

Localization and processing from a polycistronic precursor of novel snoRNAs in maize

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SUMMARY

We have shown previously that groups of U14 snoRNA genes are clustered with other, novel snoRNAs in maize. These genes are transcribed polycistronically from an upstream promoter to give a precursor snoRNA, which is processed by a splicing-independent mechanism. The clusters contain both box C/D snoRNAs, thought to guide rRNA O-ribose methylations, and the first plant box H/ACA snoRNA so far identified, thought to guide an rRNA pseudo-uridylation. Here we show that four novel snoRNAs identified as members of U14-containing gene clusters each show distinct sub-nucleolar localizations. Two of the snoRNAs (snoR2, a box H/ACA snoRNA, and snoR3, a box C/D snoRNA) colocalise closely with nucleolar rDNA transcription sites. A third box C/D snoRNA, U49, is localised to a more extended region which includes the transcription sites. On the other hand snoR1, another box C/D snoRNA, is located in a quite different region of the

nucleolus, and shows a similar distribution to that of 7-2/MRP, a snoRNA involved in the later pre-rRNA cleavage reactions. This may indicate that this snoRNA is involved at later stages of processing, whereas the other snoRNAs are involved early or cotranscriptionally. Probes to intergenic spacer regions of the precursor snoRNA have been used to determine the location of the precursor. This shows a clear labelling of both the dense fibrillar component of the nucleolus, and of coiled bodies. This distribution implies that the polycistronic precursor is imported into the nucleolus for processing to the mature snoRNAs, and that the import or processing pathway involves coiled bodies.

Key words: snoRNA, RNA processing, Nucleolus, Polycistronic, Plant

INTRODUCTION

The biogenesis of ribosomes in the nucleolus of eukaryotic organisms involves the maturation of the 45S precursor rRNA into three of the ribosomal RNA species, 18S, 5.8S and 28S/25S (Hadjiolov, 1985). Cleavage of the pre-rRNA requires several small nucleolar RNA molecules (e.g. U3, U8, U14, U17, U22, E2, E3, 7-2/MRP), which have been ascribed roles at different stages of the series of cleavage reactions (Enright et al., 1996; Lafontaine and Tollervey, 1995; Maxwell and Fournier, 1995; Mishra and Elicieri, 1997; Tollervey and Kiss, 1997). Besides cleavage and removal of the 5' and 3' external spacers and the two internal spacers, the maturation process also requires 2'-O-ribose methylation of about 100 specific nucleotides and conversion of around 100 uridine bases to pseudouridine in higher eukaryotes (Maden, 1990a,b; Smith and Steitz, 1997; Tollervey and Kiss, 1997; for recent reviews see Bachellerie and Cavaille, 1997; Ofengand and Bakin, 1997).

To date, more than 80 snoRNAs have been identified, and most are thought to be required for the post-transcriptional ribose methylation and pseudouridylation of the rRNAs

(Bachellerie et al., 1995; Balakin et al., 1996; Cavaille et al., 1996; Ganot et al., 1997a,b; Kiss-László et al., 1996; Ni et al., 1997; Nicoloso et al., 1996; Tycowski et al., 1996). All the snoRNAs, except 7-2/MRP, can be classified into two groups. Box C/D snoRNAs have box C (consensus UGAUGA) and box D (consensus CUGA) sequences adjacent to terminal inverted repeats, and are thought to form a stem/loop secondary structure involving the 5' and 3' termini and bringing boxes C and D into juxtaposition. This structure appears to be the binding site for core proteins and is required for snoRNA processing and accumulation (Caffarelli et al., 1996; Cavaille and Bachellerie, 1996; Maxwell and Fournier, 1995; Tycowski et al., 1993; Watkins et al., 1996; Xia et al., 1997). Box C/D snoRNPs also associate with the nucleolar protein fibrillarin, and most contain regions of between 10 and 21nt which are complementary to one of the rRNA species and which function in specifying the position of 2'-O-ribose methylation of the rRNAs (Bachellerie and Cavaille, 1997; Bachellerie et al., 1995; Cavaille et al., 1996; Kiss-László et al., 1996; Nicoloso et al., 1996). The other group of snoRNAs have been characterized as box H/ACA snoRNAs, and have a secondary structure comprising hairpin-hinge-hairpin-tail (Balakin et al., 1996; Ganot et al., 1997b).

The hinge region contains the box H sequence (ANANNA) and the tail region has the ACA sequence 3nt from the 3' end. These elements are essential for accumulation of the snoRNAs and form the binding site for a set of snoRNP proteins (Balakin et al., 1996; Bousquet-Antonelli et al., 1997; Ganot et al., 1997b). These snoRNAs act as guide RNAs in the pseudouridylation of rRNAs (Ganot et al., 1997a; Ni et al., 1997).

In animals and yeast, the majority of snoRNAs are encoded within introns, and are processed by a largely splicing-dependent mechanism involving exonucleolytic trimming of linearised snoRNA-containing lariats (Cavaillé and Bachellerie, 1996; Kiss and Filipowicz, 1995; Lafontaine and Tollervey, 1995; Tollervey and Kiss, 1997). In contrast, we have shown previously that groups of U14 snoRNAs genes are clustered with other, novel snoRNAs in maize (Leader et al., 1994, 1997). The novel snoRNAs included both box C/D snoRNAs and the first box H/ACA snoRNA so far identified in plants. They were non-intronic, and were transcribed polycistronically from an upstream promoter to give a precursor snoRNA (pre-snoRNA) transcript. The processing of the precursor was splicing-independent.

