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Nucleolar Targeting of 5S RNA in *Xenopus laevis* Oocytes: Somatic-Type Nucleotide Substitutions Enhance Nucleolar Localization

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Abstract In *Xenopus laevis* oocytes, 5S RNA is stored in the cytoplasm until vitellogenesis, at which time it is imported into the nucleus and targeted to nucleoli for ribosome assembly. This article shows that throughout oogenesis there is a pool of nuclear 5S RNA which is not nucleolar-associated. This distribution reflects that of oocyte-type 5S RNA, which is the major 5S RNA species in oocytes; only small amounts of somatic-type, which differs by six nucleotides, are synthesized. Indeed, ^{32}P-labeled oocyte-type 5S RNA showed a degree of nucleolar localization similar to endogenous 5S RNA (33%) after microinjection. In contrast, ^{32}P-labeled somatic-type 5S RNA showed significantly enhanced localization, whereby 70% of nuclear RNA was associated with nucleoli. A chimeric RNA molecule containing only one somatic-specific nucleotide substitution also showed enhanced localization, in addition to other somatic-specific phenotypes, including enhanced nuclear import and ribosome incorporation. The distribution of ^{35}S-labeled ribosomal protein L5 was similar to that of oocyte-type 5S RNA, even when preassembled with somatic-type 5S RNA. The distribution of a series of 5S RNA mutants was also analyzed. These mutants showed various degrees of localization, suggesting that the efficiency of nucleolar targeting can be influenced by many discrete regions of the 5S RNA molecule. J. Cell. Biochem. 69:490–505, 1998.

Key words: nucleolus; nuclear import; ribosomal protein L5; ribonucleoprotein particles; ribosome assembly; TFIIIA

In previtellogenic oocytes of *Xenopus laevis*, 5S ribosomal RNA (5S RNA) is synthesized before other components of the ribosome are available [Ford, 1971; Mairy and Denis, 1971], and stored in the cytoplasm as 7S ribonucleoprotein particles (RNPs) complexed with the 5S RNA gene-specific transcription factor IIIA (TFIIIA) [Honda and Roeder, 1980; Pelham and Brown, 1980; Picard and Wegnez, 1979], or with other nonribosomal proteins and tRNA as 42S RNPs [Picard et al., 1980]. During vitellogenesis, when the synthesis of other ribosomal components is maximal, 5S RNA forms a 5S RNP complex with ribosomal protein L5 [Allison et al., 1991]. L5 targets 5S RNA to the nucleus [Murdoch and Allison, 1996; Rudt and Pieler, 1996] for incorporation into the 60S ribosomal subunit in the amplified nucleoli [Allison et al., 1993].

In *Xenopus laevis* there are two families of 5S RNA genes, which are under developmental control and produce 5S RNAs differing in six nucleotides (Fig. 1) [Ford and Southern, 1973; Wegnez et al., 1972]. The oocyte-type family of 5S RNA genes, with 20,000 copies per haploid genome, is actively transcribed in developing oocytes, yielding large amounts of 5S RNA for ribosome stockpiling, but is relatively inactive during early embryogenesis and is switched off in somatic cells. The somatic-type 5S RNA family of genes, at 400 copies per haploid genome, is transcribed throughout development, including in oocytes [Wormington and Brown, 1983]. These two types of 5S RNA show different protein associations and localization patterns after microinjection into the cytoplasm of stage V oocytes [Allison et al., 1995]. Microinjected somatic-type 5S RNA predominantly interacts with L5 to form preribosomal 5S RNPs, while

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oocyte-type 5S RNA predominantly interacts with TFIIIA to form storage 7S RNPs. In addition, a greater amount of somatic-type 5S RNA accumulates in the nucleus and is assembled into 60S ribosomal subunits [Allison et al., 1995].

Once inside the nucleus 5S RNA must be targeted to the nucleolus for ribosome assembly. 5S RNA is thought to be incorporated into ribosomes at an early stage of assembly, as it has been detected in 55S preribosomal particles [Knight and Darnell, 1967; Warner and Soeiro, 1967], and has been mapped to both the dense fibrillar component and the granular component of the nucleolus [Raška et al., 1995]. Since the nucleolus is not enclosed by a membrane, nucleolar localization could occur by diffusion through the nucleoplasm and retention at nucleoli via interactions with other nucleolar components. This hypothesis is supported by studies on the nucleolar localization of several cellular proteins, including nucleolin [Créancier et al., 1993; Heine et al., 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993], UBF [Maeda et al., 1992], and ribosomal protein S6 [Schmidt et al., 1995], where discrete nucleolar localization sequences were not identified, but nucleolar localization was shown to be dependent on sequences involved in interactions with other nucleolar components. However, exceptions to the above have been observed for some viral regulatory proteins [Cochrane et al., 1990; Kubota et al., 1989; Mears et al., 1995; Siomi et al., 1988; Siomi et al., 1990], and for three cellular proteins, human angiogenin [Moroianu and Riordan, 1994], p120 [Valdez et al., 1994], and the constitutive nucleolar protein NO38 (B23) [Zirwes et al., 1997]. In these cases, discrete amino acid sequences were identified, which are both necessary and sufficient for the nucleolar localization of these proteins.

The nucleolar targeting of RNA molecules is less well investigated. The guanine nucleotide exchange factor, RCC1, has been shown to be involved in nucleolar localization of newly transcribed U3 small nucleolar RNA (snoRNA), suggesting that GTP hydrolysis is necessary for some step in this process [Cheng et al., 1995]. Recently, the nucleolar localization of the RNase

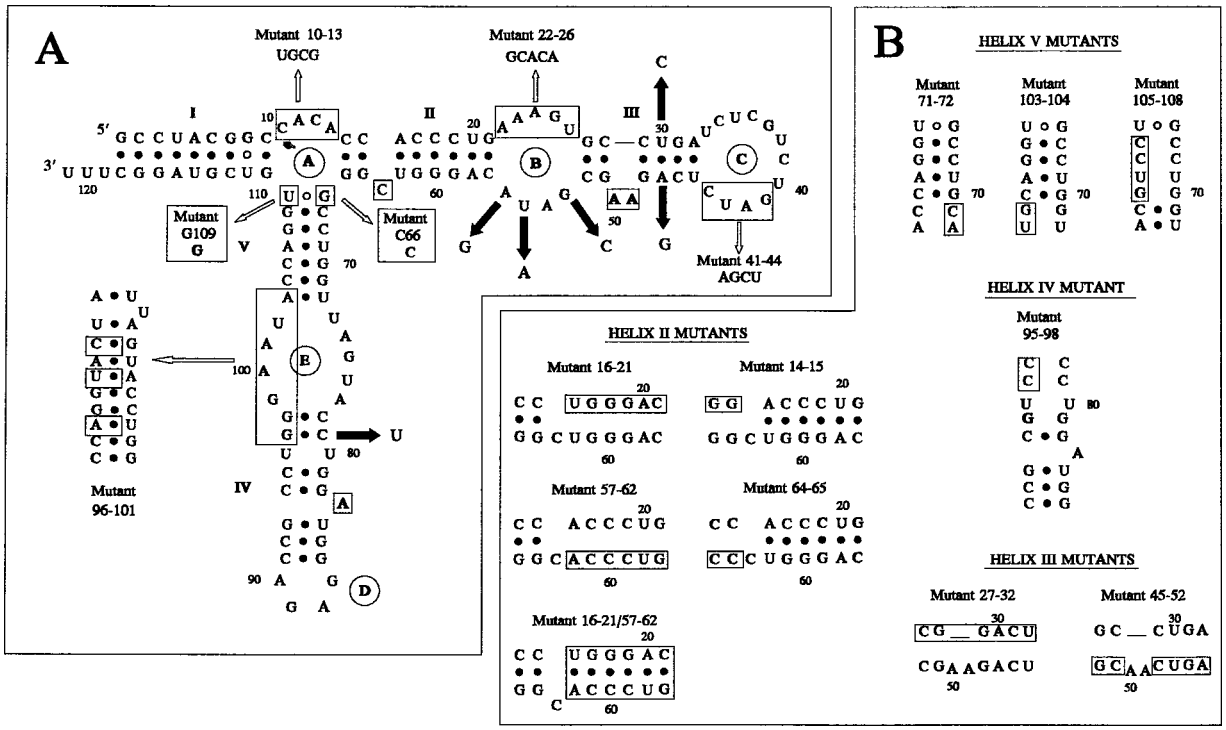


Fig. 1. Secondary structure of *X. laevis* oocyte-type 5S RNA showing somatic-specific substitutions and mutant nucleotide substitutions and deletions. **A:** Somatic-specific and single-strand substitutions. Nucleotides indicated by thick arrows at positions 30, 47, 53, 55, 56, and 79 are those present in somatic-type 5S RNA. Boxed nucleotides were replaced with the indicated nucleotides. The bulged nucleotides deleted at positions 49, 50, 63, and 83 are indicated in boxes. **B:** Helix mutants. Only the relevant region of 5S RNA is shown; substituted nucleotides are indicated in boxes.

