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U3 snoRNA genes with and without intron in the *Kluyveromyces* genus: Yeasts can accommodate great variations of the U3 snoRNA 3′-terminal domain

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ABSTRACT

The U3 snoRNA coding sequences from the genomic DNAs of *Kluyveromyces delphensis* and four variants of the *Kluyveromyces marxianus* species were cloned by PCR amplification. Nucleotide sequence analysis of the amplification products revealed a unique U3 snoRNA gene sequence in all the strains studied, except for *K. marxianus* var. *fragilis*. The *K. marxianus* U3 genes were intronless, whereas an intron similar to those of the *Saccharomyces cerevisiae* U3 genes was found in *K. delphensis*. Hence, U3 genes with and without intron are found in yeasts of the Saccharomycetoideae subfamily. The secondary structure of the *K. delphensis* pre-U3 snoRNA and of the *K. marxianus* mature snoRNAs were studied experimentally. They revealed a strong conservation in yeasts of (1) the architecture of U3 snoRNA introns, (2) the 5′-terminal domain of the mature snoRNA, and (3) the protein-anchoring regions of the U3 snoRNA 3′ domain. In contrast, stem-loop structures 2, 3, and 4 of the 3′ domain showed great variations in size, sequence, and structure. Using a genetic test, we show that, in spite of these variations, the *Kluyveromyces* U3 snoRNAs are functional in *S. cerevisiae*. We also show that *S. cerevisiae* U3A snoRNAs lacking the stem-loop structure 2 or 4 are functional. Hence, U3 snoRNA function can accommodate great variations of the RNA 3′-terminal domain.

Keywords: intron; *Kluyveromyces*; RNA evolution; RNA secondary structure; U3 snoRNA

INTRODUCTION

In eukaryotes, U3 snoRNA plays a crucial role in preribosomal RNA maturation. The eukaryotic 17/18S, 5.8S, and 25/28S ribosomal RNAs (rRNAs) are transcribed by RNA polymerase I as a single precursor molecule. A complex series of processing reactions eliminate the 5′ and 3′ external transcribed spacers (5′ ETS and 3′ ETS, respectively) and the internal transcribed spacers 1 and 2 (for review, Eichler & Craig, 1994; Raué & Planta, 1995). The primary event, A_0 cleavage within the 5′ ETS spacer, was successfully reproduced in extracts of cultured mouse cells (Craig et al., 1987) and in *Xenopus laevis* (Mougey et al., 1993), and it was found to be impaired upon U3 snoRNA depletion (Kass et al., 1990; Mougey et al., 1993). Disruption of the U3 snoRNA 5′-terminal domain in *X. laevis* oocytes was found to shift the maturation pathway involving the cleavage at a site in the ITS1 region, designated as site3, to an alternative pathway involving the cleavage at another site in the ITS1 region, designated as site2 (Savino & Gerbi, 1990). In yeast, when the expression of U3 snoRNA gene is blocked, the normal pre-rRNA intermediates in the pathway leading to the synthesis of the 17S rRNA are missing (Hughes & Ares, 1991) and some mutations in U3 snoRNA were found to impair cleavages at the site A1 and A2 located on both sides of the 17S rRNA sequence (Hughes, 1996). Altogether, this suggests that the U3 snoRNA is involved in several of the endonucleolytic steps required for the production of the small subunit rRNA.

U3 snoRNAs are characterized by the presence of six highly phylogenetically conserved boxes, namely

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A′, A, C′, B, C, and D (Wise & Weiner, 1980; Hughes et al., 1987; Myslinski et al., 1990; Tycowski et al., 1994). Boxes D and C′ are the counterparts of the boxes D and C found in several UsnoRNAs (for review, Fournier & Maxwell, 1993). The four other boxes are unique to U3 snoRNA. Within the nucleolus, U3 snoRNA is associated with at least six proteins, and boxes B, C, C′, and D were proposed to be involved in recognition and binding of the U3 snoRNP proteins (Parker & Steitz, 1987; Lübben et al., 1993; Hartshorne & Agabian, 1994; Méreau et al., 1997). The two *Saccharomyces cerevisiae* U3A and U3B snoRNAs are longer (333 nt), than all other U3 snoRNAs studied (between 142 and 279 nt) (for review, Gu & Reddy, 1996). The additional sequence folds into an additional stem-loop structure, so that the 3′-terminal domain of the *S. cerevisiae* U3 snoRNA has a cruciform structure (Ségault et al., 1992), instead of the 3-helix structure found for the vertebrates U3 snoRNAs (Parker & Steitz, 1987; Jeppesen et al., 1988). Yeast and vertebrate U3 snoRNAs also differ in the 5′ domain that forms a single stem-loop structure in vertebrates (Parker & Steitz, 1987; Jeppesen et al., 1988) and a two stem-loop structure in yeast and plant U3 snoRNAs (Porter et al., 1988; Marshallsay et al., 1990; Ségault et al., 1992; Méreau et al., 1997). The U3 snoRNA 5′-terminal domain is supposed to be the functional domain. A 10-bp complementarity between the U3 snoRNA segment from positions 39 to 48 and the pre-rRNA 5′ ETS region of *S. cerevisiae* is conserved by base-compensatory mutations in *Hansenula wingei* (Brulé et al., 1996), and it was found to be required for production of the mature 17S rRNA in *S. cerevisiae* (Beltrame & Tollervey, 1995). Based on a phylogenetic comparison and on the results of a mutational analysis, boxes A and A′ were recently proposed to interact with 17S rRNA sequences that form a pseudo-knot structure in the mature rRNA (Hughes, 1996). Using dimethylsulfate (DMS) modifications in vivo, we obtained strong arguments in favor of the formation of this base pair interaction (Méreau et al., 1997).

We previously made the unexpected observation of the presence of an intron spliced in a spliceosome in the two genes that code for U3 snoRNA in *S. cerevisiae* (U3A and U3B genes) (Myslinski et al., 1990). A canonical sequence is found at the 5′ end of the two introns, but an unusual GACTAAC sequence is present at the branch site (Myslinski et al., 1990). The introns are highly structured, with a long central stem-loop structure and 5′ and 3′ stem-loop structures that include the 5′ and 3′ functional sequences, respectively (Mougin et al., 1996). Introns were also detected in the two U3 snoRNA genes of *S. bayanus*, in the numerous U3 snoRNAs genes of *S. uvarum* (Brulé et al., 1995), and in the unique U3 snoRNA gene of another member of the Saccharomycetoideae subfamily, namely *H. wingei* (Brulé et al., 1996). However, the intron found in the *H. wingei* gene is shorter and has a canonical TACTAAC sequence at the branch site.

Up to now, the presence of spliceosomal introns in genes that code for stable RNAs seems to be restricted to yeasts and, more precisely, to yeast UsnRNA and UsnoRNA genes. Such introns were found in the U6 snRNA genes of various *Schizosaccharomyces* species, *Rhodotorula hasegawae* and *Rhodosporium dacroïdeum* (Tani & Ohshima, 1989, 1991; Frendewey et al., 1990; Reich & Wise, 1990), and in the U1, U2, and U5 snRNA genes from *R. hasegawae* (Takahashi et al., 1993, 1996). This is surprising because introns spliced in a spliceosome are rare in yeast nuclear genes coding for proteins (Woolford, 1989). It should also be pointed out that, in spite of the large variety of snoRNAs characterized in *S. cerevisiae* (for review, Tollervey, 1987; Maxwell & Fournier, 1995), only the genes coding for U3 snoRNAs were found to contain an intron. Furthermore, the presence of intron in U3 snoRNA genes is not general in yeasts, because the *S. pombe* U3 snoRNA genes have no intron (Selinger et al., 1992).

S. pombe and *S. cerevisiae* belong to two different yeast subfamilies, the Schizosaccharomycetoideae and Saccharomycetoideae subfamilies, respectively (Kreger Van Rij, 1987). One possibility was that the presence of intron in U3 snoRNA genes was a common property of members of the Saccharomycetoideae subfamily. To get more information into this problem, we cloned and sequenced the U3 snoRNA genes of five strains from the *Kluyveromyces* genus, which belong to the Saccharomycetoideae subfamily. The five strains tested were *K. delphensis* and four variants of *K. marxianus*: *K. marxianus* var. *bulgaricus*, *K. marxianus* var. *fragilis*, *K. marxianus* var. *lactis*, and *K. marxianus* var. *marxianus*. The genomic DNAs of *K. delphensis* and of three of the four *K. marxianus* variants were found to contain a single U3 snoRNA gene sequence, that of *K. marxianus* var. *fragilis* contains two U3 snoRNA genes. Whereas the *K. delphensis* U3 gene contains a 167 nt-long intron, the *K. marxianus* U3 genes had no intron. We verified that the U3 snoRNAs expressed in the five strains studied corresponded to the cloned genes. To complete the characterization of the *Kluyveromyces* U3 snoRNAs, we made an experimental study of their secondary structure. This revealed great variations in size, sequence, and structure of the 3′-terminal domain of yeast U3 snoRNAs. Nevertheless, based on a genetic test, we could show that the *Kluyveromyces* U3 snoRNAs are functional in *S. cerevisiae*. This prompted us to test by a genetic approach the functional importance of the stem-loop structures 2 and 4 of the 3′-terminal domain of the *S. cerevisiae* U3 snoRNA. The results obtained are discussed in terms of U3 snoRNA function, pre-U3 snoRNA splicing, and evolution of the U3 snoRNA genes in yeasts.

RESULTS

U3 snoRNA genes with and without introns in *Kluyveromyces*

To clone the genomic DNA sequences coding for the *Kluyveromyces* U3 snoRNAs by PCR amplification, we took advantage of the phylogenetic conservation of boxes A′ and D, which are located at the 5′ and 3′ extremities of U3 snoRNA, respectively (Hughes et al., 1987; Myslinski et al., 1990) (see Materials and Methods). In contrast to the situation found for *S. cerevisiae*, a single amplification product was obtained for *K. delphensis*, and for three of the four *K. marxianus* variants tested, namely, var. *bulgaricus*, var. *lactis*, and var. *marxianus* (Fig. 1). For *K. marxianus* var. *fragilis*, a main amplification product was found, together with a second one of lower intensity (Fig. 1). The nucleotide sequences of the unique amplification product from *K. delphensis*, *K. marxianus* var. *bulgaricus*, *K. marxianus* var. *lactis*, and *K. marxianus* var. *marxianus*, as well as that of the main amplification product obtained for *K. marxianus* var. *fragilis*, were established [EMBL databank accession numbers for these sequences are as follow: Z78433 (*K. delphensis*), Y14752 (*K. marxianus* var. *bulgaricus*), Z78437 (*K. marxianus* var. *fragilis*), Z78438 (*K. marxianus* var. *lactis*), Y14751 (*K. marxianus* var. *marxianus*)]. Only the *K. delphensis* U3 snoRNA gene (U3Kd gene) was found to contain an intron (Fig. 2). Like the introns of the *S. cerevisiae* U3A and U3B genes, the U3Kd intron has a canonical 5′ sequence and an unusual GACTAAC sequence at the branch site. The nucleotide sequence of the U3Kd intron, from the branch site to the AG terminal dinucleotide, is highly conserved compared with the U3A and U3B snoRNA introns (Fig. 2B). No intron-like element was found in the sequenced U3 snoRNA genes of the four *K. marxianus* variants. Alignment of the U3 snoRNA sequences deduced from the five cloned genes with the *S. cerevisiae* U3A and U3B snoRNA sequences (Myslinski et al., 1990) and with the *H. wingei* U3 snoRNA sequence (U3Hw) (Brulé et al., 1996) (Fig. 2A) revealed an increased size of the *Kluyveromyces* U3 snoRNAs (334–413 nt) compared with the *S. cerevisiae* and the *H. wingei* U3 snoRNAs (333 and 319 nt, respectively). The extra nucleotide sequences correspond to insertions in the 3′ domain (Fig. 2A). Whereas the nucleotide sequence corresponding to the 5′-terminal domain of U3 snoRNA is highly conserved, the conservation of the 3′ domain is poor. Only the sequences of boxes B, C, and D, of helix 5, and of a portion of helix 4 are conserved (Fig. 2A). As shown in Figure 2A, the established U3 snoRNA nucleotide sequence confirmed that *K. marxianus* var. *marxianus* and *K. marxianus* var. *bulgaricus* are two synonymous variants of the *K. marxianus* species (Van der Walt, 1971; Martini & Martini, 1987). U3 snoRNAs from the two variants *lactis* and *marxianus* show 90% similarity and the divergence is stronger for the sequenced gene of the variant *fragilis* (only 73% similarity with the U3 snoRNA of the variant *marxianus*).

