Conserved Boxes C and D are essential nucleolar localization elements of U14 and U8 snoRNAs

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Sequences necessary for nucleolar targeting were identified in Box C/D small nucleolar RNAs (snoRNAs) by fluorescence microscopy. Nucleolar preparations were examined after injecting fluorescein-labelled wild-type and mutated U14 or U8 snoRNA into Xenopus oocyte nuclei. Regions in U14 snoRNA that are complementary to 18S rRNA and necessary for rRNA processing and methylation are not required for nucleolar localization. Truncated U14 molecules containing Boxes C and D with or without the terminal stem localized efficiently. Nucleolar localization was abolished upon mutating just one or two nucleotides within Boxes C and D. Moreover, the spatial position of Boxes C or D in the molecule is essential. Mutations in Box C/D of U8 snoRNA also impaired nucleolar localization, suggesting the general importance of Boxes C and D as nucleolar localization sequences for Box C/D snoRNAs. U14 snoRNA is shown to be required for 18S rRNA production in vertebrates.

Keywords: Boxes C and D/nucleolar localization/snoRNA

Introduction

Normal cell structure and function requires that macromolecules travel to their correct subcellular destinations. A key organelle in the eukaryotic cell is the nucleolus, the site of ribosome biogenesis in eukaryotes (reviewed by Hadjiolov, 1985; Gerbi et al., 1990). However, little is known about transport of small RNAs to the nucleolus. Normal cell structure and function requires that macromolecules in this family contain Boxes C and D sequence motifs that are conserved between different organisms and also between different snoRNAs within this family. All Box C/D class members are associated with fibrillarin, a protein found predominantly in the nucleolus (Tyc and Steitz, 1989; Baserga et al., 1991; Peculis and Steitz, 1994; Watkins et al., 1996). A few snoRNA molecules in the Box C/D class (including U3, U8, U14 snoRNAs) are essential for cell growth due to their critical roles in ribosomal RNA (rRNA) processing (reviewed by Gerbi, 1995; Maxwell and Fournier, 1995; Sollner-Webb et al., 1995; Venema and Tollervey, 1995). Recently, it has been discovered that a large number of other members of the Box C/D class are non-essential and are used as guide RNAs to direct individual sites of 2'-O-ribose methylation in rRNA (Cavaillé et al., 1996; Kiss-László et al., 1996, 1998; Maden, 1996; Nicoloso et al., 1996; Tollervey, 1996; Tycowski et al., 1996; Maden and Hughes, 1997; Smith and Steitz, 1997; J.Ni and M.J.Fournier, personal communication) and snoRNA (K.T.Tycowski and J.A. Steitz, personal communication).

The second major class of snoRNAs is the Box H/ACA family (Balakin et al., 1996). These snoRNAs contain the evolutionarily conserved Box H (ANANNA) and Box ACA (Balakin et al., 1996; Bousquet-Antonelli et al., 1997; Ganot et al., 1997b), and are associated with the nucleolar protein GAR1 (Balakin et al., 1996; Ganot et al., 1997b; Bousquet-Antonelli et al., 1997; F.Dragon, V.Pogacic and W.Filipowicz, personal communication). Some snoRNAs of the Box H/ACA family are required for rRNA processing (snR30: Morrissey and Tollervey, 1993; E1, E2 and E3: Mishra and Elicieri, 1997), but the vast majority are used as guide RNAs for pseudouridine modifications in rRNA (Ganot et al., 1997a; Ni et al., 1997; Smith and Steitz, 1997). The only snoRNA that does not fit into either class is 7-2-MRP, which is required for rRNA processing (Schmitt and Clayton, 1993; Chu et al., 1994; Lygerou et al., 1996).

This paper reports results on nucleolar localization signals for snoRNAs (U8 and U14) of the Box C/D family. U8 snoRNA is transcribed from its own gene and is needed for 5.8S/28S rRNA processing (Peculis and Steitz, 1993, 1994; Peculis, 1997). U14 was the first snoRNA discovered to be encoded within the intron of another gene (Liu and Maxwell, 1990; Leverette et al., 1992; Xia et al., 1995). U14 snoRNA contains two domains (A and B) that are complementary to 18S rRNA. Mutation of domain A alone or together with mutation in domain B, or genetic depletion of U14 snoRNA block rRNA processing at sites A1 and A2 in yeast, resulting in underaccumulation of 18S rRNA (Jarmolowski et al., 1995; Venema and Tollervey, 1995). Recently, it has been discovered that a large number of other members of the Box C/D class (including U3, U8, U14 snoRNAs) are essential for cell growth due to their critical roles in ribosomal RNA (rRNA) processing (reviewed by Gerbi, 1995; Maxwell and Fournier, 1995; Sollner-Webb et al., 1995; Venema and Tollervey, 1995).
Nucleolar localization of Box C/D snoRNAs

Fig. 1. U14 snoRNA containing Boxes C and D localizes to nucleoli. Fluorescein-labelled U14 snoRNA or U2 snRNA was injected into the nuclei of Xenopus laevis oocytes. After 2 h, nucleoli were prepared. Nucleoli were analyzed by phase-contrast (PC) or fluorescence microscopy; the nucleolar rDNA is stained by DAPI. In some cases, there are multinucleolate clusters with two or more DAPI stained foci. Fluorescein-labelled wild-type U14 snoRNA and the A/V/B deletion of U14 localize to nucleoli (FL, green). U2 snRNA, a splicing RNA that is not endogenous to the nucleolus, was used as a negative control and did not localize to nucleoli. U14 snoRNA with Box C or Box D substituted by unrelated sequences did not localize to nucleoli. Bar is 10 μm.

U14 snoRNA is transcribed in the nucleoplasm (E.Bertrand, D.A.Samarsky, M.J.Fournier and R.H.Singer, personal communication). How does U14 or any other Box C/D snoRNA finally reach the nucleolus, which is its functional compartment in the cell? The results reported here demonstrate that the sequences of Boxes C and D, as well as their position in the molecule, are critical for nucleolar localization of U14 snoRNA. Boxes C and D seem to be general nucleolar localization sequences for the Box C/D snoRNA family, as they are not only essential for U14, but also for U8 snoRNA. This is the first example of common nucleolar localization sequences that target several small RNA species to the nucleolus.

