The snoRNA box C/D motif directs nucleolar targeting and also couples snoRNA synthesis and localization

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Most small nucleolar RNAs (snoRNAs) fall into two families, known as the box C/D and box H/ACA snoRNAs. The various box elements are essential for snoRNA production and for snoRNA-directed modification of rRNA nucleotides. In the case of the box C/D snoRNAs, boxes C and D and an adjoining stem form a vital structure, known as the box C/D motif. Here, we examined expression of natural and artificial box C/D snoRNAs in yeast and mammalian cells, to assess the role of the box C/D motif in snoRNA localization. The results demonstrate that the motif is necessary and sufficient for nucleolar targeting, both in yeast and mammals. Moreover, in mammalian cells, RNA is targeted to coiled bodies as well. Thus, the box C/D motif is the first intranuclear RNA trafficking signal identified for an RNA family. Remarkably, it also couples snoRNA localization with synthesis and, most likely, function. The distribution of snoRNA precursors in mammalian cells suggests that this coupling is provided by a specific protein(s) which binds the box C/D motif during or rapidly after snoRNA transcription. The conserved nature of the box C/D motif indicates that its role in coupling production and localization of snoRNAs is of ancient evolutionary origin.
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Introduction

Biogenesis of eukaryotic RNAs involves maturation of primary transcripts and targeting of product RNAs to sites where they function. Clearly, these two fundamental processes are coordinated in the cell, since precursor RNAs are usually found at the sites of synthesis, while only mature RNAs accumulate at the sites of function. This situation is believed to result from two consecutive events: (i) initial assembly of a macromolecular complex which is required for RNA maturation; and (ii) targeting of mature RNA to its site of function.

During the maturation of mRNA, precursors are retained near their site of synthesis (Singer and Green, 1997, and references therein). Subsequently, signals provided by the mature RNA direct it to the cytoplasm (Izaurralde and Mattaj, 1995, and references therein). These two basic processes have been shown to be coupled as, in some cases, efficient targeting (e.g. nuclear export) requires prior processing of the RNA (e.g. 3′ end formation) (Eckner *et al.*, 1991; Huang and Carmichael, 1996). This coupling is manifested spatially at the cellular level by localization of the precursor only at the transcription site (Zhang *et al.*, 1994).

Less is known about RNAs which are retained and targeted within the nucleus. The snoRNAs are a good example of RNAs which traffic intranuclearly. SnoRNAs exist in the cell in the form of RNP complexes (snoRNPs), and have been discovered in protozoan, fungal, plant and mammalian cells (reviewed in Gerbi, 1995; Maxwell and Fournier, 1995; Smith and Steitz, 1997; Tollervey and Kiss, 1997). Nearly all have been firmly tied to maturation of rRNA, specifically cleavage of pre-rRNA and synthesis of modified nucleotides (see also Maden, 1996; Peculis and Mount, 1996; Bachellerie and Cavaille, 1997). Based on conserved structural features, snoRNAs are now classified into three subsets (Balakin *et al.*, 1996; Ganot *et al.*, 1997; reviewed in Smith and Steitz, 1997; Tollervey and Kiss, 1997). Two of the subsets define large families known as the box C/D and box H/ACA families. These families account for all but one snoRNA; the exception is the snoRNA known as MRP (Maxwell and Fournier, 1995; Tollervey and Kiss, 1997)

Members of the box C/D snoRNA family, which are the subject of the present report, possess characteristic sequence elements known as box C (UGAUGA) and box D (GUCUGA). Most box C/D snoRNAs contain long (>12 nucleotides) sequences complementary to rRNA, which, in conjunction with adjoining box D or D′ elements, target rRNA nucleotides to be ribose methylated (reviewed in Bachellerie and Cavaille, 1997; Smith and Steitz, 1997; Tollervey and Kiss, 1997; see also Cavaille *et al.*, 1996; Kiss-Laszlo *et al.*, 1996; Nicoloso *et al.*, 1996; Tycowski *et al.*, 1996). A few box C/D snoRNAs are required for cleavage of pre-rRNA, and this essential function does not appear to be related to methylation.

The coding sequences for the box C/D snoRNAs can be found in pre-mRNA introns or in mono- or polycistronic snoRNA genes. Thus, in the early stages, transcription and processing of individual snoRNAs differ according to the nature of the coding unit and, hence, the primary transcript. However, the final maturation steps in each case are believed to be similar, and involve exonucleolytic trimming of unprotected RNA ends (reviewed in Tollervey

and Kiss, 1997). When the snoRNA possesses a 5′ cap, this structure protects the 5′ end from degradation. In all cases, the box C and D elements function in both RNA stabilization and maturation. In various organisms, mutations in these elements affect snoRNA accumulation (Baserga *et al.*, 1992; Huang *et al.*, 1992; Peculis and Steitz, 1994; Terns *et al.*, 1995; Caffarelli *et al.*, 1996; Cavaille and Bachellerie, 1996; Watkins *et al.*, 1996; Mereau *et al.*, 1997; Xia *et al.*, 1997; Samarsky and Fournier, 1998). Boxes C and D are usually flanked with inverted repeats, and the base pairing of these segments is also required for RNA accumulation. On this basis, the two boxes and the neighboring helix have been proposed to define a vital structure called the box C/D motif. This motif is believed to be a recognition signal for one or more proteins, which provide metabolic stability of RNA and define the end points in its processing (see also Tyc and Steitz, 1989; Ganot *et al.*, 1997). Evidence that the putative box C/D motif-binding protein(s) is common and conserved comes from studies showing that: (i) injection of a box C/D snoRNA into *Xenopus* oocytes inhibits accumulation of other box C/D snoRNAs (Terns *et al.*, 1995; Watkins *et al.*, 1996); and (ii) box C/D snoRNAs from distantly related organisms are stabilized and processed in yeast and animal cells (Li and Fournier, 1992; D.R.Newman, J.C.Liu, M.J.Fournier and E.S.Maxwell, unpublished data).

Another, more mysterious aspect of box C/D snoRNA biogenesis is the process by which these RNAs are localized within the nucleus. The snoRNAs are highly enriched in the nucleolus, as the name implies; however, the mechanism and signals responsible for this precise localization are unknown. This situation reflects our general lack of knowledge about intranuclear RNA trafficking. Indeed, although most eukaryotic RNAs travel through the nucleus, very few examples of intranuclear RNA targeting have yet been analyzed (Jacobson *et al.*, 1995, 1997).

The investigation described here was undertaken to gain new insight into the principles of box C/D snoRNA biogenesis. Specifically, we wished to define snoRNA elements sufficient for nucleolar targeting, and to assess the potential inter-relationship between the processes of snoRNA localization and production. Experiments were conducted in both yeast and mammalian cells, using a natural yeast snoRNA and a variety of artificial variants.

Results

Our study was initiated by analyzing the biogenesis of the U14 box C/D snoRNA (Figure 1) in the yeast *Saccharomyces cerevisiae*. U14 is essential for processing of 18S rRNA (Zagorski *et al.*, 1988; Li *et al.*, 1990; Liang and Fournier, 1995), and is involved in methylation of rRNA (Kiss-Laszlo *et al.*, 1996; Dunbar and Baserga, 1998). Homologs of U14 have been identified in >20 organisms, including fungi, plants and animals (see the electronic database by Zwieb, 1997). The U14 coding sequence is found in various genomic arrangements in different eukaryotes, including mono- and polycistronic transcription units and within introns of protein genes (Zagorski *et al.*, 1988; Liu and Maxwell, 1990; Leverette *et al.*, 1992; Leader *et al.*, 1994; Xia *et al.*, 1995; Samarsky
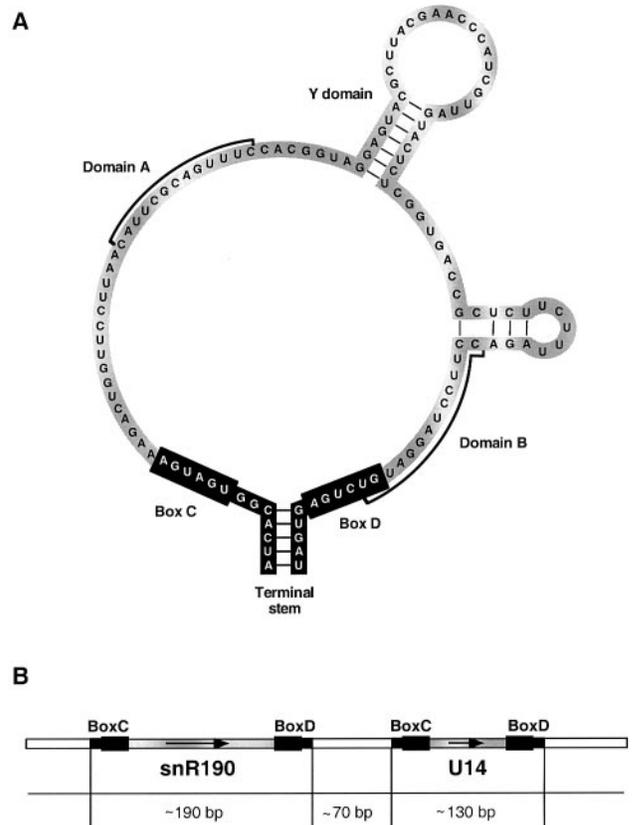

