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RESEARCH ARTICLE

# U3 snoRNA promoter reflects the RNA's function in ribosome biogenesis

**Sadeq Nabavi · Ross N. Nazar**

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**Abstract** An efficiently expressed "tagged" gene system was used to study promoter sequence elements in genes encoding the U3 snoRNAs of *Schizosaccharomyces pombe*. Transcription was found dependent on two previously thought, mutually exclusive elements, a TATA-box, as found in other *S. pombe* snRNA gene promoters, and a Homol D-box, often considered a TATA-box analogue in the promoters of genes that encode ribosomal proteins. The Homol D-box is critical while the TATA-box strongly influences transcription efficiency but is not essential. The results suggest that the U3 snoRNA promoter may represent the fusion of two promoter systems reflecting the special role of this RNA in ribosome biogenesis. Steady state measurements of cellular RNAs support such a coordinated regulation of the U3 snoRNA.

**Keywords** U3 snoRNA · Promoter · Pol II · Ribosome biogenesis

## Introduction

Perhaps reflecting their functional diversity the promoters of small nuclear RNA (snRNA) genes are surprisingly variable in both the number and nucleotide sequence of their transcriptional elements. Most snRNAs are synthesized by RNA polymerase II (pol II) but at least the U6 snRNA is synthesized by RNA polymerase III (pol III). Although snRNA promoters are usually highly conserved within a species, they do vary greatly between genera and their constitutive transcriptional elements are often unique to this gene group [reviewed in (Dahlberg and Lund 1988; Hernandez 2001; Smale and Kadonaga 2003)]. In many studies the U1 and U2 snRNA promoters have served as prototypic pol II snRNA promoters while the U6 snRNA promoter has served as the prototypic pol III snRNA promoter. Human snRNA genes are characterized by the presence of a proximal sequence element (PSE) and a distal sequence element (DSE). The human pol II promoters contain only one essential element (PSE) but the pol III snRNA core promoter contains both the PSE and a TATA-box located at a fixed distance downstream. The DSE serves to enhance transcription from the core promoter. Although initially thought only to contain one element in *S. pompe*, the U2 snRNA gene promoter is now known to contain at least two elements, the spUSE centred at −55, which functions as an activator, and a TATA-box at −26, which is the essential for basal transcription (Zhou and Lobo-Ruppert 2001). The spUSE sequence consists of two imperfect direct repeats which have been speculated to each constitute half of a protein binding site (Zhou and Lobo-Ruppert 2001).

In contrast to the snRNA pol II promoter, studies on the ribosome protein (rp) genes in fission yeast indicate that basal transcription is not based on a TATA element (Witt et al. 1995). Rather, basal transcription is promoted by a conserved sequence, CAGTCACA, or the inverted form, TGTGACTG, called the homol D-box (Witt et al. 1995). In some rp promoters an upstream tandem repeat AGG GTAGGGT or the inverted form ACCCTACCCT, called homol E, serves as an activation sequence (Gross and Kaufer 1998). Both elements have been shown to regulate the expression of ribosomal proteins in this species. The homol D-box sequence has been compared with the TATA-box functionally and the possibility has been raised that homol

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S. Nabavi · R. N. Nazar (✉)
Department of Molecular and Cellular Biology,
University of Guelph, Guelph, ON N1G 2W1, Canada
e-mail: rnnazar@uoguelph.ca

E/homol D constitutes a new type of transcription system which has evolved in parallel to the top of box system (Tanay et al. 2005). Although the actual sequence is not conserved, in *Saccharomyces cerevisiae*, a ribosomal protein module associated with RAP1 (GACATCCGTACAT) has been considered analogous to the Homol D-box found in *S. pombe* (Tanay et al. 2005) and IFH1 sites (TCCGCC TAG) have been compared with a Homol E site.

While genes encoding spliceosomal snRNAs have been studied extensively, much less is known about genes encoding nucleolar snRNAs (snoRNAs) which play important roles in rRNA processing (Hughes and Ares 1991; Kass et al. 1990; Savino and Gerbi 1990). Some U3 snoRNA promoters have been examined in vertebrates, invertebrates, yeast and plants with strikingly different results. In vertebrates, invertebrates and yeasts the RNA is transcribed by Pol II but in plants U3 snoRNA is transcribed by Pol III [reviewed in (Marshallsay et al. 1992)]. Furthermore, U3 snoRNA gene promoters of vertebrates and invertebrates have been shown to consist of a PSE at about −55 in lieu of the TATA-box with a DSE at about −200 acting as an enhancer to increase the level of transcription (Dahlberg and Lund 1988; Savino et al. 1992). While a U3 snoRNA gene of *S. cerevisiae* has been expressed experimentally in vivo (Beltrame and Tollervey 1995), the actual promoter elements have never been defined. No TATA-box has been identified and it is generally anticipated that, as observed with the spliceosomal snRNAs, a PSE further upstream of the start site initiates the transcriptional process. In *S. pombe*, two U3 snoRNA genes have been isolated and sequenced (Porter et al. 1988; Selinger et al. 1992). The encoded U3 snoRNA sequences differ at 22 positions including one additional nucleotide in the U3A snoRNA. A sequence comparison of the upstream region has suggested that a 19 bp region centred 36 nucleotides upstream of the transcriptional start site may represent an RNA polymerase II upstream regulatory element.

Because of the special role in rRNA processing and its relative abundance, the U3 snoRNA system seems an especially promising model for studies on the synthesis of snoRNAs and their roles in ribosome biogenesis. In this study we have used an efficiently expressed "tagged" U3 snoRNA gene sequence to examine promoter elements in *S. pombe*. The results suggest an unusual relationship between a Homol D element and a TATA-box, which potentially links the transcription of this critical trans-acting factor to ribosome biogenesis.