Results

Detection of snoRNA localization to nucleoli

Nucleolar localization of U14 and U8 snoRNA was assayed by injection of fluorescein-labelled snoRNA into Xenopus oocyte nuclei with subsequent fluorescence microscopy. A similar procedure has been employed previously to assess the localization of various RNA molecules in living or fixed tissue culture cells (Wang et al., 1991; Jacobson and Pederson, 1997, 1998; Jacobson et al., 1995, 1997a,b). One advantage of the method described in our study is that RNA can be recovered from the injected oocytes for determination of its stability at defined times after injection. Two hours after injection, the nuclei were manually isolated from the oocytes, the nuclear envelope was manually removed and the contents were centrifuged onto a microscope slide. This procedure was originally developed to study lampbrush chromosomes (Gall et al., 1991). In addition to chromosomes, snurposomes that contain small nuclear RNAs (snRNAs; Wu et al., 1991) and multiple nucleoli containing amplified rDNA (Painter and Taylor, 1942; Brown and Dawid, 1968; Gall, 1968; Perkowska et al., 1968) are centrifuged onto the slide. Oocyte nucleoli vary in size (Wu and Gall, 1997), and may fuse into a multinucleolar cluster that can have a vacuolated appearance (Franke et al., 1981; Shah et al., 1996). The nucleoplasm is not centrifuged onto the slide, so the injected RNA which still remains in that compartment is not visualized.

Fluorescence microscopy is valuable since it permits a direct qualitative assessment of nucleolar localization of the labelled RNA. Strong fluorescent signals depicting nucleolar localization of wild-type U14 snoRNA occurred within 2 h after injection of 1.4 ng transcript per oocyte nucleus (Figure 1). Similarly, fluorescein labelled wild-type U8 snoRNA (0.9 ng/oocyte) also localized to nucleoli within 2 h of injection into oocyte nuclei (Figure 2). In favorable preparations, signals from fluorescent U8 and U14 snoRNAs were found in ring-like structures within the nucleoli. Those structures appear to correspond to the dense fibrillar component, a compartment in nucleoli containing fibrillarin that surrounds rDNA (Shah et al., 1996). This supposition was supported by DAPI staining that shows DNA located in the center of the U8 or U14 labelled areas (e.g. Figures 1 and 2). Snurposomes do not contain DNA, and therefore the presence or absence of DNA can be assessed by microscopy to distinguish
snoRNAs from small nucleoli (Wu and Gall, 1997). Fluorescent U8 or U14 snoRNAs were never observed in structures lacking DNA. The nucleolar localization of fluorescent snoRNA was specific, as injection of the spliceosomal U2 snoRNA in the same concentration as used for U8 or U14 snoRNA did not give nucleolar signals 2 h after injection (Figure 1).

Various controls demonstrated that the fluorescent signals we observed in nucleoli are not due to degradation of fluorescent snoRNA and subsequent retrieval of the label by other nuclear components. For example, injection of fluorescent UTP alone did not label the nucleoli (data not shown). Moreover, direct assays demonstrated the stability of injected 32P-labelled wild-type U8 or U14 snoRNA, as will be described below.

The conclusions that U8 and U14 snoRNAs are transported to nucleoli after injection into the nucleoplasm, unlike U2 snoRNA that remains in the nucleoplasm, are supported by fractionation of manually isolated oocyte nuclei. Two hours after injection into oocyte nuclei, almost 40% of the U8 and U14 snoRNAs in the nuclei are found in nucleoli (U8, 37.2%; U14, 36.8%), unlike U2 snoRNA where only background levels of 3% are in nucleoli. The amount of RNA injected may have saturated the nucleolar localization machinery, thus explaining why some of the injected snoRNA remains in the nucleoplasm. Competition experiments to be described below indicate that the nucleolar localization machinery can in fact be saturated.

The cytological approach used in our experiments reveals that the injected snoRNA is in nucleoli rather than associated with chromosomes and snurposomes that also pellet with the nucleolar fraction; these components of the nucleolar fraction cannot be distinguished from one another by the nuclear fractionation described. Thus, fluorescence microscopy is an excellent way to assay the nucleolar localization of U14 and U8 snoRNAs. Moreover, the subcompartments within the nucleoli can be cytologically resolved.

**Fluorescein-labelled snoRNA can function in rRNA processing**

The data just presented indicate that fluorescein labelled U8 or U14 snoRNAs can specifically localize to nucleoli, but did not reveal whether these snoRNAs were in a functional compartment of the nucleolus and active there. Therefore, we have tested whether fluorescein-labelled snoRNA can function in rRNA processing after injection into oocyte nuclei. After *Xenopus* oocytes are depleted of endogenous U8 snoRNA by injection of an antisense oligonucleotide followed by endogenous RNase H digestion, the 28S rRNA processing pathway is hindered as previously reported (Peculis and Steitz, 1993, 1994; Peculis, 1997). 28S and 32S disappear and novel species of 32*5S* and 36*5S* appear; and 20S pre-rRNA decreased in amount. These effects of U8 snoRNA depletion have been reported by Peculis and Steitz (1993, 1994). Fluorescein-labelled or unlabelled U8, or U14 snoRNAs at 1.15 ng/oocyte could restore rRNA processing when injected after the antisense oligonucleotide.

The data just presented indicate that fluorescein labelled U8 or U14 snoRNAs are functional in rRNA processing as effectively as unlabelled U8 or U14 snoRNA. Therefore, not only are fluorescein labelled U14 or U8 snoRNAs able to localize in nucleoli (Figures 1 and 2), but they can also carry out their functions there in rRNA processing.

**A terminal core structure containing Boxes C and D is sufficient for nucleolar localization of U14 snoRNA**

To describe intrinsic elements of U14 snoRNA necessary for its nucleolar localization, substitutions, deletions or insertions in the U14 sequence were made by PCR mutagenesis. Table I summarizes the sequence of mature snoRNA/D, and the variable (V) region], the truncated U14 snoRNA containing only 39 nucleotides still localized to nucleoli as the various mutated U14 constructs used here.