We had to verify that no U3 snoRNA gene had escaped PCR amplification. For this purpose, Southern-blot analysis of the genomic DNAs was performed using various DNA probes (see Materials and Methods). Figure 3 illustrates the results obtained for the *S. cerevisiae*, *K. marxianus* var. *bulgaricus*, and *K. delphensis* genomic DNAs using the exon 2 of the *S. cerevisiae* gene as the probe (probe 1) and for the *K. marxianus* var. *fragilis*, *lactis*, and *marxianus* genomic DNAs using a DNA probe coding for the 5′-terminal 86 nt of the *S. cerevisiae* U3A snoRNA (probe 2). As shown in Figure 3, the results obtained are in perfect agreement with the presence of a unique U3 snoRNA gene in all the strains tested, except *K. marxianus* var. *fragilis* which has two U3 snoRNA genes. Indeed, for this strain, whereas no *Hin*d III and no *Pst* I restriction site was detected in the cloned gene, two distinct *Hin*d III fragments and two distinct *Pst* I fragments hybridized with the labeled probe 2 under stringent conditions and equal yield of hybridization were obtained for each band. For the four other strains, using both probe 1 and probe 2, a unique hybridization fragment was obtained for digestions with enzymes that have no recognition site in the amplified U3 snoRNA gene (*Hin*d III for *K. delphensis*, *Eco*R I and *Hin*d III for *K. marxianus* var. *lactis*, *K. marxianus* var. *marxianus*, and *K. marxianus* var. *bulgaricus*) and two hybridization fragments were obtained with probe 1 when digestion was performed with a restriction enzyme having a recognition site in the

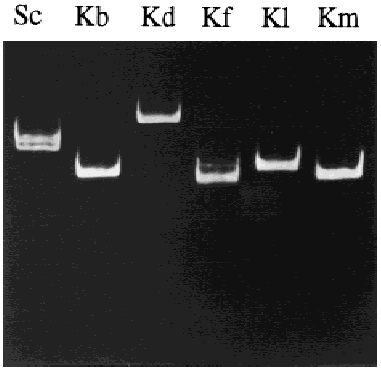


FIGURE 1. PCR amplification of *Kluyveromyces* U3 snoRNA genes. Genomic DNAs were prepared from *K. delphensis* (Kd) (CBS 2170), *K. marxianus* var. *bulgaricus* (Kb) (CBS 397), var. *fragilis* (Kf) (CBS 1555), var. *lactis* (Kl) (CBS 2360), and var. *marxianus* (Km) (CBS 397) as described in Materials and Methods. DNA sequences corresponding to the U3 snoRNA gene were PCR amplified with the oligonucleotide pair O-1, O-2. Amplified fragments were fractionated by electrophoresis on a 6% polyacrylamide gel in TBE buffer and visualized by ethidium bromide. A control amplification experiment was made with genomic DNA from the *S. cerevisiae* FL100 strain (Sc) (ATCC 28383) (Lacroute, 1968).

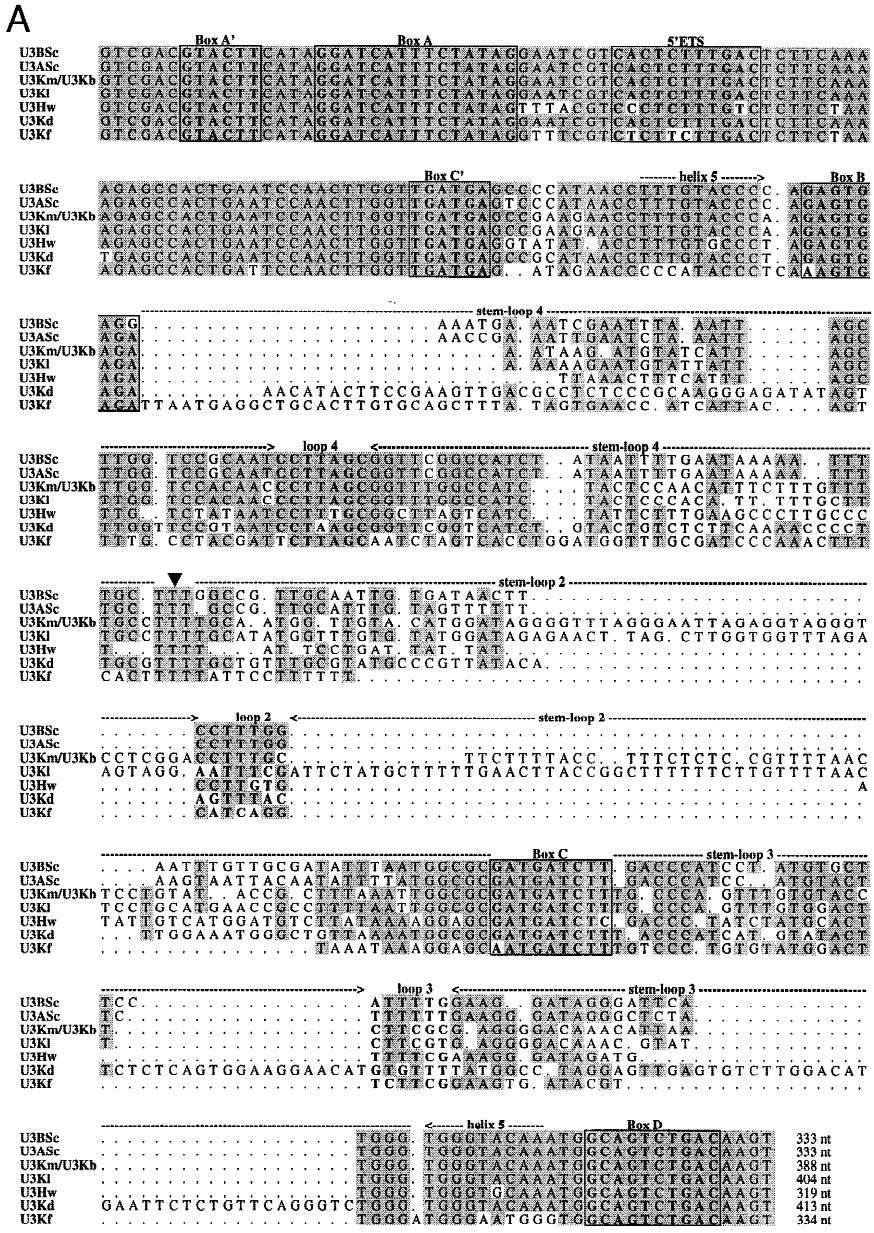


FIGURE 2. Comparative analysis of yeast U3 snoRNA genes. **A:** Alignment of yeast U3 snoRNA nucleotide sequences. The U3 snoRNA coding sequences deduced for the *K. delphensis* (Kd), *K. marxianus* var. *bulgaricus* (Kb), var. *fragilis* (Kf), var. *lactis* (Kl), and var. *marxianus* (Km) U3 snoRNA gene sequences are aligned with the *S. cerevisiae* U3A and U3B snoRNA genes coding sequences (U3ASc and U3BSc, respectively) (Myslinski et al., 1990) and with the *H. wingei* U3 snoRNA gene coding sequence (U3Hw) (Brulé et al., 1996). The 14 nt at the 3′ end of each RNA could not be determined by combination of the PCR approach and direct sequence analysis. The alignment was made with the PileUp program of the GCG Software. Positions where an identical nucleotide was found in at least four of the aligned sequences are surrounded by grey color. The phylogenetically conserved boxes A′, A, C′, B, C, D, and the 10-nt segment complementary to the 5′-ETS region (Wise & Weiner, 1980; Hughes et al., 1987; Myslinski et al., 1990; Beltrame & Tollervey, 1992; Tycowski et al., 1994) are boxed. The border between stem-loop structure 2 and stem-loop structure 4 is shown by a full triangle. Phylogenetic relationships of the various U3 genes, as deduced from the PileUp program of the GCG Software, are shown at the bottom. (*Figure continues on facing page.*)

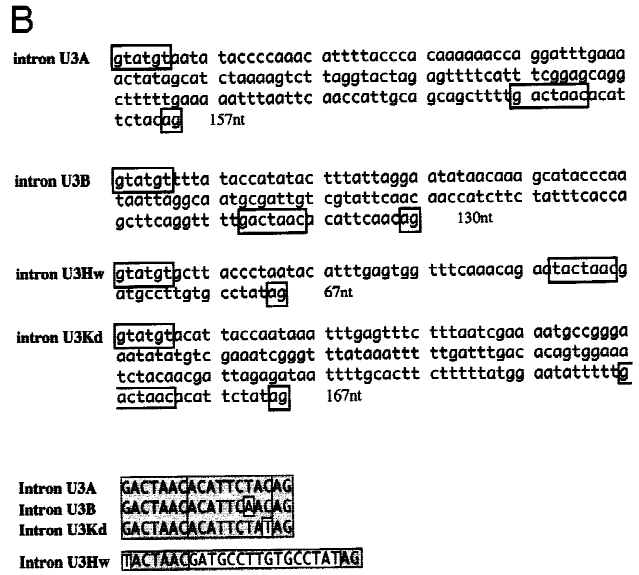


FIGURE 2. (*Continued.*) **B:** Comparison of the nucleotide sequences of the introns from yeast U3 snoRNA genes. The two introns found in *S. cerevisiae* (U3A and U3B) and the intron found in *H. wingei* (U3Hw) are shown for comparison. The 5′ sequence, the branch site sequence, and the 3′-terminal AG dinucleotide are boxed. The 3′-terminal sequences of the introns U3A, U3B, U3Kd, and U3Hw are aligned at the bottom of the figure. Identical sequences are shown in grey boxes.