We have also shown that different plant snoRNAs, known to be involved in the cleavage reactions in other eukaryotes, are concentrated in different regions of the nucleolus of plant root meristematic cells (Beven et al., 1996). U3 and U14 were found in the regions surrounding the active genes, in a domain corresponding to subregions of the dense fibrillar component (DFC) identified by electron microscopy. This region was also characterised by a high concentration of RNA species containing the 5' external transcribed spacer (5' ETS) (Shaw et al., 1995) present in the nascent and newly completed pre-rRNAs. In contrast, 7-2/MRP was concentrated in the nucleolar region surrounding this DFC region, which included the granular component. The probe to the 5' ETS did not label this region significantly, whereas a probe to ITS1 labelled it strongly (Beven et al., 1996). These results suggested that there is a spatial separation of early and late rRNA processing steps within the nucleolus, and that the snoRNAs involved in these steps are localised accordingly.

In this paper we describe the nucleolar localization of the novel maize snoRNAs and examine that of the polycistronic pre-snoRNA. We show that each of the novel snoRNAs has a characteristic nucleolar location. Most of the novel snoRNAs, including the box H/ACA snoRNA, broadly colocalize with fibrillarin, a marker for the dense fibrillar component of the nucleolus. However, one snoRNA, snoR1, is concentrated in the surrounding region, previously characterized by the presence of 7-2/MRP and ITS1 in the pre-rRNAs. Furthermore, localization of *in situ* probes to three intergenic spacer regions of the polycistronic pre-snoRNA show that the precursor is present in the nucleoli and also in coiled bodies, nuclear organelles often associated with the nucleolus but which also contain spliceosomal components (Beven et al., 1995). These results show that processing of the snoRNA precursors occurs in the nucleolus and suggests that either processing or import of the precursor may also involve the coiled bodies.

MATERIALS AND METHODS

Materials

Seeds of *Zea mays* L. (cultivar J6323C) were imbibed in aerated water

for 12 hours then germinated at 25°C for 2 days on water-soaked tissue paper.

Probes

Probes were made as described previously (Beven et al., 1996; Shaw et al., 1995) by incorporation of digoxigenin-UTP by *in vitro* transcription reactions. Probes to detect pre-snoRNA polycistronic transcripts were prepared from three intergenic regions from the snoRNA cluster of MzU14.1 (see Fig. 4). Firstly, a 286 bp *HindIII/HincII* fragment located between the snoR1.1 and U49.1 genes (Leader et al., 1994) was isolated and cloned into the *HindIII* and *SmaI* sites of pGEM3Zf(+) to produce probe PG29. Secondly, intergenic sequences between U14.1a and U14.1b, and between U14.1 ψ endo and U14.1c (Leader et al., 1994) were amplified by PCR and cloned into pGEM7Zf(-), to produce probes P7AB and P7 ψ C, respectively. Complementary probes were generated from all plasmids using T7 RNA polymerase following linearisation of the plasmids with *HindIII*.

Immunofluorescence labelling

Antibodies to fibrillarin were gifts from Dr Michael Pollard, Scripps Research Institute, La Jolla, USA and Prof. Marvin Fritzler, University of Calgary, Canada. Although we had previously used the monoclonal antibody 72B9 for pea and other dicot plants (Beven et al., 1996), this antibody failed to label maize fibrillarin, presumably because the epitope recognised is absent in this monocot. We therefore used a human autoimmune serum (#346) which did cross-react with maize fibrillarin. For immunofluorescence with anti-fibrillarin, the terminal 3-5 mm of the radicle was excised and fixed in 4% (w/v) formaldehyde (freshly made from paraformaldehyde) in PEM buffer (PEM: 50 mM Pipes/KOH, pH 6.9, 5 mM EGTA, 5 mM MgSO₄) for 1 hour at room temperature. After washing 3 times in TBS (TBS: 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM KCl), 30-40 μ m vibratome (series 1000 from TAAB) sections were cut under water and dried down on to multiwell slides coated with glutaraldehyde-activated γ -aminopropyl triethoxy silane (APTES). Sections of this thickness typically contained 2-3 layers of cells; nearly all nuclei were entirely contained within the section, and only nuclei that were clearly complete were analyzed. Sections were permeabilized by incubation with 2% (w/v) cellulase (Onozuka R-10) in TBS for 1 hour at room temperature, then incubated in antibody #346 diluted 1:100 in TBS containing 3% w/v bovine serum albumin, either for 1 hour at room temperature or overnight at 4°C, then washed and incubated in secondary antibody (goat anti-human IgG fluorescein conjugate, from Sigma). Finally, after washing, the nuclei were counterstained with the DNA dye DAPI (4',6'-diamidino-2-phenylindole).

BrUTP incorporation into tissue

For BrUTP incorporation into tissue sections, the methods previously described were used (Thompson et al., 1997). Briefly, vibratome sections of unfixed root tips were cut in 'modified physiological buffer' (Hozak et al., 1994) (MPB: 100 mM potassium acetate, 20 mM KCl, 20 mM Hepes, 1 mM MgCl₂, 1 mM ATP (disodium salt, from Sigma), 1% v/v thiodiglycol, 2 μ m/ml aprotinin, 0.5 mM PMSF (phenylmethylsulphonyl fluoride), pH 7.4 with KOH) containing 1 M hexylene glycol (2 methyl, 2,4. pentane diol). All treatments were carried out at room temperature. Tissue sections were treated with MPB/0.05%, v/v, Tween-20 (BDH) for 1 minute, then washed with MPB, before incubation with the transcription mix: 500 μ m CTP (sodium salt, Pharmacia), 500 μ m GTP (sodium salt, Pharmacia), 250 μ m BrUTP (sodium salt, Sigma), 125 μ m MgCl₂, 100 U/ml RNA Guard (Pharmacia) made up in MPB) for 3 minutes. After the transcription reaction, the sections were washed with MPB, and then fixed in 4% formaldehyde in PEM buffer for 1 hour. After fixation, the sections were washed with TBS and then with water. They were then allowed to dry down onto activated APTES-treated slides for at least 3 hours, or overnight. The sections were permeabilized by

incubation with 2% (w/v) cellulase (Onozuka R-10) in TBS for 1 hour at room temperature, then washed with TBS, before incubation for 1 hour with mouse anti-BrdU (Boehringer). For fluorescence detection, a secondary Cy3-conjugated anti-mouse (Jackson Immunoresearch) antibody was used. All antibodies were diluted into TBS + 3% bovine serum albumin (Sigma).