MRP RNA was shown to require a sequence element implicated as the binding site for the nucleolar To antigen [Jacobson et al., 1995].

The nucleolar localization of 5S RNA could be conferred by the binding of a protein, such as L5, or by sequences within the RNA molecule itself. To determine whether a discrete nucleotide sequence is responsible for targeting the molecule to the nucleolus, a series of 5S RNA mutants were tested for their ability to localize to nucleoli after microinjection into the cytoplasm of *Xenopus* oocytes. The nucleolar localization characteristics of oocyte-type 5S RNA were also compared with those of somatic-type and chimeric 5S RNAs, as well as ribosomal protein L5.

MATERIALS AND METHODS

Plasmids and Synthesis of RNA and Protein

The 5S RNA gene templates [Baudin and Romaniuk, 1989; Baudin et al., 1991; Romaniuk, 1989; Romaniuk et al., 1987] used in this study were kindly provided by P.J. Romaniuk (University of Victoria, Victoria, British Columbia, Canada), and the U1 snRNA gene template [Hamm et al., 1987] by D.S. Goldfarb (University of Rochester, Rochester, NY). The U3 snoRNA gene template (pX1U3A′) [Savino et al., 1992], produced by M. Ezrokhi (Brown University, Providence, RI) using PCR as described [Terns and Dahlberg, 1994], was provided by S. Gerbi (Brown University). Internally labeled RNAs were synthesized by in vitro transcription and purified as described [Allison et al., 1995], with the inclusion of 0.4-mM m^7G cap (New England Biolabs, Biolab Scientific, Christchurch, New Zealand) in the reaction mixture for U1 snRNA and U3 snoRNA. For in situ nucleolar localization assays, ^{33}P-labeled RNAs were synthesized in a reaction containing 50 µCi [α-^{33}P]UTP (1,000–3,000 Ci/mmol; Amersham Australia, Auckland, New Zealand) and purified as for ^{32}P-labeled RNA.

pSP6-L5b containing the *X. laevis* ribosomal protein L5 cDNA clone [Wormington, 1989] was provided by W.M. Wormington (University of Virginia, Charlottesville, VA). ^{35}S-labeled L5 was synthesized using a rabbit reticulocyte lysate-coupled transcription–translation system for templates with SP6 promoters (Promega, Dade Diagnostics, Auckland, New Zealand) as described [Murdoch and Allison, 1996]. For synthesis of ^{35}S-5S RNPs, unlabeled oocyte-type and somatic-type 5S RNAs were produced using the Ampliscribe reaction kit (Epicentre Technologies, Intermed Scientific, Auckland, New Zealand) as described [Murdoch and Allison, 1996], and 10 µg of either unlabeled RNA was included in a transcription–translation reaction to produce L5 bound to *X. laevis* oocyte-type or somatic-type 5S RNA.

Nucleolar Localization Assays

A lobe of ovary was surgically removed from an adult female *X. laevis* (Xenopus I, Ann Arbor, MI) and processed as described [Allison et al., 1991]. Twenty nanoliters (0.1- to 1.0-ng RNA) of ^{32}P-labeled RNA was microinjected into the cytoplasm of specified stages of oocytes as described [Allison et al., 1991, 1993, 1995]. Oocytes were incubated overnight at 18°C to allow nuclear import and nucleolar localization. Nucleoli were isolated using the method of Peculis and Gall [1992]. Nuclei were dissected from oocytes in nucleolar isolation buffer (83-mM KCl, 17-mM NaCl, 6.5-mM Na_2HPO_4, 10-mM $MgCl_2$, 1-mM EDTA, 1-mM DTT) using watchmaker's forceps, and collected in microfuge tubes. Isolated nuclei were sonicated in 50-µl nucleolar isolation buffer in a Branson bath sonicator (Bransonic 2) for 20 s in ice water. Samples were centrifuged for 20 min at 15,000*g* to pellet nucleoli. After drawing off the supernatant (nucleoplasmic fraction), nucleolar pellets were washed by adding 200-µl nucleolar isolation buffer, centrifuging for 5 min and discarding the supernatant. The presence of nucleoli in the pellet fraction was confirmed by two means: immunoblotting with anti-NO38 antisera [Schmidt-Zachmann et al., 1987] revealed that all of the nucleolar protein NO38 (B23), which associates with 60S preribosomal particles [Biggiogera et al., 1989; Hügle et al., 1985; Peculis and Gall, 1992; Schmidt-Zachmann et al., 1987; Spector et al., 1984; Yung et al., 1985], was present in the nucleolar fraction, and Northern blotting showed that all of the nuclear 18S and 28S rRNA was present in nucleolar fractions (data not shown). Taken together, these control assays indicate that all extrachromosomal nucleolar material was pelleted and that 60S ribosomal subunits still inside the nucleus remained associated with nucleoli during the isolation procedure.

RNA was extracted from oocyte fractions using the potassium acetate method [Peppel and Baglioni, 1990]. Samples were analyzed by 8-M urea/8% polyacrylamide gel electrophoresis

(PAGE) and autoradiography. A Zeineh analytical hand-held scanning densitometer and Biomed Image Analysis software (Advanced American Biotechnology, Fullerton, CA) were used to quantify the intensity of bands on suitable exposures of autoradiograms (within the linear range of signal intensity of the film). Alternatively, samples were resuspended in 50 µl of TE, pH 7.6, and added to 2 ml of Biodegradable Counting Scintillant (BCS_i; Amersham) for direct counting of samples in a scintillation counter (Beckman LS 2800).

For nucleolar localization of exogenous L5, 50 nl of L5 lysate mixture (approximately 100-pg L5) was injected into the cytoplasm of stage V [Dumont, 1972] oocytes. Oocytes were incubated in the presence of 100-µg/ml cycloheximide (Sigma, St. Louis, MO) to prevent incorporation of excess [^{35}S]methionine into oocyte proteins. Nucleolar pellets were dissolved directly in 10-µl SDS-PAGE sample buffer [Murdoch and Allison, 1996]. Nucleoplasmic proteins were precipitated with 5 vol of acetone overnight at −20°C, centrifuged at 15,000*g* for 10 min, and resuspended in 20-µl SDS-PAGE sample buffer. Samples were analyzed by discontinuous 12% polyacrylamide/0.1% SDS gels and fluorography as described [Murdoch and Allison, 1996]. Typical exposure times were two weeks.

In Situ Assays

For in situ nucleolar localization assays, stage V oocytes were cytoplasmically injected with ^{33}P-labeled RNA and incubated overnight. Oocytes were then fixed for 24 h in 5% acetic acid, 2% formaldehyde, 250-mM NaCl, dehydrated through an ethanol series, and then incubated in Cedarwood oil (Gurr) for 2–6 h with gentle rotation for clearing. Oocytes were embedded in paraffin by standard histological procedures and sectioned using a Beck microtome at 4–7 µm. Sections were adhered onto subbed slides [Pardue, 1985], the paraffin was removed, and slides were dipped in LM-1 emulsion (Amersham) diluted 1:1 with sterile distilled H_2O at 43°C. Slides were exposed at 4°C for four days to four weeks, then developed in D19 developer (Kodak) and stained with Giemsa [Pardue, 1985]. Slides were viewed under bright field microscopy using an Olympus BH-2 microscope and photographed with either Agfacolor Optima 125 print film using neutral density and blue filters, or with Fujichrome 64T slide film using a neutral density filter.