After half of the sequence of U14 snoRNA was deleted [A/V/B deletion construct, spanning domains A and B and the variable (V) region], the truncated U14 snoRNA containing only 39 nucleotides still localized to nucleoli as well as the full-length wild-type U14 snoRNA (Figure 1). Nucleolar localization was seen when the A/V/B deletion transcript was injected at the same concentration as used for wild-type U14. This was true even when the concentration was adjusted so that equimolar amounts were injected, compensating for its decreased molecular weight. The 49 nucleotides that had been deleted in the A/V/B construct are unimportant for nucleolar localization even though they included regions A and B that are complementary to 18S rRNA and necessary for rRNA processing and modification. The residual core structure containing the terminal stem and adjacent Boxes C and D had sufficient information for nucleolar localization of U14 snoRNA. Since the phylogenetically conserved Boxes C and D were present in the residual core structure of
Table I. Mutations in U14 and U8 snoRNAs

<table>
<thead>
<tr>
<th>U14 snoRNA</th>
<th>Region A</th>
<th>Region V</th>
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<tr>
<td>wild type mutants:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/VB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mini C/D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Box C full</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Box C-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Box D full</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Box D-5</td>
<td></td>
<td></td>
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<tr>
<td>Box D-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>open stem 1</td>
<td></td>
<td></td>
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<tr>
<td>open stem 2</td>
<td></td>
<td></td>
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<tr>
<td>open stem 3</td>
<td></td>
<td></td>
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<tr>
<td>+6 (5',3')</td>
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<table>
<thead>
<tr>
<th>U8 snoRNA</th>
<th>Box D</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type mutants:</td>
<td></td>
</tr>
<tr>
<td>A/VB</td>
<td></td>
</tr>
<tr>
<td>mini C/D</td>
<td></td>
</tr>
<tr>
<td>Box C full</td>
<td></td>
</tr>
<tr>
<td>Box C-3</td>
<td></td>
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<tr>
<td>Box D full</td>
<td></td>
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<td>Box D-5</td>
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<td>Box D-6</td>
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<tr>
<td>open stem 1</td>
<td></td>
</tr>
<tr>
<td>open stem 2</td>
<td></td>
</tr>
<tr>
<td>open stem 3</td>
<td></td>
</tr>
<tr>
<td>+6 (5',3')</td>
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</table>

The sequence of the murine processed U14 snoRNA is from Watkins et al. (1996). Boxes C and D are highly conserved in many snoRNAs and a consensus sequence has been derived for each of them (Xia et al. 1997). Regions A and B are complementary to 18S rRNA, and V is a variable region (Xia et al. 1997). One extra U (bold font) was added to the 5' end to fully close the terminal stem of U14. Nucleotides that are the same as in wild-type U14 snoRNA are shown in the sequence alignment by dots, and deletions are indicated by dashes. Substitutions or insertions are indicated by lower case letters and several are the same as used by Watkins et al. (1996) and Xia et al. (1997). In partial mutants of either Box C or Box D, the two most highly conserved nucleotides (GA) of each Box were replaced by cytosine. The double mutants Box C-2, 3 and Box D-5, 6 are not shown in this table, as they are simply the sum of the individual single mutations Box C-2 plus Box C-3 or Box D-5 plus Box D-6. Similarly, just the double mutant +6(5',3') is shown. The A/V/B deletion is the same one used by Watkins et al. (1996). The Xenopus U8 snoRNA sequence is from Peculis and Steitz (1993) and consensus sequences for conserved Boxes C and D (Xia et al., 1997) are indicated. The Box C mutation is the same one used by Peculis and Steitz (1994).

U14 snoRNA, mutations were made in Box C or D to assay their effect on nucleolar localization. Replacement of the entire Box C or Box D in full-length U14 snoRNA by unrelated sequences prevented its nucleolar localization (Figure 1). Hence, Boxes C and D are critical for nucleolar localization of U14 snoRNA.

Boxes C and D also are nucleolar localization elements for U8 snoRNA

In order to examine whether Boxes C and D are general signals for nucleolar localization of other snoRNAs of the Box C/D family, the localization of U8 snoRNA was investigated. Fluorescein-labelled wild-type U8 snoRNA that is injected into Xenopus oocyte nuclei can localize to nucleoli (Figure 2). In contrast, when the sequences of Box C or Box D in U8 snoRNA (Peculis and Steitz, 1994) were replaced by unrelated sequences, nucleolar localization was abolished (Figure 2). Therefore, Boxes C and D are necessary for nucleolar localization of both U8 snoRNA and U14 snoRNAs.

It was important to show that the failure to observe fluorescent signals after injection of Box C and D mutants...
percentage. After injection of equivalent amounts of each is expressed as a snoRNA relative to wild-type snoRNA remaining in the nucleus 2 h electrophoresis (Materials and Methods). The amount of mutant 3180 of the method used here is that the amounts of labelled h after injection into oocyte nuclei. Clearly, an advantage to have been detected, had they so localized in nucleoli 2 3185 type U14 snoRNA, they are still sufficiently stable (61, 45, 74, 92, 68, 84, 100, 60, 59, 81, 538) 325 after injection even with the 25% dilution. Other details as in Figure 1. Bar is 10 μm.

**Table II. Stability of U14 and U8 wild-type and mutant snoRNAs**

<table>
<thead>
<tr>
<th>RNA</th>
<th>Stability (% of wild-type 2 h after injection)</th>
</tr>
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<tbody>
<tr>
<td>U14 snoRNA</td>
<td></td>
</tr>
<tr>
<td>A/V/B</td>
<td>81</td>
</tr>
<tr>
<td>Box C full</td>
<td>60</td>
</tr>
<tr>
<td>Box C-2,3</td>
<td>59</td>
</tr>
<tr>
<td>Box C-2</td>
<td>100</td>
</tr>
<tr>
<td>Box C-3</td>
<td>86</td>
</tr>
<tr>
<td>Box D full</td>
<td>64</td>
</tr>
<tr>
<td>Box D-5,6</td>
<td>92</td>
</tr>
<tr>
<td>Box D-5</td>
<td>68</td>
</tr>
<tr>
<td>Box D-6</td>
<td>84</td>
</tr>
<tr>
<td>+6(5′)</td>
<td>103</td>
</tr>
<tr>
<td>+6(3′)</td>
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<tr>
<td>+6(5′,3′)</td>
<td>82</td>
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<td>open stem 1</td>
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<tr>
<td>open stem 2</td>
<td>45</td>
</tr>
<tr>
<td>open stem 3</td>
<td>43</td>
</tr>
<tr>
<td>mini C/D</td>
<td>61</td>
</tr>
<tr>
<td>U8 snoRNA</td>
<td></td>
</tr>
<tr>
<td>Box C</td>
<td>99</td>
</tr>
<tr>
<td>Box D</td>
<td>75</td>
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</tbody>
</table>

