

# Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA

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**It has long been known that U3 can be isolated hydrogen bonded to pre-ribosomal RNAs, but the sites of interaction are poorly characterized. Here we show that yeast U3 can be cross-linked to 35S pre-rRNA both in deproteinized extracts and in living cells. The sites of cross-linking were localized to the 5' external transcribed spacer (ETS) and then identified at the nucleotide level. Two regions of U3 near the 5' end are cross-linked to pre-rRNA *in vivo* and *in vitro*; the evolutionarily conserved box A region and a 10 nucleotide (nt) sequence with perfect complementarity to an ETS sequence. Two *in vivo* cross-links are detected in the ETS, at +470, within the region complementary to U3, and at +655, close to the cleavage site at the 5' end of 18S rRNA. A tagged rDNA construct was used to follow the effects of mutations in the ETS *in vivo*. A small deletion around the +470 cross-linking site in the ETS prevents the synthesis of 18S rRNA. This region is homologous to the site of vertebrate ETS cleavage. We propose that this site may be evolutionarily conserved to direct the assembly of a pre-rRNA processing complex required for the cleavages that generate 18S rRNA.**

**Key words:** pre-rRNA/ribosomes/RNA/small nucleolar RNAs/U3 binding sites

## Introduction

The synthesis of mature ribosomal RNAs in eukaryotic cells is a complex process taking place primarily in the nucleolus, where a single precursor rRNA (pre-rRNA) transcribed by RNA polymerase I undergoes base modification, methylation and a series of endo- and exonucleolytic cleavages. Although many cleavage sites have been localized at the nucleotide level (see Klootwijk and Planta, 1989 and references therein), very little is known about the mechanism and the machinery involved in the processing pathway leading from the large pre-rRNA to 18S, 5.8S and 25S rRNAs.

Eukaryotic cells contain a large number of small, nuclear RNA species. Many of these fall into two non-overlapping groups. The small nuclear RNAs (snRNAs) are nucleoplasmic, include U1, U2, U5, U4/U6 and U7 and all characterized species are involved in pre-mRNA processing. In contrast the small nucleolar RNAs (snoRNAs) are implicated in the processing of pre-rRNA. A number of different snoRNA species have been characterized from higher eukaryotes and from *Saccharomyces cerevisiae* on

basis of their nucleolar localization, association with pre-rRNA species and association with the nucleolar protein fibrillarin (NOP1 in yeast) and other nucleolar proteins (Prestayko *et al.*, 1970; Parker and Steitz, 1987; Tyc and Steitz, 1989; Tollervey, 1987; Zagorski *et al.*, 1988; Schimmang *et al.*, 1989; Clark *et al.*, 1990; Girard *et al.*, 1992). In yeast, the cloning of 10 small nucleolar RNAs (snoRNAs) has been reported (reviewed by Tollervey and Hurt, 1990). Three of these (snR10, U14 and U3) have been shown to play direct roles in pre-rRNA processing. Strains lacking snR10 are viable, but impaired in growth and specifically defective in the processing of the primary transcript, the 35S pre-rRNA (Tollervey and Guthrie, 1985; Tollervey, 1987). U14 (previously snR128) is essential for viability (Zagorski *et al.*, 1988) and its depletion results in impaired processing of pre-rRNA (Li *et al.*, 1990). The pathway leading to 18S rRNA is particularly affected: normal intermediates are missing and 18S rRNA is under-accumulated. Interestingly, conditional mutants of the nucleolar proteins NOP1 (the yeast homologue of mammalian fibrillarin) and GAR1 show very similar phenotypes under restrictive conditions (Tollervey *et al.*, 1991; Girard *et al.*, 1992).

U3 is essential for viability in yeast (Hughes *et al.*, 1987) and like U14, is required for 18S rRNA synthesis (Hughes and Ares, 1991). U3 has been shown to play a role in mammalian pre-rRNA processing *in vitro*. The first cleavage in mammalian pre-rRNA processing occurs in the 5' external transcribed spacer (ETS) and can be reproduced in a mouse *in vitro* system (Kass *et al.*, 1987). Depletion of U3 snoRNP by immunoprecipitation or oligonucleotide directed cleavage of U3 snoRNA interfere with the *in vitro* cleavage of the ETS (Kass *et al.*, 1990). Oligonucleotide directed RNase H cleavage has been used to address the function of U3 in *Xenopus* oocytes (Savino and Gerbi, 1990). Cleavage of a large fraction of *Xenopus* U3 does not prevent the synthesis of mature rRNA but, like the absence of yeast snR10, alters the pathway of pre-rRNA processing.

Both in mammalian cells and in yeast, U3 RNA co-fractionates with pre-rRNA species in deproteinized extracts (Prestayko *et al.*, 1970; Tollervey, 1987), a property consistent with hydrogen bonding. A number of different models have been proposed for the site of U3-pre-rRNA interaction (Crouch *et al.*, 1983; Bachelier *et al.*, 1983; Tague and Gerbi, 1984; Parker and Steitz, 1987; Parker *et al.*, 1988). These models were based largely on phylogenetic conservation of proposed interacting regions and on U3 secondary structure models. More recently, attempts have been made to localize *in vivo* the site of U3-pre-rRNA interaction. *In vivo* psoralen cross-linking studies indicate that in human (Maser and Calvet, 1989) and rat cells (Stroke and Weiner, 1989), U3 is associated with a region of the ETS. The exact site of interaction within the ETS was not identified, but interestingly the region of ETS cross-linked to U3 includes the U3-dependent primary

cleavage site in rat and is adjacent to it in human cells. The cross-linked residues in rat U3 RNA lie close to box A, the most highly conserved region of U3 (Stroke and Weiner, 1989; Hughes *et al.*, 1987).

Our studies, using psoralen cross-linking and exploiting molecular genetic techniques, address the question of the functional relevance of U3-pre-rRNA interaction in yeast. As in mammalian cells, U3 can be cross-linked to the ETS in yeast; the cross-linking sites in the yeast ETS have been identified and analysed.

## Results

### ***U3 can be cross-linked in vitro and in vivo to 35S pre-rRNA***

In our initial cross-linking experiments, AMT-psoralen was added to crude deproteinized extracts obtained by proteinase K-SDS treatment of cell lysates, since we knew that hydrogen bonding between U3 and pre-rRNA could be maintained under these conditions (Tollervey, 1987). Subsequently, cross-linking experiments were also performed in living cells, by addition of AMT-psoralen to intact spheroplasts. After irradiation with UV-light, total RNA extracted from the samples was denatured and separated on a 10–30% linear sucrose gradient. As a control, AMT-psoralen treated, but non-irradiated RNA was prepared and fractionated in parallel with cross-linked RNA through all mapping experiments. Fractions were recovered and aliquots were run on denaturing agarose gels. RNA was transferred to membranes which were sequentially hybridized with a probe specific for pre-rRNA species (Figure 1A and B) and with a U3 anti-sense riboprobe (Figure 1C and D).

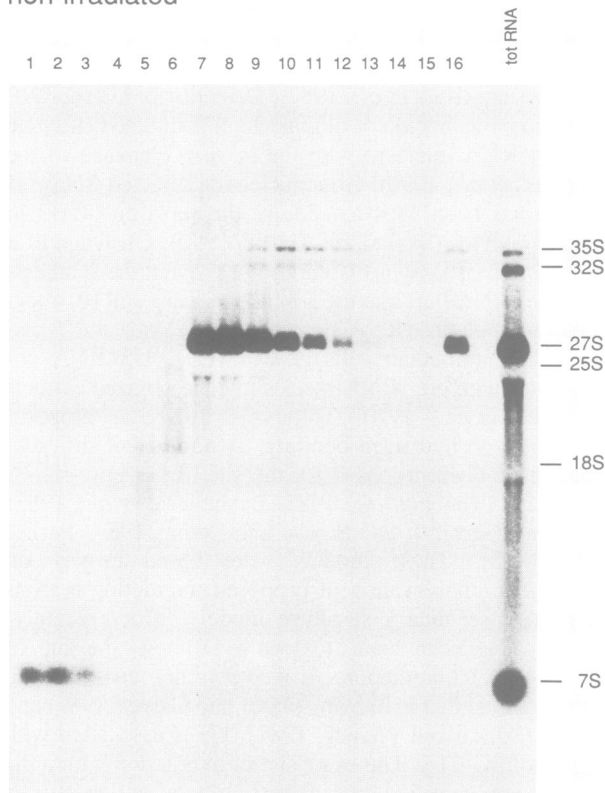
Both *in vitro* (Figure 1) and *in vivo* (data not shown), a single discrete band with a slightly lower mobility than 35S pre-rRNA hybridizes with the anti-U3 probe in the irradiated samples but not in the non-irradiated controls (Figure 1), showing that U3 can be cross-linked to the 6.7 kb long primary transcript, 35S pre-rRNA. The cross-linking efficiency is higher *in vitro* than *in vivo* under our experimental conditions. We estimate that ~5% of 35S pre-rRNA is cross-linked to U3 snoRNA *in vitro*, the percentage dropping at least 2-fold *in vivo* (data not shown). Due to the low efficiency of cross-linking and the very high abundance of mature 18S and 25S rRNA, these give relatively strong non-specific hybridization to the U3 riboprobe in the presence of both cross-linked and control RNA.