Northern Analysis

For the analysis of nucleolar localization of endogenous 5S RNA, RNA was extracted from nucleolar and nucleoplasmic fractions from 40 nuclei as described above. Samples were separated by agarose gel electrophoresis and transferred by capillary transfer to positively charged nylon membrane (Boehringer Mannheim, Auckland, New Zealand) in 20 X SSC as described [Sambrook et al., 1989]. Membranes were probed with digoxigenin (DIG)-labeled antisense 5S RNA. Antisense 5S RNA was synthesized from pXlo8G [Allison et al., 1991] in reactions containing 1 µg of template DNA, 40-U T7 RNA polymerase (Epicentre), and 0.35-mM DIG-11-UTP (Boehringer Mannheim) following the manufacturer's instructions. Membranes were prehybridized 2–4 h at 68°C in hybridization buffer (50% formamide, 5 X SSC, 2% blocking solution, 0.1% sarkosyl, 0.02% SDS). Membranes were hybridized with 50–200-ng/ml DIG-labeled RNA probe overnight at 68°C with agitation. Subsequently, membranes were washed twice in 2 X SSC and 0.1% SDS at room temperature for 5 min each, and twice in 0.1 X SSC and 0.1% SDS at 68°C for 15 min each, with agitation.

Western Analysis

For analysis of the distribution of endogenous ribosomal protein L5 and NO38, proteins were prepared from nucleolar and nucleoplasmic fractions from 50 stage V oocytes as described above. Proteins were transferred to nitrocellulose membrane (Hybond-C, Amersham) using a BioRad Mini-Trans Blot electrophoretic transfer apparatus in 25-mM Tris, 190-mM glycine, and 20% methanol for 18 h at 30 V (approximately 48 mA) at 4°C, according to the manufacturer's instructions. Membranes were blocked for 2 h at room temperature in 3% BSA (fraction V; Boehringer Mannheim) in PBS (137-mM NaCl, 2.7-mM KCl, 10.1-mM Na_2HPO_4, 1.8-mM KH_2PO_4, pH 7.2) (BSA/PBS) followed by two 5-min rinses in PBS. Anti-L5 antiserum [Kenmochi and Ogata, 1989; Murdoch and Allison, 1996] was generously donated by N. Kenmochi (University of the Ryukyus, Okinawa, Japan), and anti-NO38 (No-185) [Schmidt-Zachmann et al., 1987] by M.S.

Schmidt-Zachmann (German Cancer Research Center, Heidelberg, Germany). Antibodies were diluted 1:1,000 in 3% BSA/PBS before incubation for 1 h, followed by four 5-min washes in PBS. Membranes were then incubated in anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim) diluted 1:2,000 in 3% BSA/PBS for 1 h, followed by four washes for 5 min in PBS. For detection of proteins, membranes were incubated in 0.06% 4-chloro-1-naphthol (Sigma)/0.01% H_2O_2 until bands were suitably dark (5–30 min). The reaction was stopped by rinsing with PBS.

Analysis of Nuclear Transport and RNP Formation

After cytoplasmic microinjection of 20-nl ^{32}P-labeled 5S RNA and 20-h incubation, nuclei were manually dissected from oocytes in 1% TCA and collected for nuclear transport analysis. RNA was extracted from nuclear and cytoplasmic fractions and analyzed by denaturing PAGE as described [Allison et al., 1995]. For analysis of 5S RNP and 7S RNP formation, crude cellular lysates were prepared from samples of microinjected oocytes and separated by nondenaturing PAGE as described [Allison et al., 1995]. Anti-60S ribosomal subunit antiserum [Allison et al., 1993; Viel et al., 1990] was generously provided by M. le Maire (CEA and CNRS, Gif-sur-Yvette, France). For analysis of 60S ribosomal subunit assembly, microinjected oocytes were incubated for 48 h, immunoprecipitation assays were performed, and RNA was extracted from the immunoprecipitate and immunosupernatant fractions and analyzed by denaturing PAGE as described [Allison et al., 1993].

RESULTS

Nucleolar Localization of Endogenous 5S RNA Compared With Microinjected Oocyte-Type 5S RNA

We sought to determine whether specific sequences or secondary structures of the 5S RNA molecule are required for nucleolar targeting in *X. laevis* oocytes. To this end, a biochemical fractionation assay was used, in which sonicated nuclei were centrifuged to recover nucleoli [Peculis and Gall, 1992]. To establish a baseline from which to compare the localization of a series of mutant 5S RNAs, ^{32}P-labeled oocyte-type 5S RNA was microinjected into stage V oocytes, and assayed for nucleolar localization. The amount of 5S RNA injected (0.1 to 1.0 ng) was well below the amount of endogenous 5S RNA in the oocyte (60 ng per oocyte) [Hausen and Riebesell, 1991] and the vast excess required to saturate nuclear transport [Murdoch and Allison, 1996]. However, to avoid any possibility of overloading the nucleus and potentially saturating nucleolar binding sites, 5S RNA was injected into the oocyte cytoplasm. Subsequently, nucleolar localization was calculated as a percentage of RNA that had been imported into the nucleus. After 20-h incubation, only 33% of nuclear oocyte-type 5S RNA was associated with nucleoli (Fig. 2A, lanes 1 and 2). As a control, microinjected U1 small nuclear RNA (snRNA), which is involved in mRNA splicing, was shown to be confined to the nucleoplasmic fraction (lanes 5 and 6). In contrast to U1, U3 snoRNA, which is involved in rRNA processing in the nucleolus [Maxwell and Fournier, 1995], showed a predominantly (90%) nucleolar distribution as expected (data not shown). As an additional control, ^{32}P-labeled 5S RNA was added to isolated nuclei after sonication treatment and the nucleoli pelleted by centrifugation. All of the added 5S RNA was found in the supernatant fraction (Fig. 2A, lanes 7 and 8), indicating that the 5S RNA in nucleolar fractions from microinjected oocytes is due to specific associations with nucleoli, and not to nonspecific entrapment or sticking.

Since we previously observed that somatic-type 5S RNA shows greater levels of nuclear import and incorporation into 60S ribosomal subunits than oocyte-type 5S RNA after microinjection into the oocyte cytoplasm [Allison et al., 1995], we were interested in determining whether the nucleolar localization of somatic-type was similar to that of oocyte-type 5S RNA. Somatic-type 5S RNA showed a significantly different intranuclear distribution pattern: 70% of nuclear somatic-type 5S RNA was associated with nucleoli (Fig. 2A, lanes 3 and 4), compared with 33% for oocyte-type. This strikingly different pattern was highly reproducible in many batches of oocytes, and did not appear to depend on the amount of RNA injected; microinjecting from 0.1 to 1.0 ng of both types of RNA resulted in the same distinctive distribution (data not shown).