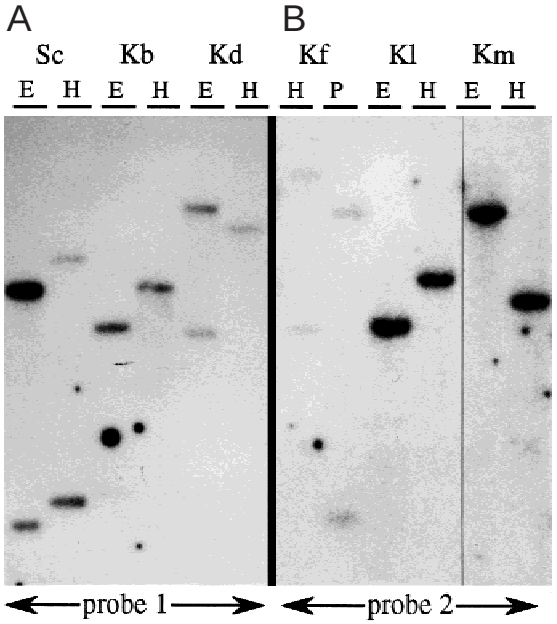


FIGURE 3. Southern-blot analysis of U3 snoRNA genes from *Kluyveromyces* genomic DNAs. Genomic DNAs from *S. cerevisiae* (Sc), *K. delphensis* (Kd), *K. marxianus* var. *bulgaricus* (Kb), *K. marxianus* var. *fragilis* (Kf), *K. marxianus* var. *lactis* (Kl), and *K. marxianus* var. *marxianus* (Km) were prepared as described in Materials and Methods, digested with the *Eco*R I (lanes marked by E), *Hin*d III (lanes marked by H), or *Pst* I (lanes marked by P) nucleases, and fractionated by electrophoresis on a 0.8% agarose gel. After transfer of the digested products on Hybond membranes, the hybridization was made with DNA probes corresponding to the exon 2 of the *S. cerevisiae* pre-snoRNA U3A (probe 1) (**A**) or to the 5′-terminal region of the *S. cerevisiae* U3A snoRNA (probe 2) (**B**). Both probes were labeled by nick translation.

amplified U3 snoRNA gene (*Eco*R I for *K. delphensis*). Control hybridizations were also performed with DNA segments coding for the U3 snoRNA of the various *Kluyveromyces* studied (not shown).

An ultimate experiment to verify that we had cloned the bona fide U3 snoRNA genes was to check whether the expressed U3 snoRNAs corresponded to the cloned genes. This was done by northern-blot analysis (Fig. 4A) and by direct sequence analysis of U3 snoRNA within total RNA extracts of each strain (Fig. 4B). For each *Kluyveromyces* strain, only one type of U3 snoRNA was detected and the nucleotide sequence was that expected from the amplified genes (for *K. marxianus* var. *fragilis*, the expressed gene was the cloned gene that we sequenced). Based on the strategy used for the amplification, the 5′ and 3′ extremities of the coding regions could not be determined. However, direct RNA sequence analysis of the *Kluyveromyces* U3 snoRNAs showed that all of them have, at their 5′ end, the same 14-nt sequence as the *S. cerevisiae* U3 snoRNAs. Only the 14-nt sequence at the 3′ end of each RNA could not be determined by combination of our PCR approach and direct sequence analysis.

Great variability of the 3′-cruciform domain of the *Kluyveromyces* U3 snoRNAs

Our previous experimental analysis of the *S. cerevisiae* U3 snoRNA (Ségault et al., 1992) and its precursor (Mougin et al., 1996) in solution showed the presence in these two RNAs of a 3′-cruciform domain. This was confirmed by our recent direct investigation of the *S. cerevisiae* U3A snoRNA structure in vivo (Méreau et al., 1997). In vivo, this cruciform domain is extended by the formation of an irregular helix including boxes C′ and D, and the 5′-terminal domain was found to be extensively base paired with pre-rRNA sequences (Méreau et al., 1997). Secondary structure analysis of the U3A snoRNA precursor also revealed a peculiar secondary structure of the intron (Mougin et al., 1996), with a central stem-loop structure that is phylogenetically conserved by compensatory base mutations in U3 snoRNA precursors of the *Saccharomyces* genus (Brulé et al., 1995). To check whether these peculiar secondary structure features of the *Saccharomyces* U3 snoRNAs and pre-U3 snoRNAs were conserved in the *Kluyveromyces* genus, we performed an experimental study of the secondary structures of the *K. delphensis* U3 snoRNA precursor and of the mature U3 snoRNAs of *K. marxianus* var. *lactis* and *K. marxianus* var. *fragilis*. For this purpose, the amplified U3 snoRNA coding sequences (with the intron in the case of *K. delphensis*) were cloned

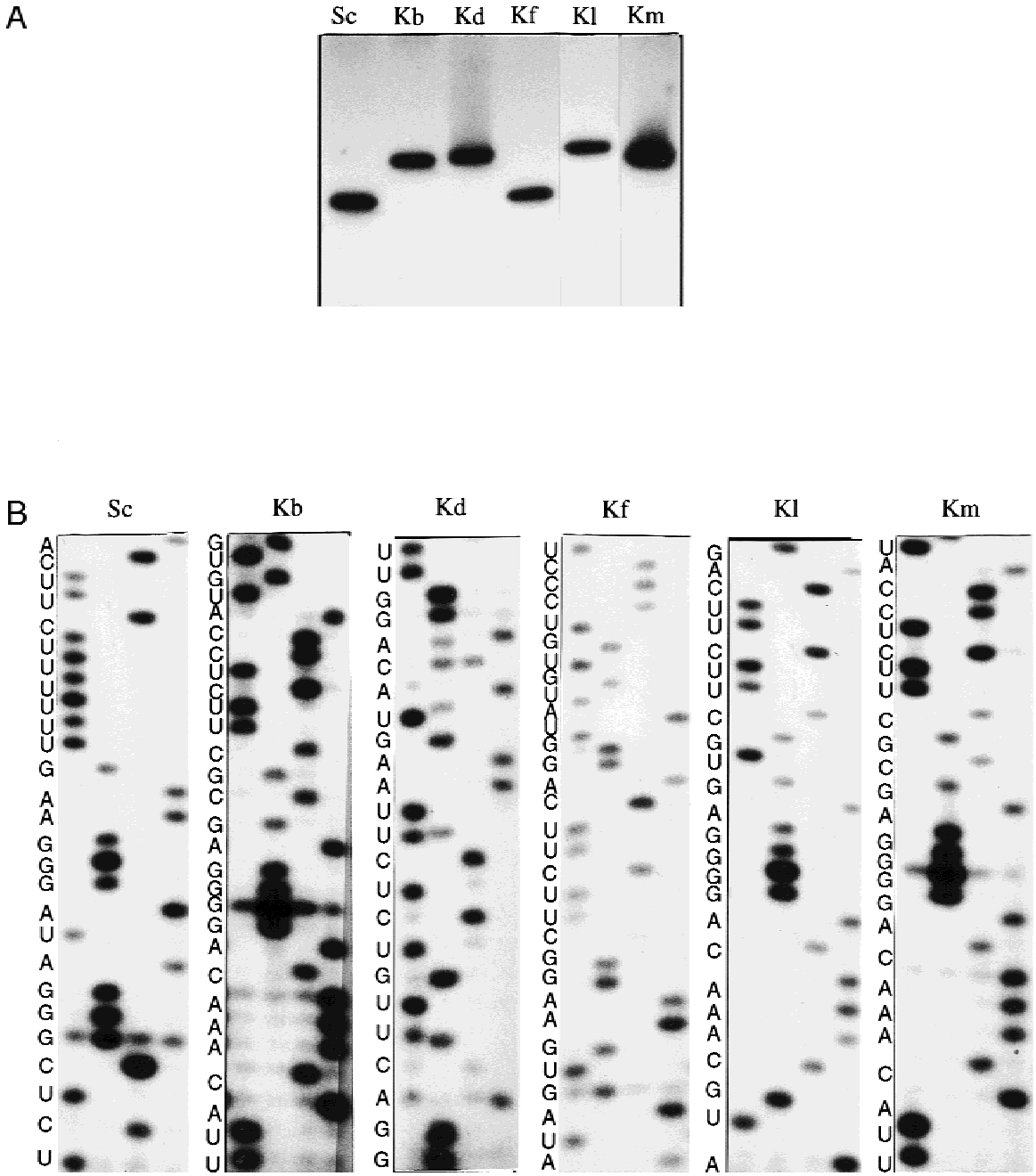


FIGURE 4. Analysis of the *Kluyveromyces* U3 snoRNAs. **A:** Northern-blot analysis of U3 snoRNA. Total RNA was prepared as described in Materials and Methods. After fractionation by electrophoresis on a 6% polyacrylamide, 8 M urea gel, and transfer on a Biodyne B membrane, hybridization was made with the 5′-end labeled oligonucleotide O-2. **B:** Direct sequence analysis of U3 snoRNA from total RNA extract. Total RNA was prepared as described in Materials and Methods and direct sequence analysis of U3 snoRNA was made with reverse transcriptase and oligonucleotide O-2 as the primer.

in plasmid pMOSBlue-T and amplifications of the genes were performed with a primer that generated a T7 RNA polymerase promoter. Using the amplification products, we produced in vitro transcripts with the same sequence as the authentic U3 snoRNAs or pre-U3 snoRNA, except for two additional G residues at the 5′ end (see Materials and Methods). After a renaturation step, the transcripts were subjected to limited digestions with T2, S1, or V1 RNAses and to partial chemical modifications with DMS or 1-cyclohexyl-3-(2-morpholino ethyl)-carbodiimide-metho-*p*-toluene sulfonate (CMCT). These were achieved in the conditions established previously for RNA secondary structure analysis (Mougin et al., 1996).

Examples of reverse-transcriptase analysis of chemical modifications and enzymatic digestions of the various RNA transcripts are shown in Figure 5, and the results obtained are represented schematically on the

proposed secondary structures in Figure 6. Whereas similar secondary structures can be proposed for the 5′-terminal regions of the two mature RNAs studied, the 3′-terminal domain of the *Kluyveromyces* snoRNAs is very different from one species to the other (*K. delphensis*, *K. marxianus*) and even from one variant of a given species to the other (*K. marxianus* var. *lactis* and var. *fragilis*). The 3′-terminal domain of the *K. marxianus* var. *lactis* U3 snoRNA has the characteristic cruciform shape found for the *S. cerevisiae* U3 snoRNAs, with boxes B and C at the center. However, one main difference with the *S. cerevisiae* snoRNAs is the long extension of stem-loop structure 2. In the *S. cerevisiae* U3 snoRNAs, two alternative base pair interactions were found to be possible between the segments that contain box B and box C, respectively. The possibility to form these two alternative interactions is conserved in the U3 snoRNAs from the two *Kluyveromyces* strains studied. However, in solution, only the interaction between the sequences located immediately downstream from helix 5 and that located immediately upstream of stem-loop structure 3 was found to be formed. Although no direct experimental analysis was performed on the U3 snoRNAs from the variant *marxianus* and *bulgaricus*, which have identical primary structure, their secondary structure could be predicted by comparison with the structure established for the U3 snoRNA of the variant *lactis*. The differences observed are either in single strands or compensate each other (not shown). In contrast to the U3 snoRNA from *K. marxianus* var. *lactis* and *K. marxianus* var. *marxianus*, the U3 snoRNAs from *K. marxianus* var. *fragilis* shows a very short stem-loop structure 2. Its stem-loop structure 4 is bifurcated and its stem-loop structure 3 is quite more regular than in all other yeast species studied (Fig. 6B). Finally, the *K. delphensis* U3 snoRNA has an usual stem-loop structure 2, but stem-loop structure 3 is replaced by a bifurcated structure and the region corresponding to stem-loop structure 4 is poorly structured (Fig. 6A).

The intron of the *K. delphensis* U3 snoRNA precursor has a secondary structure highly similar to that found for the *S. cerevisiae* pre-U3 snoRNA (Mougin et al., 1996), with a long central stem-loop structure, a base paired 5′ sequence, a single-stranded branch site, and a bulged 3′ splice site (Fig. 6A).