Fig. 1. The U14 snoRNA from *S.cerevisiae*: structure and genomic organization. U14 is a phylogenetically conserved box C/D snoRNA required for production and maturation of 18S rRNA. (**A**) The secondary structure of yeast U14 (adapted from Balakin *et al.*, 1994; Samarsky *et al.*, 1996) is based on results of *in vitro* probing, *in vivo* mutagenesis and phylogenetic sequence analyses. Domains A and B interact with pre-rRNA through complementary base pairing. Domain A is required for rRNA processing; domain B and the adjoining box D define a guide motif for 2′-*O*-methylation of 18S rRNA. The Y-domain structure (stem–loop) is conserved in yeast and plants, but not animals. It is essential for U14 function. Conserved boxes C and D and a terminal stem have been postulated to comprise a recognition motif for a hypothetical protein(s), which upon binding protects the RNA from exonucleolytic degradation (highlighted with black). (**B**) The *S.cerevisiae* U14 is encoded ~70 bp downstream of another box C/D snoRNA, snR190. Transcription from a promoter located upstream of snR190 yields a transcript containing both snoRNAs. Mature snR190 and U14 molecules are produced by endonucleolytic cleavages followed by exonucleolytic trimming.

et al., 1996; Dichtl *et al.*, 1997; Leader *et al.*, 1997; Petfalski *et al.*, 1998). In *S.cerevisiae*, U14 is encoded ~70 bp downstream of another box C/D snoRNA, snR190. Both snoRNAs are co-transcribed, and the mature molecules are derived by endonucleolytic cleavage of the dimeric precursor, followed by exonucleolytic trimming (Petfalski *et al.*, 1998). The conserved nature of U14 and the fact that it is produced from different types of precursors make this snoRNA particularly attractive for defining the general principles involved in biogenesis of box C/D snoRNAs.

The box C/D motif provides metabolic stability and nucleolar localization of snoRNA in yeast

Previous mutagenesis studies of yeast U14 yielded stable variants for an assortment of deletions and substitutions
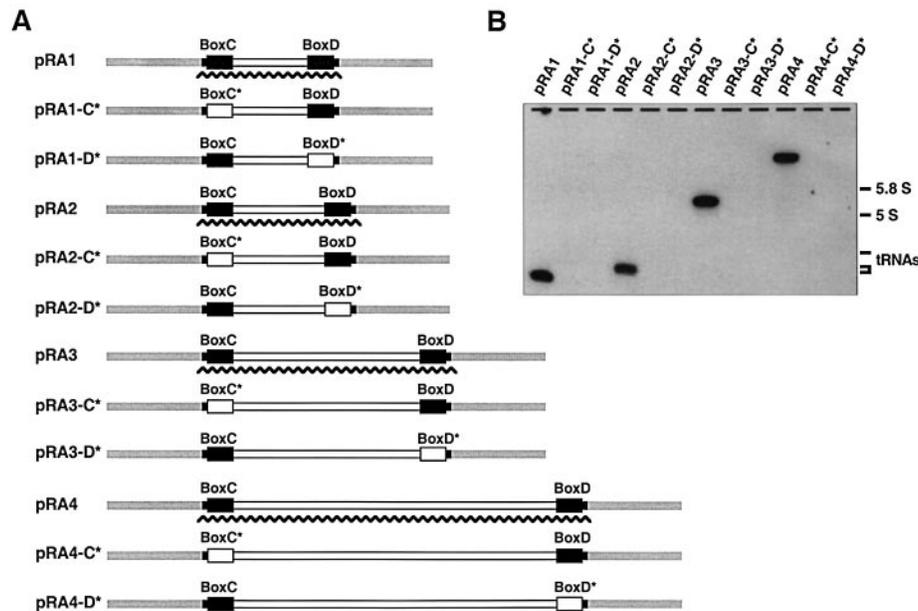
Fig. 2. Expression of modified U14 genes in yeast. (**A**) The coding sequence between boxes C and D was substituted with non-natural sequences of different length and structure, to yield plasmids pRA1–pRA4. Plasmids with C* and D* designations contain mutations in boxes C and D known to abolish accumulation of natural U14. (**B**) Production of artificial U14 derivatives was assessed by Northern blot analysis of total RNA, with equal amounts of RNA loaded in each lane. The radiolabeled oligonucleotide probe (SD18) specific for a sequence preceding box D was common to all of the artificial RNAs. The 5.8S, 5S and tRNA species are 158, 120 and 76–87 nucleotides, respectively. The major RNA species identified by hybridization are shown as black wavy lines on the maps of the individual genes.

that collectively spanned nearly all of the region between boxes C and D (Jarmolowski *et al.*, 1990). These results indicate that the internal segment does not contain information required for snoRNA production. However, in no case was the effect of a clean internal deletion or substitution examined. To resolve this issue, we created four gene constructs in which artificial sequences replaced the 104 bp segment that normally lies between the box elements (pRA1–pRA4; Figure 2A). The artificial segments were 47, 54, 120 and 228 bp in length. After expression in yeast, each new gene produced a single stable RNA of the expected size, corresponding to the artificial portion, boxes C and D and a terminal stem (~69, 76, 142 and 250 nucleotides; Figure 2B, see also Figure 1A). The artificial RNAs accumulated at levels similar to that of natural U14 expressed in the same genetic context (control data are not shown). Double point mutations in either box C or box D, known to abolish yeast U14 production (Huang *et al.*, 1992), blocked accumulation of the artificial RNAs as well (constructs C* and D*; Figure 2). These results demonstrate that the RNA region between boxes C and D does not contain information required for accumulation and processing, and that the box C/D motif is necessary and sufficient for the stability of the artificial box C/D RNAs.

To assess whether the determinants directing snoRNA accumulation are also involved in intracellular targeting, we examined the localization of an artificial box C/D RNA using fluorescent *in situ* hybridization microscopy (Figure 3). The nucleoli, which in yeast form so-called 'crescent bodies', were visualized with a probe specific for natural U14. Strikingly, the same pattern was obtained with a probe specific for the artificial RNA, and double hybridization showed that both RNAs co-localize. The specificity of the signals was verified by hybridization of

cells harboring a plasmid that does not encode any snoRNA (data not shown). These results indicate that the box C/D motif is sufficient to localize RNA to the nucleolus in yeast cells.

The box C/D motif directs snoRNA production and nucleolar localization in mammalian cells

We next asked if the *cis*-acting determinants shown to be sufficient for box C/D RNA accumulation and nucleolar localization in yeast play similar roles in mammalian cells. To this end, we analyzed expression of yeast U14 and one of the artificial box C/D RNAs in monkey cells. The artificial RNA contained a 120 nucleotide non-natural sequence between boxes C and D (from pRA3; see Figure 2). The RNAs were expressed in monkey COS-1 cells, from the human U6 snRNA gene promoter. The U6 promoter utilizes RNA polymerase III but, in contrast to conventional Pol III promoters, is located upstream of the transcribed region, rather than within. Thus, little constraint is placed on the sequence of the transcript. An added advantage of this promoter is that transcripts are not capped, which eliminates potential complications associated with a 5′ cap structure.

