ure4-D18*) as a template. The amplified DNA was digested with HindIII endonuclease and cloned in pTZ19R (upper panel). The recombinant (pTZ19U3B) was used as a template together with a megaprimer strategy (16) to remove a MboI restriction site and to add BamHI adapter sequences. As indicated in the lower panel, the 0.5-kb PCR-amplified product (U3B*_0.5) was cloned in a high copy yeast shuttle vector (pFL20) for *in vivo* expression analyses. The initial sequence was taken from contig pJ5566 as described in the *S. pombe* gene data base.

Preparation and Expression of Tagged U3 snoRNA Genes—*S. pombe* genomic and plasmid DNA were extracted from logarithmically growing cells essentially as described by Hoffman and Winston (15). Cultures (10 ml) with absorbencies of 0.4–0.6 at 550 nm were harvested by centrifugation, washed with water, and resuspended in 200 μ l of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), 200 μ l of phenol/chloroform/isoamyl alcohol (25:24:1), and 0.3 g of acid-washed glass beads. The cells were disrupted by vortex for 3 min, and after centrifugation and ethanol precipitation, the redissolved DNA was treated with RNase A (25 ng/ μ l) for 30 min. Ammonium acetate (120 mM) was added, and the DNA was again precipitated with ethanol.

The U3B snoRNA gene was prepared and tagged by PCR amplification using a “megaprimer” strategy (16) and a cloned *snU32* gene fragment template as described in Fig. 1. A single G-to-A transition mutation at nucleotide 19 initially was introduced as a neutral plasmid-derived RNA tag. Upstream (5'-GGCTGGATCCGCAAAG-3') and downstream (5'-GAACTAATCGGATCCTACACC-3') specific primers fused with BamHI adapter sequences were used to prepare a 0.5-kb fragment containing 69 bp of upstream and 198 bp of downstream sequence, which after digestion with BamHI endonuclease was cloned directly into the pFL20 yeast shuttle vector using its unique BamHI site. The mutant U3B snoRNA sequence was

confirmed by automated DNA sequencing (CBS-DNA Facility, University of Guelph), and the pFL20 recombinant was used to transform *S. pombe* cells using the methods of Okazaki *et al.* (14). To ensure a reproducible result, at least three transformants were chosen for subsequent analyses.

Preparation and Analysis of Cellular U3 snoRNAs and Precursors—To assay the total cellular U3 snoRNA or precursor RNA, logarithmically growing cells were disrupted by vortex with an equal volume of glass beads (17), and cellular RNA was prepared using the protocol of Steele *et al.* (18). The amounts of U3 snoRNA or pre-snoRNA were determined by RT-PCR after treatment with 0.1 unit/ μ l of RNase-free DNase (19). The RT reactions (20 μ l) were performed using 1–2 μ g of DNase-treated RNA, 0.2 mM deoxynucleotide triphosphate, 60 pmol of U3 snoRNA-specific (5'-ACACGTCA-GAAAACACC-3') or precursor-specific (5'-ATGCACATCAAGT-TATAAC-3') primer, and 40 units of Moloney murine leukemia virus reverse transcriptase (Fermentas Inc., Hanover, MD) incubated for

2 h at 37 °C. For PCR reactions (50 μ l), 0.1–10% of the RT reaction product was used with 0.2 mM deoxynucleotide triphosphate, 30 pmol of each primer, and 1 unit of *TaqI* polymerase for 30 cycles.

Individual U3 snoRNA components were assayed using restriction fragment length polymorphism. Whole cell nucleic acid was extracted from logarithmically growing cultures with sodium dodecyl sulfate/phenol as described previously (18), and the U3 snoRNA was purified by fractionation on 8% denaturing polyacrylamide gels and elution by homogenization in SDS buffer after staining with methylene blue (20). U3 cDNA was prepared from the gel-purified RNA by RT-PCR (21) using Moloney murine leukemia virus reverse transcriptase and primers specific for the 5' and 3' ends of the *S. pombe* U3 snoRNAs (5'-ATCGACGATACTCCATAG-3' and 5'-ACACGTCAGAAAACACC-3'), respectively, as described above. [α - 32 P]dCTP (2–5 μ Ci) was added to the PCR reaction to label the DNA products. The reaction mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), the labeled copy DNA was precipitated with ethanol and finally digested with MboI endonuclease (Invitrogen), and fragments were fractionated on 12% nondenaturing polyacrylamide gels and detected by autoradiography. For quantitative analyses, images were captured using a Umax Astra 600P scanner (Umax Technol-