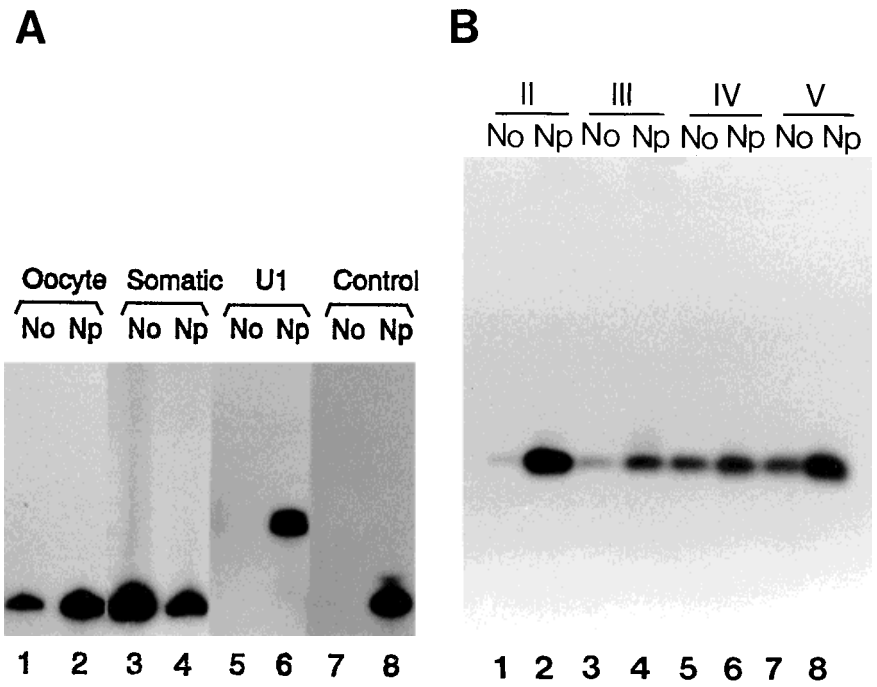


Fig. 2. Distribution of 5S RNA in biochemically fractionated *X. laevis* oocyte nuclei. **A:** Nucleolar localization of microinjected RNA. ^{32}P-labeled oocyte-type or somatic-type 5S RNA or U1 snRNA molecules were injected into the cytoplasm of stage V oocytes as indicated and incubated overnight. Ten isolated nuclei were fractionated by sonication and centrifugation to yield nucleolar (No) pellets and nucleoplasmic (Np) supernatant fractions. Total RNA was extracted from each fraction and analyzed by 8-M urea/8% PAGE and autoradiography. Control nuclei were dissected from uninjected oocytes and ^{32}P-labeled 5S RNA was added, followed by preparation of nucleolar and nucleoplasmic fractions and analysis as described above. **B:** Nucleolar localization of endogenous 5S RNA. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 40 isolated nuclei as described in Figure 2A. Total RNA was extracted and separated by agarose gel electrophoresis. The distribution of 5S RNA was determined by Northern blotting using an antisense 5S RNA probe. Oocyte stages II to V [Dumont, 1972] are indicated.

To determine whether the relatively low level of nucleolar localization of oocyte-type 5S RNA described above was simply an artifact of microinjection, the distribution of microinjected 5S RNA was compared with that of endogenous 5S RNA. Figure 2B shows the nucleolar localization of endogenous 5S RNA at various stages of oocyte development, and these results are quantified in Table I. In stage II oocytes, the majority of 5S RNA in the nucleus was not associated with nucleoli (Fig. 2B, lanes 1 and 2). The percentage of 5S RNA associated with nucleoli increased during oogenesis, peaking at 40% in stage IV oocytes (Table I), coinciding with the peak of ribosome synthesis. Similar patterns of nucleolar localization were observed in other experiments with different batches of oocytes. On average, only 33% of endogenous, nuclear 5S RNA was associated with nucleoli of stage V oocytes. Since oocyte-type 5S RNA is the predominant type of 5S RNA in oocytes [Ford and Southern, 1973; Wegnez et al., 1972; Wormington and Brown, 1983], these results primarily represent the distribution of endogenous oocyte-type 5S RNA. Thus, these findings show that throughout oogenesis there is a pool of 5S RNA within the nucleus that is not localized to nucleoli, and confirm that microinjected oocyte-type 5S RNA assumes a nuclear distribution comparable to endogenous 5S RNA.

Somatic-Type Nucleotide Substitutions Enhance Nucleolar Localization and Ribosome Assembly

To investigate further the effect of somatic-type nucleotide substitutions, the subcellular distribution and protein associations of two chimeric 5S RNAs were analyzed after microinjection into the oocyte cytoplasm. The 5′-somatic chimera, consisting of the 5′ sequence of somatic-type and the 3′ sequence of oocyte-type, contains five out of the six somatic-specific substitutions (C30, G47, C53, A55, G56; Fig. 1),

TABLE I. Nucleolar Localization of 5S RNA Throughout Oogenesis*

Stage of oogenesis	% nucleolar localization
II	6.3
III	21.6
IV	40.8
V	34.4

*Oocyte stages are according to Dumont [1972]. % nucleolar localization represents the percentage of endogenous 5S RNA within the nucleus associated with nucleoli.

whereas the 5′-oocyte chimera, consisting of the 5′ sequence of oocyte-type and the 3′ sequence of somatic-type, contains only one somatic-specific substitution (U79; Fig. 1). Both chimeric RNA molecules showed a comparable nucleocytoplasmic distribution to somatic-type 5S RNA after cytoplasmic microinjection (Fig. 3A). On average, nuclear accumulation was greater than twice that of oocyte-type 5S RNA after 20-h incubation (Table II). Analysis of RNP formation by nondenaturing polyacrylamide gel electrophoresis showed that both chimeric 5S RNAs predominantly interacted with L5 to form 5S RNPs, although more of the 5′-somatic chimera was present as unbound 5S RNA compared with somatic-type and the 5′-oocyte chimera (Fig. 3B, Table II). Chimeric 5S RNA-containing 7S RNPs were only detectable upon overexposure of the autoradiogram (data not shown). Both chimeric 5S RNAs showed enhanced nucleolar localization compared with oocyte-type 5S RNA (Fig. 3C). On average, nucleolar localization of the 5′-oocyte chimera was 86% of somatic-type, while the 5′-somatic chimera and oocyte-type were 53% and 22% of somatic-type, respectively (Table II). In the particular batches of oocytes used for these experiments, oocyte-type nucleolar localization was lower than average (compare Fig. 2 and Fig. 3C). To demonstrate that the chimeric 5S RNA molecules were targeted to sites of ribosome assembly within the nucleoli, ribosome incorporation was analyzed by immunoprecipitation with anti-60S ribosomal subunit antibodies. Both chimeric 5S RNA molecules were assembled into 60S ribosomal subunits, to a greater extent than oocyte-type, but to a lesser extent than somatic-type (Fig. 3D, Table II). These results indicate that, surprisingly, a single base substitution at position 79 can change the behavior of 5S RNA, such that it shows enhanced nuclear import, nucleolar localization, and ribosome incorporation compared with oocyte-type.

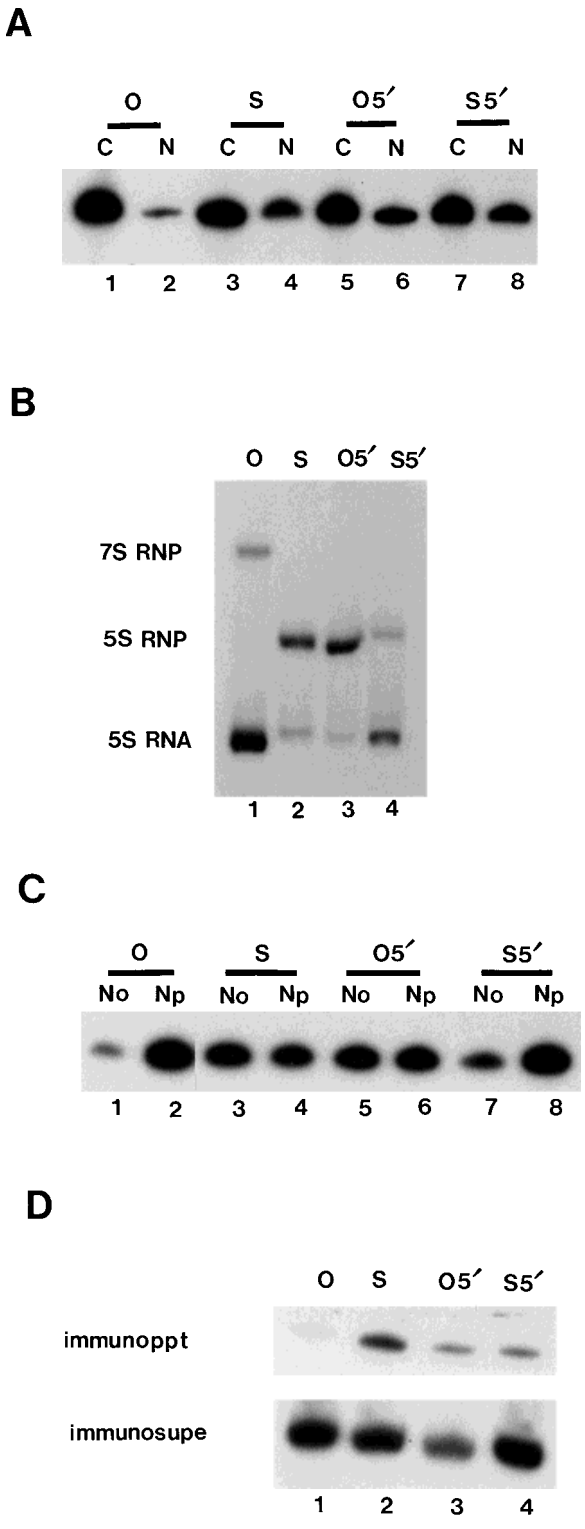
Sequence and Structural Requirements for Nucleolar Localization