### ***Two regions of U3 snoRNA interact with 35S pre-rRNA***

Cross-linking sites can be mapped by primer extension, since reverse transcriptase has been shown to stop at the nucleotide preceding a cross-linked base. To distinguish between intramolecular cross-links and monoadducts in U3 and cross-linking to pre-rRNA, cross-linked U3 was enriched prior to primer extension. To do this, denatured RNA was fractionated on 10–30% sucrose gradients, as shown in Figure 1; primer extension was performed on free U3, from the top of the sucrose gradient, and on U3 predominantly cross-linked to pre-rRNA, from the 35S pre-rRNA peak fractions. Non-irradiated samples from equivalent sucrose gradient fractions were used as controls. Primer extension was performed using several oligonucleotides complementary to different regions of U3 in order to span

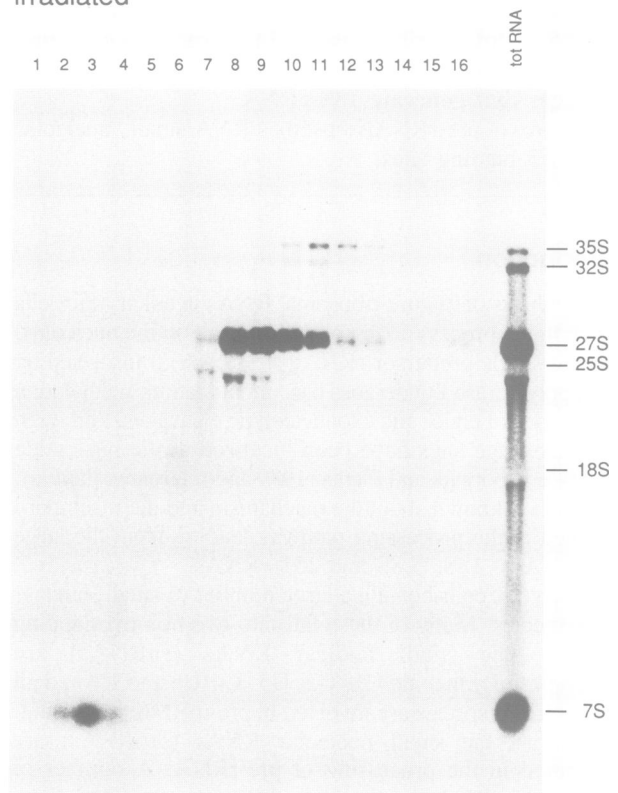
## A

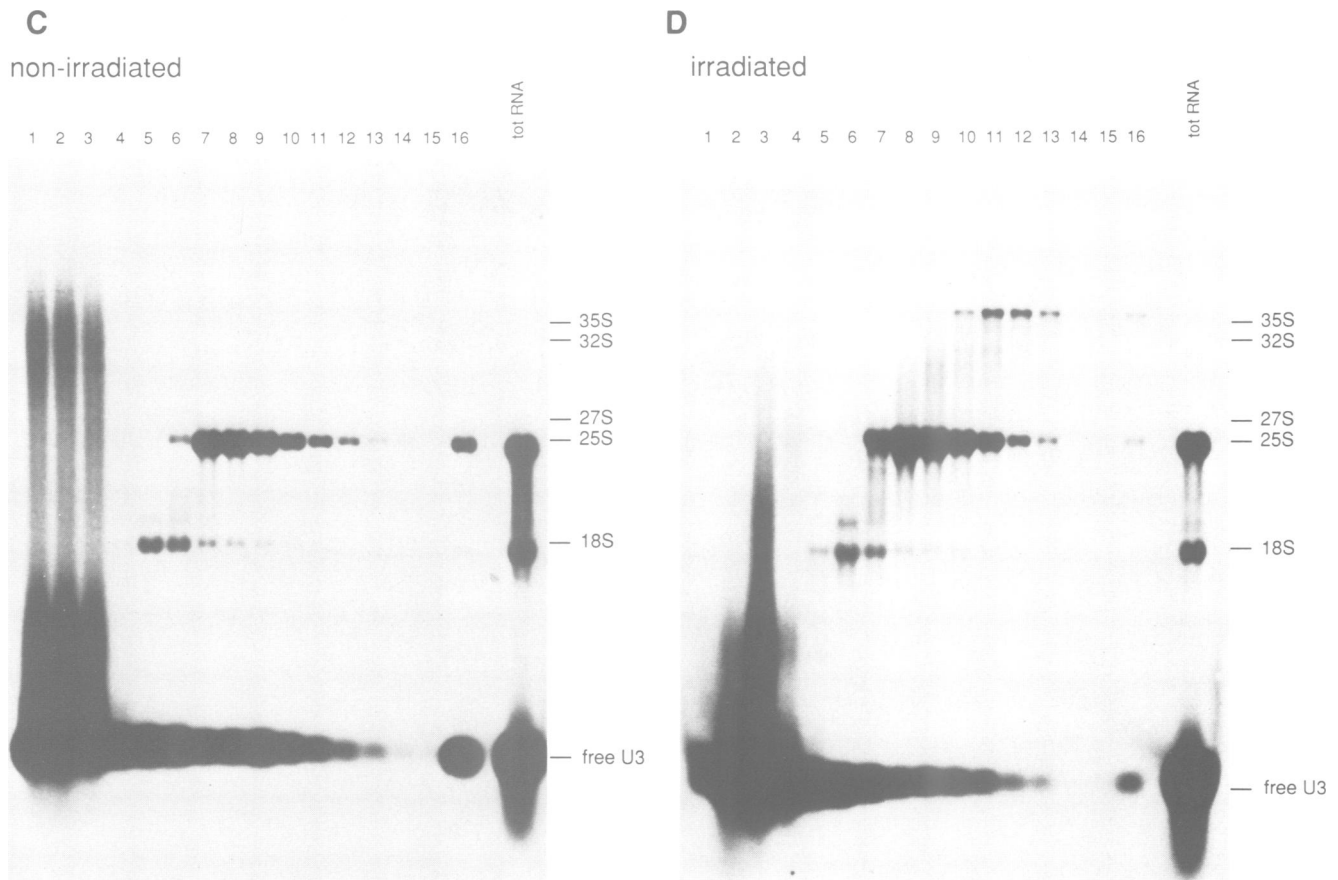
non-irradiated



## B

irradiated





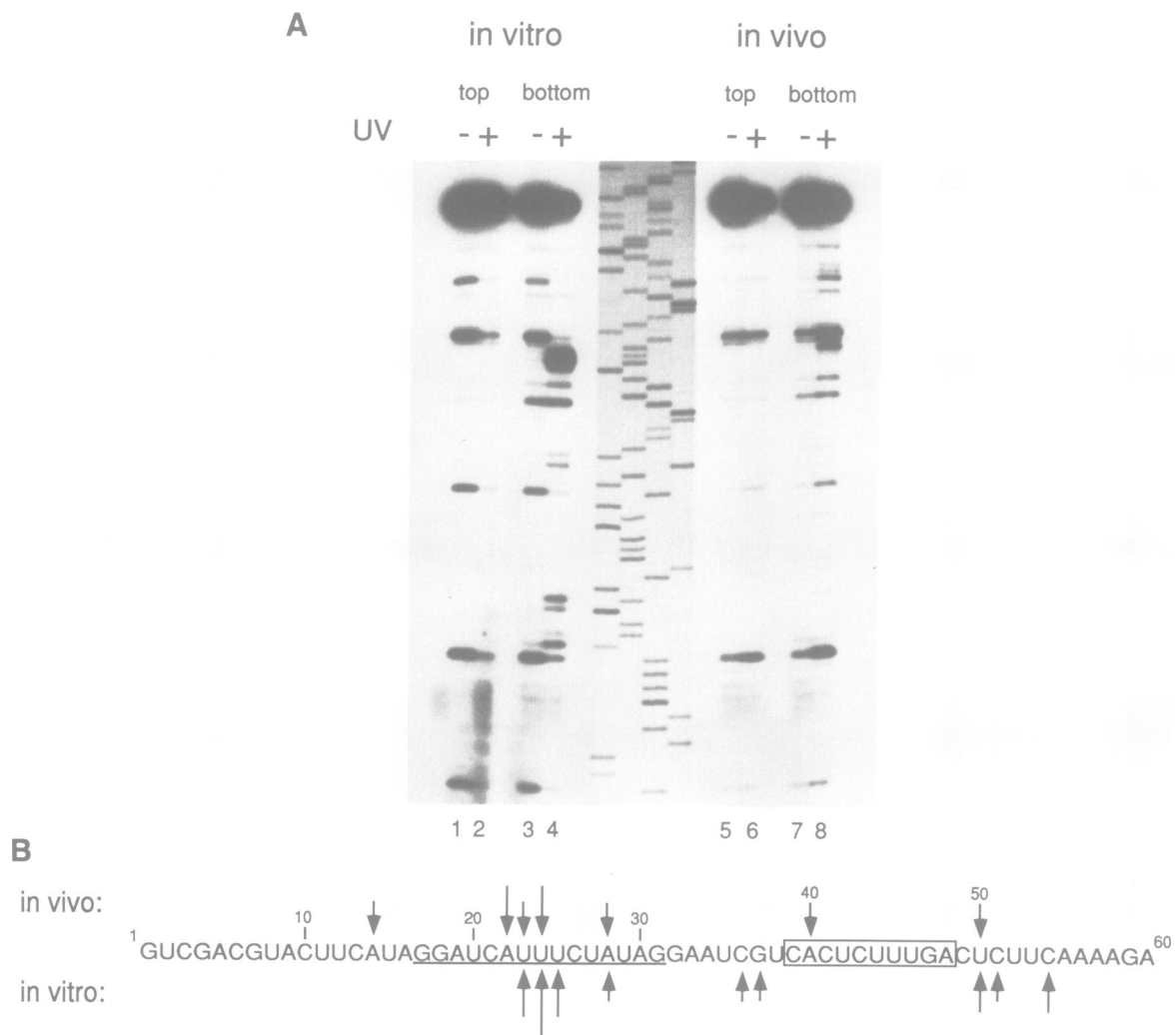
**Fig. 1.** *In vitro* cross-linking of U3 snoRNA to pre-rRNA. Total RNA was extracted from crude deproteinized lysates treated with AMT-psoralen and UV-irradiated, and from a non-irradiated control. The RNA was heat-denatured and run on sucrose gradients. Fractions were taken from the top of the gradients and numbered 1 to 16. Aliquots of each fraction (lanes 1–16) were run on agarose–formaldehyde gels alongside total untreated RNA (tot RNA) and transferred to filters. Panels A–D show Northern blots of: A and B, non-irradiated control and irradiated samples probed with an oligonucleotide specific for internal transcribed spacer 2, to detect the position of a number of pre-rRNA species; C and D, non-irradiated control and irradiated samples probed with an anti-U3 riboprobe to detect pre-rRNA species cross-linked to U3 snoRNA.