In situ labelling

For in situ labelling root tips were fixed and vibratome sections were cut as for immunofluorescence. After treatment with cellulase and washing, probe mix was applied, and the slides were incubated overnight in a moist chamber at 37°C. The probe mix contained: ~200 ng/μl digoxigenin-labelled probe; ~1,000 ng/μl unlabelled RNA transcribed from a plasmid containing an unrelated insert; 50% deionised formamide (Fisons, reagent grade), 10% dextran sulphate (Sigma, sodium salt), 300 mM NaCl, 10 mM Pipes, pH 8.0, 1 mM EDTA. After incubation the slides were washed in excess 0.1× SSC at 50°C for 1 hour. The probe was then detected by either cy3- or fluorescein-conjugated antibodies as described previously (Shaw et al., 1995). In order to combine in situ labelling with immunofluorescence detection of fibrillarin or BrUTP incorporation, the immunofluorescence detection, including the fluorescent secondary antibody stage was carried out first. The sections were then fixed again with 4% formaldehyde and washed. Probe mix was then applied and incubated, and detected as above.

Optical microscopy

Confocal optical section stacks were collected using a Bio-Rad MRC-600 or a Bio-Rad MRC-1000UV confocal scanning microscope as described previously (Beven et al., 1996). Images were transferred to a PC or a Macintosh computer and assembled into composite images using Adobe Photoshop and NIH-Image, a public domain program for the Macintosh written by Wayne Rasband and available via anonymous ftp from zippy.nimh.nih.gov. Images were printed on a Tektronix Phaser IISDX dye sublimation printer.

RESULTS

Distribution of snoRNAs

Single confocal sections from in situ images using U49 (Fig.

1A) and snoR1 (Fig. 1B) probes, counterstained with DAPI are shown in Fig. 1. The nucleoli are clearly visible as the cavities in the DAPI images, and the snoRNAs are localized to the nucleoli. However, the detailed distribution of these two snoRNAs within the nucleoli is clearly different. U49 (Fig. 1A) shows a distribution which is similar to that of U14 and the ETS probe of the pre-rRNA (Beven et al., 1996), whereas snoR1 shows a similar distribution to that we previously showed for MRP (Beven et al., 1996). To determine more precisely the localization of these and the other snoRNAs, we carried out double labelling in each case with fibrillarin, which is localized in the dense fibrillar component, in order to provide a standard 'landmark' structure within the nucleolus. The results of these double labelling experiments are shown in Fig. 2. In each case the relevant snoRNA is shown in red, fibrillarin is shown in green, and the two probes are overlaid in the right hand panels.

The distribution of U49, a box C/D snoRNA which has putative regions of complementarity to 25S RNA, is shown in Fig. 2A. This snoRNA occupies a broadly similar region of the nucleolus to fibrillarin, but is also located in the central nucleolar cavity (arrow). Overall the distribution has a speckled or punctate appearance within the regions labelled by fibrillarin. The coiled bodies, which are strongly labelled by the fibrillarin antibody (arrowhead in green panel) are only very faintly labelled by the U49 probe (red).

The distribution of snoR1 is shown in Fig. 2B. This is also a box C/D snoRNA with putative regions of complementarity to 25S RNA. The distribution within the nucleolus is quite different from that of U49. The labelling pattern is complementary to that of fibrillarin. Thus regions of high fibrillarin labelling correlate with dark regions of snoR1 labelling, and vice versa. This is clearly seen in the right hand panel of Fig. 2B showing the superposition of fibrillarin and snoR1 labelling. This is a very similar distribution to that we showed for the distribution of 7-2/MRP and includes the region labelled by a probe to the ITS1 region of the pre-rRNA (Beven et al., 1996). It includes the granular component of the