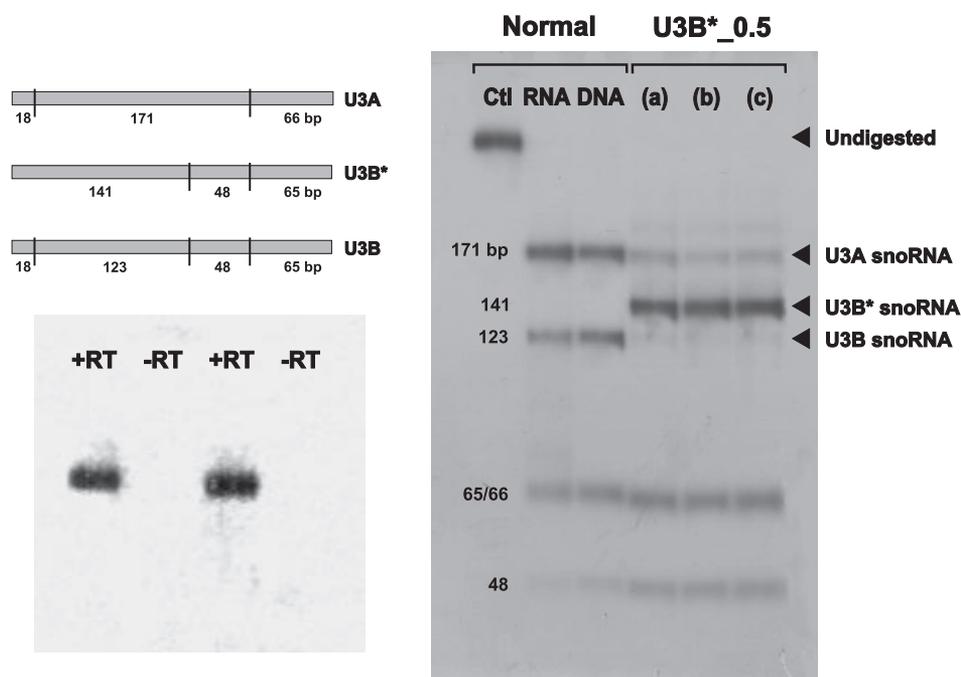


FIGURE 2. *In vivo* expression of a plasmid-associated *S. pombe* U3 snoRNA gene. U3 snoRNA was prepared from normal (RNA) or three pFL20U3B*_{0.5} transformed colonies (a–c) by SDS/phenol extraction, and the relative distribution of normal (U3A and U3B) and plasmid-derived (U3B*) U3 snoRNAs was determined by restriction fragment length polymorphism. The whole cell RNA was fractionated on an 8% denaturing polyacrylamide gel, and labeled cDNA of the U3 snoRNA fraction was prepared by RT-PCR as described under “Experimental Procedures.” The labeled DNA was digested with MboI endonuclease, and fragments were fractionated on a 12% native polyacrylamide gel. Distinct fragments for all three types of snoRNA as predicted from the nucleotide sequences are indicated on the right. An undigested sample (Ctl) and PCR-amplified genomic DNA from a normal cell (DNA) are included as controls. A restriction fragment map for all three types of DNA is shown on the left; a negative control lacking reverse transcriptase (–RT) for a normal (left) or transformed (right) cell extract is shown below.

ogies) and quantified using Molecular Analyst PC software (Bio-Rad).

In Vitro RNA Processing—A whole cell protein extract was isolated from *S. pombe* cells (strain *h⁻*, *leu1-32*, *ura4-D18*) for *in vitro* RNA processing based on the methods described by Hennighausen and Lubon (22) and Jazwinski (23). Cells were grown in a rich medium (2% dextrose, 0.5% yeast extract) of 500 ml in total volume to an absorbency of 0.4–0.6 at 550 nm and harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. After being briefly washed twice with water, the cells were resuspended in 5 ml of ice-cold breaking buffer (400 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM Hepes/KOH, pH 7.9). An equal volume of acid-washed glass beads was added to the cell suspension, and the cells were then broken by vortexing at cold room temperature (~8 cycles of 15 s alternating with 30 s on ice). The cell breakage was monitored by microscope; with 90% breakage, the glass beads and large cell debris were removed by centrifugation at 10,000 rpm, for 15 min at 4 °C (S10), and for the initial experiments, the resulting lysate was cleared further by centrifugation at 35,000 rpm in a Beckman Ti 70 rotor for 1 h at 4 °C (S100). Higher enzymatic activities were observed in the absence of the second centrifugation step, and the abbreviated procedure was used in subsequent experiments. The protein extracts (S10 or S100) were diluted further with glycerol (20%, final concentration) and divided into 50- μ l aliquots for storage at –80 °C.