## Materials and methods

### Strains and vectors

*Escherichia coli*, strain C490 (rec $A^-$, $rk^-$, $mk^-$, $thr^-$, $leu^-$, $met^-$) was used as a host for pTZ19R (Mead et al. 1986) and pFL20 (Losson and Lacroute 1983) plasmid recombinants. *Schizosaccharomyces pombe* ($h^-$, leu1-32, ura4-D18) cells were used as a host for the various 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 in MMA (medium MB w/o uracil) or on plates with 2% agar in MMA (Gutz et al. 1974; Okazaki et al. 1990).

### Preparation and expression of mutant U3 snoRNA genes

*Schizosaccharomyces pombe* genomic and plasmid DNA were extracted from logarithmically growing cells essentially as described by Hoffman and Winston (1987). Cultures (10 ml) with absorbancies of 0.4–0.6 at 550 nm were harvested by centrifugation, washed with water and resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0), 200 µl phenol:chlorophorm: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 aqueous phase was treated with RNase A (25 ng/µl) for 30 min. Ammonium acetate (120 mM) was added and the DNA was again precipitated with ethanol.

The U3B snoRNA gene regions were prepared by PCR amplification using primers complementary to spacer sequences preceding and following adjacent open reading frames as described in Fig. 1. The 4.7 kilobase (kb) amplified fragment was cloned in pTZ19R after digestion with *Hind*III restriction endonuclease and the recombinant DNA (pTZ19U3B) was used as a DNA template for further manipulations. After double digestion with *Bst*UI and *Bam*HI, the U3B gene-containing fragment was subcloned into a multicopy yeast shuttle vector, pFL20, to form a recombinant (pFL20U3B_4.2) which contained the U3B gene coding sequence together with a 1,868 base pair (bp) upstream region and a 2,133 bp downstream region. Shortened gene segments were prepared using PCR amplification and appropriate complementary primers with *Bam*HI adapter sequences at their ends. These included pFL20UB3_3.3, which contained 927 bps upstream and 2,133 bps downstream as well as pFL20U3B_0.5, which contained only 69 bps upstream and 213 bp downstream. Plasmid DNA preparations for characterization or yeast transformation, were based on the methods of Birnboim and Doly (1979).

For more detailed sequence analyses, base substitutions were introduced using a two-step PCR-based strategy as described previously (Nabavi and Nazar 2005). Again primers with *Bam*HI adapter sequences at their ends were used to prepare the final fragments, which after digestion with *Bam*HI endonuclease were cloned directly into the pFL20 yeast shuttle vector using its *Bam*HI site. A single G

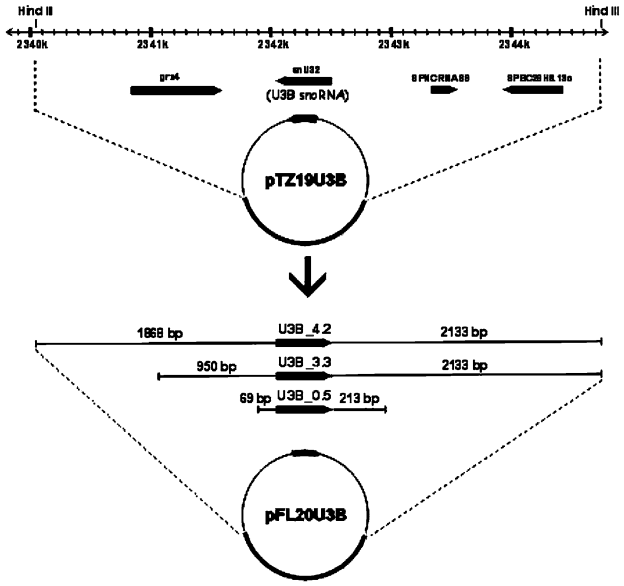



**Fig. 1** Isolation and manipulation of the U3B snoRNA gene of *S. pombe*. Primers, upstream and downstream of a *Hind*III fragment containing snU32, the gene encoding the U3B snoRNA of *S. pombe* cells, were used to amplify this region using genomic DNA prepared from *S. pombe* ($h^-$, leu1-32, ure4-D18) as a template. The amplified DNA was digested with *Hind*III endonuclease and cloned in pTZ19R. The recombinant (pTZ19U3B) was used as a template to prepare genes with varied upstream and downstream regions as indicated in the *lower panel*. The products were cloned in a high copy yeast shuttle vector (pFL20) for in vivo expression analyses. The initial sequence was taken from pJ5566 as described in the *S. pombe* gene bank

to A transition mutation at nucleotide 19 initially was introduced as a neutral plasmid-derived RNA "tag". This U3B* snoRNA sequence was used as the template for further mutations. All mutations were confirmed by automated DNA sequencing (CBS-DNA Facility, University of Guelph) and the pFL20 recombinants were used to transform *S. pombe* cells by the method of Okazaki et al. (1990). To ensure a reproducible result at least three transformants were chosen for each mutant sequence. Growth rates for the transformants were determined using the absorbancy of cultures at 550 nm.

## Preparation and analysis of the U3 snoRNAs

For all RNA analyses, whole cell nucleic acid was extracted from logarithmically growing cultures by sodium dodecyl sulfate/phenol extraction as previously described (Steele et al. 1965). The U3 snoRNA was purified by fractionation on 8% denaturing polyacrylamide gels and eluted by homogenization in SDS buffer after staining with methylene blue (Nazar 1991). Individual U3 snoRNA components were assayed using restriction fragment length polymorphism as described in Fig. 2. U3 cDNA was prepared from the gel purified U3 snoRNA by RT-PCR using a standardized protocol (Sambrook and Russell 2001), MmuLV reverse transcriptase (Fermentas Inc., Hanover, MD) and primers specific for 5′ and 3′ end of the *S. pombe* U3 snoRNAs (5′-ATCGACGATACTCCATAG-3′ and 5′-ACACGTCAGAAAACACC-3′, respectively). [$\alpha^{32}$P]dCTP (2–5 μCi) was added to the PCR reaction to label the DNA product. An initial denaturation step at 95°C for 5 min was followed with 30 cycles of 1 min at 94°C, 1 min at 47°C and 30 s at 72°C. The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), the labelled copy DNA was precipitated with ethanol and then digested with *Mbo*I endonuclease (Invitrogen Corp., Carlsbad, CA); the 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 Technologies, CA) and quantified using Molecular Analyst PC software (BioRad Laboratories, CA).