the whole snoRNA (333 nt long). Both *in vitro* and *in vivo* only the 5' region of U3 is detectably cross-linked to 35S (Figure 2). Primer extension stops specifically present on U3 associated with 35S (bottom fractions of sucrose gradient) (Figure 2, lanes 4 and 8) but not on free U3 (top fractions) (Figure 2, lanes 2 and 6) indicate the presence of cross-linking sites. It should be noted that the cross-linking sites are expected to lie 1 nt 5' to the primer extension stop sites shown in Figure 2B. Sites of cross-linking lie in the evolutionarily conserved box A (underlined sequence in Figure 2B) and flanking a region with 10 nt of perfect complementarity to a sequence in the ETS (boxed sequence in Figure 2B). An additional cross-link is found 5' to box A in RNA cross-linked *in vivo*. According to a model recently proposed for the secondary structure of yeast U3 (V.Segault, A.Mougin, A.Gregoire, J.Banroques and C.Branlant, submitted), the cross-linked nucleotides in box A (C21, A22 and U23) lie in the loop of the 5' stem of the snoRNA, while the 5' cross-linked nucleotide (C13) is bulged from this stem (see also Figure 9). A further crosslink is seen at U27; in the U3 secondary structure model, this lies at a A-U/U-A base paired sequence in the 5' stem of U3, suggesting the possibility that this may represent intramolecular cross-linking (see also Figure 9). For intramolecular cross-linking, the 5' side of the cross-link cannot be seen by primer extension.

In the U3 secondary structure (V.Segault, A.Mougin, A.Gregoire, J.Banroques and J.Branlant, submitted) the 10 nt complementary to the ETS sequence are mainly single stranded and therefore potentially available for interaction with the pre-rRNA. The patterns of primer extension stops *in vitro* and *in vivo* are similar but not identical. Stops (and therefore cross-links) are found at similar but not identical positions in U3, possibly due to distortions in the RNA structure caused by protein binding.

#### **Two regions of 35S pre-rRNA interact with U3 snoRNA**

To define the region of the 35S primary transcript interacting with U3, we used a sandwich Southern blot technique (Stroke and Weiner, 1989). RNA from sucrose gradient fractions containing the peak of 35S pre-rRNA which was cross-linked to U3 *in vitro* and from the corresponding fractions of the non-irradiated control was partially hydrolysed by treatment with alkali. Southern filters were prepared carrying different restriction digests of a plasmid containing the entire ribosomal repeat. These were first hybridized with the partially hydrolysed RNA from either cross-linked or non-irradiated samples and then probed with an anti-U3 riboprobe. Bands present only in the filter treated with irradiated RNA should correspond to DNA bands hybridizing with the region of rRNA cross-linked to U3.



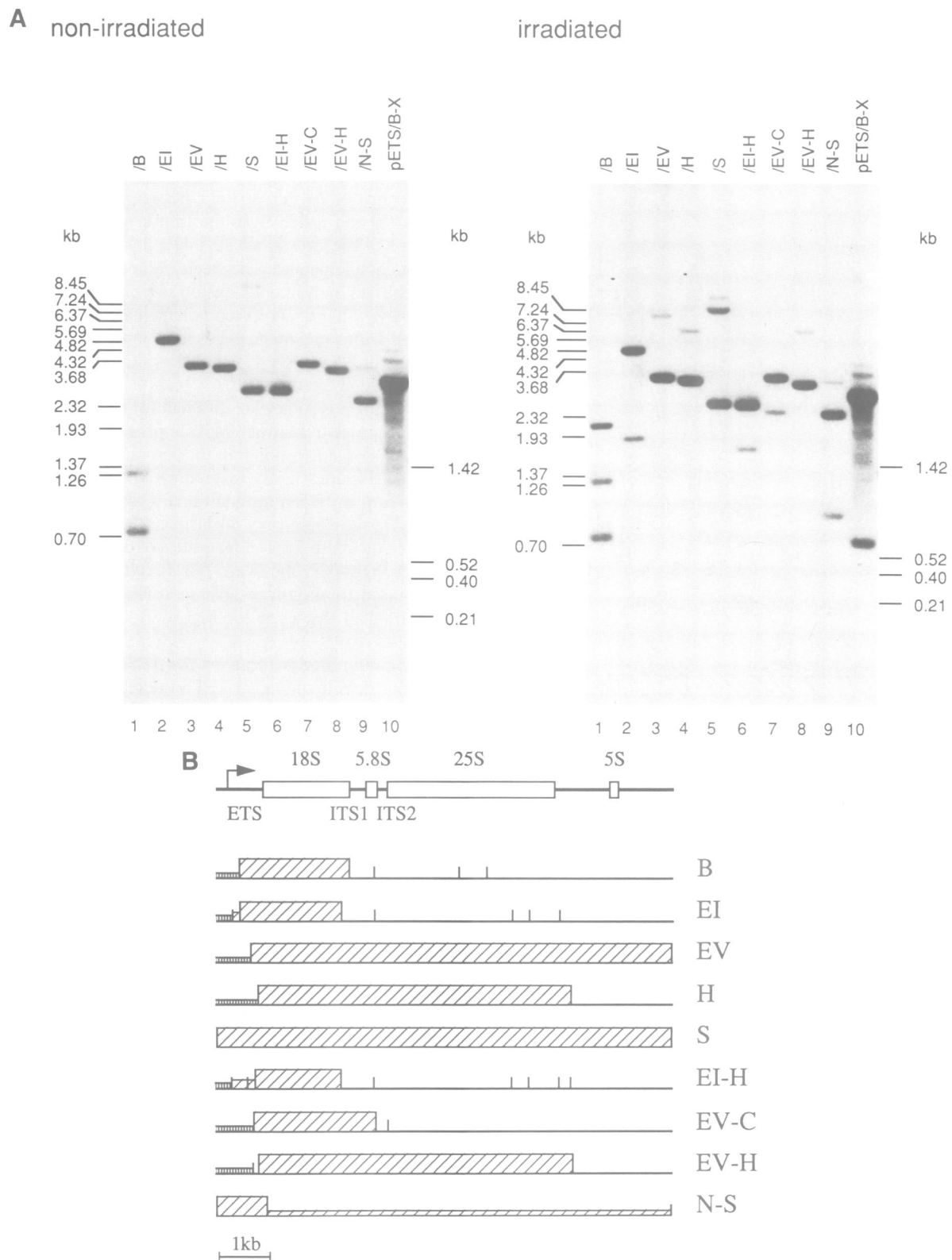
**Fig. 2.** Localization of the cross-linking sites on U3 snoRNA by primer extension. (A) Total RNA from irradiated samples (UV+) and non-irradiated controls (UV-) was fractionated on sucrose gradients as shown in Figure 1. Primer extension reactions were performed on free U3 snoRNA from top fractions (top) and on U3 snoRNA from the 35S pre-rRNA peak fractions (bottom). The samples were run on an 8% sequencing gel alongside a dideoxynucleotide sequence ladder of the SNR17A gene, which encodes U3A snoRNA (G.A.T.C from left to right). 5' to box A the sequence of the gene does not correspond to the RNA sequence due to the presence of an intron (Myslinski *et al.*, 1990). Primer extensions performed on RNA cross-linked *in vitro* are shown in lanes 1–4, the corresponding reactions on RNA cross-linked *in vivo* are shown in lanes 5–8. Lanes are: 1 and 5, top fractions from non-irradiated controls, *in vitro* and *in vivo* respectively; 2 and 6, top fractions from irradiated samples; 3 and 7, 35S peak fractions from non-irradiated controls; 4 and 8, 35S peak fractions from irradiated samples, containing U3 snoRNA cross-linked to 35S pre-rRNA. The oligonucleotide primer is complementary to nt +96 to +78 of U3. (B) Scheme of primer extension stops which are reproducibly present or enhanced in U3 snoRNA cross-linked to 35S pre-rRNA [lanes 4 and 8 of (A) of this figure]. The sequence of nt 1–60 of U3 snoRNA is shown. The evolutionarily conserved box A sequence is underlined, and the region of perfect complementarity between U3 and the ETS is boxed. *In vivo* primer extension stops are marked by arrows above the U3 RNA sequence, *in vitro* primer extension stops below it. The length of the arrows gives some indication of the relative intensity of the primer extension stops. Sites of cross-linking are expected to lie 1 nucleotide 5' to the primer extension stops.