For *in vitro* processing, U3B snoRNA precursors were prepared by *in vitro* transcription using T7 RNA polymerase (24, 25). For templates, a T7 promoter/U3B snoRNA-specific hybrid sequence (5'-CGGAATTCCGTTAATACGACTCACTATAGGATCGACGATACTCCATAG-3') was used as the forward primer, and a precursor-specific primer (5'-CCCTTACCTCCTCTCCTC-3'), beginning 22 nucleotides downstream of the U3B snoRNA sequence, was used as the reverse primer to prepare the template DNA by PCR amplification. The RNA transcripts were purified on 8% denaturing polyacrylamide gels and labeled at the 5' end using bacteriophage T4 polynucleotide kinase and [γ -³²P]ATP, after dephosphorylation with calf intestinal phosphatase (21). The phosphatase was heat-inactivated for 10 min at 75 °C in the presence of 5 mM EDTA (pH 8.0) before labeling, and the labeled RNA was again purified on an 8% denaturing polyacrylamide gel. For RNA processing reactions, 30 fmol of labeled RNA (10,000 cpm) was incubated at 30 °C

with 10 μ l of protein extract (4.3 μ g/ μ l) in a final volume of 20 μ l containing 37.5 mM NaCl, 3 mM MgCl₂, 100 mM KCl, 3 mM dithiothreitol, 10% glycerol, 0.25 mM phenylmethylsulfonyl fluoride, 50 μ M EDTA, and 30 mM Hepes/KOH, pH 7.9. Reactions were stopped by the addition of 0.4 ml of stop buffer (0.3 M sodium acetate, 0.1% SDS, 10 mM EDTA, and 40 mg/ml calf liver RNA as a carrier). The RNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol-precipitated, ethanol-washed, and vacuum-dried. For product analyses, the samples were resuspended in 5 μ l of loading buffer (formamide containing 0.03% xylene cyanol and 0.03% bromophenol blue dyes), heated for 2 min at 90 °C, and fractionated on an 8% polyacrylamide gel containing 8.3 M urea before being exposed to x-ray film for autoradiography. For quantitative analyses, images were captured and quantified as described above.

RESULTS

An efficiently expressed plasmid-associated tagged gene system was used to study the expression and maturation of U3 snoRNA encoded by the *S. pombe snU32* locus (chromosome 2, contig pJ5566, *S. pombe* gene data base). As summarized in Fig. 1, the U3B snoRNA-encoding region in the *S. pombe* genome was PCR-amplified and cloned in the pTZ19R vector. BamHI adapter sequences and a single base substitution (G-to-A transition at nucleotide 19), which resulted in one less MboI restriction site, were introduced using a megaprimer mutagenesis

strategy (16). For *in vivo* analyses, the altered gene sequence was subcloned into a multi-copy-number yeast shuttle vector (pFL20), which subsequently was used to transform *S. pombe*

TABLE 1
U3 snoRNA concentration in transformed *S. pombe* cells expressing plasmid-associated U3 snoRNA genes

Construct ^a	U3B* snoRNA ^b	U3 snoRNA ^c	Growth rate ^d
pFL20	0	0.019 ± 0.004	4.37 ± 0.15
U3B*_0.5	77.4 ± 10.3	0.025 ± 0.005	4.80 ± 0.12
U3B*_0.5-153	4.3 ± 0.7	0.022 ± 0.003	7.43 ± 0.31

^a Constructs as described in Fig. 1.

^b Relative amount of U3B* copy DNA after digestion with MboI endonuclease as described in Fig. 2.

^c Moles of U3 snoRNA per mole of ribosome determined from the 5 S and 5.8 S rRNAs as described in Fig. 4.

^d Growth rate in hours based on absorbency at 550 nm.