The genes to be tested were cloned into the mammalian pU6+1 expression vector (Bertrand *et al.*, 1997) to yield constructs pU6Y (yeast U14) and pU6A (artificial RNA; Figure 4A). Each new gene contained yeast DNA that normally occurs up- and downstream of the coding sequence for mature yeast U14, ~30 bp on the 5′ side and ~130 bp on the 3′ side. Precursor RNAs derived from these constructs were predicted to be ~225 (yeast U14) and ~250 nucleotides (artificial RNA), based on the assumption that a stretch of seven T nucleotides, located 72 bp downstream of the box D, serves as a termination signal for RNA polymerase III. To avoid the possibility
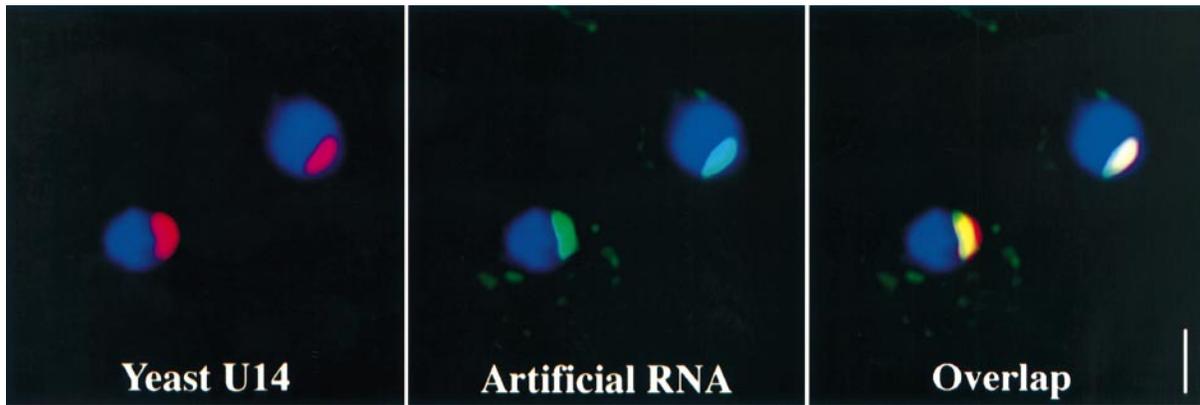
Fig. 3. The artificial box C/D RNA localizes in the nucleolus of yeast cells. Yeast cells expressing the artificial RNA from plasmid pRA3 were probed simultaneously for natural yeast U14 (red) and the artificial box C/D RNA (green). The two signals are superimposable, yielding a yellow color on the overlap. Scale bar is 2 μm. DNA was additionally stained with DAPI (blue).
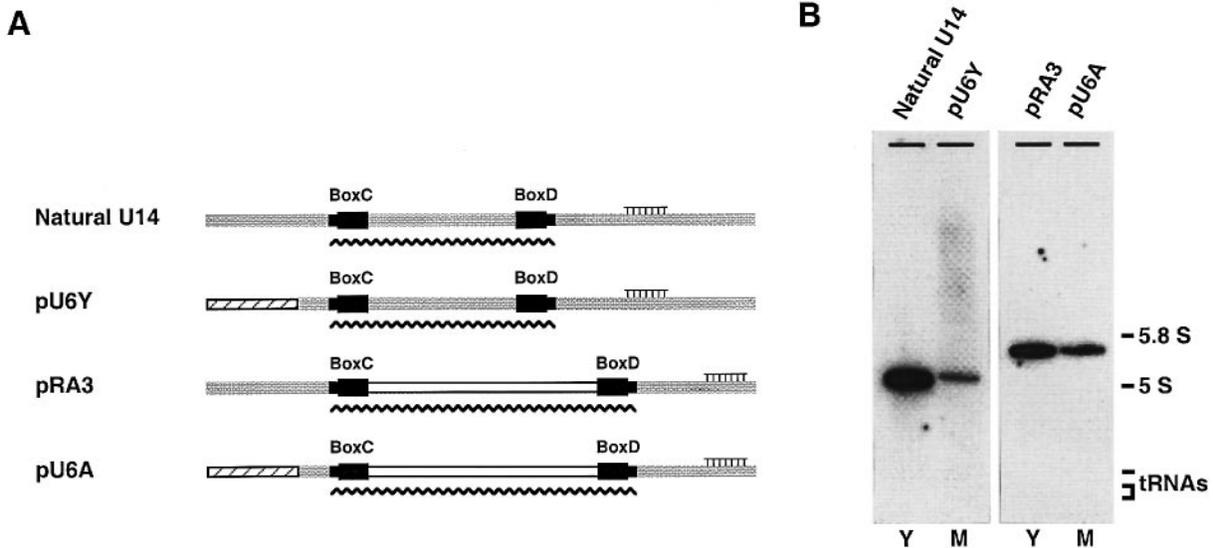

Fig. 4. Expression of experimental box C/D RNAs in mammalian cells. (**A**) The potential to produce yeast U14 and one of the artificial box C/D RNAs was evaluated by fusing the corresponding gene sequences to a human U6 snRNA gene promoter and introducing the resulting constructs (pU6Y and pU6A) into monkey COS-1 cells by transient transfection. The fused segments were derived from the wild-type U14 gene and pRA3 plasmid (Figure 2), and included ~30 bp on the 5' side and ~130 bp on the 3' side of the non-coding regions up- and downstream of the box C and D elements. The cross-hatched boxes correspond to the U6 gene promoter. The poly(T) stretches designate putative Pol III termination sites located 72 bp downstream of box D. The wavy lines represent the RNA species identified by hybridization. (**B**) Patterns of RNA production. The blots were prepared from total RNA isolated from monkey (M) and yeast (Y) cells expressing yeast U14 and/or the artificial RNA. Equal amounts of total RNA from each cell type were fractionated in neighboring lanes of the same gel. The resulting blot was cut into two halves, which were subjected to Northern hybridization with probes specific to either yeast U14 (C106) or the artificial RNA (SD121). The blot was reconstructed prior to radioautography.

of premature transcription termination of the artificial RNA gene, a run of four T residues immediately upstream of box D was modified by deleting two T nucleotides. The new genes were introduced into monkey cells by transient transfection. Stable RNAs accumulated in each case, and the major products were the same size as the corresponding RNAs produced in yeast (~130 nucleotides for yeast U14 and ~155 nucleotides for artificial RNA; Figure 4B). Minor bands corresponding in size to the precursor molecules expected were observed after longer exposure of the Northern blot (data not shown). These results show that the box C/D motif is sufficient for accumulation of box C/D RNAs in phylogenetically distant mammalian cells, as well as in yeast.

Intracellular localization of yeast U14 and the artificial box C/D RNAs was then examined in the monkey cells,

using fluorescent *in situ* hybridization analysis. A hybridization probe recognizing monkey U14 snoRNA was used to visualize the nucleolus. Both, yeast U14 and the artificial RNAs localized to the nucleoli (Figure 5A; data are shown for the artificial RNA). Expression of control RNAs lacking boxes C and D, or possessing only box C, yielded patterns of uniform distribution over the entire nucleoplasm (not shown). This last result verified that the nucleolar localization of yeast U14 and the artificial RNAs is specific and depends on the presence of a box C/D motif. Interestingly, a higher resolution analysis revealed that the artificial RNA localizes in a sub-nucleolar compartment(s), which shows partial, but not complete overlap with natural monkey U14 or the nucleolar protein fibrillarin (Figure 5B; data for fibrillarin are not shown). This pattern suggests that localization of snoRNAs within the nucleolus
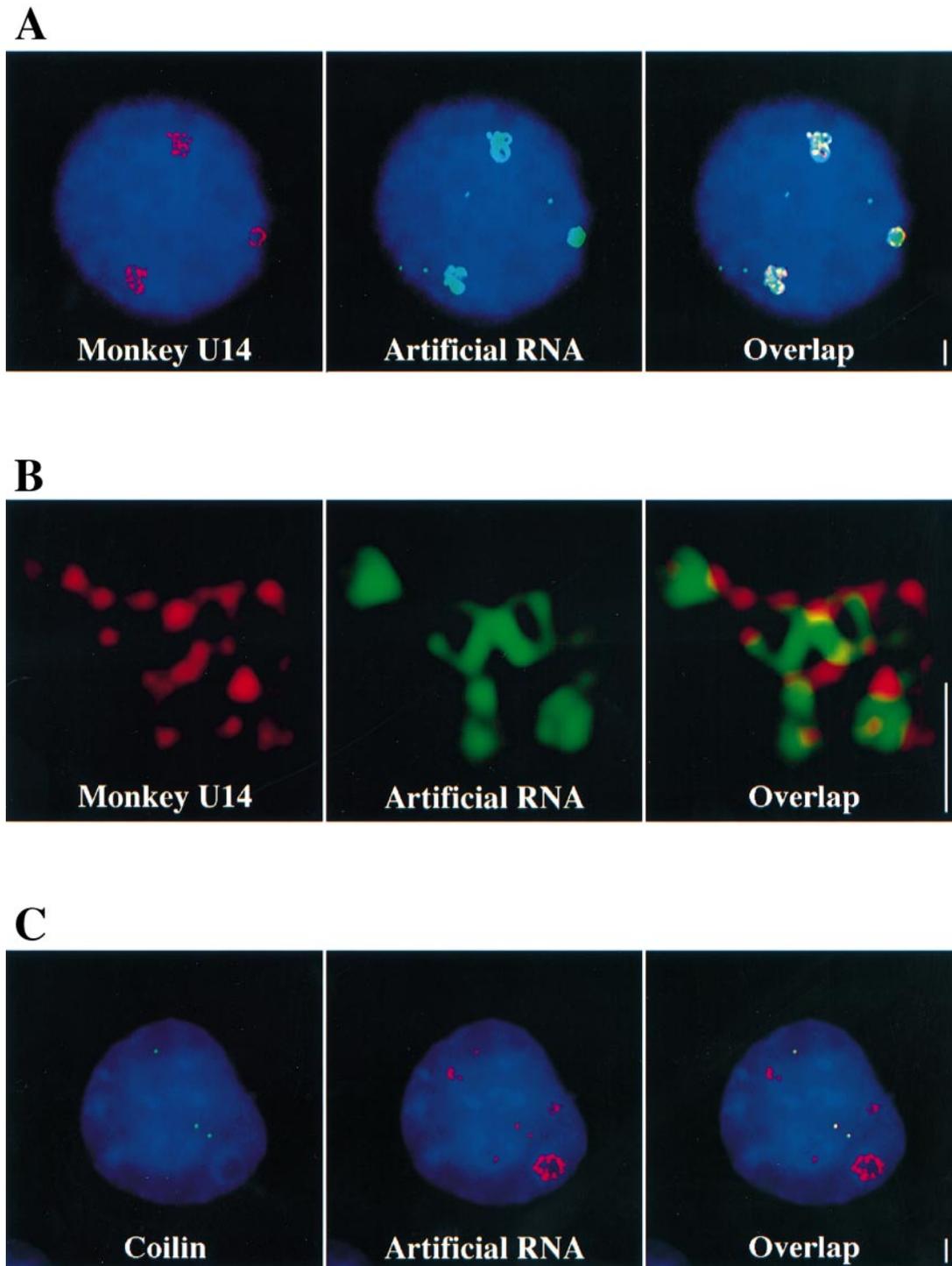
Fig. 5. Localization of artificial box C/D RNA in mammalian cells. The intranuclear location of the artificial box C/D snoRNA in monkey COS-1 cells was analyzed by fluorescent *in situ* hybridization. The scale bar is 2 μm. The DNA was stained additionally with DAPI (blue). (**A**) The artificial box C/D RNA localizes in the nucleoli. Cells transiently expressing the artificial RNA were probed simultaneously for natural monkey U14 (red) and the artificial box C/D RNA (green). The two signals are superimposable, as shown by the overlap. (**B**) High resolution analysis of the distribution of the artificial snoRNA within the nucleolus. Cells transiently expressing the artificial box C/D RNA were probed simultaneously for natural monkey U14 (red) and the artificial RNA (green). The two signals are partially superimposable, as shown by the overlap. (**C**) The artificial box C/D RNA localizes in coiled bodies of mammalian cells. Cells transiently expressing the artificial box C/D RNA were processed for immunodetection of coilin (green) and for *in situ* hybridization against the artificial RNA (red). The overlap shows that the artificial RNA accumulates in all the coiled bodies.