These experiments were performed with *in vitro* cross-linked RNA, since the cross-linking efficiency is higher *in vitro* than *in vivo*. As shown in Figure 3, the pattern of bands hybridizing with the U3 probe on the irradiated filter clearly indicates that the U3 cross-linking sites are located within the 699 nt long ETS. This is confirmed by the signal in Figure 3, lane 10 containing the whole ETS as a single fragment. The smallest region present on all strongly hybridizing fragments lies between the *NdeI* site at +724 (25 nt into 18S) and the *HindIII* site at +519 (180 nt into the ETS) of the pre-rRNA. However, fragments further into the ETS, such as the *EcoRI*–*EcoRI* and *EcoRI*–*HindIII* fragments (see Figure 3B), give relatively weaker hybridization because of their small size. In both Figures

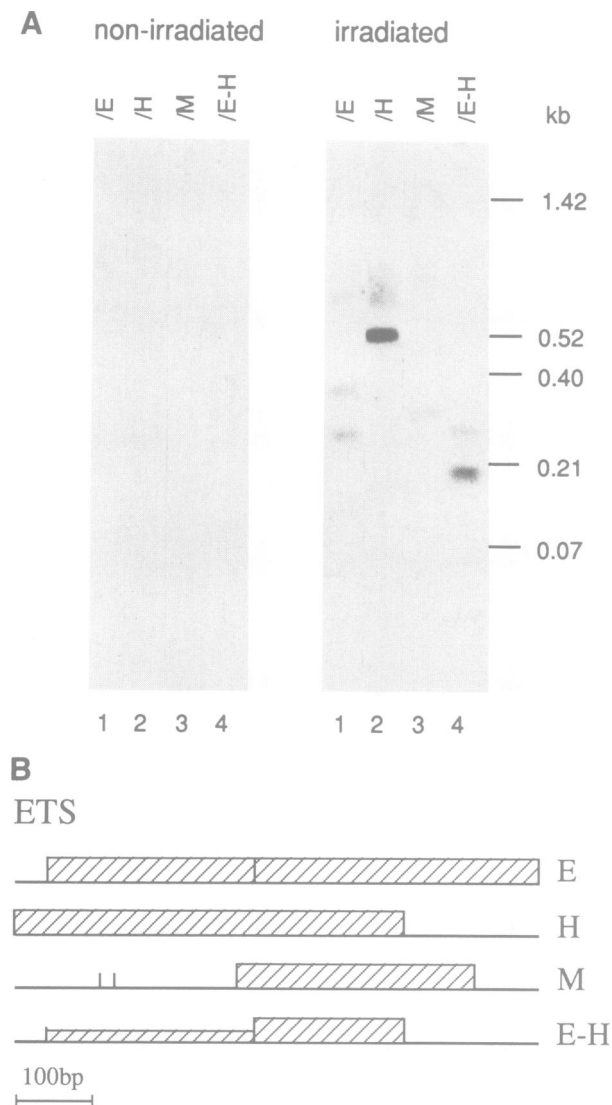
3 and 4, different digests give substantially different hybridization signals. The reason for this is not clear.

To better localize the site of cross-linking, a second sandwich Southern blot was carried out with RNAs from the same fractions but on filters containing the purified ETS region digested with several enzymes. As shown in Figure 4, we could further delimit the region of *in vitro* cross-linking to ~200 nt within the ETS.

Once the region of 35S pre-rRNA containing the U3 cross-linking sites had been defined approximately by sandwich Southern blots, we wished to identify the cross-linking sites on the ETS at the nucleotide level by primer extension. Primer extension could not be carried out on total 35S pre-rRNA, however, since only a small proportion of 35S RNA



**Fig. 3.** Sandwich Southern blot of a complete rDNA repeat. **(A)** Duplicate Southern filters containing several restriction digests of a plasmid carrying the entire rDNA repeat were hybridized with RNA from the peak 35S fractions of either an *in vitro* cross-linked sample (right panel) or a non-irradiated control (left panel). The filters were then hybridized with an anti-U3 riboprobe. Lanes 1–9 of both filters are restriction digests of the plasmid borne rDNA cut with: 1, *Bst*YI (B); 2, *Eco*RI (EI); 3, *Eco*RV (EV); 4, *Hind*III (H); 5, *Sma*I (S); 6, *Eco*RI–*Hind*III (EI-H); 7, *Eco*RV–*Cla*I (EV-C); 8, *Eco*RV–*Hind*III (EV-H); 9, *Nde*I–*Sma*I (N-S). Lane 10: *Bam*HI–*Xho*I (B-X) digest of plasmid pETS, carrying the entire ETS cloned by PCR; the double digestion precisely excises the ETS from the vector. Bands present in both panels are due to the vector, which hybridizes to polylinker sequences present on the U3 riboprobe. **(B)** Diagram of DNA bands hybridizing with the region of rRNA cross-linked to U3. Vertical lines mark the restriction sites, within the rDNA repeat, of the enzymes used in (A) lanes 1–9. Obliquely hatched boxes represent autoradiographic signals specifically present on the irradiated sample (right panel, part A) as compared with the non-irradiated control (left panel, part A); these correspond to ETS fragments hybridizing with the region of rRNA cross-linked to U3. The intensity of the autoradiographic signal roughly correlates with the thickness of the bar. Specific signals corresponding to rDNA fragments fused to vector sequences are marked with vertically hatched boxes.



**Fig. 4.** Sandwich Southern blot of the ETS. (A) Duplicate filters containing several restriction digests of a purified DNA fragment carrying the entire ETS region were hybridized with RNA from the peak 35S fractions of either an *in vitro* cross-linked sample (right panel) or a non-irradiated control (left panel). The filters were then hybridized with an anti-U3 riboprobe. Lanes 1–4 of both filters are restriction digests of the purified ETS region cut with: 1, *EcoRI* (E); 2, *HindIII* (H); 3, *MaeI* (M); 4, *EcoRI–HindIII* (E-H). (B) Diagram of DNA bands hybridizing with the region of rRNA cross-linked to U3. Vertical lines mark the restriction sites, within the ETS, of the enzymes used in (A) lanes 1–4. Obliquely hatched boxes represent autoradiographic signals specifically present on the irradiated sample (right panel, part A) as compared with the non-irradiated control (left panel, part A); these correspond to ETS fragments hybridizing with the region of rRNA cross-linked to U3. The intensity of the autoradiographic signal within each lane roughly correlates with the thickness of the bar.

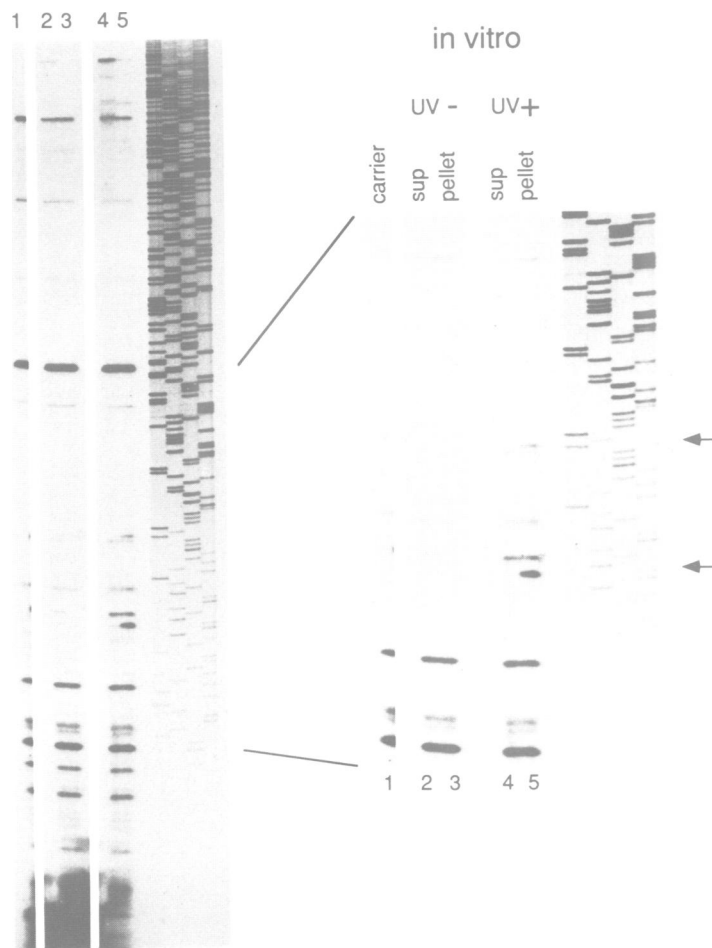
molecules are cross-linked to U3 under our experimental conditions (Figure 1). Moreover, other snoRNAs are also cross-linked to 35S pre-rRNA (J. Morrissey, M. Beltrame and D. Tollervey, unpublished). The U3-specific cross-links were enriched relative to monoadducts and intramolecular cross-links by separation of those 35S molecules cross-linked to U3 from the bulk of 35S RNA which is not cross-linked. A biotinylated oligonucleotide complementary to U3 snoRNA was used to hybrid-select 35S–U3 cross-linked

molecules from the 35S peak fractions of sucrose gradients prepared as described above. The hybrids were recovered by binding to streptavidin–agarose beads and centrifugation, leaving non-cross-linked 35S in the supernatant. Primer extensions were performed on RNA extracted from both pellet and supernatant, using oligonucleotides complementary to the ETS. In 35S RNA cross-linked *in vitro*, two primer extension stops are specifically found in the pellet indicating the presence of two cross-links between the ETS and U3 at nt +470 and +488 from the transcription initiation site (Figure 5). The cross-link at +470 lies within the region of 10 nt complementarity between U3 and pre-rRNA which was identified by primer extension stops on U3 both *in vitro* and *in vivo*. This region of complementarity can be further extended with some mismatches to nt +491, thus including the box A of U3 snoRNA, where the other primer extension stops on U3 were mapped.