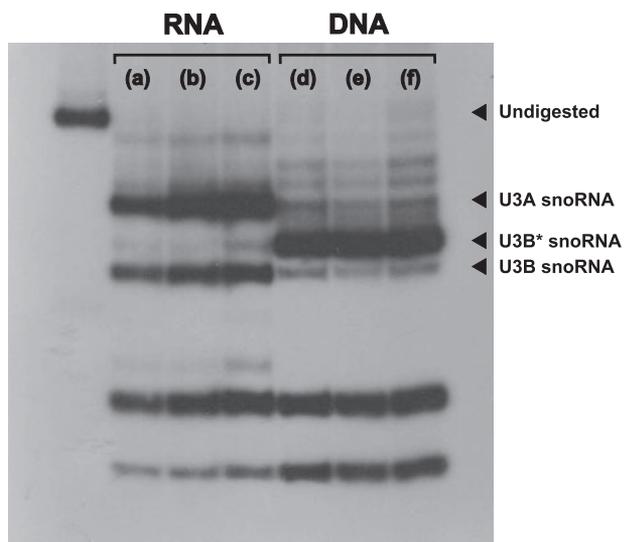


FIGURE 3. Influence of the downstream sequence on plasmid-derived *S. pombe* U3 snoRNA. The downstream sequence in the U3B*_0.5 construct (Fig. 1) was removed by PCR-based mutagenesis (U3B*_0.5-153), and the truncated sequence was recloned in the pFL20 multicopy vector and again used to transform *S. pombe* cells. U3 snoRNA was prepared and assayed from three transformed colonies (a–c) as described in Fig. 2. An undigested sample and PCR genomic DNA from the same three colonies (d–f) are included as controls. Distinct fragments for all three types of snoRNA as predicted from the nucleotide sequences are indicated on the right.

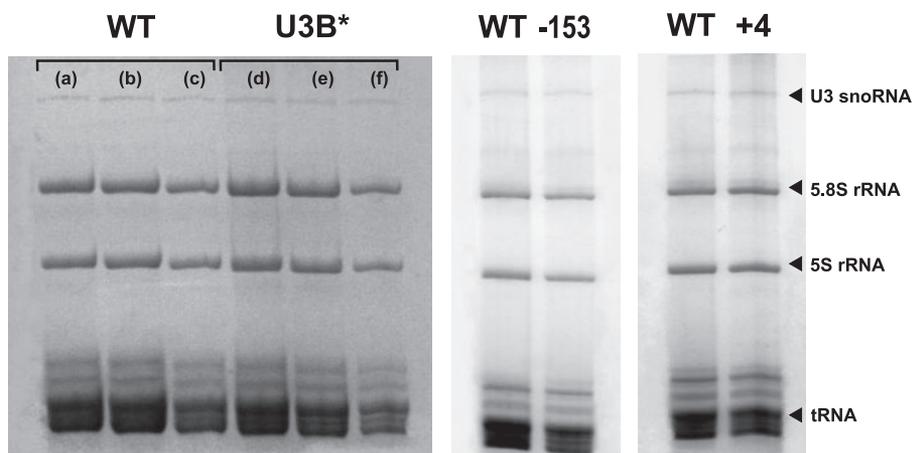


FIGURE 4. Steady state levels of U3 snoRNA in cells expressing plasmid-associated U3 snoRNA genes. Whole cell RNA was prepared directly from untransformed cells (wild-type; WT), cells transformed with normal U3 snoRNA gene pFL20U3B*_0.5 (U3B*), or with mutant genes pFL20U3*_0.5-153 (-153) or pFL20U3B*+4 (+4) by SDS/phenol extraction; fractionated on an 8% denaturing polyacrylamide gel; and stained with methylene blue. The positions of major low molecular weight RNA fractions are indicated on the right.

cultures. In this case, the U3B snoRNA-encoding gene was expressed under its own promoter elements, and the MboI sequence-tagged U3B snoRNA sequence could be readily detected using restriction fragment length polymorphism after conversion to cDNA through RT-PCR amplification. As illustrated in Fig. 2, when RNA was examined from normal and transformed cells expressing the tagged gene, the mutant RNA sequence was readily detected as a distinctly migrating band during gel electrophoresis (141-bp fragment). Three independent colonies were examined for each cell type to avoid variations. As reported previously (26), in normal cells U3A snoRNA was found to make up ~55–60% of the total U3 snoRNA population. In strong contrast, when the cells were transformed with the plasmid-associated gene, there was a dramatic change, and the tagged U3B snoRNA now made up >80% of the cellular U3 snoRNA population (lanes a–c). This strongly reflected the greatly elevated tagged gene dosage consistent with an efficient expression of the plasmid-associated sequence. The growth rate of these cells remained essentially unchanged (Table 1).