U3 snoRNAs with highly divergent 3′ domain are functional in *S. cerevisiae*

Based on the great variability of the U3 snoRNA 3′-terminal domain in *Kluyveromyces*, the question was to know whether the sequence and the size of the stem-loop structures 2, 3, and 4 of the 3′ domain had no influence on U3 snoRNA function in yeast or, on the contrary, if, for a given species, a specific sequence and a specific size of these stem-loop structures is required to get an active U3 snoRNA. To choose between these two possibilities, we checked whether the very divergent *Kluyveromyces* U3 snoRNAs could be functional in *S. cerevisiae*. The genetic test used was based on the utilization of the *S. cerevisiae* strain JH84 built by Hughes and Ares (1991). In this strain, the U3B snoRNA gene has been disrupted and the U3A snoRNA gene is placed under the control of the GAL10 upstream activation sequence (UASgal10) (Guarente et al., 1982). To grow on glucose, this strain has to be complemented by a plasmid carrying an active U3 snoRNA gene. To complement the *S. cerevisiae* strain JH84, we used the plasmid pASZ11::pU3A (Méreau et al., 1997), which contains an active U3A snoRNA gene. The cloned fragment includes the exon and intron sequences, and the transcription initiation and termination signals (Myslinski et al., 1990). When transformed with this plasmid, the *S. cerevisiae* strain JH84 grew at the same rate on glucose as on galactose. The *S. cerevisiae* pre-U3A snoRNA coding sequence from plasmid pASZ11::pU3A was replaced by the *K. delphensis* pre-U3 snoRNA coding sequence, on one hand, and the *K. marxianus* var. *fragilis* and the *K. marxianus* var. *lactis* U3 snoRNA-coding sequences on the other hand. As shown in Figure 7A, all three coding sequences produced U3 snoRNAs that were functional in *S. cerevisiae*, because they allowed growth of the JH84 strain on glucose. We verified by northern-blot analysis that the unique gene expressed by the *S. cerevisiae* JH84 strain, in these conditions, was the foreign plasmidic gene (Fig. 7B). Hence, U3 snoRNA can tolerate great nucleotide sequence variations in the 3′ domain.

These results opened a new question: are the stem-loop structures of the 3′ domain dispensable for U3 snoRNA function? To test for this possibility, plasmids pASZ11::pU3A, with a deletion of the sequence coding for the stem-loop structure 2 or for the stem-loop structure 4, were prepared by site-directed mutagenesis and their capacity to restore growth of the *S. cerevisiae* JH84 strain on glucose was tested. As shown in Figure 7, the complete deletion of stem-loop structure 2 or of stem-loop structure 4 did not impair growth of the JH84 recombinant strains on glucose (Fig. 7A), and we showed by northern-blot analysis that the U3 snoRNAs produced in these conditions were the truncated ones (Fig. 7B). Hence, the two truncations had no effect on U3 snoRNA function in *S. cerevisiae*.

DISCUSSION

The presence of an intron in U3 snoRNA genes is not a general property of the Saccharomycetoideae subfamily

Two or more U3 snoRNA genes were detected in all the *Saccharomyces* species studied up to now, and they all contain an intron spliced in a spliceosome (Myslinski et al., 1990; Brulé et al., 1995, 1996). As found for

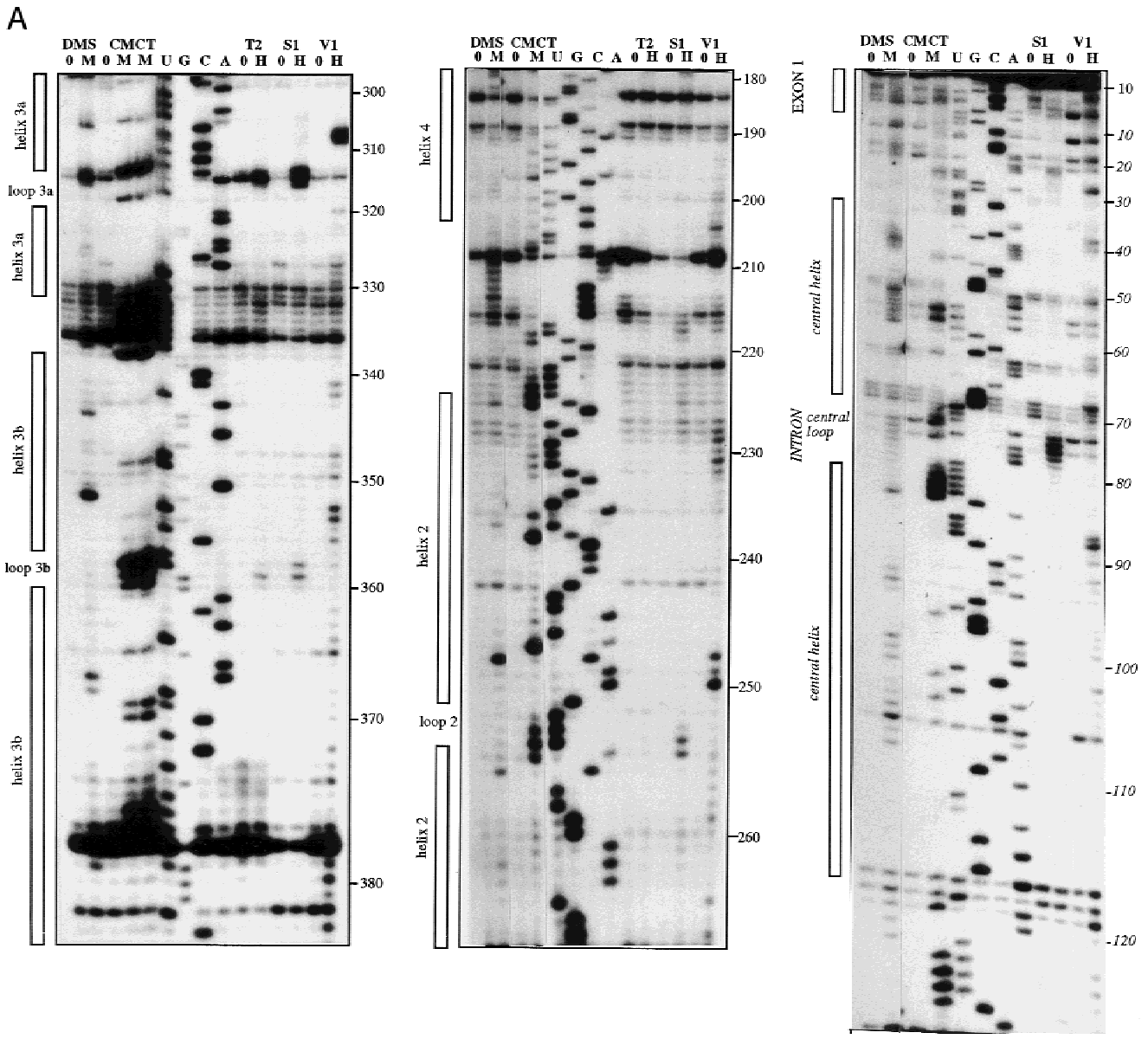


FIGURE 5. Chemical and enzymatic probing of the *Kluyveromyces* pre-U3Kd (**A**) and the mature U3Kf and U3Kl snoRNAs (**B**) U3 snoRNA transcripts were prepared as described in Materials and Methods and were modified under nondenaturing conditions with DMS or CMCT or were digested with the T2, V1, or S1 RNase. A single condition of modification or digestion was used as described in Materials and Methods (lanes marked by M for modification or H for hydrolysis). For each reaction, a control experiment was made in the absence of chemical reagent or enzyme (lanes marked by 0). Modified nucleotides and cleaved phosphodiester bonds were identified by primer-extension analysis with reverse transcriptase using oligonucleotide O-2 (**A:** left; **B:** left and middle), O-7 (**A:** middle), O-10 (**A:** right), and O-8 (**B:** right) as primers. Lanes A, C, G, and U correspond to the sequencing ladder. Positions of helices and phylogenetically conserved boxes are indicated on the left of the panels. Note that chemical modifications of bases generate stops of the reverse transcriptase one nucleotide before the modified base. (*Figure continues on facing page.*)

H. wingei (Brulé et al., 1996), four of the *Kluyveromyces* strains studied contain a unique U3 snoRNA gene. In *K. delphensis*, this gene contains an intron, whereas no intron was detected in the four sequenced *K. marxianus* U3 snoRNA genes. Hence, the presence of an intron spliced in a spliceosome in U3 snoRNA genes is not a general property of the Saccharomycetoideae subfamily.

Gene duplication is rather common in *Saccharomyces*; a large part of the *S. cerevisiae* genome seems to be duplicated (Goffeau et al., 1996). Such redundancy was not observed in *Kluyveromyces* (Aigle et al., 1983), and this is in accord with the present results. The presence of U3 snoRNA genes with and without introns in *Kluyveromyces* species opens the question of the origin of introns in U3 snoRNA genes and of the reason

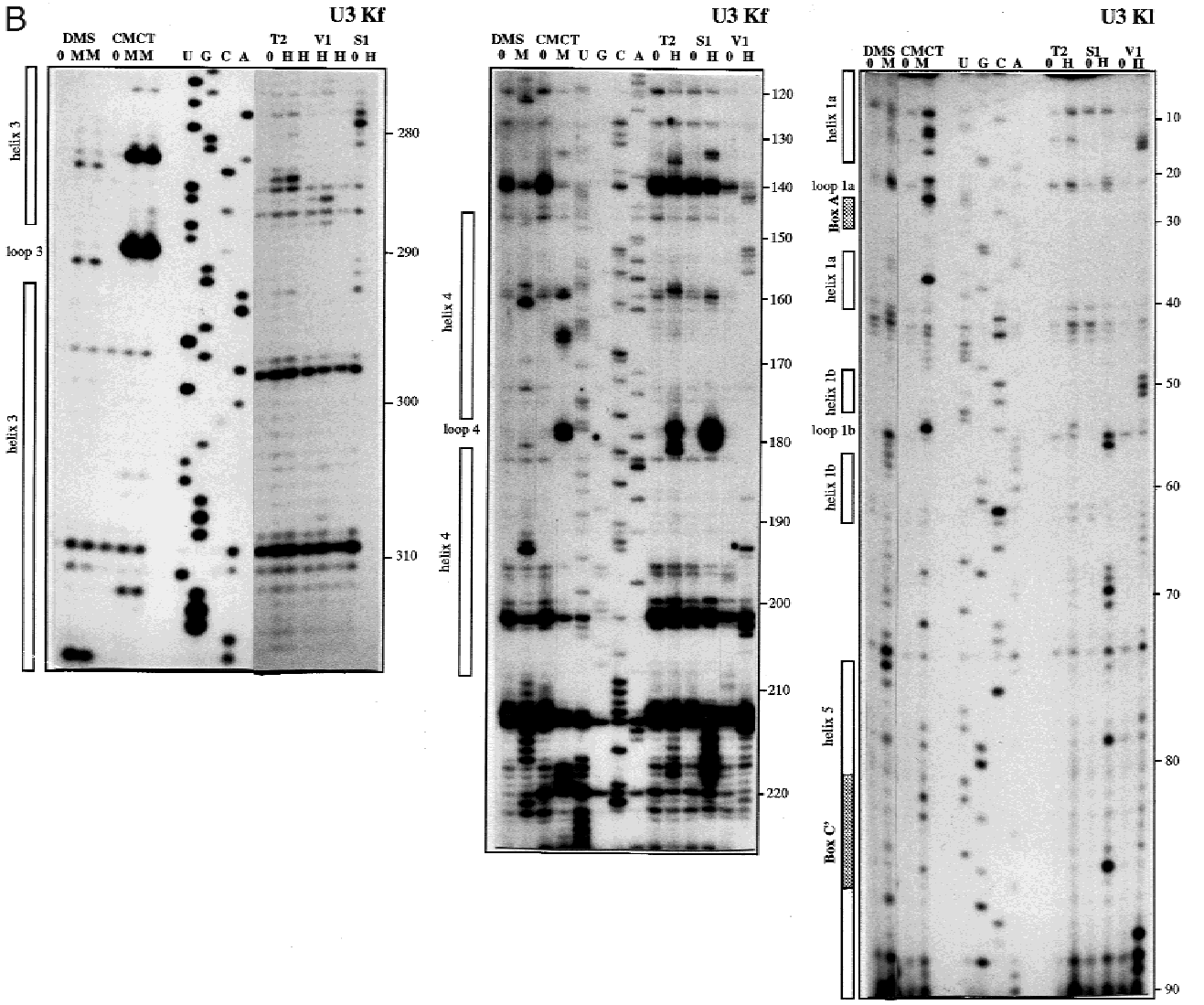


FIGURE 5. (*Continued.*)