To determine other regions of 5S RNA important for nucleolar localization, we next tested a series of mutant oocyte-type 5S RNA molecules for their localization ability. The mutants contain either substituted or deleted nucleotides (Fig. 1), and were previously tested for their ability to be imported into the oocyte nucleus, form 7S RNPs and 5S RNPs, and assemble into 60S ribosomal subunits [Allison et al., 1993; L.A. Allison, unpublished results]. Nucleolar localization for each mutant was calculated as a percentage of the RNA in the nucleus; therefore, the results are not effected by the reported differences in levels of nuclear accumulation of some of the mutants [Allison et al., 1993]. Since there is slight variation between batches of oocytes, the nucleolar localization of each mutant was compared with that of microinjected oocyte-type 5S RNA within the same batch of oocytes, and all mutants were tested in at least two different batches from different frogs.

The results from a selection of mutants are shown in Fig. 4, and a summary of all the mutants tested, made relative to oocyte-type, is presented in Fig. 5. None of the mutants tested were completely defective for nucleolar localization, but they showed a variety of localization phenotypes. The majority of 5S RNA mutants showed similar nucleolar localization to oocyte-type 5S RNA, for example, mutant 27–32 (Fig. 4, lanes 3 and 4). However, some mutants showed lower levels of nucleolar accumulation, for example, nucleolar localization of mutants with substitutions at positions 14–15 and 16–21 in helix II was reduced 1.3- and 1.7-fold, respectively (lanes 5, 6, 13, and 14). Other mutants, for example, 57–62 and 95–98, showed enhanced nucleolar localization compared with oocyte-type (1.7- and 2.0-fold enhancement, respectively; Fig. 5).

Of particular interest are four mutants previously shown to be defective for incorporation into 60S ribosomal subunits [Allison et al., 1993]. Nucleolar localization of mutant 10–13 was similar to oocyte-type (Fig. 4, lanes 15 and 16), whereas nucleolar localization of mutant 96–101 was reduced 1.6-fold compared with

oocyte-type (lanes 7 and 8). In contrast, a much greater percentage of the two deletion mutants, Δ63 (lanes 9 and 10) and Δ49,50 (lanes 11 and 12) was reproducibly found associated with the nucleolar pellet (61% and 79%, respectively).



Comparison of Nucleolar Fractionation Assay With In Situ Nucleolar Localization

Since the nucleolar isolation procedure does not yield a pure preparation of nucleoli [Peculis and Gall, 1992], the results presented above were confirmed using in situ localization assays. The distribution of oocyte-type 5S RNA in semithin sections of oocytes after microinjection was relatively homogeneous throughout the nucleus (Fig. 6A). Although silver grains were located over nucleoli, they did not appear to be enriched. Somatic-type 5S RNA, however, showed dramatic nucleolar localization (Fig. 6B), consistent with the biochemical fractionation results above. Silver grains were aggregated over nucleoli, with lesser amounts distributed throughout the nucleoplasm.

For all but two of the mutants tested in this assay, in situ nucleolar localization characteristics were consistent with the biochemical fractionation results (Fig. 6; data not shown). For example, the distribution of mutants 10–13 and 96–101 was relatively homogeneous throughout the nucleus and silver grains were not clustered over nucleoli (Fig. 6C and D), correlating with the biochemical measurement of nucleolar localization that was similar to or less than oocyte-type. However, mutants Δ49,50 and Δ63, which were previously shown to be defective for ribosome assembly [Allison et al., 1993], showed enhanced nucleolar localization according to the biochemical fractionation but did not show a corresponding distribution in situ (Fig. 6E and F). Rather, the distribution of these two

Fig. 3. Analysis of the subcellular distribution and protein associations of chimeric 5S RNA molecules. Stage V oocytes were cytoplasmically injected with ^{32}P-labeled oocyte-type (O), somatic-type (S), 5′-oocyte chimeric (O5′), or 5′-somatic chimeric (S5′) 5S RNA (see text for description of chimeric RNAs) and incubated for 20 h (A, B, C) or 48 h (D). **A:** Nuclear transport characteristics. After manual dissection, RNA was extracted from three pooled nuclear and cytoplasmic fractions and analyzed by denaturing PAGE and autoradiography. **B:** RNP distribution. Cleared homogenates from three oocytes were subjected to nondenaturing PAGE and analyzed for 5S RNP (5S RNA-ribosomal protein L5 complex) and 7S RNP (5S RNA-TFIIIA complex) formation. **C:** Nucleolar localization. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from ten isolated nuclei by sonication and centrifugation and analyzed as described in Figure 2A. **D:** Ribosome assembly. Cleared oocyte homogenates were incubated with anti-60S ribosomal subunit antibodies in immunoprecipitation assays. Labeled RNAs were recovered from the immunoprecipitate (immunoppt) and immunosupernatant (immunosupe) fractions and analyzed by denaturing PAGE and autoradiography.

TABLE II. Protein Associations and Subcellular Distribution of Chimeric 5S RNA Molecules*

Type of RNA	Nuclear transport relative to oocyte-type	Nucleolar localization relative to somatic-type	RNP assembly	60S subunit assembly
oocyte-type	1.0	0.22 ± 0.03	TFIIIA	+
somatic-type	2.10 ± 0.52 (7)	1.0	L5	+++
5′-oocyte chimera	2.43 ± 0.60 (8)	0.86 ± 0.05	L5	++
5′-somatic chimera	2.39 ± 0.81 (6)	0.53 ± 0.06	L5	++

*Nuclear transport and nucleolar localization were analyzed as described in Figure 3. Data are expressed in arbitrary units as the mean ± the standard deviation relative to oocyte-type 5S RNA for nuclear transport, and relative to somatic-type for nucleolar localization. The number of experimental repetitions is indicated in brackets. 7S RNP and 5S RNP formation were analyzed as described in Figure 3. TFIIIA represents predominantly 7S RNP formation in six experimental repetitions; L5, predominantly 5S RNP formation. 60S ribosomal subunit assembly was analyzed as described in Figure 3. Three experimental repetitions were assessed qualitatively by estimation of relative amounts of bound and free RNA; +++ denotes enhanced assembly; ++, moderate assembly; and +, reduced assembly.

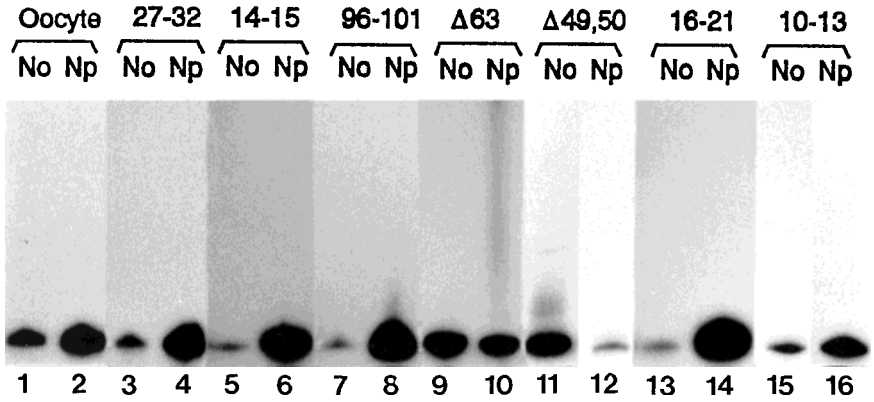


Fig. 4. Nucleolar localization of microinjected 5S RNA molecules with sequence and structural alterations. ^{32}P-labeled oocyte-type 5S RNA or mutant 5S RNA molecules were injected into the cytoplasm of stage V oocytes and assayed for nucleolar localization as described in Figure 2A. Mutant designations refer to those areas of the molecule that were substituted or deleted (Fig. 1).

mutants within the nucleus was very similar to oocyte-type 5S RNA. Silver grains were homogeneously distributed throughout the nucleus, including over nucleoli, and were not aggregated or associated with specific structures. These results imply that these two mutant RNAs are binding to other nuclear components, which are pelleted under the nucleolar isolation conditions used.