## Plasmid copy number analysis

Nucleic acid was isolated from logarithmically growing transformed cells as described by Hoffman and Winston (1987). U3 snoRNA encoding gene sequences were PCR amplified and labeled with 2–5 μCi [$\alpha^{32}$P]dCTP using common U3 snoRNA sequence primers (see above) and, after extraction with phenol: chloroform: isoamy alcohol, the products again were digested with *Mbo*I endonuclease. Digestion fragments were fractionated on 12% nondenaturing polyacrylamide gels, detected by autoradiography and images were quantified after scanning as described above.

## Cellular RNA analyses

*Schizosaccharomyces pombe* cells were grown with areation at 30°C in rich yeast extract medium (5 g/l yeast extract and 20 g/l of dextrose), minimal medium (6.4 g/l yeast nitrogen base without amino acids, 0.5 g/l potassium phosphate monobasic, 0.36 g/l potassium acetates, 0.08 g/l leucine and 0.08 g/l uracil) supplemented with 5 g/l dextrose or 5 g/l of glycerol. Growth rates were determined using the absorbency of the cultures at 550 nm. For cell number counts, liquid cell cultures were diluted 2,000 fold and plated on MB agar; colonies were counted after 10 days of incubation at 30°C.

For cellular RNA analyses, 100 ml cultures were grown to an absorbency of 0.4 and total RNA was prepared using the glass beads method of Rose et al. (1990). For total RNA content the amounts were initially determined by the absorbency at 260 nm and confirmed further by agarose gel analyses. For RNA ratios, the RNA was further fractionated on 8% polyacrylamide gels (Nazar 1991). In both cases images were captured and quantified using molecular analysts PC software as described above.

## Results

The gene sequences examined in this study initially were prepared by PCR amplification based on the *S. pombe* genome database. As indicated in Fig. 1, to initiate the gene analyses and to ensure a complete gene sequence, primers were chosen to be complementary to non-transcribed regions preceding or following open reading frames adjacent to the snU32 locus encoding the *S. pombe* U3B snoRNA. The amplified DNA was digested with *Hind* III restriction endonuclease and the resulting 4.7 Kbp fragment was cloned in pTZ19R for targeted mutagenesis and other manipulations as described in "Materials and methods". For in vivo analyses, normal or mutated gene sequences were cloned into a multicopy-number yeast shuttle vector (pFL20). Despite the fact that each of the two endogenous U3 snoRNA genes is unique in the *S. pombe* genome, a multicopy-number yeast shuttle vector was used to express the manipulated gene constructs. This largely replaced the normal transcripts with plasmid-derived transcripts without a need for potentially stressful selection condition that might be needed with a direct gene substitution strategy. It also permitted more accurate quantification when levels of expression were low.

Initially, ratios between the two U3 snoRNAs were used to quantify the plasmid-derived RNA, where the levels of one U3 snoRNA essentially served as an internal control to eliminate differences in RNA extraction. RT-PCR was used to convert RNA into cDNA and to make the assay more sensitive; sequence polymorphism was detected by restriction enzyme digestion. To eliminate contaminating genomic DNA, the U3 snoRNA template was first purified by gel electrophoresis. As indicated in Fig. 2 (left panel), with *Mbo*I endonuclease digestion, a comparison of the two *S. pombe* U3 snoRNA sequences predicted three fragments (171, 66 and 18 bp) with the U3A snoRNA sequence and four fragments (123, 65, 48 and 18 bp) with the U3B snoRNA. As also shown in Fig. 2 (left gel panel), this polymorphism was clearly evident when RNA was extracted, assayed and compared with samples from untransformed *S. pombe* cells (lanes a and b). As previously reported (Selinger et al. 1992), U3A snoRNA was found to make up approximately 60% of the U3 snoRNA population. In contrast, when cells were transformed with a plasmid-associated U3B snoRNA sequence, the ratio changed dramatically (centre gel panel) and U3B snoRNA now made up more than 80% of the cellular U3 snoRNA population (lanes c and d), closely resembling the new ratio of the gene sequences in the same cell.

To define the outer limits of the promoter, three constructs initially were compared (Fig. 1), the originally subcloned 4,232 bp *Bst*UI and *Bam*HI digestion fragment, which contained 1,868 bp upstream and 2,133 bp downstream of the coding sequence (U3B_4.2), a second, shorter construct in which the upstream region was half deleted (U3B_3.3) and a much shorter construct which contained only 69 bp upstream and 213 bp downstream U3B_0.5). As shown in Fig. 3, this comparison indicated that, unlike