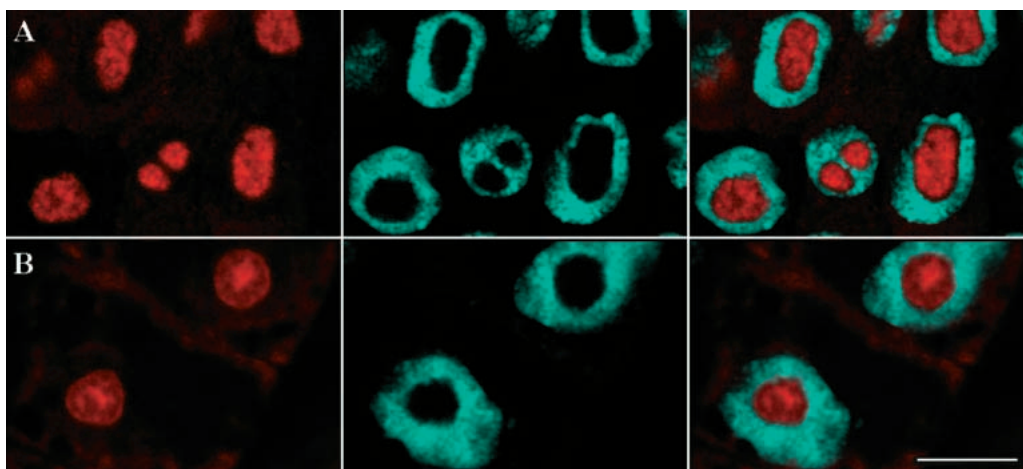
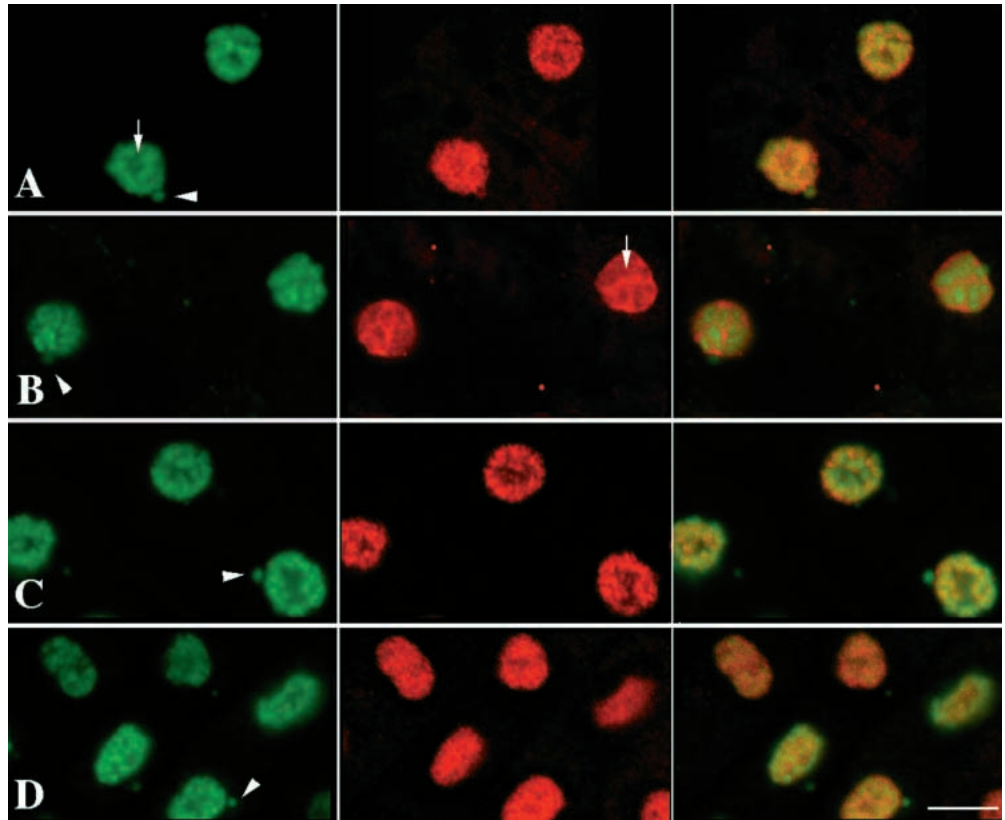


Fig. 1. Confocal image of a maize root section labelled by fluorescence in situ hybridization (left hand, red panels) and counterstained with DAPI to show the nuclear chromatin (central, blue panels). In each case the right hand panel shows the superimposition of the two labels. (A) U49 is distributed in a region of the nucleolus which broadly corresponds to the dense fibrillar component. (B) snoR1 is distributed quite differently within the nucleolus, in a region which is similar to that previously shown for 7-2/MRP, a peripheral labelling corresponding to the granular component, and labelling of the central nucleolar cavity. Bar, 10 μm.

Fig. 2. Confocal images of maize root sections labelled with an antibody to fibrillarin (green) and by fluorescence in situ hybridization to each of the four novel snoRNAs (red). In each case the right hand panel shows the superimposition of the two labels. (A) U49 (box C/D) shows a similar pattern to fibrillarin, although the nucleolar cavity region is often labelled by the snoRNA probe (arrow). The coiled bodies are clearly labelled by anti-fibrillarin, but very weakly labelled by the snoRNA probe (arrowhead). (B) snoR1 (box C/D) shows a complementary labelling pattern to fibrillarin, and the nucleolar cavity is usually strongly labelled (arrow in red panel). The coiled bodies are not labelled by the snoRNA probe (arrowhead in green panel). (C) snoR2 (box H/ACA) labels a very similar region to fibrillarin, but the coiled bodies are not labelled (arrowhead in green panel). The nucleolar cavity is unlabelled by the snoRNA probe. (D) snoR3 (box C/D) shows a very similar labelling pattern to fibrillarin, but, again, the coiled bodies are unlabelled (arrowhead in green panel). Bar, 10 μ m.



nucleolus, but there is also labelling of the central cavity (seen particularly clearly in Fig. 1B, red panel), which we observed with 7-2/MRP but not with ITS1. There is no observable labelling of the coiled bodies (arrowhead in left, green panel of Fig. 2B).

SnoR2 and snoR3 show similar labelling patterns, even though snoR2 is a box H/ACA type and snoR3 is another box C/D snoRNA. Both show a distribution quite closely correlated with fibrillarin, but comprising many small foci within the fibrillarin zone (the dense fibrillar component, Fig. 2C and D). The nucleolar cavity regions are sometimes labelled by the snoR3 probe, but less brightly than the dense fibrillar

component. Very faint labelling of the coiled bodies can sometimes be seen, but is usually not visible.