is influenced by RNA structural elements in addition to the terminal box C/D motif.

The distribution patterns of the experimental RNAs also showed the presence of sharp, dot-like signals in the nucleoplasm (see Figure 5A). These dots could correspond to RNA transcription sites (see below), but also to coiled bodies, since at least one box C/D snoRNA, U3, has been found in coiled bodies of mammalian and plant cells (see

Jimenez-Garcia *et al.*, 1994; Raska, 1995; Olmedilla *et al.*, 1997). The latter suggestion was examined by double *in situ* hybridization with probes for the test RNAs and antibodies against coilin, a specific protein component of coiled bodies (Figure 5C; data are shown for the artificial RNA). The results showed that, indeed, yeast U14 and the artificial RNAs accumulate in coiled bodies as well as in the nucleolus.

To determine where transcription of the test snoRNAs occurs in the nucleus, we performed an *in situ* hybridization analysis of transfected monkey cells with: (i) a probe specific for a non-transcribed sequence of the plasmid; and (ii) probes used previously which recognize the mature artificial and yeast U14 snoRNA molecules, respectively. The plasmid probe yielded sharp dots in the nucleoplasm in several places. Differences in signal intensity in the various dots are presumed to reflect differences in gene copy number at the respective sites. Although the plasmid could sometimes be found at the edge of the nucleolus, it was always excluded from this region (data for yeast U14 are shown in Figure 6A, upper panel). Double labeling with coilin antibodies did not reveal co-localization of plasmids with coiled bodies (not shown). Most of the plasmid sites are transcriptionally active, as revealed by double hybridization with the plasmid and mature RNA probes (Figure 6A, upper panel). These results indicate that yeast U14 and the artificial RNAs are initially produced in the nucleoplasm and targeted to the nucleolus post-transcriptionally.

Taken together, these results demonstrate that, as in yeast, the box C/D motif is sufficient for RNA accumulation and nucleolar localization in mammalian cells. Thus, in mammals, and presumably in yeast, snoRNAs are transcribed in the nucleoplasm and then transported to the nucleolus. Interestingly, in mammalian cells, the box C/D motif also targets RNAs to the coiled bodies.

Processing of experimental box C/D RNAs occurs in the nucleoplasm of mammalian cells

To determine the sites of box C/D snoRNA processing in the mammalian nucleus, we performed a localization analysis of RNA precursors, once again using yeast U14 and the artificial RNA featured earlier. The precursor probe selected hybridizes specifically to a sequence downstream of box D (see Materials and methods). This probe was used in conjunction with the probes used previously for the mature RNAs and the corresponding plasmid DNA. *In situ* hybridization analyses showed that precursors

Fig. 6. Transcription and processing sites of the artificial box C/D RNAs in mammalian cells. COS-1 cells transiently transfected with a plasmid expressing yeast U14 were hybridized *in situ* with various combinations of probes specific for mature RNA, precursor RNA or the transfected plasmid (see Materials and methods). (**A**) Only mature RNA is present in the nucleolus. Cells were transfected with a non-replicative plasmid. The top panel shows a cell hybridized with the plasmid probe (red) and with the mature RNA probe (green). The overlap shows that the plasmid is excluded from the nucleolus, and represents a *bona fide* transcription site since it co-localizes with the RNA. The middle panel shows a cell hybridized with the precursor probe (green), and with a probe for monkey U3, which is a nucleolar box C/D snoRNA (red). The overlap shows that the precursor is absent in the nucleolus. The bottom panel shows a cell hybridized with the plasmid probe (red) and with the precursor probe (green). The overlap shows that the precursor is associated predominantly with the plasmid, i.e. at its transcription site. (**B**) Overexpression yields only mature RNA throughout the nucleoplasm and in the nucleolus. Cells were transfected with a replicative plasmid. The nucleolus was visualized with DAPI staining (blue), and is marked with arrows. The top panel shows a cell hybridized with a probe for the mature RNA (green) and with the plasmid probe (red). The plasmid is excluded from the nucleolus (arrows) and forms a speckled pattern, while the mature RNA is present in the nucleolus (arrows), at the transcription sites, and also at a low level throughout the entire nucleoplasm. The middle panel shows a cell hybridized with the plasmid probe (red) and with the precursor probe (green). Both plasmid and precursor RNA are excluded from the nucleolus and form a speckled pattern (arrows). The bottom panel shows a cell hybridized with a probe for the mature RNA (red) and for the precursor (green). The precursor is excluded from the nucleolus (arrows) and forms a speckled pattern, while the mature RNA is found in the nucleolus (arrows), and also at a lower level through the entire nucleoplasm.
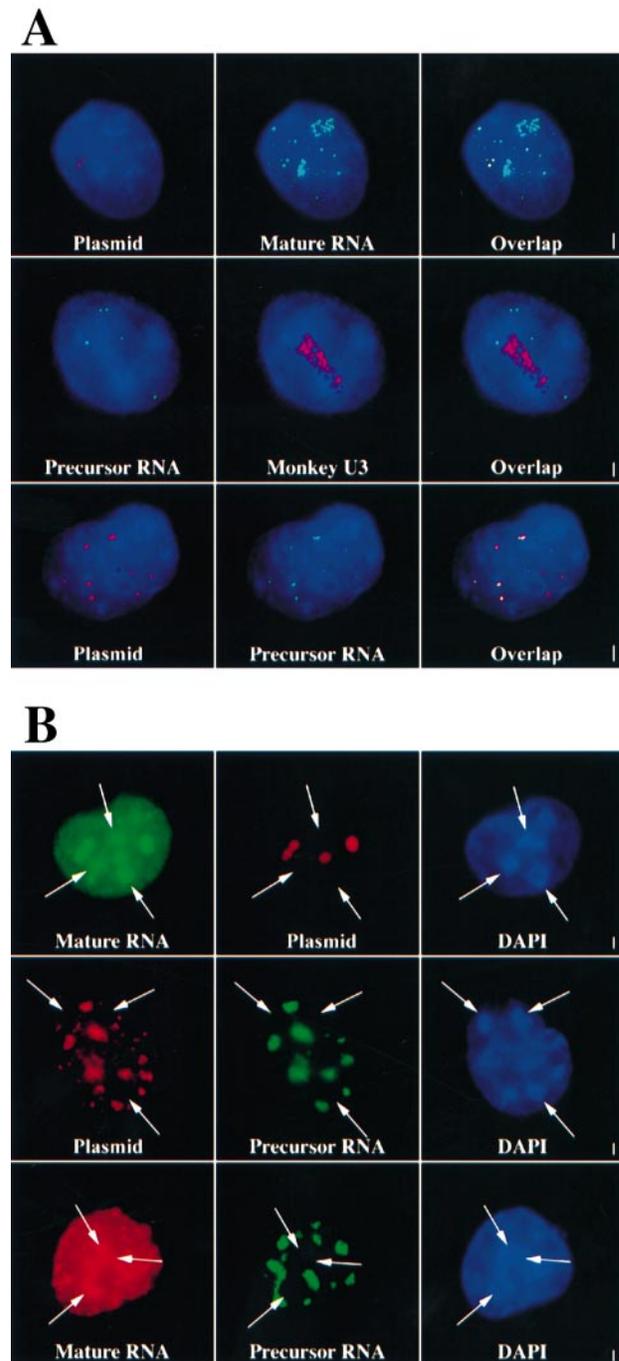
localized to distinct areas outside the nucleolus (Figure 6A, central panel; results are shown for yeast U14 only, but are identical for the artificial snoRNA). Double *in situ* hybridizations with the precursor and plasmid probes showed that the precursors localized predominantly to the same regions as the transfected plasmids (Figure 6A, lower panel).