The situation is somewhat different for *in vivo* cross-linked 35S. The cross-link at nt +470 is present, but no primer extension stop corresponding to a cross-link at nt +488 is detected (Figure 6). By extending the analysis further downstream on ETS, an additional cross-linking site was detected at nt +655, only 44 nt from the 5' end of mature 18S rRNA (Figure 7). The intensities of the primer extension stops in Figures 6 and 7 are not directly comparable; the primer used for Figure 6 gives consistently greater signal strengths than that used for Figure 7.

#### A U3 binding site is required for 18S rRNA synthesis

To assess the role of the U3–ETS interactions in pre-rRNA processing, we have mutagenized the U3 binding sites on ETS. Mutagenesis of rDNA presents some problems due to the fact that yeast cells contain 150–200 copies of the ribosomal repeat unit. It would therefore be difficult to replace all units by mutated copies. To follow the processing of mutated pre-rRNAs, we inserted short oligonucleotide tags within the mature 18S and 25S sequences (Musters *et al.*, 1989, 1990). The regions of insertions were selected as being variable regions, which would carry the tags as neutral mutations. A 16 nt long tag was inserted by PCR at position +190 of the 18S rRNA coding sequence. This tag is predicted to extend a stem and loop structure which is already present in yeast 18S rRNA; since rat 18S rRNA has a more extended stem at the corresponding site, we reasoned that this mutation would be unlikely to disrupt the overall 18S rRNA structure. For the insertion of a tag in 25S, we followed the procedure described by Musters *et al.* (1989). The two tags were introduced in a plasmid borne rDNA unit containing the selectable marker URA3 in the non-transcribed spacer. After mutagenesis of the ETS, the tagged rDNA was excised from the plasmid and transformed as a linear fragment into yeast cells. Selection for URA3<sup>+</sup> transformants should result in the precise replacement of a copy of the rDNA repeat by the mutated copy. Gene replacement was used instead of introducing the mutant rDNAs on a replicating plasmid, to ensure that they would have a nucleolar localization. Both 18S and 25S tagged rRNAs were normally expressed and processed from a wild-type tagged rDNA construct used as a control (Figure 8, lane 1). A 23 nt deletion (+469 to +491) was constructed around the cross-link at +470 which is present both *in vitro* and *in vivo*. This deletion eliminates the extended region of complementarity between U3 and ETS. As shown in



**Fig. 5.** Localization of the *in vitro* cross-linking sites on the ETS by primer extension. Pre-rRNA molecules cross-linked to U3 *in vitro* were hybrid-selected using a biotinylated anti-U3 oligonucleotide, recovered with streptavidin-agarose beads and pelleted. Primer extension reactions were performed on RNA extracted from both pellet and supernatant fractions of the selection using an oligonucleotide complementary to the ETS. Both selection and primer extension were performed in the presence of *E. coli* tRNA as carrier. The samples were run on a 6% sequencing gel alongside a dideoxynucleotide sequence of the ETS region (G.A.T.C from left to right). The left panel shows full length primer extensions; the region containing the stops due to cross-links is expanded in the right panel. Lanes 1–5 are primer extensions with *E. coli* tRNA as carrier plus: 1. no RNA; 2. supernatant RNA from non-irradiated control; 3. pellet RNA from non-irradiated control; 4. supernatant RNA from irradiated sample; 5. pellet RNA from irradiated sample. The arrows mark primer extension stops at +471 (upper arrow) and +489 (lower arrow) specifically present in the pellet fraction of the irradiated sample (lane 5) indicating the presence of U3–35S cross-links at nt +470 and +488 respectively. The oligonucleotide primer is complementary to nt +562 to +546 of the pre-rRNA.

Figure 8 (lane 3), the  $\Delta 23$  deletion has a severe effect on 18S rRNA synthesis: the accumulation of 18S rRNA is dramatically reduced while the level of 25S rRNA is unaffected. The region of base-pairing between U3 and pre-rRNA around position +470 appears therefore to be essential for normal processing events that lead to 18S synthesis, but not for 25S synthesis. A more detailed mutagenesis of this region will be needed to assess the role played by U3–pre-rRNA complementarity.

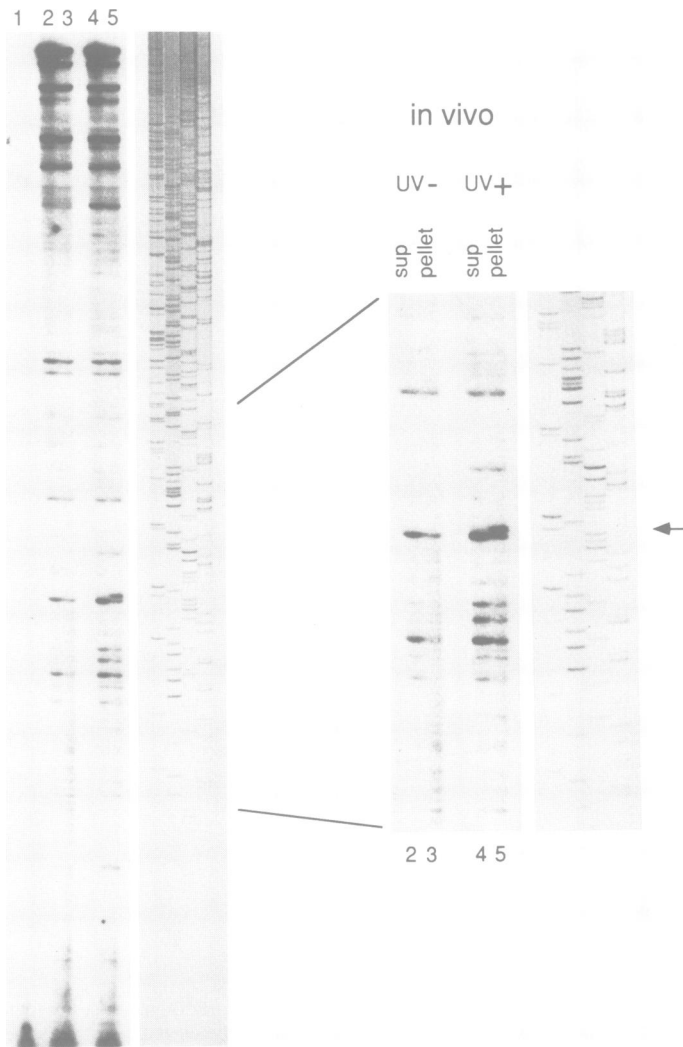
The effects of the deletion of 18 nt (+644 to +661) and the substitution of nt +650 to +655, including the cross-link at position +655, were also tested in the tagged rDNA construct. Unexpectedly, neither mutation detectably affects the accumulation of 18S or 25S rRNA (data not shown).

## Discussion

We have shown that yeast U3 snoRNA can be cross-linked to the 35S primary transcript, both in deproteinized cell extracts and in living cells (Figure 1). Although ~50% of

U3 snoRNA appears to be associated with the 35S pre-rRNA, as judged by co-sedimentation in non-denaturing sucrose gradients (Tollervey, 1987), the proportion of U3 that can be cross-linked to 35S is significantly lower. Since the efficiency of cross-linking is higher in the absence of proteins, we initially mapped the cross-linking sites in deproteinized cell extracts, exploiting a sandwich Southern hybridization technique. We found that U3 is cross-linked to the external transcribed spacer (ETS) (Figures 3 and 4).