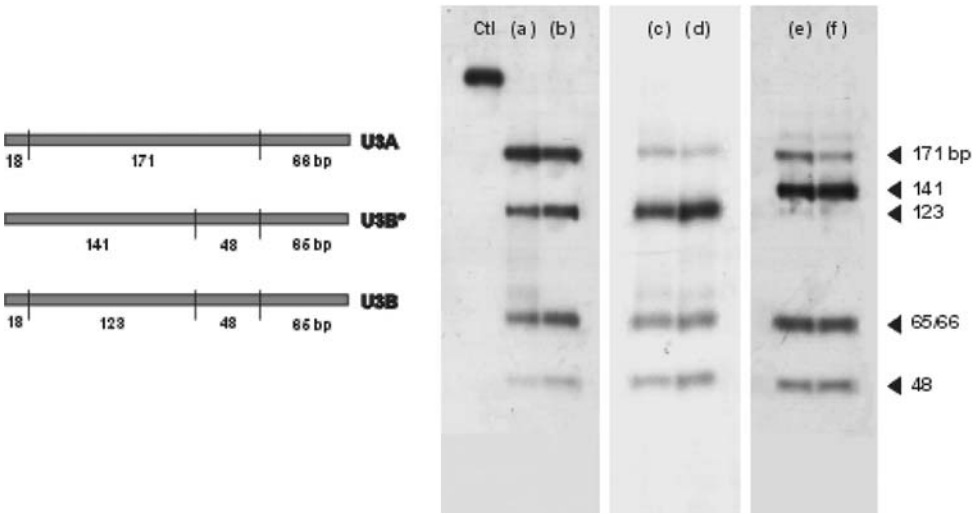



**Fig. 2** RT-PCR-based assay for *S. pombe* U3 snoRNAs. U3 snoRNA was prepared from normal or transformed cells by SDS/phenol extraction and the relative distribution of normal "tagged" (U3B*) U3 snoRNAs was the determined by restriction fragment length polymorphism. The whole cell RNA was fractionated on an 8% denaturing polyacrylamide gel and labeled cDNA of the U3 snoRNA fraction was prepared by RT-PCR as described in "Materials and methods". The labeled DNA was digested with *Mbo*I endonuclease and fragments were fractionated on 12% native polyacrylamide gels as illustrated on the *right*. Fragment sizes for all three types of snoRNA as predicted from the nucleotide sequences are indicated on the *left*; the gels represent examples of RNA from untransformed cells (*lanes a* and *b*), cells transformed with a normal U3B snoRNA gene (*lanes c* and *d*) and cells transformed with a "tagged" U3B snoRNA gene (*lanes e* and *f*). An undigested sample is also included (Ctl)

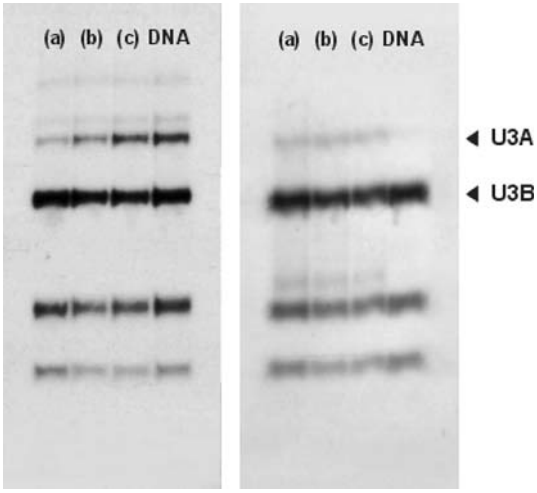



**Fig. 3** Influence of the upstream sequence on *S. pombe* U3 snoRNA gene expression. U3 snoRNA genes with upstream regions containing 1,868 bp (*left panel*) or 69 bp (*right panel*) of sequence were prepared and expressed in *S. pombe* cells as described in Fig. 1. Whole cell RNA was extracted and the relative concentrations of the U3 snoRNAs were determined by restriction fragment length polymorphism after *Mbo*I endonuclease digestion as described in Fig. 2. The results with three transformants (*a–c*) are shown together with a similar assay of the DNA from one of the transformants. Fragments, exclusive to either the U3A or U3B snoRNA sequence are indicated on the *right*

other U3 snoRNA gene promoters (Savino et al. 1992), in *S. pombe* the promoter region was relatively short with 69 or fewer base pair being sufficient to fully express the plasmid-associated gene. Levels of U3B snoRNA from three U3B_4.2 transformants (left panel) clearly are very comparable to that observed with RNA from three U3B_0.5 gene transformants (right panel). Both closely refelected the gene ratio as determined by PCR amplification of the whole cell extracts (DNA). This was confirmed further when gel images were captured and quantified. As summarized in Table 1, all three types of transformant indicated very similar amounts of plasmid-derived U3 snoRNA.

Since low levels of plasmid-derived U3 snoRNA were anticipated with critical mutations, a neutral tag also was introduced into the U3B snoRNA sequence (a single G to A transition mutation at nucleotide 19) and selected to produce plasmid-derived RNA (U3B* snoRNA) that could be unambiguously distinguished from the genome encoded RNAs. As also indicated in Fig. 2, this single base substitution (lanes e and f) removed a *Mbo*I cleavage site in the U3B snoRNA sequence resulting in three fragments (141, 65 and 48 bp) with one (141 bp) being a new clearly distinguishable band. Subsequent comparisons with this new sequence showed it to be neutral with equivalent amounts of plasmid-derived RNA being detected in transformed cells. The growth rate with the new "tagged" sequence remained entirely comparable with that which was observed with the normal U3 snoRNA sequence or even cells transformed with only pFL20.