for their disappearance in some species. Interestingly, the intron detected in the *K. delphensis* gene is different from that found in the *H. wingei* U3 snoRNA gene (Brulé et al., 1996), but it shares common properties with those found in the U3 snoRNA genes from the *Saccharomyces* genus (Brulé et al., 1995; Mougin et al., 1996). First, it is 167-nt long, compared with 157-nt and 130-nt long for the U3A and U3B introns of *S. cerevisiae*, respectively, and 67 nt for the *H. wingei* intron. Second, the *K. delphensis* U3 snoRNA intron has a GACUAAC sequence at the branch site, as does the intron from *Saccharomyces* species, and this is in contrast to the *H. wingei* intron, which has a canonical branch-site sequence. The nucleotide sequence from the entire 3′-terminal region of the *K. delphensis* intron, including the branch-site sequence and the AG-terminal dinucleotide, shows only one and two base substitutions compared with the 3′-terminal sequences of the U3A and U3B introns of *S. cerevisiae*, respectively (Fig. 2B). The corresponding nucleotide sequence of the *H. wingei* intron shows a higher degree of divergence (Fig. 2B). Hence, there seems to be at least two categories of introns in the U3 snoRNA genes from the yeast Saccharomycetoideae subfamily.

Comparison of the *Kluyveromyces*, *Saccharomyces*, and *Hansenula* U3 snoRNA nucleotide sequences shows that, in spite of several insertions, the *K. delphensis* U3 snoRNA displays greater similarity with the *S. cerevisiae* U3A and U3B snoRNAs (82% and 83%, respectively), than with the *H. wingei* U3 snoRNA (74%). Thus, similar phylogenetic relationships are found for the introns and the coding sequences. This suggests a common ancestor of the *Saccharomyces* and *K. delphensis* U3 snoRNA genes that contained an intron. In contrast, based on U3 snoRNA nucleotide sequence alignment (Fig. 2), the intronless *K.*

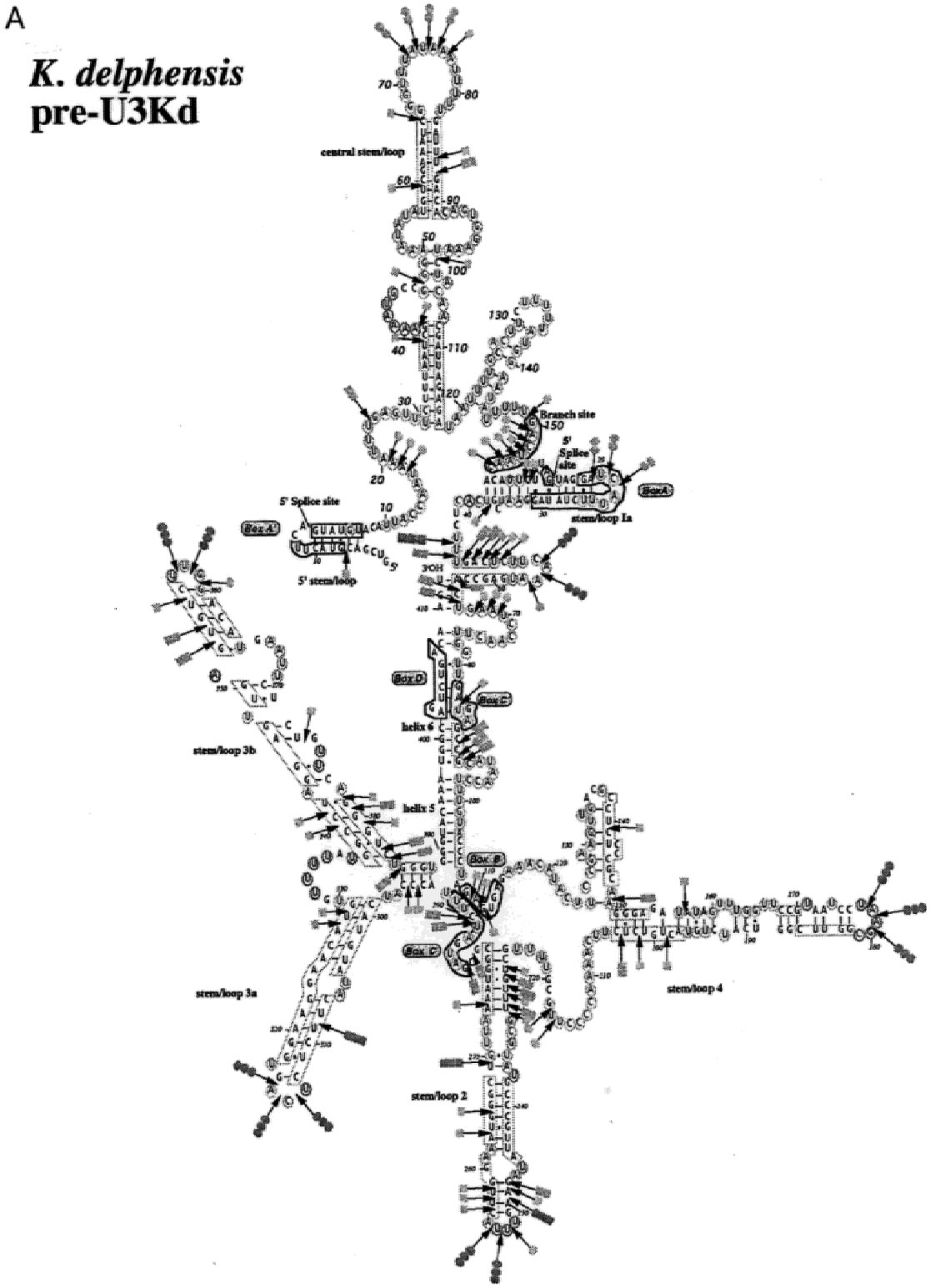
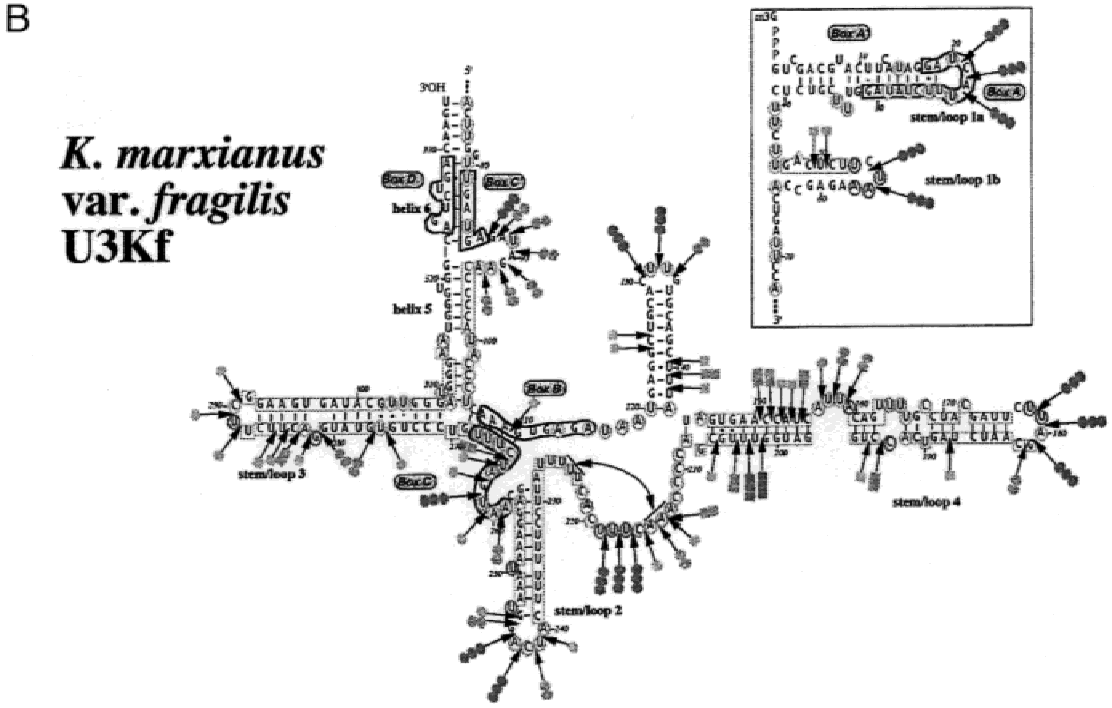


FIGURE 6. Schematic representation of the results of chemical and enzymatic probing on the secondary structure proposed for the (**A**) pre-U3Kd snoRNA, (**B**) U3Kf snoRNA, and (**C**) U3Kl snoRNA. The proposed secondary structures are adaptated from the *S. cerevisiae* U3A pre-snoRNA (Mougin et al., 1996) and the *S. cerevisiae* U3 snoRNA secondary structure (Méreau et al., 1997). Nucleotides modified by DMS or CMCT are circled. Colors of circles indicate the intensity of modification (green, orange, and red for low, medium, and strong modification, respectively). Nucleotides in blue squares were not modified by chemical reagents. Nucleotides that are not squared correspond either to pause of reverse transcriptase or to sites of primer hybridization in the analysis of chemical modifications and enzymatic cleavages. Arrows linked to symbols indicate RNase cleavages: squares for V1 (double-stranded regions), circles for S1 and T2 nucleases (single-stranded regions). Colors of symbols show the yield of cleavage (one green symbol, two orange symbols, and three red symbols for low, medium, and strong cleavage, respectively). The phylogenetically conserved boxes A′, A, B, C′, C, and D are indicated and stem-loop structures are numbered as previously (Ségault et al., 1992; Méreau et al., 1997). In panel B, a double arrow-headed line indicates a possible base pairing between nt 213 to 215 and nt 223 to 225. (*Figure continues on facing page.*)