32P-labelled capped U14 or U8 snoRNA were isolated 2 h after injection into Xenopus oocyte nuclei and quantified after gel electrophoresis (Materials and Methods). The amount of mutant snoRNA relative to wild-type snoRNA remaining in the nucleus 2 h after injection of equivalent amounts of each is expressed as a percentage. was not due to their lack of stability. We ruled out this trivial explanation for Box C and D mutants of U8 and U14 and all other U14 mutants studied here as follows. A dilution series shows that just 25% of the amount of wild-type U14 snoRNA usually injected (1.4 ng/oocyte) can still be detected (Figure 4). The stability was determined for 32P-snoRNA constructs 2 h after their injection into oocyte nuclei (Table II). Although the mutated U14 snoRNAs are generally somewhat less stable than wild-type U14 snoRNA, they are still sufficiently stable (>25%) to have been detected, had they so localized in nucleoli 2 h after injection into oocyte nuclei. Clearly, an advantage of the method used here is that the amounts of labelled mutant U14 snoRNAs can be determined in the oocyte following injection.

**Competition reveals that U14 snoRNA nucleolar localization can be saturated**

Competition experiments were carried out using U14 snoRNA as the fluorescent probe and other unlabelled transcripts as the competitor. Since a 100-fold molar excess of competitor was used, the amount of fluorescent probe was decreased to 0.5 ng/oocyte (about one third of the usual amount) to reduce the total amount of RNA being injected to a net excess of 35 times the usual amount. Unlabelled wild-type U14 snoRNA competes effectively with fluorescein-labelled U14 snoRNA for nucleolar localization, indicating that limiting components needed for its nucleolar localization become saturated (Table III). U2 snoRNA is unable to compete with fluorescent U14 snoRNA for its nucleolar localization. However, the U8 snoRNA is a competitor for U14 nucleolar localization, suggesting that the sequences they share in common, namely Boxes C and D, may be responsible for the competition. This is supported by the observation that U14 snoRNA carrying a double mutation (U14 Box C/D) that substitutes sequences in both Boxes C and D does not appreciably hinder the nucleolar localization of fluorescent U14 wild-type snoRNA. Finally, a truncated and mutated construct of U14 (mini C/D to be discussed later), which has just Boxes C and D and no other wild-type sequences, can compete the nucleolar localization of fluorescent U14 snoRNA. Therefore, to sum up, the components needed for U14 snoRNA localization can be saturated by an excess of any molecule tested that has Boxes C and D.

**Table III. Competition of U14 snoRNA nucleolar localization**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Competitor</th>
<th>Competition</th>
</tr>
</thead>
<tbody>
<tr>
<td>U14</td>
<td>U14</td>
<td>strong</td>
</tr>
<tr>
<td>U14</td>
<td>U2</td>
<td>none</td>
</tr>
<tr>
<td>U14</td>
<td>U8</td>
<td>moderate</td>
</tr>
<tr>
<td>U14</td>
<td>U14 Box C/D</td>
<td>weak</td>
</tr>
<tr>
<td>U14</td>
<td>U14 mini C/D</td>
<td>strong</td>
</tr>
</tbody>
</table>

0.5 ng/oocyte of fluorescein-labelled wild-type U14 snoRNA was co-injected into Xenopus oocyte nuclei with a 100-fold molar excess of unlabelled transcripts (competitor), and slides with nucleoli were prepared 2 h later. The qualitative degree of competition is indicated.

**Importance of the spatial position of Boxes C and D for nucleolar localization of U14**

Is the spatial relationship or position of Boxes C and D with respect to the 5′- and 3′-termini of U14 snoRNA important for nucleolar localization? Increasing the distance of Box C from the 5′ end [+6(5′)] or Box D from the 3′ end [+6(3′)] by six nucleotides prevents processing of U14 snoRNA (Xia et al., 1997). These same insertions abolished localization of U14 snoRNA to nucleoli 2 h after oocyte injection (Figure 5). This effect could be due to the staggered alignment of Box C sequences relative to Box D sequences within the secondary structure of U14 snoRNA after inserting six nucleotides between the terminal stem and either Box C or Box D. To attempt to realign Box C with Box D, a double mutant [+6(5′/3′)] was made from the single mutations just

![Fig. 4. Dilution of injected snoRNA. Wild-type U14 snoRNA was diluted to 50% or 25% of the amount generally used (1.4 ng/oocyte) prior to injection into oocyte nuclei. Nucleolar labelling is seen 2 h after injection even with the 25% dilution. Other details as in Figure 1. Bar is 10 μm.](image-url)
in Figure 1. Bar is 10 μm.

The stem of the terminal core structure is not critical for nucleolar localization

As described above (Figure 1), a residual core structure lacking regions A/V/B is still able to localize to nucleoli. Which sequences and structures of this core structure are essential for nucleolar localization? The A/V/B deletion mutant has a terminal stem bounded by Box C on one side and Box D on the other side, and connected by a small loop. We created three different mutations to open the terminal stem: open stem 1, open stem 2, and open stem 3 above, the terminal stem was opened by substituting both sides of the stem with guanosine residues. The small internal loop was also replaced by unrelated nucleotides. Therefore, the only wild-type sequences that remain in this mini C/D construct of 35 nucleotides are Boxes C and D (Table I). Amazingly, the mini C/D construct could still localize to nucleoli, though not as well as wild-type U14 snoRNA (Figure 5).