Distribution of snoR2 and snoR3 correlates with sites of rRNA transcription

The pattern of foci seen for snoR2 and snoR3 was very reminiscent of that seen for transcription sites by incorporation of BrUTP (Thompson et al., 1997). We therefore carried out experiments to compare directly the location of these snoRNAs with the sites of transcription. Unfixed maize root sections were briefly permeabilised and then incubated in a transcription mix including BrUTP. After fixation and antibody

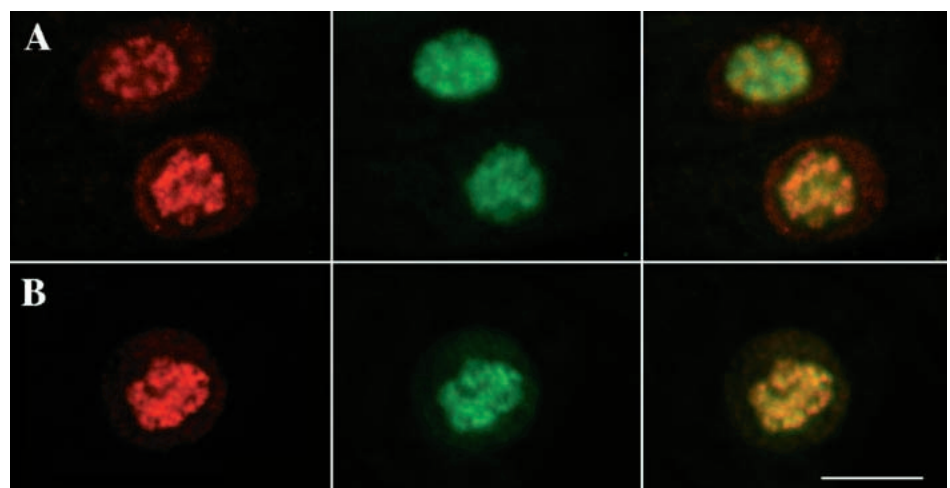


Fig. 3. Comparison of snoR2 (A) and snoR3 (B) localization with transcription sites. In each case, BrUTP incorporation is shown in red in the left hand panel, snoRNA in situ hybridization in green in the central panel, and the superimposition of the two labels in the right hand panel. In both cases, the snoRNA location corresponds closely with the labelled nucleolar transcription sites. Bar, 10 μ m.



Fig. 4. Diagram of the maize MzU14.1 gene cluster, showing the positions of three novel snoRNAs (snoR1, U49 and snoR2), followed by 4 variants of U14. Probes to the intergenic sequences indicated were made to localise the polycistronic precursor RNA (PG29, P7AB and P7ΨC).

detection of the incorporated BrUTP, the snoRNAs were detected by standard fluorescence in situ hybridization. The sites of transcription comprised many small foci within the DFC in the nucleolus, and also, generally, more faintly labelled sites in the nucleoplasm (Thompson et al., 1997). This is shown in Fig. 3A and B in red. The location of the snoRNAs corresponded very closely with the sites of BrUTP incorporation in the nucleolus for both snoR2 and snoR3, but without the labelling of the nucleoplasm (see Fig. 3A and B, respectively). Thus in both cases the snoRNAs were concentrated at the transcription sites.

Nucleolar distribution of precursor snoRNAs

We showed previously that the above snoRNAs together with copies of U14 are transcribed as a polycistronic precursor RNA (Leader et al., 1997). In order to examine the distribution of the pre-snoRNA to address where the processing of the precursor may occur, we made probes to three intergenic sequences between the mature snoRNAs in the MzU14.1 gene cluster, which would be expected only to be present on the precursor and processing intermediates. This gene cluster and the position of the intergenic sequence probes are shown in a schematic diagram in Fig. 4. Examples of the labelling patterns seen for two of these probes (P7AB and PG29) are shown in Fig. 5, again counter-labelled with an antibody to fibrillarin to show the nucleolar architecture (the third probe, P7ΨC, is not shown but gave a very similar labelling pattern to the other two). The overall level of label was much lower than with the probes to the mature snoRNAs, as would be expected for a

precursor present in relatively low amounts. All three spacer probes showed specific labelling of the nucleolus, in a more punctate distribution than fibrillarin, but within the same region of the nucleolus (Fig. 5, red). The spacer probes showed little labelling of the nucleolar cavity, but did label the coiled bodies clearly (arrows in fibrillarin-labelled, green panel in Fig. 5). The level of labelling of the coiled bodies was comparable to that of the nucleolus, whereas when mature snoRNAs were detectable in the coiled bodies, the labelling was very much fainter than in the nucleoli (see Fig. 2). A gallery showing a number of examples of nucleoli labelled with probe P7AB which clearly labelled coiled bodies is shown in Fig. 6. Thus the spacer probes showed an enrichment in the coiled bodies relative to all of the mature snoRNAs. As the intergenic regions were AU-rich, control experiments were carried out using a probe generated from an AU-rich (72% AU) intron of the pea legumin gene, to show that the labelling obtained was not due to non-specific cross-hybridization. This showed no labelling of nucleoli or coiled bodies (data not shown).

DISCUSSION

We have shown that several snoRNAs with different putative functions are transcribed as polycistronic precursor RNAs. Thus in the maize clone MzU14.1 there are four copies of U14, a box C/D snoRNA which is involved in early pre-rRNA cleavage reactions as well as 2'-O-ribose methylation; snoR1 and U49, box C/D snoRNAs also involved in specifying methylation sites; and snoR2, a box H/ACA snoRNA thought to be involved in specifying a pseudouridylation site. Similarly, the maize clone MzU14.4 contains gene variants of U14, U49, snoR1, snoR2 and a further box C/D snoRNA, snoR3 (Leader et al., 1997). Isolation of snoRNA genes from other plant species indicates that this genomic organization, although so far unique to plants, may be a common arrangement of plant snoRNA genes (J. W. S. Brown, unpublished). In this paper we have shown that the different snoRNAs have different patterns of localization within the nucleolus. U14, snoR2 and snoR3 are closely associated with rDNA transcription sites in foci within the nucleolar dense