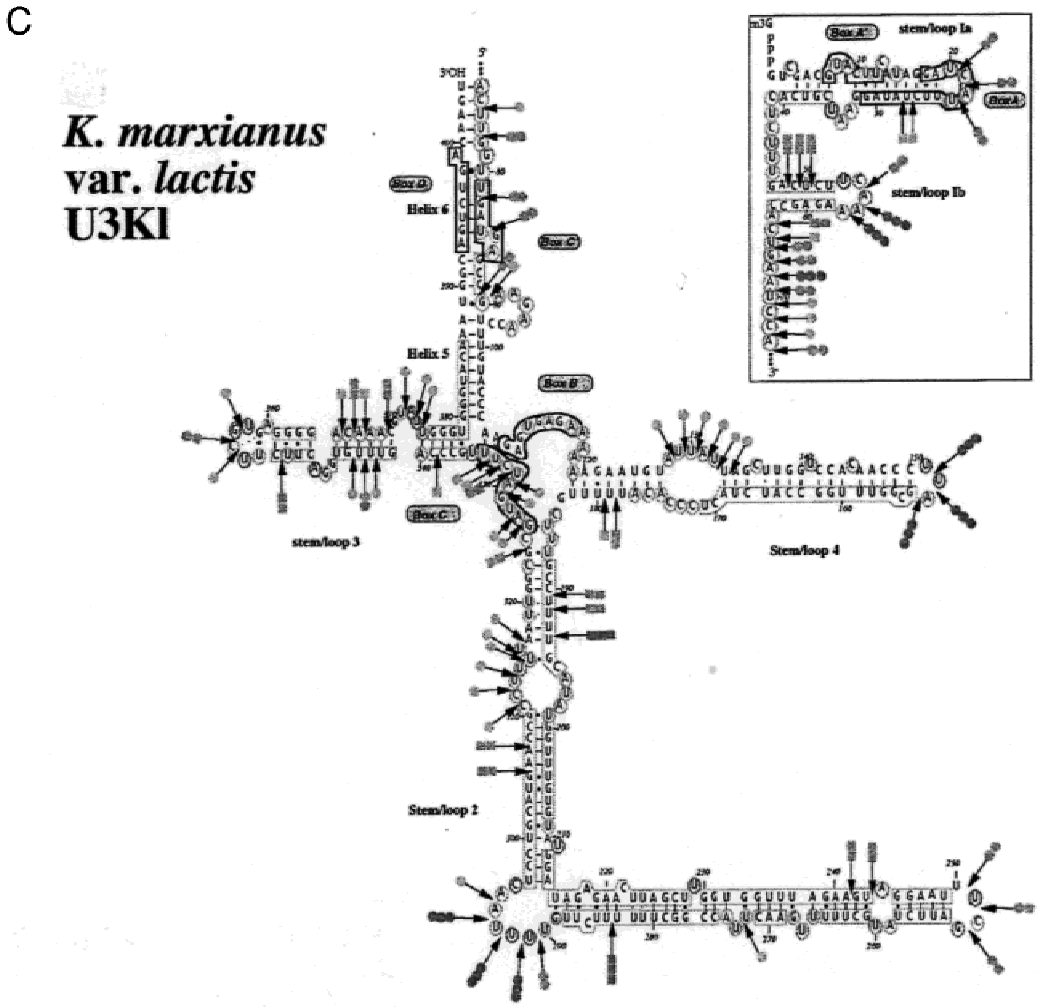
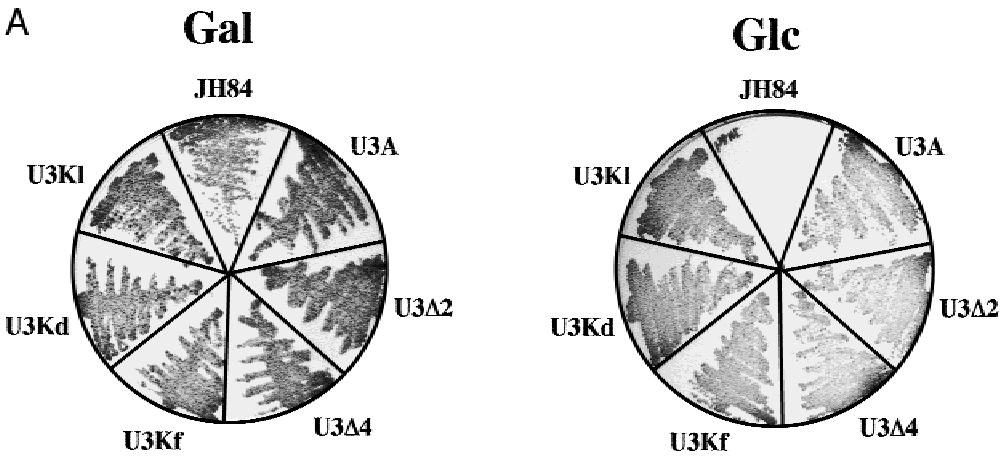
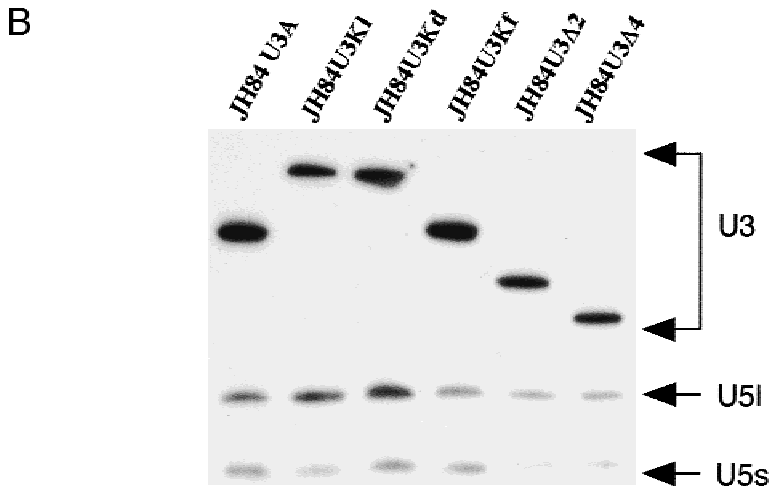
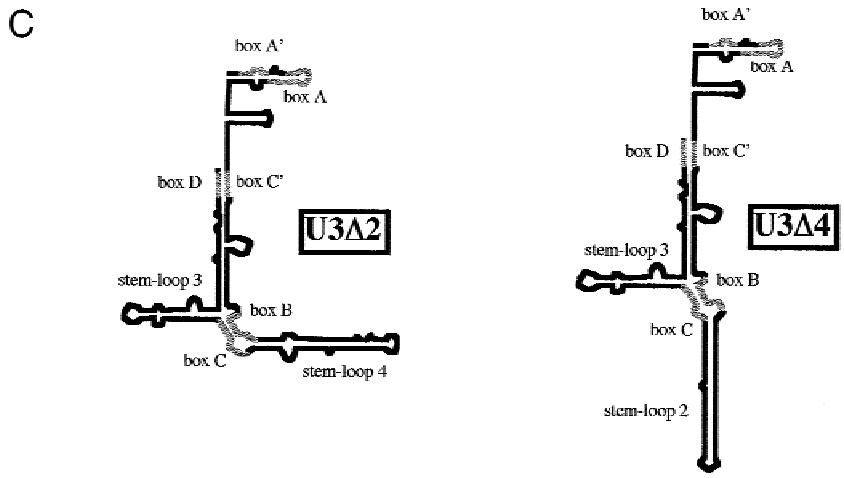


FIGURE 6. (*Continued.*)

marxianus U3 snoRNA genes show a strong divergence with the *S. cerevisiae* U3A and U3B snoRNA genes and the *K. delphensis* U3 snoRNA gene. Hence, there are different types of U3 snoRNA genes in yeasts, that seem to derive from different ancestors. Puzzlingly, the distribution of these genes among yeast species does not parallel the presently accepted yeast classification (Kurtzman, 1984, 1993). It should be







mentioned that the yeast classification has been the subject of several modifications, especially for the *K. marxianus* var. *fragilis*, var. *lactis*, and var. *marxianus*. After being considered as *Saccharomyces* species (*S. lactis*, *S. fragilis*, and *S. marxianus*, respectively) (Lodder & Kreger Van Rij, 1952), they were reclassified as distinct *Kluyveromyces* species (*K. marxianus*, *K. fragilis*, and *K. lactis*) (Van der Walt, 1970) and finally classified as variants of the same species, *K. marxianus* (Bicknell & Douglas, 1970; Campbell, 1972; Van der Walt & Yarrow, 1984). Based on their U3 snoRNA gene sequences, the *K. marxianus* var. *fragilis*, var. *lactis*, and var. *marxianus* clearly do not belong to the *Saccharomyces* genus. The question is opened for *K. delphensis*, whose U3 snoRNA gene shows similarity with those of *S. cerevisiae*. The presence of identical U3 snoRNA genes in the *K. marxianus* variants *marxianus* and *bulgaricus* is in agreement with their classification as two synonymous variants of the same species. However, the important differences that we observed between U3 snoRNAs of the *K. marxianus* variants *marxianus*, *lactis*, and especially *fragilis* open the question of their belonging to a unique species. The analysis of U3 snoRNA genes of a larger number of *Kluyveromyces* species should help to answer these various phylogenetic questions.

Great conservation of the yeast U3 snoRNA 5′ domain and great variability of the 3′ domain

The 5′-terminal 64 nt of U3 snoRNAs are highly conserved in the yeast Saccharomycetoideae subfamily (85% of conservation as referred to all the yeast U3 snoRNAs studied up to now). Such a strong conservation is in accord with the idea that the 5′-terminal region of U3 snoRNA plays a crucial role in the pre-rRNA maturation process leading to the 17S rRNA. Several previous results substantiated this hypothesis: (1) cleavage of the U3 snoRNA 5′ domain abolished the in vitro maturation at site A0 in a mouse cell extract (Kass et al., 1990); (2) deletion of this domain in the *X. laevis* U3 snoRNA from oocytes modified the cleavages in the ITS1 region (Savino & Gerbi, 1990); (3) mutations in the 10-bp segment of the *S. cerevisiae* U3 snoRNA 5′ domain, which is complementary to the pre-rRNA 5′-ETS region, abolished 17S rRNA production (Beltrame & Tollervey, 1992) and some mutations in the phylogenetically conserved box A of the *S. cerevisiae* U3 snoRNA were found to impair cleavages of the pre-rRNA at sites A1 and A2 (Hughes, 1996). In *H. wingei*, compared with *S. cerevisiae*, two base substitutions were detected in the U3 snoRNA 10-nt sequence complementary to the 5′-ETS region of the pre-rRNA, and they are compensated by two base substitutions in the 5′-ETS region (Brulé et al., 1996). In the *K. delphensis*, *K. marxianus* var. *lactis*, var. *bulgaricus*, and var. *marxianus* U3 snoRNAs, this 10-nt element has the same sequence as in the *S. cerevisiae* U3 snoRNAs. However, two base substitutions are observed in the *K. marxianus* var. *fragilis*. It will be interesting to check whether compensatory mutations are present in the 5′-ETS region of this variant. This observation reinforces the idea of an important phylogenetic distance between the *K. marxianus* strains studied.

More recently, the 5′-terminal region of U3 snoRNA was proposed to interact with 17S rRNA sequences of the pre-rRNA (Hughes, 1996), and we brought strong arguments in favor of the formation of this interaction in vivo (Méreau et al., 1997). Altogether, five distinct heterologous helices can be formed between the U3 snoRNA and the pre-rRNA. Interestingly, positions with variable residues are located outside of the segments involved in the formation of the helices I, II, III, and IV (Fig. 8A). They are clustered in the segments that join helix I to helix V and helix V to helix IV, respectively (Fig. 8A). By site-directed mutagenesis, we recently showed that deletions within the segment linking helix I to helix V strongly affects growth (Méreau et al., 1997). The fact that U3 snoRNAs having a linker of the same length, but with different sequences, are functional in *S. cerevisiae*, as revealed by the genetic experiments described in this paper, shows that the length, but not the sequence of this linker region, is important for U3 snoRNA function.