Specific nucleotides within Boxes C and D are essential localization elements in U14 snoRNA

Recently it was shown that consensus sequences of both Box C and Box D also are required for U14 processing (Watkins et al., 1996) and that certain single highly conserved nucleotides within both Boxes are critical for its processing (Xia et al., 1997). We have tested point substitutions of the most highly conserved and important nucleotides in Boxes C and D for their effect on nucleolar localization of U14 snoRNA. Among snoRNAs of the Box C/D class of numerous eukaryotic organisms, the nucleotides in position 2 (G) and 3 (A) of Box C are 100% conserved (Xia et al., 1997). We replaced these nucleotides, individually (Table I) or both, by cytosine. Both the double and single substitutions within Box C of U14 snoRNA greatly reduced localization to nucleoli (Figure 6). Similarly, positions 6 (A) and 5 (G) of Box D are conserved 100% and 99.3%, respectively (Xia et al., 1997). These nucleotides were also replaced by cytosine (Table I). Again, the double and single substitutions within Box D greatly diminished nucleolar localization (Figure 6). Thus, the GA nucleotides in Box C (2,3) and Box D (5,6) are necessary for nucleolar localization, and their substitution compromises this localization. Overall, the data from this study show that the sequences of Boxes C and D are crucial elements for nucleolar localization of Box C/D snoRNAs.

Discussion

Injected snoRNA is localized to the nucleolus and is functional in rRNA processing

We have injected fluorescent-labelled snoRNA into *Xenopus* oocyte nuclei, and have determined certain principles for nucleolar localization of Box C/D snoRNAs by fluorescence microscopy. In favorable preparations, fluorescent-labelled U8 or U14 snoRNA localized to ring-like structures within the oocyte nucleoli. These rings surround rDNA that was detected by DAPI staining; the rings probably correspond to the dense fibrillar component described for *Xenopus* oocyte nucleoli (Shah et al., 1996).
where early rRNA processing events occur (Hadjiovol, 1985; Gerbi et al., 1990). Depletion and rescue experiments reported here show that the injected fluorescein-labelled U14 and U8 snoRNAs are fully functional in 18S and 28S rRNA processing, respectively. Therefore, the injected snoRNAs can localize to nucleoli where they are functionally active.

Moreover, our data indicate that U14 snoRNA plays a role in 18S rRNA production in metazoans. When U14 snoRNA was depleted by injection of an antisense oligonucleotide, 20S pre-rRNA could not be processed and no 18S rRNA product was made. As a result, almost no newly synthesized 18S rRNA is seen, and the level of its 20S precursor is enhanced relative to 28S and 32S (Figure 3). Furthermore, we found that the underaccumulation of 18S rRNA in Xenopus oocytes after depletion of intact U14 snoRNA could be rescued by U14 snoRNA, with or without fluorescein label (Figure 3). Somewhat different conclusions were drawn by Dunbar and Baserga (1998), who found that newly synthesized 18S rRNA was decreased by two-thirds and that there was no increase in 20S pre-rRNA (the precursor to 18S rRNA). They concluded that U14 snoRNA does not affect pre-rRNA cleavages, but simply stabilizes newly synthesized 18S rRNA. In contrast, since we see a complete disappearance in new 18S rRNA and a relative accumulation of its 20S precursor, we conclude that metazoan U14 snoRNA is needed for cleavage at sites 1 and 2 that flank each side of 18S rRNA, just as has been found for yeast (Jarmolowski et al., 1990; Li et al., 1990; Li and Fournier, 1992; Liang and Fournier, 1995). The quantitative differences between Dunbar and Baserga's (1998) results and ours probably reflect differences in experimental procedures (they injected a lower amount of different oligonucleotides than used here, and their injection was into the cytoplasm, unlike our nuclear injections; they injected radioactivity to label newly synthesized rRNA 1 day later, whereas we added it with the second injection of depleting oligonucleotide), suggesting that U14 snoRNA depletion was more complete in our case.

### Nucleolar localization elements (NoLEs) in U14 snoRNA

Targeting of macromolecules to their correct destination in the cell is extremely important. Nuclear localization elements (NLE) are beginning to be defined for nuclear import of RNA (Grimm et al., 1997). However, little is known about nucleolar localization elements (NoLEs), although the nucleolus contains a vast array of snoRNAs needed for ribosome biogenesis. In 7-2/MRP RNA, nucleotides 23–62, which contain the To antigen binding site, are required for nucleolar localization (Jacobson et al., 1995), but the only other small RNA that may also use the To binding site for nucleolar localization is the RNA of RNase P (Jacobson et al., 1997a). Three other snoRNAs (E1, E2 and E3) were shown to localize to nucleoli after injection into Xenopus oocytes (Selvanuragan et al., 1997), but the NoLE used for that localization has not yet been elucidated. A small 56 nucleotide fragment of U3 snoRNA containing Boxes C' and D and the terminal stem had sufficient information for nuclear import from the cytoplasm (Baserga et al., 1992), but whether it could reach its nucleolar destination was not tested. Similarly, a truncated version of U20 snoRNA still containing Boxes C and D could be detected in a nucleolar fraction of a mouse L929 cell line (Cavaille et al., 1996).

We have identified the intrinsic sequences of the NoLE used by U14 snoRNA, and these features may be generalized to other members comprising the Box C/D family, since they were found to also be important for U8 snoRNA. U14 snoRNA that carried a large deletion (A/V/B) spanning domains A and B and the variable (V) region localized to nucleoli in a comparable manner to wild-type U14. This indicates that the regions of U14 known to be important for 18S rRNA processing and modification (Jarmolowski et al., 1990; Li et al., 1990; Li and Fournier, 1992; Liang and Fournier, 1995; Morrissey and Tollervey, 1997; Dunbar and Baserga, 1998) are not necessary for nucleolar localization. Similarly, the functional regions of U8 snoRNA required for interactions with pre-rRNA for rRNA processing are not needed for nucleolar targeting of these molecules (T.S.Lange, A.Borovjagin and S.A.Gerbi, submitted). Thus, snoRNAs probably are not localized to nucleoli simply by their hydrogen bond interactions with pre-rRNA there. This is supported by the finding that in
Xenopus kidney cells in culture, 68% of endogenous U14 snoRNA and all of U8 snoRNA remain in the nucleolus after actinomycin D depletion of early rRNA precursors (Rivera-León and Gerbi, 1997).