As noted earlier, past studies on the expression of both the 5 S rRNA genes and even rDNA have indicated that levels of the mature RNAs were strongly dependent on transcript termination and processing (3). To evaluate this requirement in respect to U3 snoRNA biosynthesis, a truncated U3 snoRNA gene (U3B*_0.5-153) was prepared in which the last 153 nucleotides of the 198-nucleotide downstream sequence were removed. As shown in Fig. 3, when expressed in transformed *S. pombe* cells, the presence of the tagged gene sequence could easily be demonstrated after PCR amplification (lanes d–f), but essentially no mature tagged U3B* snoRNA was formed (lanes a–c). Clearly the termination and maturation processes are equally important for U3 snoRNA biosynthesis. In addition, unlike the tagged normal gene (U3B*_0.5), the growth rate of cells transformed with the truncated construct (U3B*_0.5-153) was significantly depressed (Table 1).

Although these comparisons reflected the relative expression of the U3 snoRNA genes, they did not provide a measure of actual cellular RNA yield. Instead, a direct method was used to compare the actual amounts of U3 snoRNA in the transformed

and untransformed cells. As shown in Fig. 4, when total RNA was extracted from normal (lanes a–c) and transformed (lanes d–f) cultures and fractionated on an 8% polyacrylamide gel, the results were surprisingly similar, reflecting little or no change in the cellular U3 snoRNA concentration. Even when accurately expressed (Table 1) as a ratio to the ribosomal small RNAs (5.8 and 5 S rRNAs), the changes were very small, and on average the total cellular U3 snoRNA population was observed to increase by only a modest amount (Table 1). In normal cells there is about one U3 snoRNA molecule per 56 ribosomes, whereas in the transformed cells the concentration had only

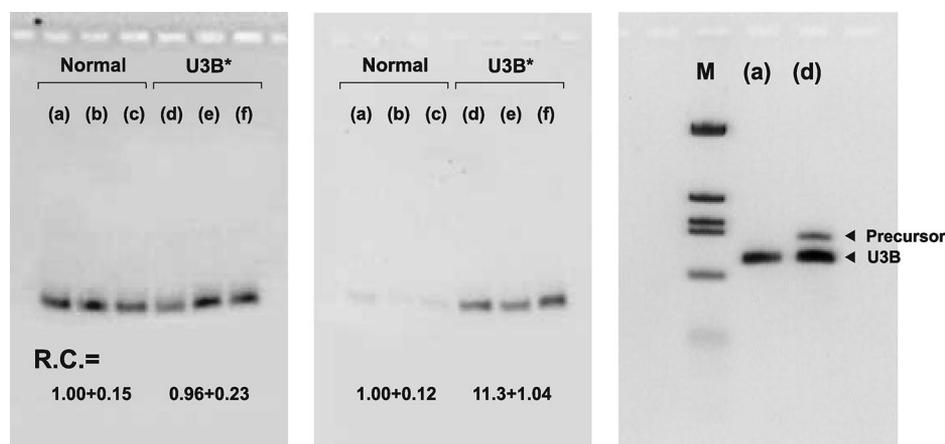


FIGURE 5. A comparison of U3 snoRNA and precursor levels in *S. pombe* cells expressing plasmid-associated U3 snoRNA genes. Whole cell RNA was prepared from three disrupted normal (a–c) or pFL20U3*_{0.5}-transformed colonies (d–f) and treated with RNase-free DNase, and levels of mature U3 snoRNA (left panel) or precursor (center panel) were assayed by RT-PCR using mature RNA- or precursor-specific primers, respectively. The products were fractionated on 2% agarose gels and visualized by ethidium bromide staining; RNA concentrations (R.C.) in pFL20U3*_{0.5}-transformed cells relative to cells expressing normal genes are indicated below. RNA from one of the normal (a) or transformed (d) colonies was assayed further by competitive RT-PCR (right panel) using the same three primers to simultaneously determine levels of both the precursor and mature RNA. A *Hinf*I digest of pTZ19R is included as a size marker (M).