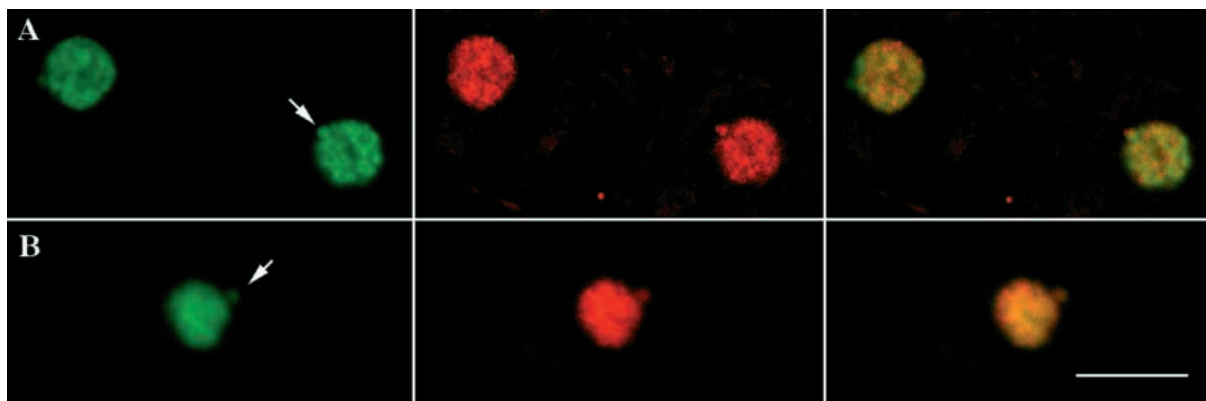


Fig. 5. In situ hybridization with intergenic spacer probes to P7AB (A) and PG29 (B). In each case the intergenic spacer probe is shown in red, and antibody labelling with anti-fibrillarin is shown in green. The third intergenic spacer probe (P7ΨC) showed very similar labelling to the two probes shown here. In each case the intergenic spacer probe was restricted to the nucleolus, and labelled similar regions of the nucleolus to fibrillarin. In contrast to the mature snoRNAs, the intergenic spacers labelled the coiled bodies clearly (arrows). Bar, 10 μm.

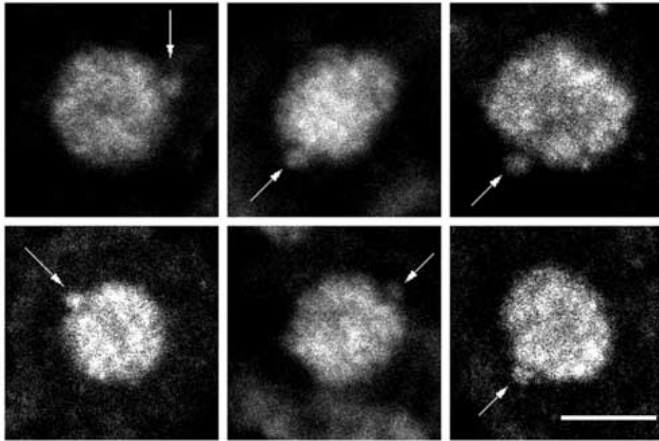


Fig. 6. Gallery of nucleoli labelled with intergenic spacer probe P7AB (fibrillarin double labelling was not used for these images). In each case, a coiled body is seen adjacent to the nucleolus, as strongly labelled as the nucleolus. Bar, 5 μ m.

fibrillar component, whereas U49 is slightly more widely distributed, but is also present in the same regions as the preceding snoRNAs. SnoR1, on the other hand, is concentrated in the surrounding nucleolar region, which includes the granular component. The organization of the novel snoRNAs and their relation to other nucleolar structures and components are shown in diagrammatic form in Fig. 7.

We have shown previously that the pre-rRNA transcripts progress from the transcription sites outwards to the dense fibrillar component, where the 5' ETS cleavage occurs, then to the surrounding region of the nucleolus, approximating the granular component, where the cleavage of ITS1 and subsequent rRNA cleavages occur. The simplest interpretation of the present localization results of the novel snoRNAs would be that, as with the pre-rRNA cleavage reactions, the methylation and pseudouridylation reactions in which the snoRNAs are involved are partitioned within the nucleolus according to the temporal stage at which they occur in the maturation of pre-rRNA. Earlier biochemical data (Maden, 1990b) suggested that most methylation and pseudouridylation reactions occurred very early in the processing pathway and probably co-transcriptionally. Co-transcriptional modification, or at least modification prior to excision of the 5' ETS cleavage, would be consistent with the localization we have demonstrated for U49, snoR2 and snoR3. However, it would not be easy to reconcile early involvement in processing with the localization we have found for snoR1. The localization of snoR1 to the GC regions surrounding the transcription sites suggests that this snoRNA is involved later in the processing pathway. We have shown previously that 7-2/MRP, which is involved in ITS1 cleavage, and ITS1-containing pre-rRNAs are also concentrated in the GC region. As some modifications are thought to occur later in the pathway, snoR1 may determine such a modification. An alternative explanation would be that association of the various snoRNAs with the maturing RNP complexes is not restricted to the time when they are functionally active in guiding the modification reactions. Thus, snoR1 may accumulate in the GC because it remains associated longer