**Table 1** Influence of the upstream sequence on *S. pombe* U3 snoRNA gene expression

| Mutation[a] | Upstream sequence (bp) | Growth rate[b] | Amount of RNA[c] (%) |
|---|---|---|---|
| pFL20 | | 4.25 ± 0.15 | 43.3 ± 4.5 |
| U3B_4.2 | 1,868 | 4.50 ± 0.17 | 80.3 ± 5.9 |
| U3B_3.3 | 950 | 4.78 ± 0.16 | 83.6 ± 6.1 |
| U3B_0.5 | 69 | 4.57 ± 0.05 | 86.0 ± 5.9 |

[a] Constructs as described in Fig. 1

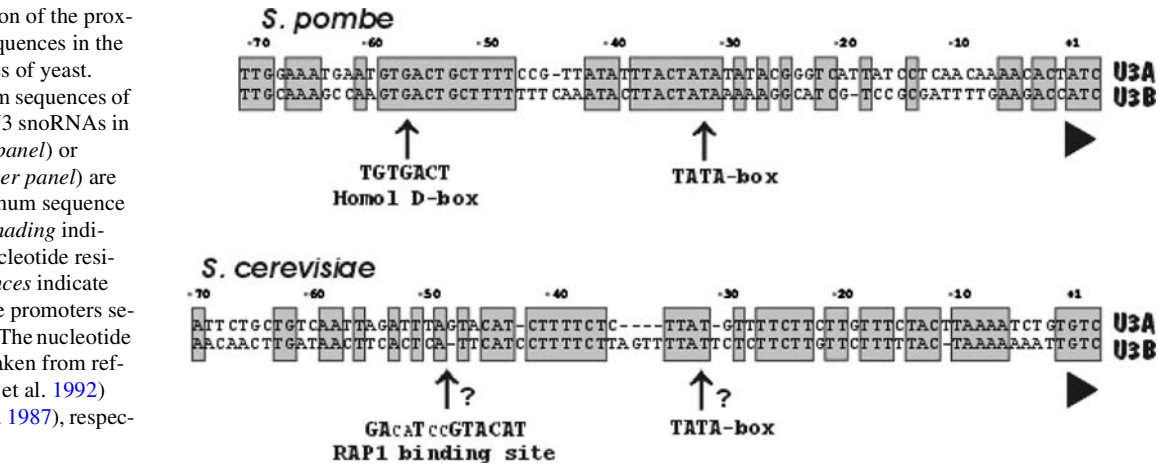
[b] Growth rate in hours based on absorbancy at 550 nm

[c] Relative amount of U3B copy DNA after digestion with *Mbo*I endonuclease as described in Fig. 2

To determine critical sequence elements in this relatively short upstream region, the two *S. pombe* sequences were first reexamined for conserved elements. As shown in Fig. 4, 39 of the 69 nucleotides or about 56% of the sequence is conserved with two large blocks centred at about −34 and −53, respectively. The eight nucleotide block at −33 contains a TATA sequence element but even more interesting was the fact that the twelve nucleotide block at −53 contained a Homol D-box like sequence, an element not previously associated with snoRNAs.

To evaluate the significance of these sequences, changes were introduced (Fig. 5) in each element (mutants P1 and P4), immediately adjacent to each element (mutants P2 and P3), one in the much less conserved region (mutant P5) and one at the transcription start site (mutant P6). The new sequences were prepared using a PCR-based strategy (Nabavi and Nazar 2005) and pTZ19U3B_0.5 as a DNA template. For expression in vivo, each sequence was inserted into the unique *Bam*HI site of the pFL20 shuttle vector using *Bam*HI adapter sequences which also were introduced during PCR-based mutagenesis. To ensure accurate nucleotide sequences, each recombinant shuttle vector construct was subjected to sequence analysis prior to yeast cell transformation. To exclude significant differences in the plasmid copy number, which might be the true cause of RNA level changes, the relative DNA copy number with the alternate constructs also was determined (e.g., DNA lanes in Fig. 6); again three transformants were examined for each construct to ensure a reproducible result.

As illustrated in Fig. 6, the results differed greatly depending on the mutant construct. The Homol D-box element (mutant P1) was found to be absolutely critical for transcription (left panel). Some transcription still occurred (about 20%) with changes to the TATA-box (mutant P4) but clearly it was essential for efficient gene expression (right panel). In contrast, the other changes had little or no effect. As also shown in Fig. 6, a change in the conserved extension to the TATA-box (mutant P3) was observed to be




| Mutation | Growth Rate | Amount of RNA |
|---|---|---|
| P1 | 4.82 ± 0.18 | n.d. |
| P2 | 4.33 ± 0.03 | 79 ± 3.6% |
| P3 | 4.48 ± 0.09 | 100 ± 0.3% |
| P4 | 4.60 ± 0.21 | 20 ± 8.1% |
| P5 | 4.30 ± 0.14 | 100 ± 0.5% |
| P6 | 5.03 ± 0.19 | 96 ± 2.3% |

**Fig. 4** Comparison of the proximal upstream sequences in the U3 snoRNA genes of yeast. Proximal upstream sequences of genes encoding U3 snoRNAs in *S. pombe* (*upper panel*) or *S. cerevisiae* (*lower panel*) are aligned for maximum sequence homology. The *shading* indicates identical nucleotide residues; *bold sequences* indicate known or putative promoters sequence elements. The nucleotide sequences were taken from references (Selinger et al. 1992) and (Hughes et al. 1987), respectively

**Fig. 5** Effect of proximal upstream mutations on *S. pombe* U3 snoRNA expression. Mutant U3 snoRNA genes were prepared by a PCR-based mutagenesis strategy (Nabavi and Nazar 2005) as indicated in *bold letters* (*P1–P6*) and expressed in vivo. Whole cell RNA was prepared from three transformants for each mutant and the relative amount (expressed as a percentage of the level with a normal "tagged" sequence) of plasmid-encoded RNA was determined by restriction fragment length polymorphism as described in Fig. 2. RNA and growth rate values are averages for three different transformants ± SD

expressed at normal levels and, after scanning and quantification, the conserved thymidylic acid residue extension to the Homol D-box (mutant P2) was found to have only a modest effect with a 20% reduction in transcription (Fig. 5). Equally, there was little or no effect with a change in the less conserved region (mutant P5) or the start site (mutant P6). In both cases essentially normal levels of transcription were observed.