Detection of RNA precursors at the putative transcription sites suggests that processing of immature RNAs takes place in the nucleoplasm, immediately after transcription. It is possible, though less likely, that maturation occurs in the nucleolus after targeting. In this case, processing would have to occur so rapidly that precursors cannot be detected by our assay. An attempt to resolve these two alternatives was made by analyzing the distribution patterns of non-processed and processed RNAs in monkey cells transfected with replicative plasmids. These vectors occur at a higher copy number and were expected to yield higher levels of precursors. We reasoned that this strategy could allow unprocessed RNAs to be detected at other nuclear locations.

Overproduction of RNAs was achieved when the box C/D RNA genes were expressed from the replicative plasmids pU6Yrep and pU6Arep (Figure 6B; results are shown for yeast U14, but are identical for the artificial RNA). *In situ* hybridization demonstrated that despite a much higher expression level, the precursor was still detected only at the transcription sites. Interestingly, in cells expressing the highest levels of experimental RNAs, signals were distributed over the entire nucleoplasm, in addition to the nucleolus. This last result most likely reflects saturation of box C/D RNA uptake and/or retention sites in the nucleolus, leading to nucleoplasmic accumulation of RNAs. This view is supported by our observation that natural monkey U14 snoRNA also accumulates in the nucleoplasm of cells overproducing the experimental box C/D RNAs (data not shown). Parallel hybridizations with precursor- and plasmid-specific probes showed that in overexpressing cells, the precursor RNAs localize at the transcription sites, which form a characteristic speckled pattern, and that the remaining population of RNAs in the nucleoplasm represents processed RNAs (Figure 6B).

Taken together, these results argue that in mammalian cells, precursors to the box C/D snoRNAs are produced in the nucleoplasm, processed at or near the sites of transcription and only then are localized to the nucleolus.

Discussion

The expression and localization results provide new insights into the biogenesis of the box C/D snoRNAs in eukaryotic cells. In particular, the simple *cis*-acting box C/D motif was shown to direct intranuclear trafficking of box C/D snoRNAs, from the nucleoplasmic sites of synthesis to the nucleolus. The new results also argue strongly that production and intracellular targeting of the box C/D snoRNAs are tightly coupled.

Principles of intranuclear localization of box C/D snoRNAs

The localization results obtained with the artificial box C/D snoRNAs showed that, both in yeast and mammalian cells, nucleolar targeting is mediated by the the box C/D motif. Since the box C/D motif defines one of the two major snoRNA families, we predict that it is used as a nucleolar localization signal for all box C/D snoRNAs, i.e. for scores of RNA species in various eukaryotes. In this regard, it is similar to the m⁷G cap and the poly(A) tail or the Sm-binding site, which define whole classes of RNAs and also function as determinants of RNA localization (see Hamm *et al.*, 1990; Marshallsay and Luhrmann, 1994; Huang and Carmichael, 1996; Lewis and Izaurralde, 1997). It is tempting to speculate that the unknown localization signals in other RNA families might also be shared by all family members. If true, this common property could greatly facilitate identification of such signals in the future. Thus, it is reasonable to expect that the conserved box elements which define the H/ACA snoRNA family also function in RNA targeting, as well as in RNA production.

In mammalian cells, the artificial RNAs were also detected in the coiled bodies. This last finding is consistent with earlier observations of U3 box C/D snoRNAs in the coiled bodies of animal (human HeLa) and plant (olive) cells (Jimenez-Garcia *et al.*, 1994; Raska, 1995; Olmedilla *et al.*, 1997), and reinforces striking similarities between the coiled bodies and the nucleolus. Indeed, in addition to snoRNAs, both compartments contain common proteins, including fibrillarin, Nopp140, Nap57 and Sp6 (reviewed in Brasch and Ochs, 1992). In light of the recent discovery that a box C/D snoRNA guides methylation of a splicing snRNA (K.T.Tycowski, Z.-H.You, P.Graham and J.A. Steitz, personal communication), and the fact that coiled bodies also contain splicing snRNAs (see Brasch and Ochs, 1992; Carmo-Fonseca *et al.*, 1992; Matera and Ward, 1993; Gall *et al.*, 1995), our results raise the fascinating possibility that methylation of splicing snRNAs occurs in coiled bodies.

Detection of the box C/D RNA precursors at the transcription sites, but never in the nucleolus, suggests that processing occurs at or near the sites of transcription, and the RNAs are targeted to the nucleolus as mature-length molecules. When expressed at high levels, processed RNAs accumulated throughout the entire nucleus, indicating saturation of nucleolar uptake/retention sites. Importantly, in these cells, the precursor RNAs were still confined to the transcription sites, suggesting that precursor processing was not a limiting factor, and that processing occurs in the nucleoplasm during, or soon after transcription.

A general model of box C/D snoRNA biogenesis

Both RNA maturation and nucleolar targeting appear to be mediated by the same RNA structure, the box C/D motif. Because it seems certain that this motif is a protein recognition signal, and because of its small size, it seems likely that: (i) these functions are mediated by the same protein or a small set of core proteins and (ii) both motif-dependent processes require formation of the same initial complex.

Accordingly, the major principles of box C/D snoRNA synthesis can be summarized in a simple model (Figure 7). Regardless of the nature of the primary transcript and its initial processing events, it will fold in such a way that boxes C and D are brought together to form the box C/D motif. The motif is then rapidly recognized and bound by a
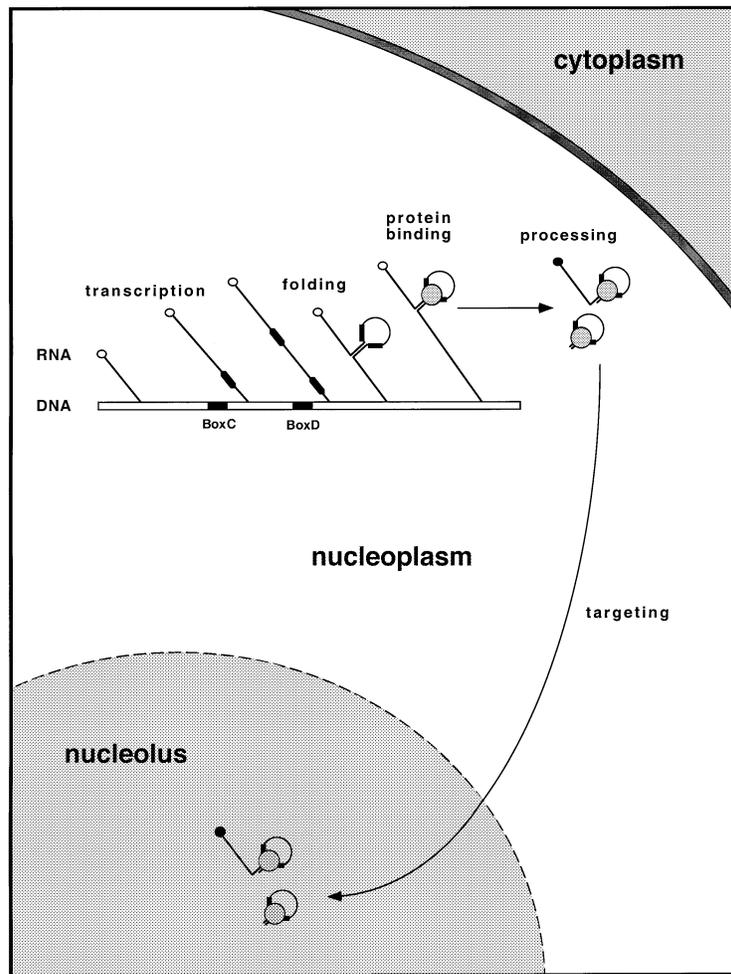
Fig. 7. General model for box C/D snoRNA biogenesis in eukaryotic cells. The model proposed is based on expression results obtained here and earlier with various box C/D snoRNAs in different cells and in different genetic contexts. The major features are predicted to be universal for all box C/D snoRNAs. Variations are likely to occur in the early steps, due to differences in genomic arrangements, i.e. introns of protein genes, or mono- or polycistronic snoRNA transcription units. Transcription occurs in the nucleoplasm. Folding of the precursor produces a functional box C/D motif. This motif is then recognized by a box C/D snoRNA family-specific binding protein(s). These events occur during (as depicted), or rapidly after transcription. A transcript then follows one of two possible pathways: (i) a precursor capped with monomethylguanosine (open circle) is hypermethylated to yield trimethylguanosine (closed circle) and trimmed at the 3′ end by exonucleases, or (ii) if uncapped, the precursor is trimmed at both ends. In both cases, protein binding via the box C/D motif is then responsible for the delivery of the snoRNA to the nucleolus.

nucleoplasmic protein which alone, or with other proteins, protects the snoRNA from degradation by exonucleases that specify the ends of the mature molecule. This initial RNP complex alone, or in conjunction with additional nucleoplasmic factors, is then delivered to the nucleolus. Translocation could occur by active transport or by simple diffusion and subsequent nucleolar binding through common protein components.