The A/V/B deletion of U14 snoRNA indicated that the remaining Box C/D core structure is not only necessary but sufficient for nucleolar localization of U14 snoRNA. We used various mutations to identify features used as the NoLE within this core structure. The conserved sequences of Boxes C and D are critical elements, as shown by the mini C/D construct where these are the only wild-type sequences that remain. Moreover, even a single point mutation in Box C or Box D can hinder nucleolar localization of an otherwise wild-type, full-length U14 molecule. The position of Boxes C and D relative to the ends of the molecule is also an important feature of the NoLE. We have shown here that Boxes C and D are also necessary for nucleolar localization of U8 snoRNA, indicating that Boxes C and D are essential whether the snoRNA was transcribed from its own gene (U8) or as part of the intron of another gene (U14). Thus, we can generalize and conclude that snoRNAs in the Box C/D family utilize Boxes C and D as critical features for nucleolar localization. It will be interesting to learn from future studies if snoRNAs of the Box H/ACA family utilize Boxes H and ACA as their NoLE. If so, we would predict that snoRNA members of the Box C/D family would not cross-compete with members of the Box H/ACA family nor with the minor 7-2/MRP family for factors needed for their nucleolar localization. Instead, each family would rely on its own NoLE that may interact with a family-specific targeting factor(s).

Boxes C and D can have multiple functions. For example, they are necessary for U14 processing to liberate it from its intronic location in the precursor (Watkins et al., 1996; Xia et al., 1997). However, other snoRNAs such as U8 are transcribed from free-standing genes, and do not need the intronic processing function of Boxes C and D. Box D is also needed for 5′ cap hypermethylation (Terns et al., 1995), but this does not occur in the intronic members of the Box C/D snoRNA family. Additionally, Box D is required for nuclear retention of U8 but apparently not for U3 snoRNA (Terns et al., 1995). The function of Boxes C and D for rRNA processing is unknown, but Box D is important for 2′-0′-ribose methylation where it guides the modification on a complementary stretch of rRNA 5 nucleotides from the end of the Box D (Cavaillé et al., 1996; Kiss-Lásló et al., 1996, 1998; Maden, 1996; Nicoloso et al., 1996; Tollerey, 1996; Tycowski et al., 1996; Maden and Hughes, 1997; Smith and Steitz, 1997; J.Ni and M.J.Fournier, personal communication). However, some Box C/D snoRNAs, such as U8, do not act as guide RNAs for methylation. Therefore, the only function of Boxes C and D shared by all snoRNAs in the Box C/D family is that of nucleolar localization, since only a subset of these snoRNAs also utilize these regions for intronic processing, 5′ cap trimethylation, nuclear retention or rRNA methylation.

Candidate factors that may interact with the Box C/D NoLE

The competition experiments reported here demonstrate that the nucleolar localization of U14 snoRNA can be competed for by any of the transcripts tested that have Boxes C and D. This implies that the machinery needed for nucleolar localization of U14 snoRNA can be saturated. Similarly, others concluded that snoRNA nuclear retention is due to a saturable component, since after injection of more than 25 fmole (~0.2 ng/oocyte) into nuclei, some U3 and U8 are found in the cytoplasm (Terns et al., 1995).

Proteins that interact with the Box C/D NoLE are candidates as saturable factors needed for localization of the snoRNA to the nucleolus. One such protein that has been extensively studied is fibrillarin, a 34–46 kDa nucleolar protein conserved in yeast through humans (Lapeyre et al., 1990 and references therein) which is associated with snoRNAs of the Box C/D family. Fibrillarin needs Box C in U3 snoRNA (Baserga et al., 1991) and also adjacent sequences and Box D in U8 snoRNA (Peculis and Steitz, 1994) and additional regions in U14 snoRNA (Watkins et al., 1996) for binding. The general consensus has been that fibrillarin association with the Box C/D snoRNAs is indirect and occurs via protein–protein interactions with other snoRNP proteins. However, a cross-link of fibrillarin to U16 snoRNA has just been reported and this cross-link requires Boxes C and D (Caffarelli et al., 1998). As a common protein of the Box C/D snoRNA family, fibrillarin is certainly an appealing candidate for snoRNA transport. Yet, some evidence suggests that this nucleolar protein might not be responsible for snoRNA localization to the nucleolus. First, yeast strains genetically depleted of fibrillarin contain nucleoli that still stain with antibody against the TMG cap (Tollervey et al., 1991). This implies that snoRNAs are still localized in the nucleolus, although a direct demonstration by in situ hybridization was not carried out. Secondly, and more compelling, U14 immunoprecipitation experiments using fibrillarin antibody have demonstrated the absence of fibrillarin binding to the A/V/B deletion mutant in Xenopus oocytes (Watkins et al., 1996). Since this truncated Box C/D snoRNA is efficiently transported to the nucleolus as seen in the present study, it can be deduced that fibrillarin might not be required for nucleolar transport.

It is intriguing to note that all of the mutations in Boxes C and D of U14 snoRNA that prevent nucleolar localization also disrupt intronic processing of U14 snoRNA (Watkins et al., 1996; Xia et al., 1997). Is the same Box C/D binding protein used for nucleolar localization and snoRNA intronic processing? Recent work has defined a Box C/D-binding protein that is 65 kDa in mouse nuclear extracts (Watkins et al., 1998) and 68 kDa in Xenopus oocytes (Caffarelli et al., 1998). The cross-linking of the 68 kDa protein to U16 snoRNA requires the terminal stem in addition to Boxes C and D (Caffarelli et al., 1998). The terminal stem is also necessary for intronic processing of U14 snoRNA (Xia et al., 1997). However, the conclusion that the terminal stem is not an essential NoLE common to snoRNAs of the Box C/D family is supported by the fact that some members of the Box C/D snoRNA family (U8, U13 and U18; summarized in Watkins et al., 1996) lack a terminal stem. Thus, it is not clear if the 65 or 68 kDa protein is utilized for snoRNA nucleolar localization.