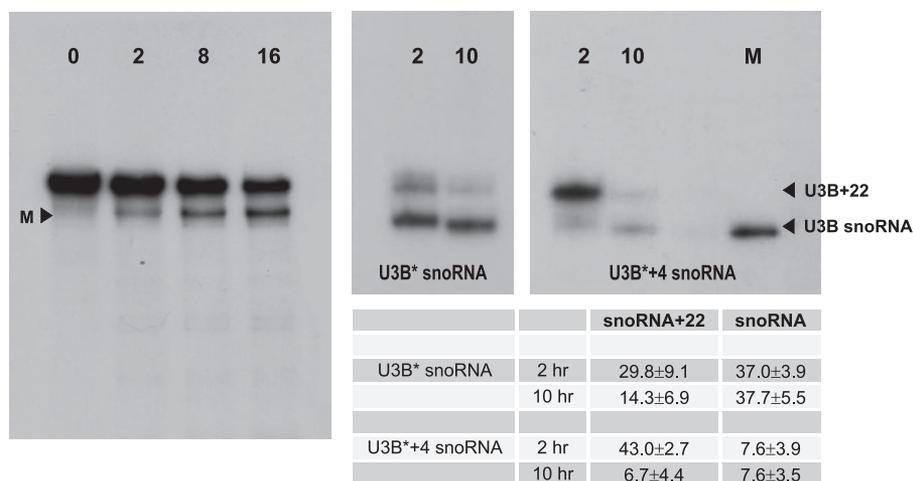


FIGURE 6. *In vitro* processing of 3' extended precursor U3B snoRNA. U3B snoRNA precursor was prepared by runoff transcription using T7 RNA polymerase, labeled at the 5' end, and purified by gel electrophoresis as described under "Experimental Procedures." Aliquots of RNA were incubated for 0–16 h with protein extract (S100); the RNA and any fragments were then extracted with SDS/phenol, fractionated by gel electrophoresis, and visualized by autoradiography (left panel). The position of unlabeled mature RNA marker (M) is indicated on the left. Normal (U3B snoRNA) or a mutant RNA containing a 4-nucleotide insert in the 3' end (U3B*+4 snoRNA) was prepared, labeled, incubated for 2 or 10 h with protein extract (S10), and fractionated by gel electrophoresis as described above (right panels). The positions of the precursor and mature RNA are indicated on the right; labeled U3B snoRNA is included as a marker. For quantitative analyses, the amount of RNA as a percentage of the substrate at time 0 for three replicate experiments was determined with averages tabulated together with their S.D.

increased to approximately one molecule per 41 ribosomes. Despite the introduction of more than 50 copies of the gene and a clearly indicated efficient expression of the tagged RNA (Fig. 2), the cell was observed to tightly regulate the final concentration.

The results presented in Fig. 4 actually raise two possibilities regarding the regulation mechanism; either it is tightly regulated at the transcription level or the access RNA is rapidly degraded at the post-transcriptional level. These possibilities were examined further when levels of both the mature and precursor U3 snoRNAs were simultaneously assessed using RT-PCR amplification. To amplify precursor molecules, we used a

primer in the reverse transcription reaction, which could bind downstream of the coding region in the nascent transcripts instead of the 3' end of the mature U3 snoRNA. Three independent colonies were again examined to eliminate variations. As shown in Fig. 5, left panel, the overall levels of the mature U3 snoRNA remain virtually unchanged between the wild-type cells (lanes a–c) and the transformants (lanes d–f), as observed in the direct analyses (e.g. Fig. 4) using acrylamide gels. However, in contrast, the level of precursor was very different, with at least an 11-fold increase in the transformed cells (middle panel). Equally, when competitive RT-PCR was applied to normal or transformed cells to ensure a quantitative assay, the results were again striking. As also shown in Fig. 5 (right panel), in competition with mature RNA, the level of precursor was too low to be detectable in transformed cells (lane a), but it was very evident in cells expressing multiple copies of plasmid-associated genes (lane d). Clearly, the U3 snoRNA is very substantially overproduced in the transformed cells but is unstable and rapidly degraded at the post-transcriptional level, resulting in unchanged steady state levels of mature U3 snoRNA.

In past studies on the 5 S rRNA (2), maturation at the 3' end of the precursor molecules could be readily demonstrated *in vitro* with the binding of cellular protein limiting the extent of trimming. Studies on the U3 snoRNA in *S. cerevisiae* as well as several snRNAs have demonstrated that similar precursor intermediates are formed by endonuclease cleavage (e.g. Refs. 9 and 27–30). Indeed, as observed in *S. cerevisiae*, studies with the Pac1 RNase III-like endonuclease in *S. pombe* indicate that a 3' end hairpin structure, which follows the mature snoRNA sequence, is efficiently cut at nucleotide C⁺²² (31). To examine RNA trimming in *S. pombe* cells, U3B snoRNA precursor with the 22 additional nucleotides at the 3' end was prepared using T7 RNA polymerase. As indicated in Fig. 6, when this precursor was incubated with a crude cell protein extract (S100), the results were entirely reminiscent of those observed with the 5 S rRNA (2). The extra nucleotides were clearly trimmed away while the extent of trimming was dramatically limited to form the mature RNA.