In spite of the few sequence differences observed, the 5′-terminal segment of the five studied *Kluyveromyces* U3 snoRNAs can be folded into a two stem-loop structure, identical to that we proposed recently for the *S. cerevisiae* U3 snoRNAs (Méreau et al., 1997) (Fig. 6). In our revised version of the *S. cerevisiae* U3 snoRNA secondary structure, the helix 5 of the cruciform structure is extended by a base pair interaction that involves boxes D and C′ (helix 6). As shown in Figure 6A, B, and C, formation of this interaction is

FIGURE 7. Test of the functionality of heterologous U3 snoRNA genes and variant U3A snoRNA genes in *S. cerevisiae*. The *S. cerevisiae* JH84 strain (Hughes & Ares, 1991) was used as the host strain. Plasmid pASZ11::pU3A contains the wild-type pre-U3A snoRNA coding sequence. This coding sequence was replaced by those coding for the U3Kl, pU3Kd, and U3Kf snoRNAs in plasmid pASZ11::U3Kl, pASZ11::pU3Kd, and pASZ11::U3Kf, respectively, and by truncated pre-U3A snoRNA coding sequences in plasmids pASZ11::pU3AΔ2, pASZ11::pU3AΔ4. **A:** Growth on YPD solid medium of the untransformed JH84 strain and of the JH84 strain transformed with the plasmids listed above. **B:** Northern-blot analysis of U3 snoRNA. Total RNA was prepared as described in Materials and Methods. The 5′-end labeled oligonucleotide O-3 was used as the probe for U3 snoRNA, and an oligonucleotide complementary to the U51 and U5s RNAs was used to get a control (see Materials and Methods). **C:** Schematic representation of the truncation in the U3AΔ2 and U3AΔ4 snoRNAs.

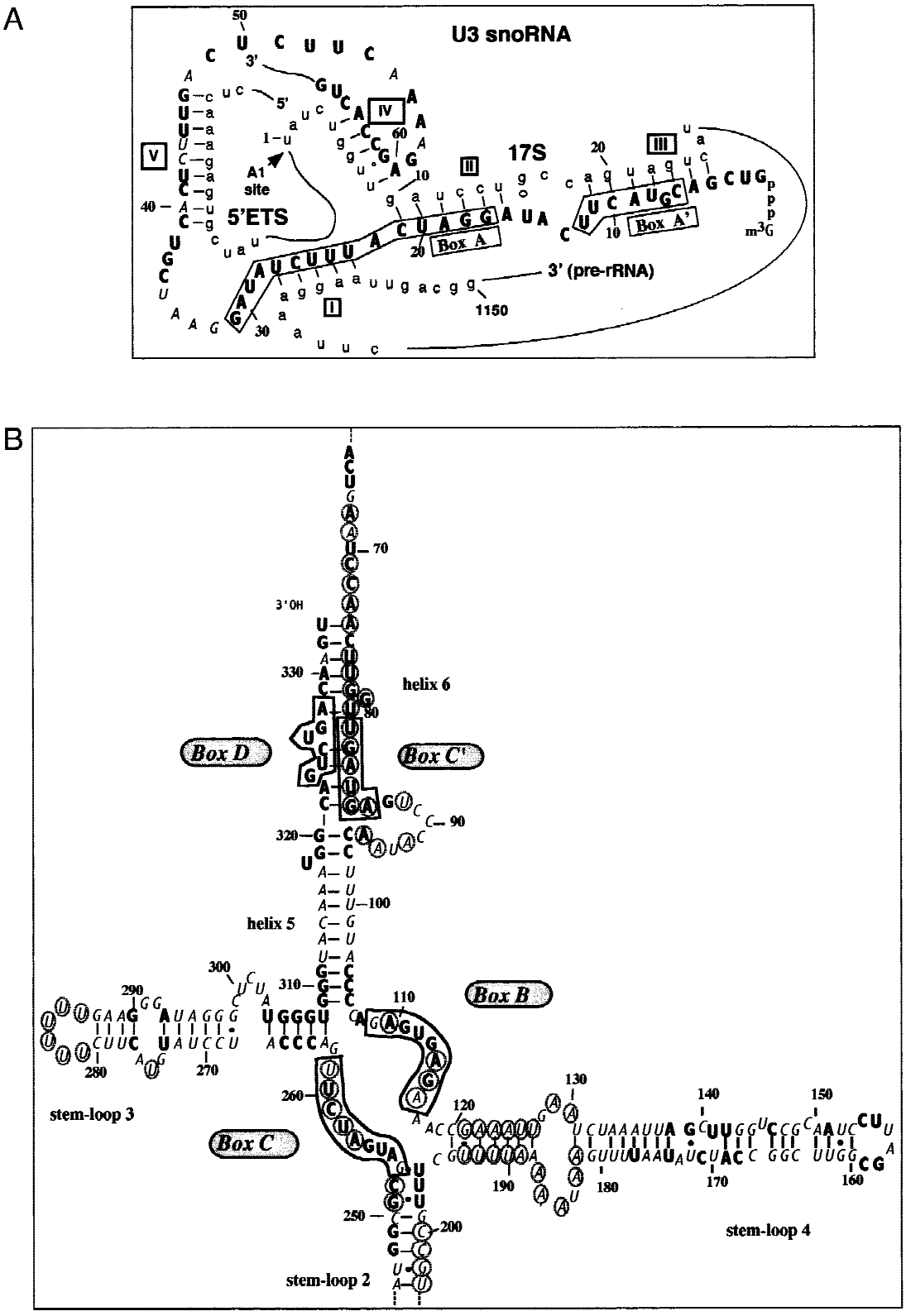


FIGURE 8. Nucleotide sequence conservation in yeast U3 snoRNAs compared with the pattern of protection in the *S. cerevisiae* U3A snoRNP. **A:** Conservation of the nucleotide sequence in the U3 snoRNA 5′ domain of *S. cerevisiae*, *H. wingei*, and the five *Kluyveromyces* strains studied. The 5′-terminal domain of the *S. cerevisiae* U3A snoRNA is shown base paired with the 5′-ETS and 17S regions of the pre-rRNA, as proposed by Méreau et al. (1997). Nucleotides that are conserved in all the species studied are represented by bold characters. The pre-rRNA sequence is represented by lowercase letters, the U3A snoRNA sequence by uppercase letters. **B:** Conservation of the nucleotide sequence in the U3 snoRNA 3′ domain of *S. cerevisiae*, *H. wingei*, and the five *Kluyveromyces* strains studied. As above, the nucleotide sequence of the *S. cerevisiae* U3A snoRNA is shown, from position 64 to the 3′ extremity. Nucleotides conserved in all species from the Saccharomycetoideae subfamily that have been studied are represented by bold letters. Nucleotides protected in the snoRNP (Méreau et al., 1997) are circled. The phylogenetically conserved boxes C′, B, C, and D are shown.

compatible with the nucleotide sequences established for the five *Kluyveromyces* U3 snoRNAs. The nucleotide sequence of helix 6 is highly conserved in the Saccharomycetoideae subfamily. Nucleotide sequence variations are clustered in the bulged loop located between helix 5 and helix 6 (Fig. 8B). In the U3 snoRNP, we showed a strong protection of helix 6 against ribonuclease attacks and chemical modifications, and we concluded that U3 snoRNP proteins are tightly bound to this U3 snoRNA region (Méreau et al., 1997). As shown in Figure 8B, in this area of U3 snoRNA, there is a great similarity between the pattern of nucleotide protection in the U3A snoRNP and the pattern of nucleotide conservation in yeasts. The part of the bulged loop that has a variable sequence in yeast is highly accessible within the U3 snoRNP. Hence, most of the protected nucleotides are probably required for association with the snoRNP proteins.

The second U3 snoRNA area that was found to be protected by the proteins in the U3A snoRNP consists of the segments containing box B and box C and of the bottom part of the helicoidal regions of the stem-loop structures 2 and 4 (Méreau et al., 1997). For this second protein-anchoring region, there is a great similarity between the pattern of protection and the pattern of nucleotide conservation only in the segments containing box B and box C (Fig. 8B). The correspondence is less strong for the bottom parts of the stem-loop structures 2 and 4. This suggests that the protein binding sites are located in boxes B and C. The protection of the helicoidal regions of the stem-loop structures 2 and 4 may be due to steric hindrances in the presence of the proteins or to electrostatic interactions between RNA and proteins that are independent of the nucleotide sequence of the RNA chain.

Some regions of the cruciform domain that are not protected by the proteins are, however, conserved in yeasts. This is the case for the two clusters of G-C base pairs present, one in helix 5, the other one in the helicoidal region of the stem-loop structure 3. Their conservation may reflect an implication in the 3D conformation of the U3 snoRNA 3′-terminal domain. Indeed, in this domain, helix 5 and the helicoidal region of the stem-loop structure 3 are likely to be stacked together, forming a highly stable structure with six G-C base pairs. The nucleotide sequence of the terminal loop and bordering sequences in stem-loop structure 4 is also conserved and this may reflect an interaction with other nucleolar components. But, as discussed later, if such an interaction exists, it should not be essential.

Apart from these conserved sequences, the helicoidal regions of the stem-loop structures 2, 3, and 4 are subjected to great variations of size and sequence in yeasts. The nucleotide sequence of the upper part of helix 5 is also variable. Interestingly, in spite of these variations, the three *Kluyveromyces* U3 snoRNAs tested were functional in *S. cerevisiae*. This means that, in the 3′ domain, except for the center of the cruciform structure and helix 6 that both interact with the U3 snoRNP proteins, great variations are tolerated. We could even show that U3 snoRNAs lacking the stem-loop structure 2 or 4 are functional in *S. cerevisiae*. This reinforces the idea that protection in the stem-loop structures 2 and 4, within the snoRNP, results from steric hindrance, the proteins being bound to the segments containing box B and box C. Furthermore, in spite of the observed nucleotide sequence conservation of the top part of the stem-loop structure 4, this area of U3 snoRNA is not essential for U3 snoRNA function.

In vertebrates, the segments containing box B and box C, respectively, are separated by a unique stem-loop structure and, in the *Trypanosoma* U3 snoRNA, they are contiguous in a large loop (Hartshorne & Agabian, 1994). The present results show that *Trypanosoma* is not a special case, because the two stem-loop structures between box B and box C are dispensable in yeasts.

MATERIALS AND METHODS

Strains and growth conditions

Five yeast strains were used in this study: *K. delphensis* (CBS 2170), *K. marxianus* var. *bulgaricus* (CBS 397), *K. marxianus* var. *fragilis* (CBS 1555), *K. marxianus* var. *lactis* (CBS 2360), and *K. marxianus* var. *marxianus* (CBS 397). For genomic DNA or total RNA preparation, yeasts were grown for 12 h in 10 mL of standard YPD medium (1% yeast extract, 1% bactopeptone, 2% glucose), at 30 °C. The *S. cerevisiae* strain JH84 (Mata snr17a.Gald:*URA*3 snr17b::*LEU*2 his3 ade2 can1) (Hughes & Ares, 1991), generously provided by J. Hughes, was used to test for the functionality of U3 snoRNA genes. It was grown on YPG medium (1% yeast extract, 1% bactopeptone, 2% galactose) or on YPD medium. The *Escherichia coli* strain TG1 (Gibson, 1984) was used as the host strain for production of recombinant DNA. It was grown at 37 °C, in the Luria Broth medium, with 100 μg/mL of ampicillin added when necessary.

Yeast genomic DNA preparations and PCR amplification

For genomic DNA preparations, yeast cells grown to stationary phase were ground with glass beads and DNA was phenol extracted as previously described by Brulé et al. (1996).