Nucleolar Localization of Ribosomal Protein L5

Since ribosomal protein L5 plays a role in mediating 5S RNA nuclear import [Allison et al., 1991, 1995; Murdoch and Allison, 1996; Rudt and Pieler, 1996] and is postulated to target 5S RNA to the nucleolus in HeLa cells [Steitz et al., 1988], it was of interest to compare the intranuclear distribution of microinjected L5 with the distribution pattern of microinjected 5S RNA. Quantification of results indicated that approximately 40% of cytoplasmically microinjected ^{35}S-labeled L5 in the nucleus was nucleolar-associated (Fig. 7A, lanes 1 and 2). These results were also confirmed using in situ localization assays. Semithin sections of microinjected oocytes showed distinct accumulations of silver grains over and around nucleoli, although not to the extent shown by somatic-type 5S RNA (Fig. 7C). Since the ^{35}S-labeled L5 was not purified from unincorporated [^{35}S]methionine in the lysate mixture, controls were performed in which oocytes were injected with the product of a transcription–translation reaction that was primed with H_2O instead of template DNA. The pattern of distribution was distinct from ^{35}S-L5: most of the [^{35}S]methionine was localized in the cytoplasm, with only a small number of silver grains in the nucleus, which appeared to be randomly distributed (Fig. 7B).

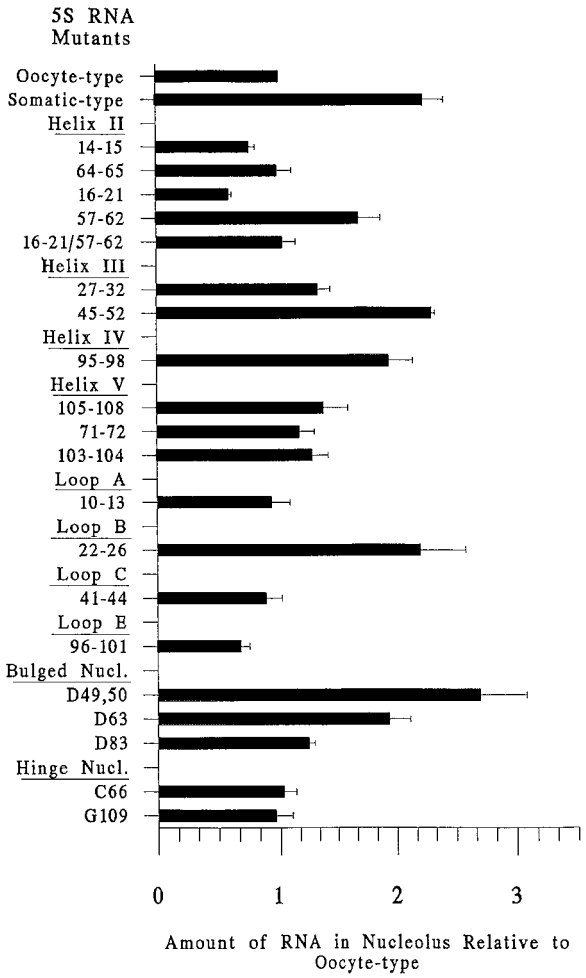


Fig. 5. Summary of nucleolar localization of 5S RNA mutants. Nucleolar localization was analyzed as described in Figure 4. Nucleolar localization was calculated as a percentage of the RNA in the nucleus, and expressed relative to the nucleolar localization of oocyte-type 5S RNA within the same batch of oocytes, as quantified by densitometry or scintillation counting. All mutants were tested in at least two different batches from different frogs. The bars indicate the standard errors of the means from six to 16 replicate samples. Mutant designations refer to those areas of the 5S RNA molecule that were substituted or deleted (Fig. 1).

The rabbit reticulocyte lysate used to synthesize ^{35}S-labeled L5 contains excess 5S RNA [Zehavi-Willner and Danon, 1972], which binds to the nascent L5, forming 5S RNPs [Murdoch and Allison, 1996]. Thus, the distribution of labeled L5 in the nucleus may reflect the distribution of 5S RNPs containing rabbit reticulocyte 5S RNA. To determine whether the sequence of the 5S RNA that is bound to L5 alters the nucleolar localization, L5 was synthesized in the presence of excess unlabeled oocyte-type or somatic-type 5S RNA, thus forming oocyte- or somatic-type 5S RNA-containing 5S RNPs [Murdoch and Allison, 1996]. The amount of labeled L5 recovered from nucleolar pellets was identical for L5 synthesized in the presence of either oocyte-type 5S RNA, somatic-type 5S RNA, or only rabbit reticulocyte components (Fig. 7A, lanes 3 to 6). It is not known, however, whether injected in vitro-generated RNPs are stable in the oocyte, or whether dissociation, followed by reassociation with endogenous 5S RNA, occurs by an exchange reaction [Allison et al., 1995; Huber and Wool, 1986; Nazar and Wildeman, 1983]. Finally, the nuclear distribution of endogenous L5 was assayed by Western analysis. Due to limitations in antibody sensitivity, only faint amounts of endogenous L5 were reproducibly detected in nucleolar fractions (Fig. 7A, lanes 7 and 8). The presence of nucleolar proteins in the pellet fraction was confirmed by immunodetection of the nucleolar protein NO38 (B23) [Biggiogera et al., 1989; Hügle et al., 1985; Peculis and Gall, 1992; Schmidt-Zachmann et al., 1987; Spector et al., 1984; Yung et al., 1985]. As expected, NO38 was present in the nucleolar fraction (Fig. 7D, lane 2) but absent from the cytoplasmic and nucleoplasmic fractions (Fig. 7D, lanes 1 and 3).

DISCUSSION

In this work we have investigated the nucleolar localization characteristics of 5S RNA in *Xenopus* oocytes. We found that throughout oogenesis there is a pool of nuclear 5S RNA that is not nucleolar-associated. In addition, we have shown that efficient nucleolar targeting is not conferred by one discrete region of the 5S RNA molecule; however, nucleolar localization is enhanced by somatic-type 5S RNA-specific nucleotide substitutions.

Behavior of Oocyte-Type and Somatic-Type 5S RNA

We found that only 33% of endogenous 5S RNA within the nucleus is associated with nucleoli. In addition, only 30–40% of microinjected ^{32}P-labeled oocyte-type 5S RNA and ^{35}S-labeled ribosomal protein L5 in the nucleus are localized to the nucleolus and therefore being assembled into ribosomal subunits. The consistency between the results for endogenous and microinjected 5S RNA, as well as between the two different techniques utilized, suggests that this pattern reflects the situation in vivo. Even in stage IV oocytes, where ribosome assembly is