By primer extension, we were able to identify residues involved in the interaction both on U3 snoRNA and on pre-rRNA. The efficiency of cross-linking at different sites varies considerably. These differences are, however, likely to reflect the stereochemical requirements for psoralen/RNA interaction rather than the functional importance of the contacts. Both in deproteinized cell extracts and in living cells, two regions of U3 are cross-linked to 35S pre-rRNA (Figure 2); these are within and 5' to the evolutionarily conserved box A and in a region of perfect complementarity between U3 and the ETS. As initially postulated by Porter



**Fig. 6.** Localization of an *in vivo* cross-linking site at +470 in the ETS by primer extension. Pre-rRNA molecules cross-linked to U3 in living cells were hybrid-selected using a biotinylated anti-U3 oligonucleotide, recovered with streptavidin-agarose beads and pelleted. Primer extension reactions were performed on RNA extracted from both pellet and supernatant fractions of the selection using an oligonucleotide complementary to the ETS. The samples were run on a 6% sequencing gel alongside a dideoxynucleotide sequence of the ETS region (G.A.T.C from left to right). The left panel shows full length primer extensions; the region containing the stop due to cross-link is expanded in the right panel. Lanes 1-5 are primer extensions with: 1, no RNA; 2, supernatant RNA from non-irradiated control; 3, pellet RNA from non-irradiated control; 4, supernatant RNA from irradiated sample; 5, pellet RNA from irradiated sample. The arrow marks a primer extension stop at +471 specifically present in the pellet fraction of the irradiated sample (lane 5) indicating the presence of a U3-35S cross-link at nt +470. The oligonucleotide primer is complementary to nt +562 to +546 of the pre-rRNA.

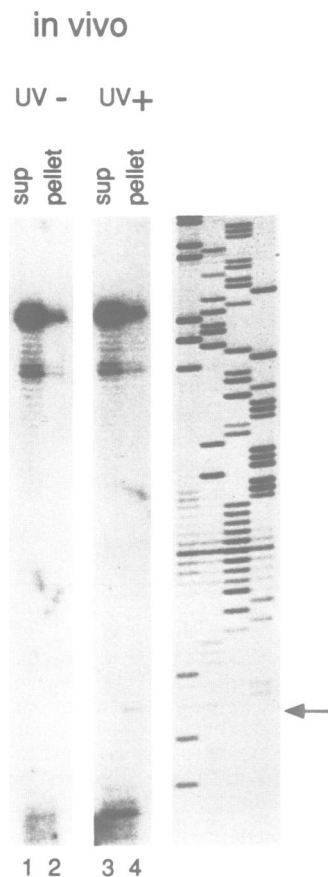
*et al.* (1988) and more recently confirmed (V. Segault, A. Mougín, A. Gregoire, J. Banroques and C. Branlant, submitted) on the basis of the revised U3 5' end sequence (Myslinski *et al.*, 1990), yeast U3 appears to have a distinctly different secondary structure as compared with vertebrate U3. The U3 secondary structure proposed for lower eukaryotes contains two small hairpins at the 5' end instead of the single hairpin proposed for higher eukaryotes. The box A region, which is highly conserved throughout evolution and therefore postulated to play a functionally important role, is predicted to include the loop of the 5' stem. The box A nucleotides (C21, A22 and U23) that we find to be cross-linked to pre-rRNA in yeast, lie in the 4 nt loop of this predicted stem (see Figure 9). Interestingly, although most of box A is predicted to lie along one side of the longer 5' stem in mammalian U3 and to be base-paired, these 4 nt are bulged (Parker and Steitz, 1987; Stroke and Weiner, 1989). The box A cross-linking site detected in rat U3 lies

in this bulged loop (Stroke and Weiner, 1989), suggesting that the box A regions of yeast and rat U3 make functionally similar contacts with the pre-rRNA. 5' to box A, we see cross-linking to C13 in yeast U3 (see Figure 9). Strikingly, rat U3 is also cross-linked to pre-rRNA via U13 and C14 (Stroke and Weiner, 1989), indicating that this site of interaction may also be functionally conserved.

The other region of yeast U3 pinpointed by our cross-linking data is a sequence of 10 nt of perfect complementarity between U3 and the ETS, that lies only a few nucleotides apart from box A. Like the box A loop sequence, this region is also potentially available for interaction with the pre-rRNA since it is predicted to be mainly single stranded (V. Segault, A. Mougín, A. Gregoire, J. Banroques and C. Branlant, submitted).

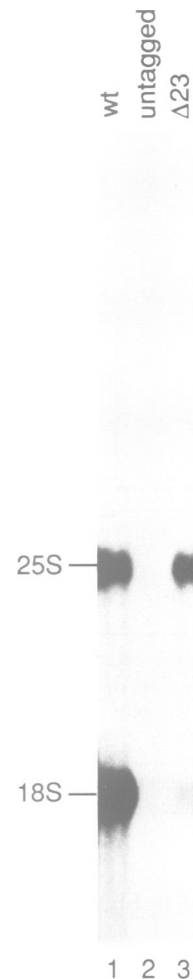
*In vivo* psoralen cross-linking studies in human (Maser and Calvet, 1989) and rat cells (Stroke and Weiner, 1989) have localized a U3-pre-rRNA interaction to within a few





**Fig. 7.** Localization of an *in vivo* cross-linking site at +655 in the ETS by primer extension. Pre-rRNA molecules cross-linked to U3 in living cells were hybrid-selected using a biotinylated anti-U3 oligonucleotide, recovered with streptavidin-agarose beads and pelleted. Primer extension reactions were performed on RNA extracted from both pellet and supernatant fractions of the selection using an oligonucleotide complementary to the ETS-18S boundary. The samples were run on a 6% sequencing gel alongside a dideoxynucleotide sequence ladder of the ETS region (G.A.T.C from left to right). Lanes 1-4 are primer extensions with: 1, supernatant RNA from non-irradiated control; 2, pellet RNA from non-irradiated control; 3, supernatant RNA from irradiated sample; 4, pellet RNA from irradiated sample. The arrow marks a primer extension stop at +656 specifically present in the pellet fraction of the irradiated sample (lane 5) indicating the presence of a U3-35S cross-link at nt +655. The oligonucleotide primer is complementary to nt +706 to +687 of the pre-rRNA.

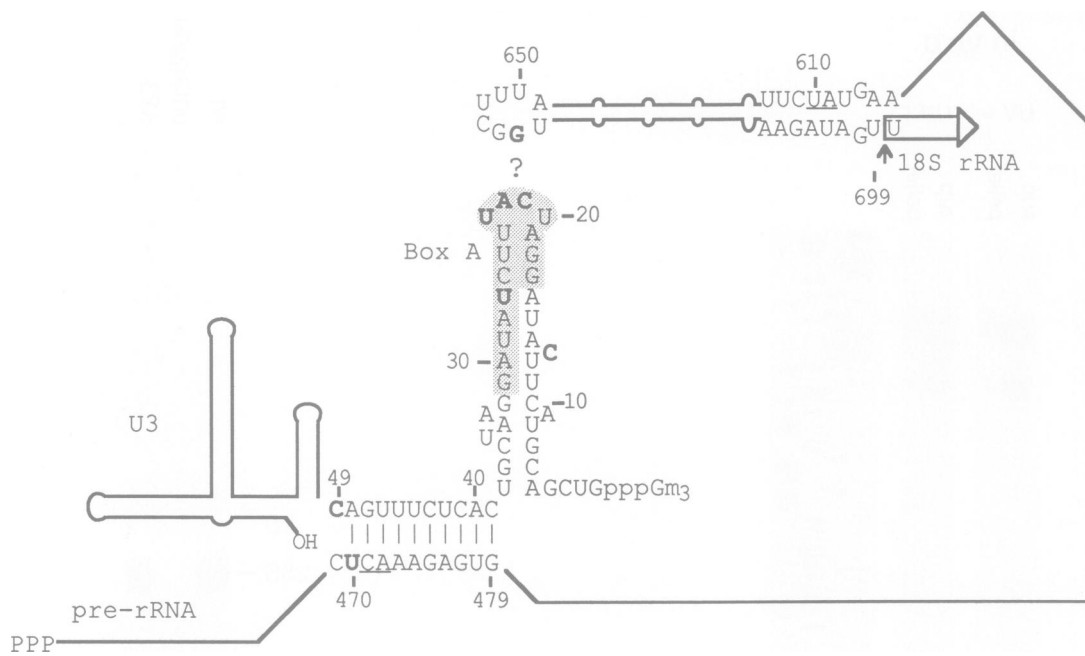
hundred nucleotides in the 5' ETS region, but the cross-linking sites could not be more precisely mapped. By hybrid-selection of 35S pre-rRNA cross-linked to U3, we could identify at the nucleotide level the sites of U3 interaction within the yeast ETS. In deproteinized extracts, two cross-links are found at nt +470 and +488 from the transcription initiation site (Figure 5). The ETS region from +470 to +479 is perfectly complementary to U3, and the interaction can be further extended to +491 involving U3 box A, forming a complementary region of 22 bases. This extended interaction is very likely to exist in deproteinized extracts; the cross-links in U3 and the ETS can be perfectly aligned and the ability of deproteinized U3 to remain base-paired to the pre-rRNA during sucrose gradient centrifugation (Prestayko *et al.*, 1970; Tollervey, 1987) indicates that they make a substantial number of base pairs. The situation is somewhat different in living cells, where only the +470



**Fig. 8.** Effect of the deletion of the +470 U3 binding site in the ETS on the levels of 18S and 25S rRNAs. Total RNA was extracted from yeast transformants carrying a tagged rDNA repeat with a deletion of 23 nt (+469 to +491) around the cross-link at +470, or a wild-type tagged repeat. Total RNA from an untagged strain was used as a negative control. A Northern filter was prepared from the RNAs run on an agarose-formaldehyde gel, and hybridized with oligonucleotide probes complementary to the tags. Lanes are: 1, wild-type tagged RNA; 2, untagged RNA; 3, tagged RNA from the  $\Delta 23$  mutant.

cross-link can be detected in this region (Figure 6). It therefore seems likely that only the contacts between the U3 and ETS sequences showing perfect complementarity are made *in vivo* in this region. As expected for psoralen cross-linking, cross-linked nucleotides in U3 and the ETS lie diagonally opposite each other. An additional *in vivo* cross-linked nucleotide can be detected downstream in the ETS at position +655, just 44 nt upstream of the 5' end of 18S rRNA (Figure 7). Computer folding of the ETS predicts that this site lies in the loop of an extended stem, with the 5' end of 18S rRNA at the base of the stem. This suggests that the single stranded region of U3 may recognize and bind to the pre-rRNA at +470 by sequence complementarity. This could then be followed by an interaction between the box A region of U3 and the ETS at +655. A possible model for the interaction between U3 and the pre-rRNA is shown in Figure 9.