The dominant role of the Homol D-box, an element previously linked to the coordinated control of ribosomal protein, raises the possibility of a more extended regulatory network in respect to ribosome biogenesis, linking not only the synthesis of the ribosomal constituents but also trans-acting factors which act in rRNA maturation and protein integration. To explore this possibility further an analysis was undertaken of relative RNA concentrations under different growth conditions. Because protein synthesis is essential for cell growth, cellular levels of rRNA and the ribosomal proteins have been shown to be closely co regulated in many organisms (Kief and Warner 1981; Rudra and Warner 2004). In the present study levels of the U3 snoRNA were examined in comparison to ribosomal and tRNAs. As illustrated in the gel analysis shown in Fig. 7 and summarized in the resultant table, as anticipated from past studies, significant differences in growth rates and the corresponding cellular RNA contents were clearly apparent. In contrast, the results demonstrate a fixed relationship in the relative concentration of the U3 snoRNA when compared with the ribosomal or tRNAs. Consistent with ribosome structure the 5 and 5.8S rRNAs were present in equimolar amounts and consistent with past analyses in many organisms much less U3 snoRNA was present. More important, however, the relative concentration of the U3 snoRNA remained constant with essentially 1 molecule of U3 snoRNA per 18 ribosomes under all three growth conditions. Clearly the level is regulated with respect to ribosome concentration much as has previously been demonstrated for ribosomal proteins.

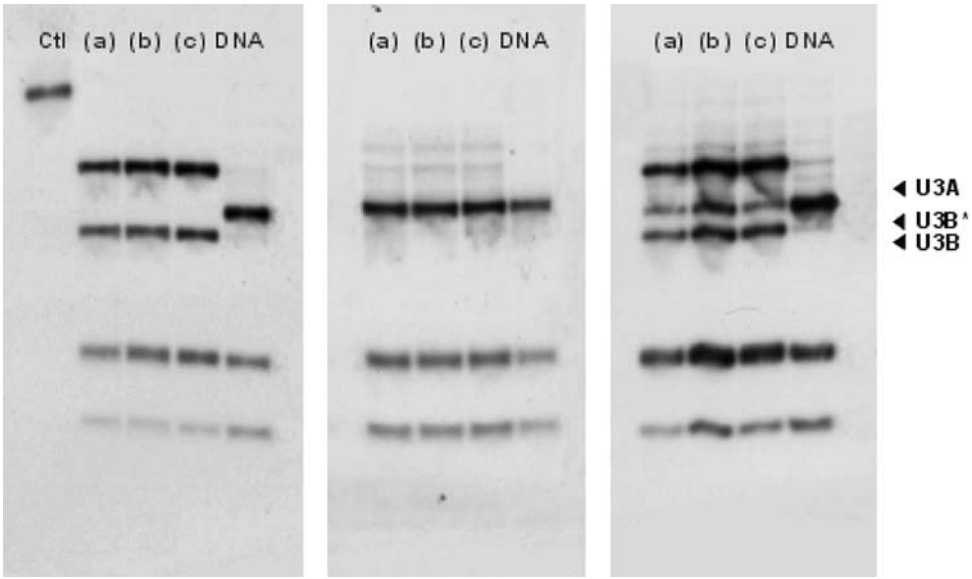
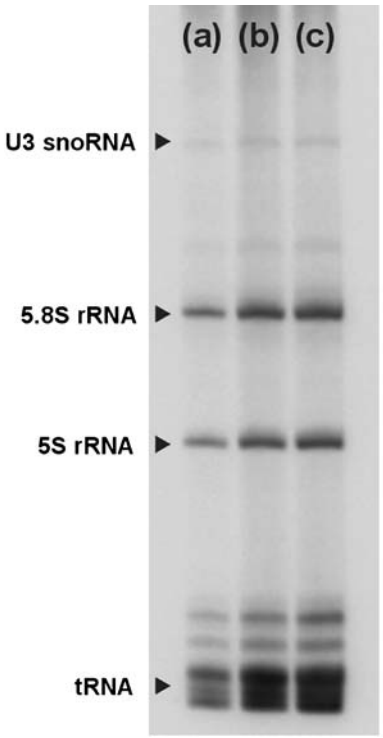



**Fig. 6** Critical proximal upstream sequence elements in the U3B snoRNA gene promoter of *S. pombe*. Mutant U3 snoRNA genes were prepared as described in Figs. 1 and 5 and expressed in vivo. Whole cell RNA was prepared from three different transformants (*a–c*) for each mutant and the relative concentrations of the U3 snoRNAs were determined by restriction fragment polymorphism as described in Fig. 2. Effects of changes in the Homol D-box (*left panel*) and TATA-box (*right panel*) are shown together with a mutation in an intervening (P3) sequence (*centre panel*). Example DNAs for each type of mutant cell (DNA) also were assayed. Fragments exclusive to the U3A, U3B or plasmid-derived (U3B*) snoRNAs are indicated on the *right*




| | YNB-glycerol | YNB-dextrose | YE-dextrose |
|---|---|---|---|
| Growth Rate (hrs) | 8.5±0.4 | 4.2±0.2 | 3.4±0.2 |
| RNA/Cell (pg) | 1.24±0.10 | 2.16±0.08 | 2.32±0.13 |
| U3 snoRNA (moles$^{-1}$) | 18.8±3.2 | 19.8±2.1 | 16.7±0.6 |
| 5.8S rRNA (moles) | 1.02±0.04 | 1.04±0.03 | 0.99±0.03 |
| 5S rRNA (moles) | 0.98±0.04 | 0.96±0.03 | 1.01±0.03 |
| tRNA (moles) | 13.0±3.5 | 12.6±1.6 | 12.6±2.4 |