It could be that other proteins that bind the core structure containing Boxes C and D are mediators of snoRNA localization.
nucleolar localization. Biochemical fractionation revealed that the U14 core structure forms a complex containing 50 and 55 kDa proteins in addition to the 65 kDa protein mentioned above (Watkins et al., 1998). The 55 kDa protein seems to be distinct from the U3 specific protein of the same size studied by Lübben et al. (1993), and therefore is unlikely to be a universal mediator of snoRNA nucleolar localization. Whether the 50 kDa protein is the same as the one also found in a complex with U3 snoRNA (Lübben et al., 1993) remains to be elucidated. Finally, a 38 kDa protein is cross-linked with the U14 core structure in addition to the 65 kDa protein discussed above (Watkins et al., 1998), but the former was not thought to be fibrillarin, although fibrillarin was reported to cross-link to U16 snoRNA along with the 68 kDa protein in Xenopus oocytes as discussed above (Caffarelli et al., 1998).

Future research needs to focus on which proteins mediate snoRNA nucleolar localization, and whether they are the same or different from those needed for intronic processing of snoRNAs. In any case, it is appealing to think that a common protein may be used to target a family of small RNAs to their correct cellular destination. Just as Sm proteins are needed for nuclear import of snRNAs (Mattaj and DeRobertis, 1985; Hamm et al., 1990; Fischer et al., 1991, 1993; Marshall and Lührmann, 1994; Grimm et al., 1997), so too, nucleolar targeting may utilize specific proteins for each family of snoRNAs. The NoLEs described here appear in snoRNAs of the Box C/D family and may be recognized by a protein(s) specific for that family. Such a protein(s) may either transport the snoRNA from the nucleoplasm to the nucleolus and/or anchor it within the nucleolus.

Materials and methods

Plasmids, in vitro transcription and labelling of RNA

U14 templates for in vitro transcription reactions were constructed by PCR. We used murine U14 snoRNA from hsp70 intron 5 (Liu and Maxwell, 1990; Leverette et al., 1992), as the sequences and structures needed for its removal from the intron have been extensively studied (Watkins et al., 1995; Xia et al., 1997). The nucleotides that differ between mouse and Xenopus wild-type U14 snoRNAs (Xia et al., 1995) are found primarily in the variable (V) region (between regions A and B); pilot experiments showed that mouse and Xenopus snoRNAs localized equally well to nucleoli of Xenopus oocytes (data not shown).

For all (except mini C/D) U14 snoRNA constructs, the template was BamHI restricted vector pSP64T7 carrying EcoRI–T7 promoter–gggccc–I–BamHI. The U14 insert for all PCR constructs (except mini C/D) used as the starting template in the present study was the murine wild-type U14 snoRNA from hsp 70 intron 5 (Leverette et al., 1992) or ΔAV for the A/V/B deletion of U14. Primers annealing to the 5′ end included the T7 promoter sequence in front of the U14 sequence to eliminate the following: ggcgcc between the insert and the T7 promoter, the flanking intronic sequences and linker sequences already present in pSP64T7. The primers used in this study are listed below:

U14 5′-end primers:

WT (5′): 5′-TAA TAC GAC TCA CTA TAG GGT TCG CTG TGA TGG ATT CCA AAA-3′

Box C: 5′-TAA TAC GAC TCA CTA TAG GGT TCG CTG TGA TGG ATT CCA AAA-3′

Box C-2: 5′-TAA TAC GAC TCA CTA TAG GGT TCG CTG TCA TGA TGG ATT CCA AAA-3′

Box C-3: 5′-TAA TAC GAC TCA CTA TAG GGT TCG CTG TGA TGG ATT CCA AAA-3′

+6 (5′): 5′-TAA TAC GAC TCA CTA TAG GGT TCG Ccc ccc cTG TGA TGG ATT CCA AAA-3′

open stem 1: 5′-TAA TAC GAC TCA CTA TAG GGa age gTG TGA TGG ATT-3′

open stem 2: 5′-TAA TAC GAC TCA CTA TAG GGe ccc ccc TGA TGA TGG ATT CAA AAA-3′

open stem 3: 5′-TAA TAC GAC TCA CTA TAG GgG ggg ggg TGA TGA TGG ATT CCA AAA-3′

U14 3′-end primers

WT (3′): 5′-TTC GCT CAG ACA TC-3′

Box D: 5′-TTC GCg ggg gaA TCC AAG GAA GGA A-3′

Box D–5′: 5′-TTC GCg gAg ACG ACA TCC AAG GAA GGA A-3′

Box D–5′: 5′-TTC GCT gAC ACG ACA TCC AAG GAA GGA A-3′

Box D–6′: 5′-TTC GCg CAC ACG ACA TCC AAG GAA GGA A-3′

+6 (3′): 5′-TTC GCg ggG ggT CAG ACA TCC AAG G-3′

open stem 2: 5′-ggg ggT CAG ACA TCC AAG G-3′

open stem 3: 5′-ccc ccT CAG ACA TCC AAG G-3′

Mutated bases in the primers are indicated by lower case letters. When a mutated primer is not listed for a construct, the wild-type primer was used (i.e. mutant primer at one end and wild-type primer at the other end). For PCR cloning of the mini C/D construct (see sequence in Table 1), an oligo containing this sequence and the T7 promoter was used as the template. The primers for the mini C/D construct were: 5′- end primer: 5′-TAA TAC GAC TCA CTA TAG GGT TCG CTG TGc-3′; 3′-end primer: 5′-ccc ccT CAG ACt aga-3′. The PCR products were cloned into pcR3.1 (Invitrogen, Carlsbad, CA) and their sequences were confirmed.

U8 snoRNA sense strand transcripts were prepared from BsuRI digested pSP64 plasmid carrying full-length wild-type or a Box C mutant U8 snoRNA (Peculis and Steitz, 1994), using the T7 transcription and 5′ cap conditions listed below. The Box D mutant of U8 was created using pSP64/WT U8 as the template (Peculis and Steitz, 1994) and the primers listed below:

U8 5′-end primer: 5′-TAA TAC GAC TCA CTA TAG GgG TCG TCA GTG AGG TTA-3′

U8 3′-end primer: 5′-AAC gcG ggT GAT CCT GCC-3′

The T7 promoter sequence was added to cloned U2 DNA (pXIU2 plasmid of Mattaj and Zeller, 1985) by PCR using the following primers:

U2 5′-end primer: 5′-TAA TAC GAC TCA CTA TAG GGA TCG CTG TCG CTC CCT GGC CTT TTG GC-3′

U2 3′-end primer: 5′-AAG TGC ACC GGT CCT GGA GG-3′

Labelling reactions for U14, U8 or U2 transcripts were either incubated for 2 h (fluorescein) or 1 h ([32P]UTP) at 37°C. All transcripts were obtained using a T7 megascript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer’s instructions. To label the transcripts, the reactions included either 0.2 mM fluorescein-12-UTP (DuPont New England Nuclear, Boston, MA) or 1 mM (10 nCi) [α-32P]UTP (Du Pont New England Nuclear, Boston, MA) in addition to 1 mM ATP, 1 mM CTP, 1 mM GTP and 0.2 mM UTP.