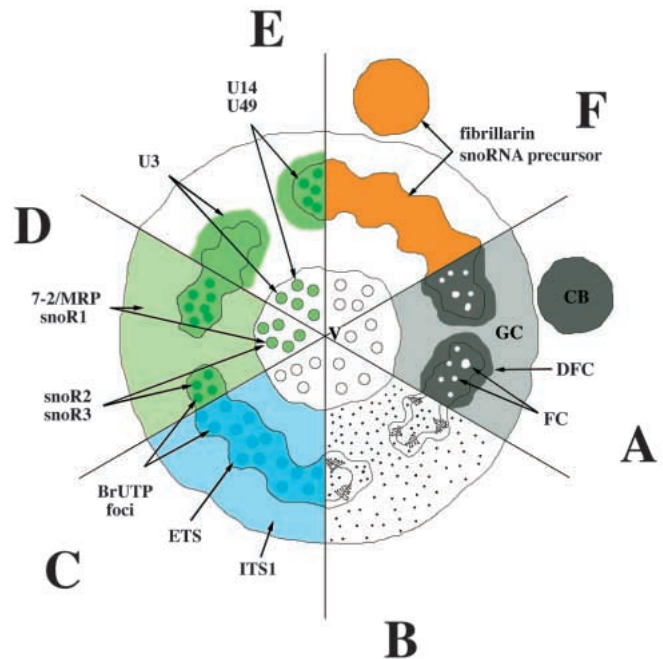


Fig. 7. Schematic diagram of the observed localization of various components in plant nucleoli (modified from Beven et al., 1996). In each segment of the diagram outlines corresponding to the ETS region, the nucleolar cavity, and the nucleolar periphery are shown. (A) The observed EM thin section ultrastructure: V – nucleolar vacuole or cavity; DFC – dense fibrillar component; GC – granular component; CB – coiled body; FC – fibrillar centres. (B) Highly stylized diagram of a possible molecular organization (not to scale), showing the location of the transcription complexes within the zone defined by the ETS. (C) BrUTP is incorporated into many small foci (dark blue) within the DFC, most of which probably correspond to single gene copies. These foci are surrounded by an enveloping layer of nascent and newly completed transcripts, as shown by an anti-sense probe to the ETS region of the pre-rRNA transcripts (mid-blue). In turn, surrounding this region is a layer broadly corresponding to the GC, in which the ETS has been removed but ITS1 is still present (light blue). Thus the stages of processing after ETS cleavage leading to pre-ribosome assembly must take place in this region. (D) 7-2/MRP and snoR1 are localized to the GC (light green) and to foci in the nucleolar cavity (mid-green). snoR2 and snoR3 are located at the transcription foci (dark green) and the nucleolar cavity (mid-green). (E) U3 labels a zone which includes the ETS region, but is more diffuse, whereas U14 and U49 colocalise with the transcription foci within this region (mid-green). All three snoRNAs also label the nucleolar cavity. (F) The nucleolar protein fibrillarin is localized to the DFC and to the coiled bodies, in contrast to the snoRNAs, which are barely detectable in coiled bodies. We have not detected fibrillarin in the nucleolar cavity.

with the maturing RNP complexes. Currently, there is little data about these post-transcriptional modifications in plants, and resolution of this question will require detailed analysis of methylation and pseudouridylation in plant rRNAs and the various processing intermediates.

The general features of tightly clustered snoRNA genes in plants and their polycistronic transcription raises questions about where and how these transcripts are processed. Another major conclusion from the work presented here is that the

precursor snoRNA, which we assume is transcribed, in common with other similar precursors, at various sites in the nucleus, must be imported into the nucleolus and coiled bodies before cleavage and processing into the mature snoRNAs. We base this on localization of three separate probes to spacer regions between the snoRNA sequences. These sequences are expected to be present only in the precursor and possible processing intermediates. It might be argued that processing of the precursor could involve excision and subsequent degradation of the intergenic sequences. If the degradation was not immediate, then our localization would reflect the distribution of the excised intergenic sequences as well as the precursors. However we consider this is unlikely, because current data suggest that processing of plant polycistronic pre-snoRNAs involves endonucleolytic cleavage, followed by exonucleolytic trimming (found with intron-encoded snoRNAs in animals and yeast) to the correct end sequences (Leader et al., 1997). If this is the case, the intergenic sequences would not be present as separate cleavage products, and our localization would indeed represent the location of the precursor and possibly processing intermediates.

Compared to the mature snoRNAs, which are at most barely detectable in coiled bodies, the snoRNA precursor is relatively enriched in these structures. The very low levels of labelling occasionally seen in the coiled bodies with (mature) snoRNA probes may indeed also represent labelling of the precursor, which contains the sequences of the mature snoRNAs. However, the precursor is much less abundant than the mature snoRNAs, and so, in contrast to the strong nucleolar labelling with probes to the mature snoRNAs, the labelling in the coiled bodies is faint or undetectable. The precursor is detected with intergenic sequence probes at about the same level in both the nucleolus and the coiled bodies. The simplest hypothesis to explain this labelling pattern would be that the precursor passes first through the coiled bodies before being localized to the nucleolus. However, we cannot eliminate more complicated schemes, in which the precursor is first transported to the nucleolus, then is exported to the coiled bodies before finally returning either intact or after cleavage to the nucleolus where the snoRNAs accumulate and function. In either case, these results suggest a role for the coiled bodies in the transport or processing of the precursor RNA, either in the early cleavage stages or in other post-transcriptional modification of the precursor RNA itself, such as methylation or pseudouridylation. In contrast with the labelling in the nucleolar cavity seen with U49 and snoR1, and with results we obtained previously using probes to U3, U14 and 7-2/MRP, we do not see a concentration of the precursor in the nucleolar cavity. This suggests that this region of the nucleolus, where the snoRNAs cannot be functionally active, represents a site of storage or recycling, rather than of precursor processing. In summary, these results show that in addition to being the site of ribosome biogenesis and maturation of rRNAs, the nucleolus also has a role in the maturation of components required for these processes.