**Fig. 7** Coordinated RNA regulation at different growth rates. *S. pombe* cells were grown in yeast nitrogen base (YNB) +glycerol or dextrose or a yeast extract (YE)-based medium as described in "Materials and methods". The growth rates in hours were based on the absorbency of cultures at 550 nm and the RNA/cell was based upon the absorbency of RNA extracts at 260 nm. RNA molar ratios for the three types of extract were based on gel electrophoretic analyses (*a–c*) assuming a 5/5.8 S rRNA ratio of 1. Moles of tRNA per mole of 5S or 5.8 S rRNA were based on an average 75-nucleotide sequence. Values represent averages for three replicate experiments ± SD

To confirm this correlation further, two additional comparisons were undertaken as shown in Fig. 8. Since the U3 snoRNA can be subject to post-transcriptional regulation (Nabavi et al. 2008) a comparison was made of both the mature and precursor RNA levels with a RT-PCR assay using a common forward primer complementary to the mature snoRNA sequence with primers specific for the mature (right panel) and precursor (left panel) RNAs, respectively. As indicated in Fig. 8, the correlation which was observed with methylene blue stained gels (Fig. 7) was

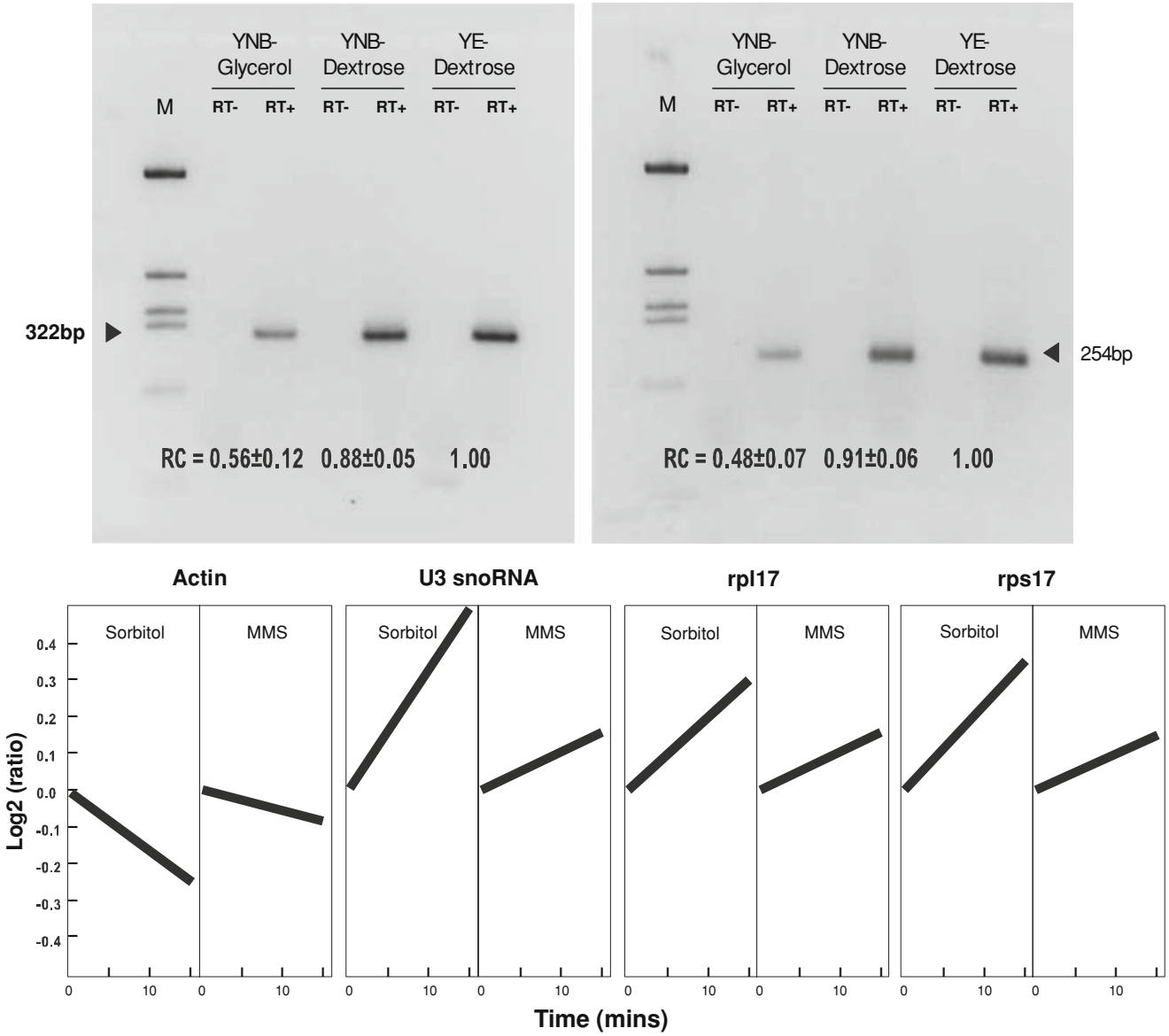



**Fig. 8** Comparisons of gene expression analyses. Levels of U3 snoRNA were compared with the levels of U3 snoRNA precursor or other genes under alternative growth conditions. For precursor analyses (*upper panels*) cellular RNA was prepared as described in Fig. 7 and the U3 snoRNA (*right*) or precursor (*left*) was assayed using RT-PCR with a common U3 snoRNA gene-specific forward primer and primers specific for the mature (254 bp) or precursor (322 bp) RNAs, respectively. The amplified DNA fragments were fractionated on 2% agarose gels. A *Hinf*I digest of pTZ19R is included as a fragment length marker (M). The average amounts of RNA relative (RC) to levels with cells grown in yeast extract/dextrose are indicates at the *bottom* of each panel. For general transcription comparisons, data were taken from the Sanger Institute fission yeast functional genomics gene expression viewer and are based on microarray analyses by Bahler and coworkers (Chen et al. 2003). Changes are indicated in actin (cytoskeletal protein Syp1) mRNA, U3 snoRNA (snu3), ribosomal large subunit protein 17 mRNA (rpl17) and ribosomal small subunit protein 17 (rps17) under stressed conditions after treatment with sorbitol (osmotic stress) or methyl methane sulfate (MMS) which methylates DNA and protein