Maturation of box C/D snoRNAs can also include: (i) modification of internal nucleotides, about which little is known (see Reddy *et al.*, 1979; Wise *et al.*, 1983; Reddy and Busch, 1988); and (ii) formation of a 5′ trimethylguanosine (TMG) cap. TMG cap formation is believed to result from hypermethylation of the m^7G cap normally found in RNA polymerase II transcripts. Interestingly, hypermethylation of the *Xenopus* U3 and U8 caps occurs in the nucleoplasm, and has been shown to depend on box D and the adjoining terminal stem (Terns *et al.*, 1995). This last finding raises the possibility that hypermethylation also depends on the box C/D

motif, i.e. that TMG synthesis is activated shortly after transcription by the same motif-specific protein(s).

It seems certain that direct binding of a motif-specific protein(s) and conversion of naked RNA to a core snoRNP complex is a pivotal step in both production and localization of the box C/D snoRNAs. This arrangement provides a reliable and efficient means of integrating these two major aspects of snoRNA biogenesis, since common factors are required for both processes and render them inseparable. Such coupling might be common for other classes of eukaryotic RNA as well. For example, both processing and nuclear export of mRNAs are believed to be affected by some hnRNP proteins and components of the nuclear cap-binding complex (reviewed in Weighardt *et al.*, 1996; Lewis and Izaurralde, 1997).

In addition to integrating the various aspects of snoRNA synthesis (maturation, stability and localization), there is the intriguing possibility that the box C/D motif also links snoRNA synthesis with function. In particular, this possibility applies to the snoRNAs which guide methyl-

ation of rRNA. The guide function is mediated by a long sequence complementary to the rRNA segment to be modified, and site selection depends on a box D or D-like element (CUGA, box D′) located immediately downstream of the guide sequence. Methylation occurs in the complementary rRNA sequence, precisely five nucleotides from box D/D′. Some guide sequences occur near the 3′ end, immediately upstream of the canonical box D, whereas others occur in the interior of the RNA adjoined to box D′. Some snoRNAs contain guide sequences in both arrangements. Interestingly, we and others noted that snoRNAs with internal guide sequences and box D′ also contain an additional box C-like sequence (UGAU) at a modest distance downstream of box D′, suggesting that these RNAs have a second box C/D motif, in addition to the canonical one (Kiss-Laszlo *et al.*, 1998; D.A.Samarsky, unpublished). Results from recent mutational studies have demonstrated that the novel box C-like element (called box C′) is essential for the methylation reaction, but not for snoRNA accumulation (Kiss-Laszlo *et al.*, 1998). This finding argues that one or more proteins involved in the methylation function also bind directly or indirectly to the simple box C/D motif, and that snoRNA production and function may be connected at this level. It will be interesting to learn if the motif-binding protein(s) includes a ribose methylase enzyme.

The strict conservation of the box C/D motif among different snoRNAs in evolutionarily divergent eukaryotes indicates that the principles of box C/D snoRNA biogenesis, in particular synthesis and intranuclear targeting, are universal to all RNAs in this family and, hence, are as ancient as the eukaryotic world itself.

Materials and methods

Yeast strains and mammalian cell lines
All yeast experiments were carried out with the *S.cerevisiae* haploid strain YSD92 (*ura3-167*). This strain was prepared by sporulating strain YS152 (a/α *his3Δ/HIS3 his4-280/HIS4 trp1/TRP1 ura3-167/URA3 GAL1::U14:HIS3/U14*) described previously as a diploid obtained after mating of haploid strains YS133 and E280 (Jarmolowski *et al.*, 1990). Yeast transformants were grown on YNBD (0.67% yeast nitrogen base, 2% glucose) medium at 30°C.

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Transfections were performed by a DNA–calcium phosphate co-precipitation method (Sambrook *et al.*, 1989), using 10 μg of DNA per 3 cm dish. Cells were fixed 36 h after addition of the precipitate.

Plasmid construction
The artificial snoRNA sequences were derived from *Escherichia coli* vector pUC18 and newly created plasmids pBl-B, pBl-KS and pBl-C. The latter constructs were prepared from the pBluescript IISK(–) *E.coli* vector by deleting a *Bss*HII restriction fragment (pBl-B), deleting a *Kpn*I–*Sac*I fragment (pBl-KS) or removing a *Cla*I restriction site (pBl-C).

Yeast plasmids pRA1, pRA2, pRA3 and pRA4 were prepared from a previously created plasmid pCer (Samarsky *et al.*, 1996). This parental plasmid contains a 1.3 kb *Cla*I *S.cerevisiae* genomic DNA fragment with genes for snR190 and U14 snoRNAs, cloned into a *Cla*I site of yeast vector pRS316. The pRA1–pRA4 derivatives were made by substituting the DNA between U14 boxes C and D with DNA fragments located between and encompassing the universal primer regions of plasmids pUC18, pBl(-B), pBl(-KS) and pBl(-C), respectively. The DNA replacement fragments were prepared by PCR amplification with oligonucleotide primers SD15 (ATTTATGATCACGGTGATGAGG-AAACAGCTATGACCAT) and SD18 (CTACAGTATACGATCACTCA-GACGTAAAACGACGGCCAGTGA). Oligonucleotides SD49 (TATA-TATAATTTATGATCACGGTCTTGAGG) and SD50 (AAGATACTA-CAGTATACGATCACAGAGACG) were used for making pRA1–pRA4

derivatives containing mutant box C (box C*) or box D (box D*) elements.

Plasmid pU6Y and pU6A were obtained by cloning into the pU6+1 vector (Bertrand *et al.*, 1997) the PCR products obtained from plasmids pCer and pRA3 with the oligonucleotides SD24 and SD81 (GTGGGTAATTTGAGTTAACAGATAATATAT). Plasmids pU6Yrep and pU6Arep were obtained by cloning the SV40 origin of replication into the pU6Y and pU6A plasmids. Detailed restriction maps are available upon request.

RNA preparation and Northern blot analysis
Total RNA from yeast cells was isolated using an acidic phenol/glass bead procedure (Kohrer and Domdey, 1991). RNA from mammalian cells was prepared using the guanidine-HCl method (Sambrook *et al.*, 1989). Northern analysis was performed as described previously (Balakin *et al.*, 1993; Samarsky *et al.*, 1995). Oligonucleotide probes used for hybridization were C106 (CGATGGGTTCGTAAGCGTACTCCTACC-GTGG), specific for yeast U14 snoRNA, and SD18 (see above), specific for all artificial RNAs, except RNA expressed in mammalian cells. Oligonucleotide SD121 (GCGTAATACGACTCACTATAGGGCG-AATTGGCC) was used to probe artificial RNA expressed in mammalian cells.

In situ hybridization
Mammalian cells were fixed for 10 min at room temperature in 4% formaldehyde, 10% acetic acid, phosphate-buffered saline (PBS; 100 mM Na$_2$HPO$_4$, 20 mM KH$_2$PO$_4$, 137 mM NaCl, 27 mM KCl, pH 7.4). After two washes in PBS, cells were permeabilized by treatment with 70% ethanol for at least overnight. After rehydration in 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), 50% formamide, cells were hybridized overnight at 37°C in 40 μl of a mixture containing 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNase-free bovine seum albumin (BSA), 40 μg of *E.coli* tRNA, 2× SSC, 50% formamide, 30 ng of probe. Cells were then washed twice for 30 min at the appropriate stringency (2× SSC, 50% formamide, 37°C for oligonucleotide probes; 0.1× SSC, 50% formamide, 50°C for RNA probes). When required, slides were then processed for immunofluorescence. Digoxigenin-labeled probes were detected with sheep anti-digoxigenin antibodies (1/200, Boehringer Mannheim), and then with donkey anti-sheep antibodies conjugated to fluorescein (1/150, Sigma). Slides were incubated for 1 h at 37°C in 2× SSC, 8% formamide, 2 mM vanadyl-ribonucleoside complex, 0.2% RNase-free BSA, and washed twice for 15 min in 2× SSC, 8% formamide at room temperature.

Yeast cells were prepared for *in situ* hybridization as previously described (Long *et al.*, 1995), except that cells were fixed for 10 min at room temperature by addition of 10 ml of 2% formaldehyde, 5% acetic acid, to 45 ml of culture medium. Hybridization was performed as for mammalian cells.

Slides were mounted in 90% glycerol, PBS, 1 mg/ml *p*-phenylendiamine, 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI).