Some snoRNAs (e.g. U3 and U8 snoRNAs) that are transcribed from their own genes normally contain a monomethyl G cap that is subsequently converted to a trimethyl G cap (Terns and Dahlberg, 1994; Terns et al., 1995). In contrast, endogenous U14 snoRNA lacks a 5′ cap, since it is transcribed and processed from the intron of another gene. Although wild-type U8 or U14 snoRNA was stable with or without a monomethyl G cap added to its 5′ end, much degradation was observed immediately after injection into oocytes of mutated U14 transcripts with an unprotected 5′ end (data not shown). This observation is consistent with reports of others that Boxes C and D are needed for stability of U8 and U14 and the terminal stem for stability of U14 (in Gerbi, 1995: references in Table 2). This degradation was prevented by capping the 5′ ends by addition of 4 mM m7G(5′)ppp(5′)G cap analog (Ambion, Austin, TX) to the transcription reactions. When a monomethyl G cap was added, all mutated U14 and U8 snoRNAs were sufficiently stable to be within the range of concentrations that would be detectable by fluorescence microscopy if they had localized to nucleoli (Figure 4;
Table II). The presence or absence of a cap did not affect wild-type snoRNA nuclear localization (data not shown).

Following the removal of template DNA by a 10 min incubation with 0.1 units/ml DNase I (Ambion, Austin, TX), RNA was purified by gel filtration with Sephadex G-50 beads (Pharmacia, Uppsala, Sweden), phenol/chloroform (1:1, v/v) extractions, and ethanol precipitation. The integrity of the in vitro RNA transcripts was confirmed by 8% polyacrylamide/8M urea gel electrophoresis. The amount of fluorescent transcript was determined as follows. The concentration of in vitro transcripts of unlabelled wild-type snoRNA was calculated by spectrophotometry at 260 nm and formed the basis for a dilution series of known concentrations of snoRNA run on a gel stained with methylene blue. Fluorescein-labelled in vitro transcripts of the mutated snoRNAs were run on the same gel and their intensity compared with that of the wild-type snoRNA. Concentrations were adjusted accordingly so that equivalent amounts of mutated and wild-type snoRNAs were injected.

**Oocyte microinjections and fractionation**

A portion of the ovary was surgically removed from female Xenopus laevis (Nasco, Fort Atkinson, WI) following NIH IACUC-approved procedures, and transferred to OR2 saline buffer (Wallace et al., 1973).

Single oocytes were obtained by digesting the connective tissue with collagenase type I and II (3000 units/ml each) (Sigma, St Louis, MO) in OR2 for 2 h at room temperature. Stage V oocyte nuclei were injected 16–40 h after isolation (Drummond ‘Nanoject’, Broomall, PA).

For snoRNA depletion and rescue experiments, snoRNA was disrupted by two nuclear injections spaced 4 h apart of the antisense oligonucleotide TCTGGTGAGAACACTA-G/CGAAGTG-3' complementary to nucleotides 26–45 (similar to an oligonucleotide used by Enright et al., 1996) at a concentration of 10 μg/μl (9.2 nl = 92 ng/oocyte) for U14 snoRNA disruption, and 5'-GCTGGTTTC-3' complementary to nucleotides 39–48 (as in Peculis and Steitz, 1993) at 5 μg/μl for U8 snoRNA disruption. In order to use in vitro label RNA, [α-32P]UTP at 50 μCi/μl was added to the second injection of antisense oligonucleotide. Rescue was achieved by injecting 9.2 nl of 0.125 μg/μl in vitro-synthesized snoRNA (with or without fluorescein label) 4 h after the antisense oligonucleotide injection. Oocytes were incubated at 20°C for 16 h after the rescue injection. Subsequently, the nuclei were manually isolated and total RNA extracted using an RNA extraction kit (5 μl oocyte equivalent, 1/1000). The presence or absence of a cap did not affect wild-type snoRNA. Concentrations were adjusted accordingly so that equivalent amounts of mutated and wild-type snoRNAs were injected.

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**Nucleolar localization of Box C/D snoRNAs**

Following the method of Gall et al. (1991), originally designed for lampbrush chromosome preparations, the nuclear content of one oocyte was dispersed in a chamber on a gelatinized slide containing a solution of 20.75 mM KCl, 4.25 mM NaCl, 0.5 mM MgCl₂, 10 mM CaCl₂, 0.1% paraformaldehyde, 6.5 mM NaHPO₄ and 3.5 mM KH₂PO₄ pH 7.2. The slides were then centrifuged at 4000 g for 40 min at 4°C, incubated in distilled water and RNA was isolated from the fractions by an RNA extraction kit (5'-3' Inc, Boulder, CO). Gel electrophoresis and quantification was the same as described below for stability assays.

**Analysis by fluorescence microscopy**

Following the method of Gall et al. (1991), originally designed for lampbrush chromosome preparations, the nuclear content of one oocyte was dispersed in a chamber on a gelatinized slide containing a solution of 20.75 mM KCl, 4.25 mM NaCl, 0.5 mM MgCl₂, 10 mM CaCl₂, 0.1% paraformaldehyde, 6.5 mM NaHPO₄ and 3.5 mM KH₂PO₄ pH 7.2. The slides were then centrifuged at 4000 g for 40 min at 4°C, incubated in distilled water and RNA was isolated from the fractions by an RNA extraction kit (5'-3' Inc, Boulder, CO). Gel electrophoresis and quantification was the same as described below for stability assays.

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