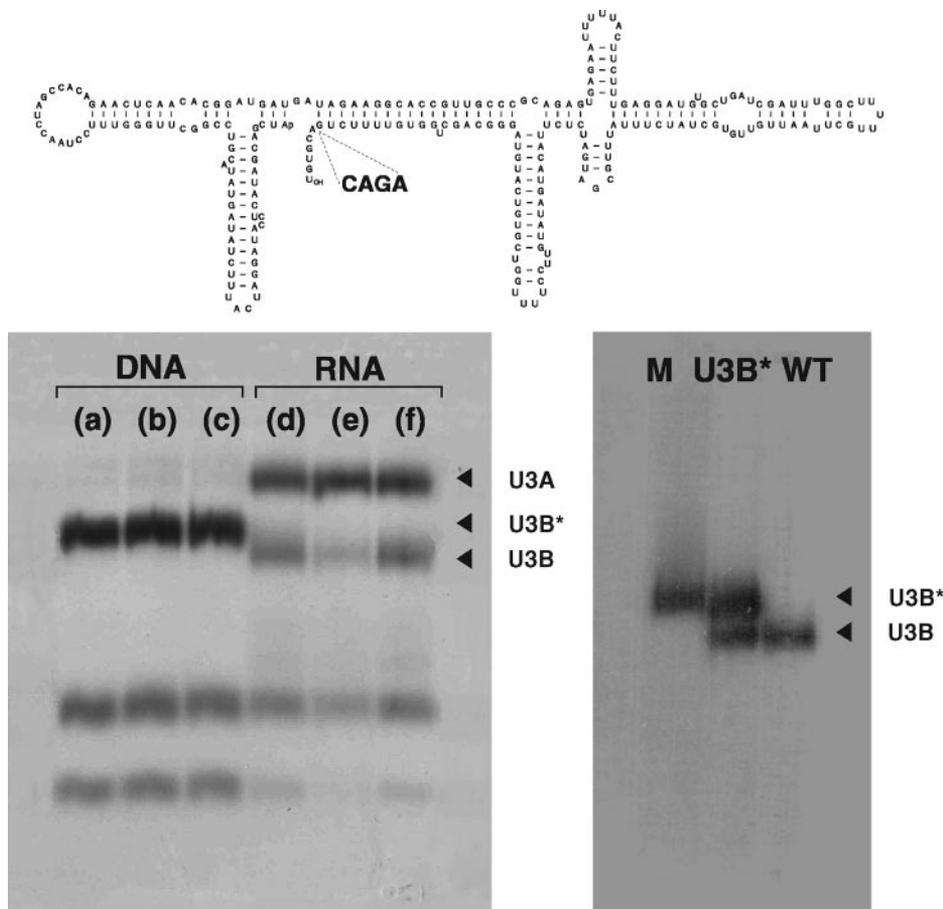


FIGURE 7. Effect of a 3' end mutation on the stability of the *S. pombe* U3B snoRNA *in vivo*. A 4-nucleotide insertion (CAGA) was made in the 3' end of the Mbol-tagged U3 snoRNA gene (pFL20U3B*+4) and used to transform *S. pombe* cells for expression *in vivo*. Genomic DNA (a–c) or U3 snoRNA (d–f) was purified from three colonies, and the relative copy number of each gene or amount of each RNA was determined by restriction fragment length polymorphism (left panel) as described under “Experimental Procedures.” Distinct fragments for the three types of snoRNA sequence are indicated on the right. Precursor levels were determined by competitive RT-PCR (right panel). Whole cell nucleic acid was prepared from one of the mutant colonies (U3B*) or untransformed cells (wild-type; WT), and the amount of mutant RNA precursor relative to normal, host cell-derived precursor was determined using a U3 snoRNA-specific forward primer together with primers specific for the 3' extended precursor sequence. A marker (M) for the precursor was prepared by PCR using a plasmid template and the precursor-specific primer.

In the case of the 5 S rRNA and some snoRNAs, past studies also showed that mutations at known protein binding sites close to the termini had striking effects on the processing and/or stability of precursor transcripts (2, 32, 33). As shown in Fig. 7, upper panel, to further evaluate the general nature of this observation, a 4-nucleotide insertion was made near the 3' end of the MboI-tagged *S. pombe* U3B snoRNA gene, and RNA production was examined *in vivo*. As also shown in Fig. 7, left panel, although the gene itself was easily detected by PCR (lanes a–c), little or no plasmid-derived mature RNA was observed using RT-PCR and the same U3 snoRNA-specific primers (lanes d–f). This was equally true when the steady state level was assessed as illustrated in Fig. 4 (right panel). In strong contrast, when precursor levels were determined by competitive RT-PCR, simultaneously using precursor- and mature RNA-specific primers (right panel), the elevated level of precursor was easily detected (U3B*). Clearly the transcript was now highly unstable and readily degraded as reported previously with the 5 S rRNA.