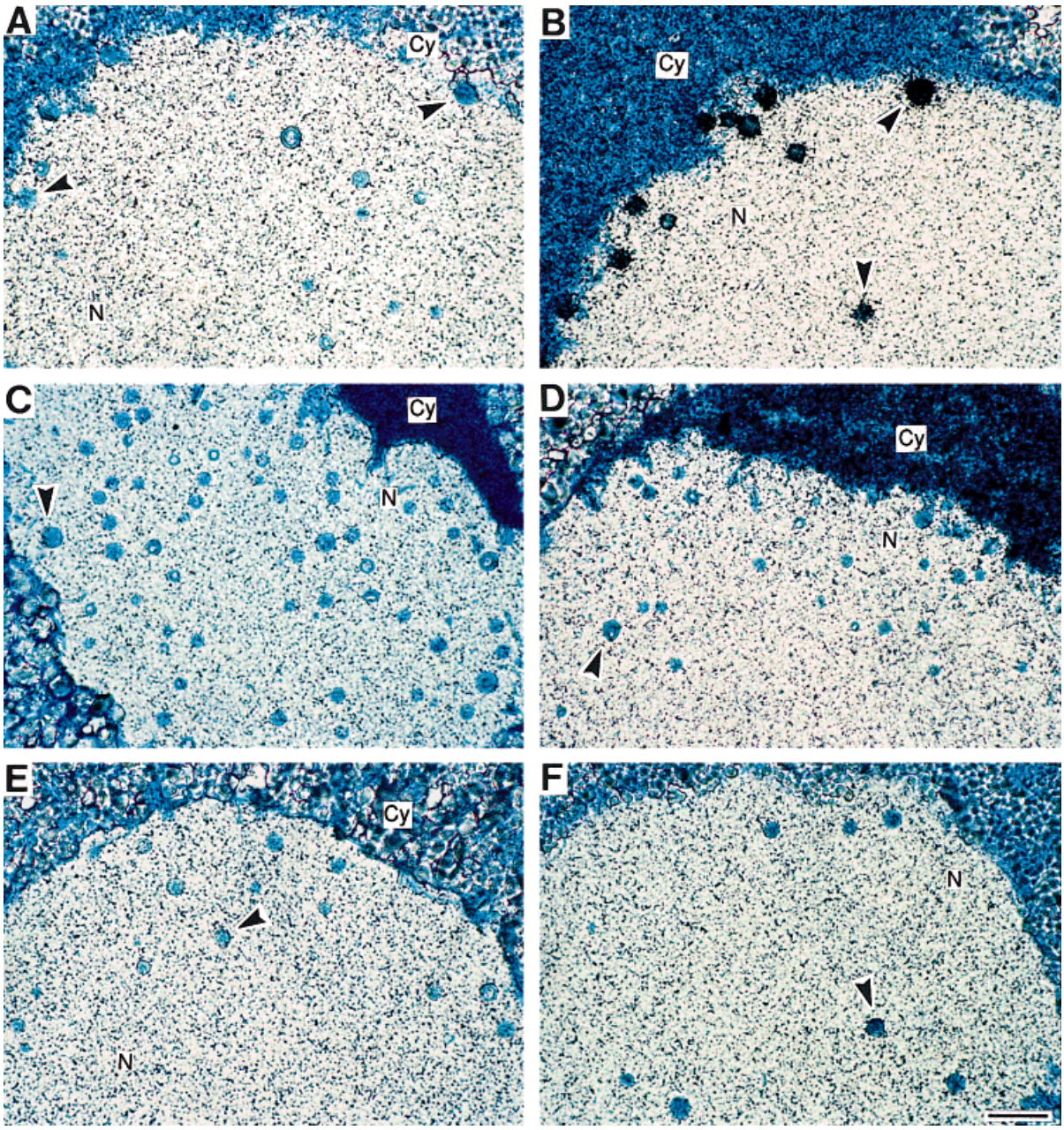


Fig. 6. In situ nucleolar localization of exogenous RNAs in *X. laevis* oocytes. ^{33}P-labeled 5S RNA was injected into the cytoplasm of stage V oocytes, which were then incubated overnight. Oocytes were fixed and sectioned at 4–7 μm, subject to autoradiography and stained with Giemsa. **A:** Oocyte injected with oocyte-type 5S RNA. **B:** Oocyte injected with somatic-type 5S RNA. **C:** Oocyte injected with mutant 10–13. **D:** Oocyte injected with mutant 96–101. **E:** Oocyte injected with mutant Δ49,50. **F:** Oocyte injected with mutant Δ63. Cy denotes cytoplasm; N, nucleus; arrows indicate some of the extrachromosomal nucleoli. Scale bar = 20 μm.

occurring at a maximum rate, only 40% of the endogenous, nuclear 5S RNA was localized to nucleoli. These results are consistent with previous in situ hybridization studies, which showed that the localization of 5S RNA over nucleoli peaked in stage IV oocytes [Allison et al., 1991].

In contrast to oocyte-type 5S RNA, 70% of microinjected somatic-type 5S RNA in the nucleus was associated with nucleoli. Thus, the six nucleotide differences between oocyte-type and somatic-type 5S RNA in the nucleus not only affect nuclear import and ribosome incorporation, but also the intranuclear distribution. A