equally evident with the RT-PCR analyses and applied to both the mature and precursor RNAs. The amounts of RNA with cells grown in YNB/glycerol or YNB/dextrose relative to cells grown in yeast extract/dextrose (RC), as measured by RT-PCR, closely reflected each other and were very similar to the values with methylene blue staining. Equally, when published data on global analyses of gene expression in *S. pombe* were examined using the Sanger Institute fission yeast functional genomics gene expression viewer (http://www.sanger.ac.uk/perl/SPGE/geexview), example changes again were found to correlate strongly. As also shown in Fig. 8 (lower panels), when cells were stressed using sorbitol or methylmethane sulphonate and transcription was measured using microarray hybridization (Chen et al. 2003) changes in the U3 snoRNA, ribosomal large subunit protein 17 (rpl17) and ribosomal small subunit

protein (rps17) all reflected very similar upregulation in transcription. In strong contrast, downregulation was clearly evident with the actin gene further supporting the special relationship suggested by the promoter analyses.

## Discussion

While the yeast U3 snoRNA is the most abundant and well studied nucleolar trans-acting RNA factor, surprisingly little is known about its control and biosynthesis. Nevertheless, its critical role in ribosome biogenesis (Hughes and Ares 1991; Kass et al. 1990; Savino and Gerbi 1990) continues to invite further experimentation. The present study describes an effective "tagged"system for experimentation with this RNA in *S. pombe*, including its biosynthesis and function structure relationships. This system permits the plasmid-derived "tagged" RNA to largely replace all cellular U3 snoRNA and to accurately quantify it at both high and low levels (e.g., Fig. 5). The growth rate and RNA analysis (e.g., Table 1 and Fig. 5) indicate the system can be applied without negative consequences to cell growth and survival.

Targeted mutations in the upstream region and the subsequent expression of these mutant sequences in vivo have revealed a number of unanticipated properties of the *S. pombe* U3 snoRNA promoter. The two important elements, a Homol D-box and a TATA-box, are clustered relatively close to the start site, starting at −60 and −35, respectively. This is similar to the spUSE and TATA-box configuration in other snRNA promoters of *S. pombe* but unlike the spUSE element in the spliceosomal snRNAs, the Homol D-box sequence does not function as an activator of basal transcription based on a TATA sequence element. In the case of the U3 snoRNA promoter the Homol D-box is critical and some transcriptional activity remains even when the TATA-box sequence is disrupted (Fig. 6). This is somewhat reminiscent of the ribosomal protein genes in this fission yeast, where basal transcription also is not based on a TATA sequence element; instead, Homol E seems to activate basal transcription based on the Homol D-box.

The results raise an intriguing question regarding the evolution of the U3 snoRNA promoter and its relationship to ribosome biogenesis. Past studies of the ribosomal protein genes in *S. pombe* (Gross and Kaufer 1998; Witt et al. 1995) have clearly documented the importance of the Homol D-box as a TATA-analogue involved in determining transcriptional start sites and a target of a protein binding factor essential to ribosomal protein synthesis and regulation (Tanay et al. 2005). However, the Homol D-specific proteins appear not to compete with TATA-box specific factors leading to speculation that the systems are parallel. Having both in a single promoter in which Homol D appears to actually be dominant raises the possibility of a two promoter fusion which leaves the U3 snoRNA potentially of a "servant of two masters" perhaps linking ribosome biogenesis to other RNA metabolism. The resolution of this question will, undoubtedly, require a full dissection of all factors interacting with this promoter. In the interim, studies of cellular RNA levels provide direct evidence for coordinated regulation of U3 snoRNA synthesis with ribosomal components, a relationship indirectly suggested by the dominant role of the Homol D-box in the U3 snoRNA promoter. As shown in Fig. 7, cellular levels of the U3 snoRNA, while changing with growth rate, closely correlate with the rRNA levels, reflecting its role in rRNA processing and ribosome biogenesis. This correlation is strongly supported further in Fig. 8, which extends the analyses to U3 snoRNA precursor and examples of ribosomal proteins and clearly documents a close relationship which is very different from that observed with an unrelated protein gene such as actin.

The current observations in *S. pombe* also raise similar questions in other yeasts and perhaps U3 snoRNAs, in general. While sequence homology is not evident, as noted earlier, in *S. cerevisiae* a RAP1 binding site (GACATCCGTACAT) has been considered analogous to the Homol D-box. Sequence analyses have not identified a TATA-box in the U3 snoRNA promoter of this yeast and an upstream element has been anticipated but never identified (Hernandez 2001; Smale and Kadonaga 2003). In view of our results in *S. pombe*, a further re-examination of equivalent sequence regions was undertaken. As also indicated in Fig. 4, the proximal regions in the *S. cerevisiae* U3 snoRNA promoters show little conservation with those of *S. pombe* but are homologous with each other to a similar degree; 41 of the 69 nucleotides or about 59% of the sequence is identical. A TTAT sequence replaces the TATA-box starting at −31 but more interesting may be the thymidylic acid residue cluster at about −40 and the preceding GATTTAGTACAT sequence in the *S. cerevisiae* U3A snoRNA which represents about 90 percent of the U3 snoRNA population in this organism (Hughes et al. 1987). Eight of the nucleotides beginning and ending this 12 nucleotide block are identical to the RAP1 binding sequence. Whether this homology is significant, clearly will depend on more detailed studies in the budding yeast. In the interim the present results and comparisons raise new questions about the evolution of pol II promoters including the interchange of basal transcriptional elements to ultimately reflect gene function.

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