Primer extension analysis of the ETS region using a primer within the 18S rRNA sequence (which reveals events prior to cleavage at the 5' end of 18S rRNA) shows two major



**Fig. 9.** Possible interaction between U3 and pre-rRNA. The U3 snoRNA secondary structure is drawn according to the recently revised model (V. Segault, A. Mouglin, A. Gregoire, J. Banroques and C. Branlant, submitted). Nucleotides in bold indicate sites of *in vivo* cross-linking. The evolutionarily conserved box A is indicated by a shaded region. In the arbitrarily drawn ETS, the region of complementarity with U3 is shown, and the complementarity is marked by vertical bars. The 18S rRNA sequence is boxed and the start site is marked with an arrow. A computer predicted stem and loop structure adjacent to the ETS-18S boundary is drawn, but only the sequences of the loop and base of the stem are shown. Underlined nucleotides indicate the sites of primer extension stops between nt +471/472 and +609/610.

stop sites which we map between nt +471/472 and +609/610 (underlined in Figures 9 and 10) (Hughes and Ares, 1991; our unpublished results—the site designated A<sub>0</sub> by Hughes and Ares lies 2 nt 3' to the site we identify as +609/610 apparently due to heterogeneity in the rDNA sequences used as references). In cells depleted of U3 snoRNA (Hughes and Ares, 1991) or of NOP1 (our unpublished results), the primer extension stop at +610 disappears. It is not at present clear whether this stop corresponds to a cleavage site or to a site of base modification, but it is tempting to speculate that whatever is happening at this position may be linked to the processing of the 5' end of 18S rRNA, since the two sites lie in close proximity in the predicted stem and loop structure.

To assess the functional role of the two regions of interaction between U3 and pre-rRNA, we have mutagenized the U3 cross-linking sites in the ETS. The results indicate that the two ETS regions interacting with U3 play different roles in pre-rRNA processing. The effect of a deletion of 23 nt (+469 to +491), that eliminates the whole region of extended complementarity between U3 and the ETS, is dramatic; the level of 18S rRNA is severely reduced, while 25S rRNA accumulation is unaffected (Figure 8). The phenotypes of a number of large deletions in the ETS have been reported (Musters *et al.*, 1990). All of these mutations affect the +470 region and, consistent with our results, all abolish 18S, but not 25S rRNA synthesis. In contrast mutations altering sequences including the *in vivo* cross-link at position +655 have no detectable effect on the levels of 18S or 25S rRNA. This downstream site of interaction between U3 and pre-rRNA does not therefore appear to be required for normal accumulation of rRNA. It should be noted, however, that more subtle effects on processing, such as those caused by RNase H cleavage of *Xenopus* U3 (Savino

Human	GGCUCUUCGUGAUCGAUG...UGGUGA..CGUCGUGCUCUC
412	
Yeast	AAC <b>UCAAGAGUG</b> CUAUGGUAUGGUGA..CGGAGUGCUCUC
467	
Mouse	CACUCUU..AGAUCAUG...UGGUCUCCGAGUUCUCUU
649	
Xenopus	99CGCUC <sup>CGG</sup> CCCCGGAUCGAUC...UGGCAACCCGCGCCCGGGCC

**Fig. 10.** Alignment of U3 cross-linking site in the yeast ETS with vertebrate ETS sequences. The sequence of the yeast ETS around the U3 cross-linking site at +470 (shown in bold) is aligned with the sequences around the human and mouse ETS cleavage sites (underlined) (Kass *et al.*, 1987), and with the homologous region of the ETS from *Xenopus laevis* (Maden *et al.*, 1982). The yeast ETS sequence which is complementary to U3 is boxed and a primer extension stop observed between nt +471 and +472 is underlined.

and Gerbi, 1990) or the absence of yeast snR10 (Tollervey, 1987), would not have been detected.

The effects of deletion of the +470 U3 binding site are strikingly similar to the effects of depletion of U3 itself (Hughes and Ares, 1991), but also to the effects of the depletion of the snoRNA U14 (Li *et al.*, 1990) and the nucleolar, snoRNA associated proteins NOP1 (Tollervey *et al.*, 1991) and GARI (Girard *et al.*, 1992). The similarities in the phenotypes of mutations in different nucleolar components suggests the possibility that they function in a common step, perhaps all being required for the function of a large pre-rRNA processing complex (Hughes and Ares, 1991; Girard *et al.*, 1992). If this is the case, the +470 region is likely to be an important assembly site for this complex.

A primary event in mammalian pre-rRNA processing is a cleavage in the ETS, that can be reproduced *in vitro* (Kass *et al.*, 1987). This event has been shown to be U3 dependent

*in vitro* (Kass *et al.*, 1990), and to occur in a large complex including at least six polypeptides which are not part of the U3 snoRNP (Kass and Sollner-Webb, 1990). Complex formation appears to be a prerequisite for processing, since it can be observed in the absence of cleavage whereas the reverse is not true (Kass and Sollner-Webb, 1990). This region of the mouse and human pre-rRNA is required for ETS cleavage *in vitro*, but has not been shown to play a role in the synthesis of mature rRNA. Interestingly, although ETS sequences are generally highly divergent, the sequences of the human and mouse ETS surrounding the processing sites can be aligned with a sequence in the *Xenopus* ETS (Michot and Bachellerie, 1991). Alignment of the primer extension stop at +471/472 in the yeast ETS (see above) with the cleavage sites in the mouse and human ETS shows a degree of homology similar to that of the *Xenopus* sequence (Figure 10). We conclude that this site has been evolutionarily conserved between yeast and vertebrates to direct the assembly of a pre-rRNA processing complex which includes U3 snoRNA and is required, at least, for the cleavages that generate 18S rRNA. The conservation of these *cis*-acting sequences, together with the functional conservation of the *trans*-acting factor fibrillarlin (Jansen *et al.*, 1991), provides strong evidence for the conservation of the mechanism of pre-rRNA processing between vertebrates and yeast.

## Materials and methods

### Yeast strains

Yeast strain BWG1-7A (*MATa*, *ura3-52*, *leu2-3.112*, *ade1-100*, *his4-519*, *GAL<sup>+</sup>*), generously provided by L. Guarente, was used for all experiments except cross-linking. For cross-linking experiments, D80 (*MATa*, *ura3-52*, *leu2-3.112*, *gal4-Δ537*, *gal7-Δ2*) was used since spheroplasts from this strain are obtained more readily.

### Cross-linking experiments

AMT-psoralen (4'-aminomethyl-4,5',8-trimethylpsoralen) was kindly provided by U. Pielers and B.S. Sproat or purchased from HRI Associates, Inc.

For *in vitro* cross-linking experiments, cells were grown in minimal medium supplemented with leucine and uracil at 30°C to OD<sub>600</sub> 0.5, resuspended in 0.1 vol of spheroplasting buffer (1.1 M sorbitol, 20 mM Na-phosphate pH 6.5, 20 mM DTT) and treated with zymolyase at 30°C until spheroplasted. After 90 min recovery in minimal medium containing 1.1 M sorbitol, spheroplasts were rapidly pelleted and frozen. Frozen pellets were resuspended in buffer containing 150 mM Na-acetate, 20 mM Tris-acetate, 5 mM Mg-acetate, 10 mM DTT, 2% SDS and 2 mg/ml proteinase K, and incubated at 18°C for 20 min. The lysate was stored on ice for 10 min and cleared by centrifugation. AMT-psoralen was added to the deproteinized extract at 80 μg/ml. After a 1 h incubation on ice, half of the extract was irradiated at 4°C with UV-light for 15 min, while the other half was kept on ice in the dark as a non-irradiated control. An HRI UVA reaction chamber (Ultralum, Inc.) was used as UV-light source at 365 nm with an intensity of ~20 mW/cm<sup>2</sup>. Total RNA was extracted from the irradiated and non-irradiated samples as described by Tollervy and Mattaj (1987), resuspended in 10 mM Tris-HCl pH 8, 10 mM NaCl, 2 mM EDTA, 0.5% SDS, heat denatured for 2 min at 100°C and loaded on 10–30% linear sucrose gradients containing the same buffer. The gradients were centrifuged at 19°C for 20 h at 22 000 r.p.m. in an SW40 rotor.