In view of the similarities with studies on the 5 S rRNA, the maturation of the mutant transcript also was examined, *in vitro*,

as illustrated in Fig. 6 (right panels). Because U3 snoRNA processing in *S. cerevisiae* has been linked to the exosome, a large protein complex of exoribonuclease activities (28), extracts containing higher molecular components (S10) were also examined. Indeed, this alternative extract was more efficient, and as shown in Fig. 6, essentially complete maturation was now observed after 2 h of incubation (middle panel). In sharp contrast, the results with the mutant precursor RNA were very different. Much less mature RNA was produced, and by 10 h much of the mature RNA or precursor was degraded with little of either remaining. The results were fully consistent with the observations *in vivo* (Fig. 7) and again underline the significance of the 3' end sequence/structure with respect to RNA processing and stability.

DISCUSSION

As noted above, past studies on 5 S rRNA have shown that termination and processing are critical to rRNA stability and utilization (3). Early protein interactions limit the extent of 3' end processing and protect the nascent RNA from random degradation. This represents a post-transcriptional mechanism that both prevents excess steady state levels of the RNA and perhaps a quality control mechanism that

helps assure that only functional RNA is integrated into ribosomes (2). In contrast, limited past studies on other small nuclear or nucleolar RNAs such as the U-rich spliceosomal snRNAs generally have not reported such mechanisms. Only the U1 snRNA of mouse cells (34) and the U6 snRNA of a human cell line (10) have been shown to be subject to any gene dosage compensation, and even then, post-transcriptional regulation has only been demonstrated in the human system. Other U3 snoRNAs have been expressed *in vivo* (e.g. Refs. 9 and 35), but the possibility of gene compensation appears not to have been examined. The present study shows that like the rRNAs, excess U3 snoRNA is effectively regulated at the post-transcriptional level, and precursor processing again appears to act as a quality control mechanism. The level of expression as established by precursor level measurements largely reflected the gene dosage indicating little or no control at the transcription level. Despite these far higher levels of U3 snoRNA transcription (see Fig. 5), the cellular levels of mature RNA remained essentially constant (see Fig. 4). Furthermore, when the 3' end sequence was modified (see Fig. 7), all of the mutant

RNA was completely or almost completely eliminated. As reported previously for the pre-rRNA (1) and the 5 S rRNA (2), this basically represents a quality control mechanism that helps assure that only functional RNA is incorporated into ribonucleoprotein and subsequently utilized in cellular function. Accordingly, it is attractive to speculate that, as illustrated by the present examples, many other 3' end extended RNAs similarly may be subject to such post-transcriptional quality and level control. Searches for further examples of what appears to be an important general regulatory approach are warranted.

Although the mechanism underlying the targeted degradation is unclear, it is likely to be connected to saturable protein(s) that specifically bind to the nascent RNA and protect the transcript from housekeeping nucleases. In the case of 5 S rRNA, a specific ribosomal protein, YL3 in yeast, has been identified and shown to essentially "cap" the termini (24). Although structural analyses (32) indicate that the 5' and 3' ends of the U3 snoRNA do not interact to form a terminal helix, which might limit the enzymatic trimming, clearly a simple cellular protein extract provides both trimming activity and factors that can limit it at the mature 3' end (Fig. 6). As observed previously with the 5 S rRNA, mutations at known protein binding sites close to the termini of snoRNAs also have been reported to impair the processing of precursor transcripts (32, 33), but again, proteins that actually stabilize the RNAs are not known. In the present study, a mutation in the 3' end of the *S. pombe* U3 snoRNA clearly had a comparable effect, both *in vivo* and *in vitro* (Figs. 6 and 7), further underlining similarities in the RNAs with 3' end extended precursors. Depletion of U3 snoRNA-specific binding proteins in *S. cerevisiae* appear not to affect U3 snoRNA levels (7, 5, 36, 37), but because the proteins were depleted individually, and the U3 small nucleolar ribonucleoprotein has been reported to contain many protein constituents, it is possible that stability is actually maintained by multiple protein interactions. Alternatively, the critical protein simply may not have been identified. Whatever the case, the present study illustrates a more general application for the previous observations with rRNAs. The expression system described in this study remains as an attractive vehicle for further studies on the contributing protein factors, including their roles in non-polyadenylated polymerase II termination and precursor processing.

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