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REFERENCES

- Bachelierie, J. P., Nicoloso, M., Qu, L. H., Michot, B., Caizerguesferrer, M., Cavaille, J. and Renalier, M. H. (1995). Novel intron-encoded small nucleolar RNAs with long sequence complementarities to mature ribosomal RNAs involved in ribosome biogenesis. *Biochem. Cell Biol.* **73**, 835-843.
- Bachelierie, J.-P. and Cavaille, J. (1997). Guiding ribose methylation of rRNA. *Trends Biochem. Sci.* **22**, 257-261.
- 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.
- Beven, A. F., Simpson, G. G., Brown, J. W. S. and Shaw, P. J. (1995). The organization of spliceosomal components in the nuclei of higher-plants. *J. Cell Sci.* **108**, 509-518.
- Beven, A. F., Lee, R., Razaz, M., Leader, D. J., Brown, J. W. S. and Shaw, P. J. (1996). The organization of ribosomal-RNA processing correlates with the distribution of nucleolar snRNAs. *J. Cell Sci.* **109**, 1241-1251.
- Bousquet-Antonelli, C., Henry, Y., Gélunge, J.-P., Caizergues-Ferrer, M. and Kiss, T. (1997). A small nucleolar RNP protein is required for pseudouridylation of eukaryotic ribosomal RNAs. *EMBO J.* **16**, 4769-4775.
- Caffarelli, E., Fatica, A., Prislei, S., Degregorio, E., Fragapane, P. and Bozzoni, I. (1996). Processing of the intron-encoded U16 and U18 snoRNAs – the conserved C-box and D-box control both the processing reaction and the stability of the mature snoRNA. *EMBO J.* **15**, 1121-1131.
- Cavaille, J. and Bachelierie, J.-P. (1996). Processing of fibrillar-in-associated snoRNAs from pre-mRNA introns: An exonucleolytic process exclusively directed by the common stem-box terminal structure. *Biochimie* **78**, 443-456.
- Cavaille, J., Nicoloso, M. and Bachelierie, J.-P. (1996). Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. *Nature* **383**, 732-735.
- Enright, C. A., Maxwell, E. S. and Sollner-Webb, B. (1996). 5'ETS rRNA processing facilitated by four small RNAs: U14, E3, U17 and U3. *RNA* **2**, 1094-1099.
- Ganot, P., Bortolin, M.-L. and Kiss, T. (1997a). Site-specific pseudouridine formation in pre-ribosomal RNA is guided by small nucleolar RNAs. *Cell* **89**, 799-809.
- Ganot, P., Caizergues-Ferrer, M. and Kiss, T. (1997b). The family of box ACA small nucleolar RNAs is defined by an evolutionarily defined secondary structure and ubiquitous sequence elements essential for RNA accumulation. *Genes Dev.* **11**, 941-956.
- Hadjilov, A. A. (1985). The nucleolus and ribosome biogenesis. *Cell Biology Monographs*, vol. 12. Wien, New York: Springer Verlag.
- Hozak, P., Cook, P. R., Schofer, C., Mosgoller, W. and Wachtler, F. (1994). Site of transcription of ribosomal-RNA and intranucleolar structure in HeLa cells. *J. Cell Sci.* **107**, 639-648.
- Kiss, T. and Filipowicz, W. (1995). Exonucleolytic processing of small nucleolar RNAs from pre-messenger-RNA introns. *Genes Dev.* **9**, 1411-1424.
- Kiss-László, Z., Henry, Y., Bachelierie, 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.
- Lafontaine, D. and Tollervey, D. (1995). Trans-acting factors in yeast pre-ribosomal-RNA AND pre-snoRNA processing. *Biochem. Cell Biol.* **73**, 803-812.
- Leader, D. J., Sanders, J. F., Waugh, R., Shaw, P. and Brown, J. W. S. (1994). Molecular characterization of plant U14 small nucleolar RNA genes – closely linked genes are transcribed as polycistronic U14 transcripts. *Nucl. 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.
- Maden, B. E. H. (1990a). The modified nucleotides in ribosomal RNA of man and other eukaryotes. In *Chromatography and Modification of Nucleosides* (ed. C. W. Gehrke and K. C. T. Kuo), pp. 265-301. Amsterdam: Elsevier.
- Maden, B. E. H. (1990b). The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog. Nucl. Acid Res.* **39**, 241-303.
- Maxwell, E. S. and Fournier, M. J. (1995). The small nucleolar RNAs. *Annu. Rev. Biochem.* **64**, 897-934.
- Mishra, R. K. and Elicieri, G. L. (1997). Three small nucleolar RNAs that are involved in ribosomal RNA precursor processing. *Proc. Nat. Acad. Sci. USA* **94**, 4972-4977.
- Ni, J., Tien, A. L. and Fournier, M. J. (1997). Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* **89**, 565-573.

- Nicoloso, M., Qu, L.-H., Michot, B. and Bachellerie, J.-P.** (1996). Intron-encoded, antisense small nucleolar RNAs: The characterisation 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.
- Ofengand, J. and Bakin, A.** (1997). Mapping to nucleotide resolution of pseudouridine residues in large subunit ribosomal RNAs from representative eukaryotes, prokaryotes, Archaeobacteria, mitochondria and chloroplasts. *J. Mol. Biol.* **266**, 246-268.
- Shaw, P. J., Highett, M. I., Beven, A. F. and Jordan, E. G.** (1995). The nucleolar architecture of polymerase I transcription and processing. *EMBO J.* **14**, 2896-2906.
- Smith, C. M. and Steitz, J. A.** (1997). Sno storm in the nucleolus: new role for myriad small RNPs. *Cell* **89**, 669-672.
- Thompson, W. F., Beven, A. F., Wells, B. and Shaw, P. J.** (1997). Sites of rDNA transcription are widely dispersed through the nucleolus in *Pisum sativum* and can comprise single genes. *Plant J.* **12**, 571-582.
- Tollervey, D. and Kiss, T.** (1997). Function and synthesis of small nucleolar RNAs. *Curr. Opin. Cell Biol.* **3**, 337-342.
- Tycowski, K. T., Shu, M. D. and Steitz, J. A.** (1993). A small nucleolar RNA is processed from an intron of the human gene encoding ribosomal protein-S3. *Genes Dev.* **7**, 1176-1190.
- 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. Nat. Acad. Sci. USA* **93**, 14480-14485.
- 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 box-C and box-D. *RNA* **2**, 118-133.
- Xia, L., Watkins, N. J. and Maxwell, E. S.** (1997). Identification of specific nucleotide sequences and structural elements required for intronic U14snoRNA processing. *RNA* **3**, 17-26.