Image acquisition and processing
Images were captured using a CellScan system (Scanalytics, Fairfax, VA), with a CH250 CCD camera (Photometrics, Tucson, AZ), mounted on a Provis AX70 epifluorescence microscope (Olympus, Melville, NY). Except for Figure 6B, where only a single plane was recorded, red- and green-filtered images were captured every 200 nm on the *z*-axis, for a total of 20–25 images, and were then deconvolved using EPR (exhaustive photon reassignment) software (Scanalytics). A single median plane was recorded for blue-filtered images. Restored three-dimensional images were observed to select one or a few consecutive planes which were projected onto the *z*-axis. The resulting two-dimensional images were then scaled so that the signal occupies the full dynamic range of light intensities, pseudocolored and superimposed.

Preparation of probes for in situ hybridization
The probes used to detect natural monkey snoRNAs were amino-modified DNA oligonucleotides of the following sequences: T*AT-CCAAGGAAGGT*AGTTGCCAACAT*AAGACTTTCTGGT*GGAA-ACTACGAAT*T for U14 and T*CTTCCTCGTGGTTTT*CGGTG-CTCTACACGT*T for U3 snoRNAs (the amino-modified T nucleotides are marked with asterisks). These oligonucleotides were synthesized and labeled with Cy3 (U14) or Cy2 (U3; Amersham), as previously described (Kislauskis *et al.*, 1993).

All other probes were RNAs synthesized *in vitro* with T7 or T3 RNA polymerase. The DNA templates were: PCR products encompassing the RNA coding sequence for the artificial box C/D RNA and yeast U14

(yielding probes of 130 and 120 nt, respectively); a plasmid containing the yeast U14 transcribed sequences 3′ to box D for the precursor probe (linearized to yield an ~130 nucleotide transcript); and pBluescript SK(+) deleted of its polylinker for the plasmid-specific probe (linearized with *Pvu*II to yield an ~230 nucleotide probe).

Except for the precursor probe, the RNA probes were labeled with a novel procedure. This protocol was developed to allow for high incorporation of the fluorescent label (typically 5–10%, which is 5–10 times higher than can be obtained by labeling during transcription), and for quantitative studies, since it renders the use of antibodies for signal amplification unnecessary. It was developed as a versatile, low-cost alternative to amino-modified synthetic oligonucleotides (Kislauskis *et al*., 1993), and was derived from an existing protocol used to label RNA for microinjection (Wang *et al*., 1991). Details of the procedure are available on the web site http://Singerlab.aecom.yu.edu. Briefly, RNAs were synthesized using amino-allyl UTP instead of UTP during transcription. Transcription reactions were phenol extracted, RNA was precipitated, resuspended in 1× SSC, and unincorporated nucleotides were removed by gel filtration (1× SSC-buffered P30 micro-spin column, Bio-Rad). RNA was again ethanol precipitated and resuspended in water. Labeling was initiated by mixing 4–50 µg of RNA (depending on the final specific activity desired), resuspended in 70 µl of 0.1 M NaHCO$_3$ buffer, pH 8.8, with one vial of Cy3, or 1 mg of Oregon green 488 (Molecular Probes) resuspended in 30 µl of dimethylsulfoxide (DMSO). Labeling was performed for 48 h in the dark, at room temperature, with occasional vortexing. Unreacted dye was removed by two rounds of ethanol precipitation and washing. Specific activity of the probes was calculated by absorption spectroscopy.

The probes for the artificial RNA and yeast U14 were labeled with a specific activity 20 times lower than for the plasmid probe. This ensured that these probes detected RNA preferentially and not the plasmid. Indeed, a labeled plasmid probe of similar specific activity gave signals much fainter than the ones detected with the artificial and yeast U14 probes (data not shown). The probe for the precursor was labeled with digoxigenin-UTP during transcription. This probe detected predominantly RNA, not the plasmid, since an antisense precursor probe failed to give signals (data not shown).

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References

Bachellerie,J.-P. and Cavaille,J. (1997) Guiding ribose methylation of rRNA. *Trends Biochem. Sci*., **22**, 257–261.

Balakin,A.G., Schneider,G.S., Corbett,M.S., Ni,J. and Fournier,M.J. (1993) SnR31, snR32, and snR33: three novel, non-essential snRNAs from *Saccharomyces cerevisiae*. *Nucleic Acids Res*., **21**, 5391–5397.

Balakin,A.G., Lempicki,R.A., Huang,G.M. and Fournier,M.J. (1994) *Saccharomyces cerevisiae* U14 small nuclear RNA has little secondary structure and appears to be produced by post-transcriptional processing. *J. Biol. Chem*., **269**, 739–746.

Balakin,A.G., Smith,L. and Fournier,M.J. (1996) The RNA world of the nucleolus: two major families of small RNAs defined by different box elements with related functions. *Cell*, **86**, 823–834.

Baserga,S.J., Gilmore-Hebert,M. and Yang,X.W. (1992) Distinct molecular signals for nuclear import of the nucleolar snRNA, U3. *Genes Dev*., **6**, 1120–1130.

Bertrand,E. *et al*. (1997) The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA*, **3**, 75–88.

Brasch,K. and Ochs,R.L. (1992) Nuclear bodies (NBs): a newly 'rediscovered' organelle. *Exp. Cell Res*., **202**, 211–223.

Caffarelli,E., Fatica,A., Prislei,S., De Gregorio,E., Fragapane,P. and Bozzoni,I. (1996) Processing of the intron-encoded U16 and U18 snoRNAs: the conserved C and D boxes control both the processing reaction and the stability of the mature snoRNA. *EMBO J*., **15**, 1121–1131.

Carmo-Fonseca,M., Pepperkok,R., Carvalho,M.T. and Lamond,A.I. (1992) Transcription-dependent colocalization of the U1, U2, U4/U6, and U5 snRNPs in coiled bodies. *J. Cell Biol*., **117**, 1–14.

Cavaille,J. and Bachellerie,J.-P. (1996) Processing of fibrillarin-associated snoRNAs from pre-mRNA introns: an exonucleolytic process exclusively directed by a common stem-box terminal structure. *Biochimie*, **78**, 443–456.

Cavaille,J., Nicoloso,M. and Bachellerie,J.-P. (1996) Targeted ribose methylation of RNA *in vivo* directed by tailored antisense RNA guides. *Nature*, **383**, 732–735.

Dichtl,B., Stevens,A. and Tollervey,D. (1997) Lithium toxicity in yeast is due to the inhibition of RNA processing enzymes. *EMBO J*., **16**, 7184–7195.

Dunbar,D.A. and Baserga,S.J. (1998) The U14 snoRNA is required for 2′-*O*-methylation of the pre-18S rRNA in *Xenopus* oocytes. *RNA*, **4**, 195–204.

Eckner,R., Ellmeier,W. and Birnstiel,M.L. (1991) Mature mRNA 3′ end formation stimulates RNA export from the nucleus. *EMBO J*., **10**, 3513–3522.

Gall,J.G., Tsvetkov,A., Wu,Z. and Murphy,C. (1995) Is the sphere organelle/coiled body a universal nuclear component? *Dev. Genet*., **16**, 25–35.

Ganot,P., Caizergues-Ferrer,M. and Kiss,T. (1997) The family of box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation. *Genes Dev*., **11**, 941–956.

Gerbi,S.A. (1995) Small nucleolar RNA. *Biochem. Cell. Biol*., **73**, 845–858.

Hamm,J., Darzynkiewicz,E., Tahara,S.M. and Mattaj,I.W. (1990) The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell*, **62**, 569–577.

Huang,G.M., Jarmolowski,A., Struck,J.C.R. and Fournier,M.J. (1992) Accumulation of U14 small nuclear RNA in *Saccharomyces cerevisiae* requires box C, box D, and a 5′, 3′ terminal stem. *Mol. Cell. Biol*., **12**, 4456–4463.

Huang,Y. and Carmichael,G.C. (1996) Role of polyadenylation in nucleocytoplasmic transport of mRNA. *Mol. Cell. Biol*., **16**, 1534–1542.

Izaurralde,E. and Mattaj,I.W. (1995) RNA export. *Cell*, **81**, 153–159.

Jacobson,M.R., Cao,L.G., Wang,Y.L. and Pederson,T. (1995) Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. *J. Cell Biol*., **131**, 1649–1658.

Jacobson,M.R., Cao,L.G., Taneja,K., Singer,R.H., Wang,Y.L. and Pederson,T. (1997) Nuclear domains of the RNA subunit of RNase P. *J. Cell. Sci*., **110**, 829–837.

Jarmolowski,A., Zagorski,J., Li,H.V. and Fournier,M.J. (1990) Identification of essential elements in U14 RNA of *Saccharomyces cerevisiae*. *EMBO J*., **9**, 4503–4509.

Jimenez-Garcia,L.F., Segura-Valdez,M.L., Ochs,R.L., Rothblum,L.I., Hannan,R. and Spector,D.L. (1994) Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell*, **5**, 955–966.

Kislauskis,E.H., Li,Z., Singer,R.H. and Taneja,K.L. (1993) Isoform-specific 3′-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J. Cell Biol*., **123**, 165–172.