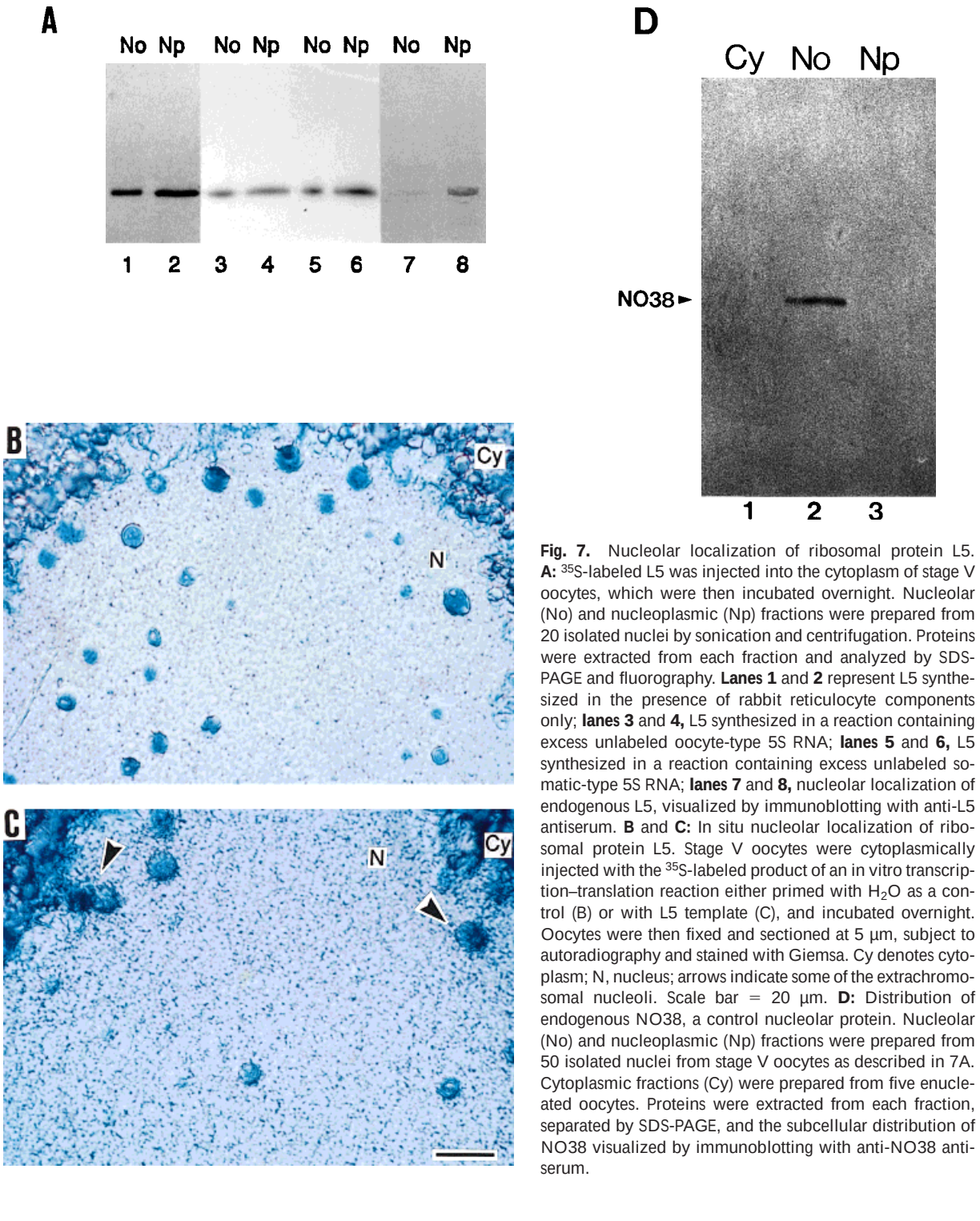


Fig. 7. Nucleolar localization of ribosomal protein L5. **A:** ^{35}S-labeled L5 was injected into the cytoplasm of stage V oocytes, which were then incubated overnight. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 20 isolated nuclei by sonication and centrifugation. Proteins were extracted from each fraction and analyzed by SDS-PAGE and fluorography. **Lanes 1** and **2** represent L5 synthesized in the presence of rabbit reticulocyte components only; **lanes 3** and **4,** L5 synthesized in a reaction containing excess unlabeled oocyte-type 5S RNA; **lanes 5** and **6,** L5 synthesized in a reaction containing excess unlabeled somatic-type 5S RNA; **lanes 7** and **8,** nucleolar localization of endogenous L5, visualized by immunoblotting with anti-L5 antiserum. **B** and **C:** In situ nucleolar localization of ribosomal protein L5. Stage V oocytes were cytoplasmically injected with the ^{35}S-labeled product of an in vitro transcription–translation reaction either primed with H_2O as a control (B) or with L5 template (C), and incubated overnight. Oocytes were then fixed and sectioned at 5 µm, subject to autoradiography and stained with Giemsa. Cy denotes cytoplasm; N, nucleus; arrows indicate some of the extrachromosomal nucleoli. Scale bar = 20 µm. **D:** Distribution of endogenous NO38, a control nucleolar protein. Nucleolar (No) and nucleoplasmic (Np) fractions were prepared from 50 isolated nuclei from stage V oocytes as described in 7A. Cytoplasmic fractions (Cy) were prepared from five enucleated oocytes. Proteins were extracted from each fraction, separated by SDS-PAGE, and the subcellular distribution of NO38 visualized by immunoblotting with anti-NO38 antiserum.

similarly high degree of nucleolar localization was also observed when HeLa cell 5S RNA was microinjected into *Xenopus* oocytes [De Robertis et al., 1982]. Interestingly, somatic-type 5S RNA bears a closer resemblance to HeLa cell 5S RNA in sequence than oocyte-type [Ford and Southern, 1973]. Surprisingly, a chimeric 5S RNA molecule containing only one somatic-specific nucleotide substitution in helix IV (U79) also showed enhanced nucleolar localization, as well as enhanced nuclear import and ribosome incorporation. Thus, a single nucleotide substi-

tution at this position is able to confer somatic-type characteristics to a 5S RNA molecule after microinjection into the oocyte cytoplasm.

Five of the six nucleotides that differ between oocyte-type and somatic-type 5S RNA are located in the hairpin structure composed of helix III and loop C, which is the major determinant of L5 recognition [Scripture and Huber, 1995]. In addition, after injection into the cytoplasm, somatic-type 5S RNA predominantly associates with ribosomal protein L5, whereas oocyte-type preferentially associates with TFIIIA, forming storage 7S RNPs [Allison et al., 1995]. It could therefore be hypothesized that all of the behavioral differences shown by oocyte-type and somatic-type 5S RNA are a consequence of their initial protein-binding preferences in the oocyte cytoplasm. In the case of nuclear import, this is indeed likely, since L5 is thought to play a key role in the transport of 5S RNA into the nucleus [Murdoch and Allison, 1996; Rudt and Pieler, 1996]. The enhanced ribosome incorporation of somatic-type 5S RNA likely reflects both this increased level of nuclear import and the observed enhanced nucleolar localization. However, if nuclear import is mediated by L5, it would therefore follow that all of the 5S RNA in the nucleus would be bound to L5, suggesting that the differences in nucleolar localization are not due to the different protein binding characteristics of the two RNAs.

The results therefore suggest that the sequence and/or conformational differences between the two types of 5S RNA are recognized by other nuclear factors. These could either be components of the nucleolus or nucleolar targeting machinery, which have a higher affinity for somatic-type 5S RNA, or nucleoplasmic factors, which have a higher affinity for oocyte-type 5S RNA. The role of putative 5S RNA-binding factors in the nucleus could be to prevent TFIIIA-mediated efflux of 5S RNA back to the cytoplasm [Guddat et al., 1990], or to regulate the amount of 5S RNA targeted to nucleoli at one time. Retention of excess 5S RNA in the nucleus would ensure continued availability for ribosome synthesis.

Requirements for Nucleolar Localization of 5S RNA

The mutant 5S RNA molecules tested showed various degrees of nucleolar localization after cytoplasmic microinjection. Since mutations in most regions of the molecule were tested, these results imply that a specific sequence of the 5S RNA molecule is not responsible for this variable nucleolar targeting efficiency. As all of the 5S RNA mutants tested are capable of binding L5 to form 5S RNPs [Allison et al., 1993; L.A. Allison, unpublished results], a critical role for L5 in 5S RNA nucleolar localization cannot be ruled out. However, in HeLa cells 5S RNA binding and nucleolar localization activities were mapped to different domains of rat L5 [Michael and Dreyfuss, 1996]. Further experimentation is clearly necessary to determine the roles of protein and RNA in the targeting of 5S RNPs to the nucleolus.

Four of the 5S RNA mutants were previously characterized as being defective for ribosome incorporation [Allison et al., 1993]. Since it was not known if these mutants were capable of nucleolar localization, the results presented here provide more information on which step in the pathway is blocked. One of these four mutants, mutant 10–13, showed similar nucleolar localization characteristics compared with oocyte-type 5S RNA, suggesting that the defect occurs at some later step in the ribosome assembly pathway but allows some nucleolar retention. Mutant 10–13 possesses low binding affinity for TFIIIA (17% of oocyte-type) and 7S RNP formation is not detectable by electrophoretic mobility shift assays [Allison et al., 1993]. These findings suggest that TFIIIA binding is not a prerequisite for nucleolar targeting. Instead, by sequestering the RNA in storage particles, TFIIIA restricts oocyte-type 5S RNA from the nucleolus. Nucleolar localization of another mutant defective for ribosome assembly, mutant 96–101 [Allison et al., 1993], was reduced compared with oocyte-type. This mutant forms detectable 5S RNPs in vivo [Allison et al., 1993], but other changes that alter the conformation of loop E have been shown to reduce the binding affinity of L5 in vitro [Scripture and Huber, 1995]. Thus, the 5S RNP formed may have an altered conformation, reducing nucleolar localization and ribosome incorporation.

Deletion of the bulged nucleotides at positions 49 and 50 or 63 also resulted in 5S RNA molecules defective for ribosome assembly. Comparison of biochemical fractionation and in situ nucleolar localization assays indicated that the high percentage of mutants Δ49,50 and Δ63 in the nucleolar pellet must be due to binding to nuclear components other than nucleoli, which are large enough to be pelleted during the as-

say. These interactions, which could be either normal nuclear retention processes or nonspecific artifacts, may thereby prevent these mutants from being integrated into ribosomal subunits. Similarly, two other mutant RNAs, one derived from $tRNA^{met}$ and the other from U1 snRNA, have been shown to be defective in nuclear export due to interactions with unidentified, saturable binding sites within the oocyte nucleus [Boelens et al., 1995]. Deletion of two bulged nucleotides in *E. coli* ribosomal protein S8 mRNA was recently shown to increase the binding of S8 to its own transcript fivefold, thus increasing translational repression [Wu et al., 1994]. Thus, it is possible that deletion of the bulged nucleotides in 5S RNA has similarly increased the binding affinity of the resulting RNAs to an unknown nucleoplasmic component.

The results presented here suggest that the degree to which 5S RNA is localized to nucleoli or retained in the nucleoplasm in *Xenopus* oocytes is regulated by dynamic and complex molecular interactions between the RNA molecule and other nucleoplasmic and nucleolar components with differential binding affinities for 5S RNA. Further characterization of these putative binding sites may provide insight into the enigmatic way in which ribosomes are assembled in the nucleoli of eukaryotic cells.

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