For *in vivo* cross-linking experiments, cells were grown to OD<sub>600</sub> 0.8 at 30°C in YPD liquid medium, quickly chilled and harvested by centrifugation at 4°C. Cells were resuspended in spheroplasting buffer containing zymolyase 100T and shaken gently at 4°C until spheroplasting was complete. AMT-psoralen was added at 100 μg/ml and samples were incubated on ice in the dark for 15 min. Irradiation was carried out as for deproteinized extracts. Spheroplasts were harvested by centrifugation and frozen. For RNA extraction and sucrose gradients, the procedure described above was followed.

### Sandwich Southern blots

Sandwich blot hybridization was modified from the technique described by Stroke and Weiner (1989). An *Xho*I fragment containing the whole rDNA

repeat was cloned into pUC8 and the plasmid was digested with several restriction enzymes. Cut DNA was run on 0.8% agarose gels and transferred to Gene Screen Plus membranes. RNA from the peak 35S fractions of irradiated and non-irradiated samples was incubated at 95°C in 50 mM Tris-HCl pH 9.5 for 1 h to give fragments of mean size of 500 nt. Duplicate Southern filters were prehybridized at 65°C in 50% formamide, 5×Denhardt's solution, 5×SSPE, 1% SDS and 200 μg/ml herring sperm DNA for 30 min. Partially hydrolysed RNA from either irradiated or non-irradiated samples was added and hybridization was carried out at 42°C for 24 h. A labelled U3 anti-sense riboprobe was added to each filter and hybridization was continued at 42°C for 24 h. Filters were separately washed at room temperature in 1×SSPE twice for 5 min and once for 15 min, then at 60°C for 15 min in 0.1×SSPE, 0.1% SDS. To reduce the background the filters were subsequently washed up to 70°C in 0.1×SSPE, 0.1% SDS.

For finer mapping on the ETS (Figure 4), a cloned ETS fragment was gel purified and digested with restriction enzymes. The cut DNA was separated on 2% agarose gels and blotted to Gene Screen Plus membranes. RNA from the same fractions as above was hydrolysed at 95°C in 50 mM Tris-HCl pH 9.5 for 150 min. After prehybridization, duplicate filters were incubated with partially degraded RNA from either irradiated sample or non-irradiated control at 42°C for 24 h. A U3 riboprobe was added and hybridization and washes were performed as described above.

### Construction of tagged rDNA

A 9.1 kb *Xho*I fragment corresponding to an entire rDNA repeat was cloned into the polylinker of pUC8. The insertion of a tag into the 25S coding region was carried out essentially as described by Musters *et al.* (1989). The plasmid was linearized with *Kpn*I at a unique restriction site within the 25S coding region. Two complementary oligonucleotides of the same sequence as those used by Musters *et al.* (1989) were annealed and ligated into the *Kpn*I site, thus destroying the *Kpn*I site and creating an *Xho*I restriction site. After transformation into *Escherichia coli* cells, plasmids containing the *Xho*I site were sequenced in order to establish the orientation of the inserted oligonucleotides. A plasmid containing the oligonucleotides inserted in the opposite orientation to the clone described by Musters *et al.* (1989) was also used. The tagged rDNA was excised from the vector by *Xho*I digestion and inserted into the *Xho*I site in the polylinker of pBluescript KS<sup>+</sup> (Stratagene). The orientation of the insert in the resulting plasmid, pBSrDNAtag, was assessed with restriction enzymes: the *Nor*I site of the polylinker lies upstream of the 5' end of the rDNA repeat. The plasmid was partially digested with *Hind*III and ligated with the *URA3* gene from YE24, a 1.1 kb *Hind*III fragment. Resulting plasmids were screened for the insertion of the *URA3* marker at the *Hind*III site of the NTS, 3' to the 25S coding region. A plasmid containing *URA3* in this position, pBSrDNAtagURA3-51, was subsequently used as template DNA for the insertion of a tag in the 18S coding sequence by PCR. The 18S tag sequence, creating a *Bam*HI restriction site, is the following: 5'-AAAGCCTGGAT-CCTCG-3'. The 18S tag was inserted at nt +190 of the 18S coding sequence by a mutagenesis procedure involving a double PCR step. An oligonucleotide containing the tag sequence in the middle flanked by sequences complementary to 18S DNA was used as a primer for PCR together with a primer on the opposite strand complementary to polylinker sequence 5' to the rDNA insert. Another PCR was driven by a primer complementary to the 3' end of the tag containing oligonucleotide and by a primer complementary to a downstream region of 18S DNA containing a unique *Sfi*I restriction site. The two fragments generated by this first round of PCR can be annealed together since they are complementary at one end. A second round of PCR was carried out using the two fragments generated by the first round of PCR as DNA template and polymerizing from the two external primers. A fragment was generated in this way carrying the tag insertion in the middle, pBS polylinker sequence at one end and an *Sfi*I site at the other end. The PCR fragment was cut with *Nor*I and *Sfi*I, gel purified and used to replace the wild-type *Nor*I–*Sfi*I fragment of pBSrDNAtagURA3-51. The resulting plasmid, pBSrDNA2×tagURA3-6, contains the entire rDNA with an 18S tag, a 25S tag and a *URA3* gene.

### Construction of mutations in the ETS

Mutagenesis was carried out by double PCR on the tagged rDNA plasmid, pBSrDNA2×tagURA3-6. Because of the lack of unique restriction sites, base substitutions and deletions were performed by the PCR technique described above for the insertion of the tag in the 18S coding sequence, except that the mutations were carried by the oligonucleotide with the non-coding strand sequence. The mutated fragment generated in this way was cut with *Nor*I and *Sfi*I, gel purified and used to replace the *Nor*I–*Sfi*I fragment of pBSrDNA2×tagURA3-6.

The entire tagged rDNA containing the mutation in the ETS was excised from the plasmid by a *Nor*I–*Sfi*I double digestion. DNA was briefly treated with nuclease *Bal*31 to eliminate polylinker sequences at the ends and

therefore favour the integration of the rDNA repeat into the chromosome. 1–2 µg of DNA were used to transform yeast competent cells by the Li-acetate method (Ito *et al.*, 1983). Total RNA and genomic DNA were prepared from URA3<sup>+</sup> transformants grown under selective conditions. The presence of both tags and the ETS mutation in the transformants was checked by Southern hybridization or PCR. Total RNA from the mutants and a control tagged wild type was run on 1.2% agarose–formaldehyde gels and transferred to Hybond membranes as described by Tollervey (1987). Northern blots were subsequently hybridized with labelled oligonucleotides complementary to the tags and with a probe specific for pre-rRNA species.

The sequences of mutagenic oligos are as follows: 5'-GGAACCTGATTAGAGGAAGACGGAGTGCCTG-3' for deletion of nt +469 to +491; 5'-GAATGGGTGGGAAAAAATCTTTTCTGCTTGTG-3' for deletion of nt +644 to +661; and 5'-GAATGGGTGGGAAAAAAGAGAAGATCTTTCTTTTCTTTTACTGC-3' for base substitution of nt +650 to +655.

#### Primer extension

Reactions were performed in molar excess of primer essentially as described (Borstein and Craig, 1989) except that actinomycin D was omitted. After extension with AMV reverse transcriptase, RNA was hydrolysed and the samples were precipitated and run on sequencing gels. The oligonucleotides used for the experiments shown in the figures were: 5'-GTTATGGGACTCATCAACC-3' complementary to U3 snoRNA (Figure 2); 5'-TCGGGTCTCTCTGCTGC-3' complementary to the ETS (Figures 5 and 6); 5'-CCAGATAACTATCTTAAAAG-3' complementary to the ETS–18S boundary (Figure 7). The same oligonucleotides were also kinased with unlabelled ATP and used to generate dideoxy sequence ladders from plasmid DNA templates, which were run alongside the primer extension samples.

#### Selection of cross-linked RNA

U3 cross-linked 35S was selected at 37°C using a biotinylated, 2'-O-allyl RNA oligonucleotide complementary to U3 snoRNA (5'-GGUUAUGGG-ACUCCU-3'; biotinylated residues are in bold). Streptavidin–agarose beads (Sigma) were equilibrated with selection buffer containing 100 mM KCl, 1 mM DTT, 20 mM Tris–HCl pH 8, 5 mM MgCl<sub>2</sub>, 0.1 U/µl RNasin. The beads were incubated with 0.1 mg/ml *E. coli* tRNA as carrier when specified and washed three times with selection buffer. RNA from sucrose gradient fractions containing 35S pre-rRNA was incubated with the biotinylated anti-U3 oligo in selection buffer for 4 min at 80°C followed by 30 min at 37°C to allow annealing of U3-cross-linked 35S with the oligonucleotide. The annealed RNA was then incubated with the streptavidin–agarose beads for 1 h at 37°C. The beads were pelleted by centrifugation, the supernatant was removed and the pellet was washed three times with selection buffer. RNA was recovered from both supernatant and pellet by phenol–guanidinium extraction (Tollervey and Mattaj, 1987); the pellet fraction was heated for 5 min at 68°C in guanidinium thiocyanate/phenol to detach the biotinylated oligonucleotide from the streptavidin–agarose beads. After precipitation, RNA from pellet and supernatant fractions was used for primer extension. Since a small percentage of 35S is cross-linked to U3, only a portion of supernatant RNA was used for primer extension. In order to get comparable backgrounds with U3-selected pellet, 5% of the supernatant RNA was used for RNA cross-linked *in vitro* and 1–3% for RNA cross-linked *in vivo*.

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