Kiss-Laszlo,Z., Henry,Y., Bachellerie,J.-P., Caizergues-Ferrer,M. and Kiss,T. (1996) Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. *Cell*, **85**, 1077–1088.

Kiss-Laszlo,Z., Henry,Y. and Kiss,T. (1998) Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. *EMBO J*., **17**, 797–807.

Kohrer,K. and Domdey,H. (1991) Preparation of high molecular weight RNA. *Methods Enzymol*., **194**, 398–405.

Leader,D.J., Sanders,J.F., Waugh,R., Shaw,P. and Brown,J.W.S. (1994) Molecular characterisation of plant U14 small nucleolar RNA genes: closely linked genes are transcribed as polycistronic U14 transcripts. *Nucleic Acids Res*., **22**, 5196–5203.

Leader,D.J., Clark,G.P., Watters,J., Beven,A.F., Shaw,P.J. and Brown,J.W.S. (1997) Clusters of multiple different small nucleolar RNA genes in plants are expressed as and processed from polycistronic pre-snoRNAs. *EMBO J*., **16**, 5742–5751.

Leverette,R.D., Andrews,M.T. and Maxwell,E.S. (1992) Mouse U14 snRNA is processed intron of the cognate hsc70 heat shock pre-messenger RNA. *Cell*, **71**, 1215–1221.

Lewis,J.D. and Izaurralde,E. (1997) The role of the cap structure in RNA processing and nuclear export. *Eur. J. Biochem*., **247**, 461–469.

Li,H.V. and Fournier,M.J. (1992) U14 function in *Saccharomyces cerevisiae* can be provided by large deletion variants of yeast U14 and hybrid mouse–yeast U14 RNAs. *EMBO J.*, **11**, 683–689.

Li,H.V., Zagorski,J. and Fournier,M.J. (1990) Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae. Mol. Cell. Biol.*, **10**, 1145–1152.

Liang,W.Q. and Fournier,M.J. (1995) U14 base-pairs with 18S rRNA: a novel snoRNA interaction required for rRNA processing. *Genes Dev.*, **9**, 2433–2443.

Liu,J.C. and Maxwell,E.S. (1990) Mouse U14 snRNA is encoded in an intron of the mouse cognate hsc70 heat shock gene. *Nucleic Acids Res.*, **18**, 6565–6571.

Long,R.M., Elliott,D.J., Stutz,F., Rosbash,M. and Singer,R.H. (1995) Spatial consequences of defective processing of specific yeast mRNAs revealed by fluorescent *in situ* hybridization. *RNA*, **1**, 1071–1078.

Maden,T. (1996) Click here for methylation. *Nature*, **383**, 675–676.

Marshallsay,C. and Luhrmann,R. (1994) *In vitro* nuclear import of snRNPs: cytosolic factors mediate m3G-cap dependence of U1 and U2 snRNP transport. *EMBO J.*, **13**, 222–231.

Matera,A.G. and Ward,D.C. (1993) Nucleoplasmic organization of small nuclear ribonucleoproteins in cultured human cells. *J. Cell Biol.*, **121**, 715–727.

Maxwell,E.S. and Fournier,M.J. (1995) The small nucleolar RNAs. *Annu. Rev. Biochem.*, **35**, 897–934.

Mereau,A., Fournier,R., Gregoire,A., Mougin,A., Fabrizio,P., Luhrmann,R. and Branlant,C. (1997) An *in vivo* and *in vitro* structure–function analysis of the *Saccharomyces cerevisiae* U3A snoRNP: protein–RNA contacts and base-pair interaction with the pre-ribosomal RNA. *J. Mol. Biol.*, **273**, 552–571.

Nicoloso,M., Qu,L.H., Michot,B. and Bachellerie,J.-P. (1996) Intron-encoded, antisense small nucleolar RNAs: the characterization of nine novel species points to their direct role as guides for the 2′-O-ribose methylation of rRNAs. *J. Mol. Biol.*, **260**, 178–195.

Olmedilla,A., de Dios Alche,J. and Rodriguez-Garcia,M.I. (1997) Nucleolar evolution and coiled bodies during meiotic prophase in *Olea europaea*: differential localization of nucleic acids. *Eur. J. Cell Biol.*, **74**, 181–189.

Peculis,B.A. and Mount,S.M. (1996) Ribosomal RNA: small nucleolar RNAs make their mark. *Curr. Opin. Cell Biol.*, **6**, 1413–1415.

Peculis,B.A. and Steitz,J.A. (1994) Sequence and structural elements critical for U8 snRNP function in *Xenopus* oocytes are evolutionarily conserved. *Genes Dev.*, **8**, 2241–2255.

Petfalski,E., Dandekar,T., Henry,Y. and Tollervey,D. (1998) Processing of the precursor to small nucleolar RNAs and rRNAs requires common components. *Mol. Cell. Biol.*, **18**, 1181–1189.

Raska,I. (1995) Nuclear ultrastructures associated with the RNA synthesis and processing. *J. Cell Biochem.*, **59**, 11–26.

Reddy,R. and Busch,H. (1988) Small nuclear RNAs: RNA sequences, structure, and modifications. In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, New York, NY, pp. 1–37.

Reddy,R., Henning,D. and Busch,H. (1979) Nucleotide sequence of nucleolar U3B RNA. *J. Biol. Chem.*, **254**, 11097–11105.

Samarsky,D.A. and Fournier,M.J. (1998) Functional mapping of the U3 small nucleolar RNA from the yeast *Saccharomyces cerevisiae. Mol. Cell. Biol.*, **18**, 3431–3444.

Samarsky,D.A., Balakin,A.G. and Fournier,M.J. (1995) Characterization of three new snRNAs from *Saccharomyces cerevisiae*: snR34, snR35 and snR36. *Nucleic Acids Res.*, **23**, 2548–2554.

Samarsky,D.A., Schneider,G.S. and Fournier,M.J. (1996) An essential domain in *Saccharomyces cerevisiae* U14 snoRNA is absent in vertebrates, but conserved in other yeasts. *Nucleic Acids Res.*, **24**, 2059–2066.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Singer,R.H. and Green,M.R. (1997) Compartmentalization of eukaryotic gene expression: causes and effects. *Cell*, **91**, 291–294.

Smith,C.M. and Steitz,J.A. (1997) Sno storm in the nucleolus: new roles for myriad small RNPs. *Cell*, **89**, 669–672.

Terns,M.P., Grimm,C., Lund,E. and Dahlberg,J. (1995) A common maturation pathway for small nucleolar RNAs. *EMBO J.*, **14**, 4860–4871.

Tollervey,D. and Kiss,T. (1997) Function and synthesis of small nucleolar RNAs. *Curr. Opin. Cell Biol.*, **9**, 337–342.

Tyc,K. and Steitz,J.A. (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *EMBO J.*, **8**, 3113–3119.

Tycowski,K.T., Smith,C.M., Shu,M.D. and Steitz,J.A. (1996) A small nucleolar RNA requirement for site-specific ribose methylation of rRNA in *Xenopus. Proc. Natl Acad. Sci. USA*, **93**, 14480–14485.

Wang,J., Cao,L.G., Wang,Y.L. and Pederson,T. (1991) Localization of pre-messenger RNA at discrete nuclear sites. *Proc. Natl Acad. Sci. USA*, **88**, 7391–7395.

Watkins,N.J., Leverette,R.D., Xia,L., Andrews,M.T. and Maxwell,E.S. (1996) Elements essential for processing intronic U14 snoRNA are located at the termini of the mature snoRNA sequence and include conserved nucleotide boxes C and D. *RNA*, **2**, 118–133.

Weighardt,F., Biamonti,G. and Riva,S. (1996) The roles of heterogeneous nuclear ribonucleoproteins (hnRNP) in RNA metabolism. *BioEssays*, **18**, 747–756.

Wise,J.A., Tollervey,D., Maloney,D., Swerdlow,H., Dunn,E.J. and Guthrie,C. (1983) Yeast contains small nuclear RNAs encoded by single copy genes. *Cell*, **35**, 743–751.

Xia,L., Liu,J.C., Sage,C., Trexler,E.B., Andrews,M.T. and Maxwell,E.S. (1995) Intronic U14 snoRNAs of *Xenopus laevis* are located in two different parent genes and can be processed from introns during early oogenesis. *Nucleic Acids Res.*, **23**, 4844–4849.

Xia,L., Watkins,N.J. and Maxwell,E.S. (1997) Identification of specific nucleotide sequences and structural elements required for intronic U14 snoRNA processing. *RNA*, **3**, 17–26.

Zagorski,J., Tollervey,D. and Fournier,M.J. (1988) Characterization of an SNR gene locus in *Saccharomyces cerevisiae* that specifies both dispensable and essential small nuclear RNAs. *Mol. Cell. Biol.*, **8**, 3282–3290.

Zhang,G., Taneja,K.L., Singer,R.H. and Green,M.R. (1994) Localization of pre-mRNA splicing in mammalian nuclei. *Nature*, **372**, 809–812.

Zwieb,C. (1997) The uRNA database. *Nucleic Acids Res.*, **25**, 102–103.