The oligonucleotide pairs O-1, 5′-AGCTCGTCGACGTA CTTCA-3′, with the same sequence as the 14 nt at the 5′ end of the *S. cerevisiae* U3A and U3B snoRNAs (Myslinski et al., 1990), and O-2, 5′-ACTTGTCAGACTGCC-3′, complementary to the 3′ end of the U3 snoRNAs and to the phylogenetically conserved box D (Myslinski et al., 1990), were used to amplify the coding sequence of U3 snoRNA genes using PCR in the conditions described previously (Brulé et al., 1996).

Cloning and sequencing of the amplified DNA fragments

Amplified DNA fragments were purified by electrophoresis on 6% nondenaturing acrylamide gel in 0.5× TBE buffer (Sambrook et al., 1989). After elution, the amplified DNA fragments were cloned into the plasmid pMOSBlue-T using the commercial kit of Amersham (RPN 1719). Several recombinant plasmids were prepared from each ligation, and their inserts were sequenced as described by Tabor and Richardson (1987). Nucleotide sequence alignments were made with the PileUp program of the GCG software Version 8.1 Unix (1995). The Alscript program (Barton, 1993) was used for schematic representation of sequence similarities. The GenBank database accession numbers of the sequences established in this paper are as follows: Z78433 (*K. delphensis*), Y14752 (*K. marxianus* var. *bulgaricus*), Z78437 (*K. marxianus* var. *fragilis*), Z78438 (*K. marxianus* var. *lactis*), Y14751 (*K. marxianus* var. *marxianus*).

Southern-blot analysis

For Southern-blot analysis, genomic DNAs were digested with the *Eco*R I, *Hin*d III, or *Pst* I nucleases. The digestion products were fractionated by electrophoresis on a 0.8% agarose gel made up in 0.5× TBE buffer and transferred onto a nitrocellulose Hybond N+ filter (Amersham) (Sambrook et al., 1989). To prepare probe 1, the exon 2 DNA was recovered by digestion with the *Bam*H I and *Hpa* I nucleases of the recombinant plasmid pASZ11::pU3Av490 (Méreau et al., 1997) followed by electrophoresis on an 0.8% agarose gel in 0.5× TBE buffer. To prepare probe 2, the 86-bp DNA fragment corresponding to the 5′ extremity of the U3A snoRNA coding sequence was amplified by PCR using the recombinant plasmid pVS1::snR17A (Ségault et al., 1992) as the template and oligonucleotides O-1 and O-3 (5′-TCATCAACCAAGTTGG-3′) as the primers. The resulting amplified products were labeled by nick translation using the commercial kit of Boehringer and [α32P]dATP (Amersham). Hybridizations were performed overnight at 65 °C in 6× SSC, 0.1% SDS, 5× Denhardt's reagent, 5 mM EDTA, 10 μg/mL of sonicated and denatured calf thymus DNA (Sambrook et al., 1989). The filters were then washed twice for 30 min at 37 °C and once for 30 min at 55 °C in 6× SSC buffer containing 0.5% SDS (w/v). Autoradiography of the membranes were at −80 °C with an intensifying screen.

Construction of plasmid pASZ11::pU3A and its derivatives

Plasmid pASZ11::pU3A (Méreau et al., 1997) contains the 3.3-kb *Eco*R I–*Pst* I fragment from plasmid pFL1::snR17A (Myslinski et al., 1990), including the U3A snoRNA gene with its intron, the U3 snoRNA gene promoter and its termination signal. To substitute the *S. cerevisiae* pre-U3A snoRNA coding sequence by the *K. delphensis* pre-U3 snoRNA, *K. marxianus* var. *lactis* U3 snoRNA and *K. marxianus* var. *fragilis* U3 snoRNA coding sequences, the 1.5-kb *Hin*d III–*Hin*d III fragment from plasmid pFL1::snR17A was subcloned into plasmid pBluescript and the *Hpa* I site located 250 nt downstream from the pre-U3A snoRNA coding sequence was destroyed by site-directed mutagenesis and converted into an *Apa* I site. Then, the 0.49-kb *Sal* I–*Hpa* I fragment from this mutated plasmid was substituted by the *Sal* I–*Hpa* I fragments obtained upon digestion with these two enzymes of the amplification products corresponding to the *Kluyveromyces* U3 snoRNA coding sequences listed above. Finally, the 1.5-kb *Kpn* I–*Pst* I fragments from the recombinant pBluescript plasmids obtained in this way were subcloned into the centromeric plasmid pASZ11. The constructs obtained were designated as pASZ11::pU3Kd, pASZ11::U3Kl, and pASZ11::U3Kf, for *K. delphensis*, *K. marxianus* var. *lactis*, and *K. marxianus* var. *fragilis*, respectively.

For production of plasmid pASZ11::pU3A derivatives carrying mutated pre-U3A snoRNA coding sequences, site-directed mutageneses of the pre-U3A snoRNA coding sequence were achieved on the recombinant phage M13mp9::T7-snR17A, which contains the 0.95-kb *Sal* I–*Bam*H I fragment from plasmid pFL1::snR17A (Myslinski et al., 1990). The method of Kramer et al. (1984) was used. Oligonucleotide O-4, 5′-GGGTCAAGATCATCGCGCAAAG CAAAATTTTTATTC-3′, complementary to the pre-U3A snoRNA sequences between positions 181–199 and 250–266, was utilized to delete the sequence coding for stem-loop structure 2, and oligonucleotide O-5, 5′-CTACAAATGCAA CGGCGCCGGTTTCTCACTCTGGGG-3′, complementary to the pre-U3A snoRNA sequence between positions 104 and 122 and 199 and 214, was used to delete the stem-loop structure 4 coding sequence. After site-directed mutagenesis, the 0.95-kb *Sal* I–*Eco*R I fragment of plasmid pASZ11::pU3A containing the wild-type U3A snoRNA coding sequence was substituted by the 0.95-kb *Sal* I–*Eco*R I fragments from the mutated M13mp9::T7snR17A phage DNAs. The resulting plasmids were designated as pASZ11::pU3AΔ2 for deletion of stem-loop structure 2 and pASZ11::pU3AΔ4 for the deletion of stem-loop structure 4.

RNA preparation, northern blot and nucleotide sequence analysis

Total RNA was phenol extracted from yeast cells ground with glass beads in the presence of 50 mM AcONa, pH 5.0, 10 mM EDTA, 1% SDS as described by Domdey et al. (1984). Five micrograms of total RNA prepared were subjected to a 6% polyacrylamide (38:2) gel electrophoresis in the presence of 8 M urea and 0.5× TBE buffer. After 6 h electrophoresis at 10 V/cm, RNA was electroblotted on Biodyne B transfer membrane (Pall) and hybridized together with the 5′-end labeled oligonucleotide O-3 and with a 5′-labeled oligonucleotide, 5′-GTTTTTTAGTTCCAAAAAATATGGCAA-3′, complementary to *Saccharomyces cerevisiae* U5 snoRNAs (L and S) from position 1 to 20 (Guthrie & Patterson, 1988), as described previously (Méreau et al., 1997). Direct sequence analysis of U3 snoRNAs in the total RNA was performed on 5 μg of total RNA mixture in the conditions that we described previously with oligonucleotide O-2 as the primer (Méreau et al., 1997).

In vitro transcription

PCR amplification products were used for in vitro transcription of the *K. delphensis* pre-U3 snoRNA and of *K. marxianus*

var. *lactis* and var. *fragilis* U3 snoRNAs. For this purpose, the DNA sequence coding for the *K. delphensis* U3 snoRNA precursor, and the *K. marxianus* var. *lactis* and var. *fragilis* U3 snoRNAs, cloned in plasmid pMOSBlue-T as described above, were PCR amplified with oligonucleotide O-6, 5′-AAATTtaat acgactcactataGGGTCGACGTACTTCA-3′, which generates a T7 RNA polymerase recognition site (lower case letters) and contains the 14-nt sequence at the 5′ extremity of the *K. delphensis* pre-U3 snoRNA, *K. marxianus* var. *lactis* and var. *fragilis* U3 snoRNAs (underlined), and the oligonucleotide O-2, described above. PCR was performed as described above.

In vitro transcriptions were performed 2 h at 37 °C, in 20 μL of 6 mM $MgCl_2$, 10 mM DTT, 40 mM Tris-HCl, pH 7.5, containing 250 ng of amplified fragment, 12.5 nmol of each ribonucleoside triphosphate, 20 units of RNase Guard™ (Pharmacia), 140 units of T7 RNA polymerase (Amersham). After DNase I digestion, phenol extraction, and ethanol precipitation, the RNA (about 10 μg) was dissolved in 120 μL of sterile water; 2 μL of this solution was used for each chemical reaction or enzymatic digestion.

Chemical modifications and enzymatic cleavages

Chemical modifications were performed under nondenaturing conditions using DMS or CMCT as described by Mougin et al. (1996). Enzymatic digestions were performed with V1 RNase (Pharmacia) and S1 nuclease (Pharmacia), as described previously (Mougin et al., 1996). Modified RNAs or cleaved RNAs were dissolved in 2 μL of sterile water. Positions of enzymatic cleavages and chemical modifications in the U3 snoRNA transcripts were analyzed by primer-extension analysis with Avian Myeloblastosis Virus Reverse Transcriptase (Life Science), in the conditions described above using 1 μL of modified or cleaved transcript. T2 RNase digestions were performed on T7 RNA transcripts mixed with 5 μg of commercial tRNA (Boehringer). The RNA transcripts were pre-incubated 10 min at 20 °C in 10 μL of reaction buffer (50 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5). Incubation was for 6 min at 20 °C with 2 units of enzyme. The reaction was stopped as described by Mougin et al. (1996).

The following oligonucleotides were used as primers: O-2, complementary to the 3′-end of all yeast U3 snoRNAs; O-7 (5′-GGGTCAAGATCATCGCGC-3′), complementary to nucleotide positions 341–317 of the *K. marxianus* var. *lactis* U3 snoRNAs and 296–279 of the *K. delphensis* pre-U3 snoRNA; O-8 (5′-TCTCACTCTTGGGTACAAAGG-3′), complementary to nucleotide positions 116–96 of the *K. marxianus* var. *lactis* and 116–96 of the *K. delphensis* pre-U3 snoRNA; O-9 (5′-TCCTATAGAAATGATCCTA-3′), complementary to nucleotide positions 33–15 of the *K. delphensis* pre-U3 snoRNA; and O-10 (5′-ATAGAATGTGTTAGTCA-3′), complementary to nucleotide positions 164–147 of the *K. delphensis* pre-U3 snoRNA intron.

Test of U3 snoRNA functionality

Using the LiCl transformation method (Ito et al., 1983), the strain JH84 was transformed with plasmid pASZ11 or plasmid pASZ11::pU3A or one of its derivative. Selection of transformants was made on minimum medium (YNB 0,67%, galactose 2%, histidine 20 mg/L). The selected recombinant JH84 strains were grown on YPG medium (1% yeast extract, 1% bactopeptone, 2% galactose). Recombinant JH84 yeast strains were tested on plates in conditions of U3A gene expression [YPG medium] or gene repression [YPD medium]. Strain growth was checked after 24 h at 30 °C. Repression of the U3A snoRNA gene was achieved as described by Hughes and Ares (1991): freshly grown galactose cultures were either washed once in glucose medium and used to inoculate fresh pre-warmed glucose medium, or diluted directly into fresh pre-warmed galactose medium as controls. To maintain exponential growth, the cultures were successively diluted. Dilutions were made in order to get an A_{600} of about 0.5 at the zero time point. At each dilution step, a cell aliquot was collected for analysis of U3 snoRNA by northern-blot analysis.

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