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The sphere organelle contains small nuclear ribonucleoproteins

(germinal vesicle/oocyte/lampbrush chromosome)

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ABSTRACT **We show by immunofluorescence microscopy of amphibian oocyte nuclei that small nuclear ribonucleoproteins (snRNPs) occur in lampbrush chromosome loops, in a few dozen extrachromosomal organelles previously described as "spheres," and in thousands of smaller granules. Spheres are variable in size (up to ≈20 μm in diameter in the newt *Notophthalmus* and ≈10 μm in the frog *Xenopus*) and are easily distinguishable from nucleoli by morphology and composition. Spheres occur both free in the nucleoplasm and attached to specific chromosome loci, the sphere organizers. Oocyte nuclei of a cricket and a spider contain essentially similar organelles, suggesting that spheres may be common throughout the animal kingdom. We suggest that spheres play a role in the assembly of snRNP complexes for the nucleus comparable to the way that nucleoli assemble ribosomal RNP complexes for the cytoplasm.**

Cytological observations dating back to the 19th century suggested that oocyte nuclei contain more than one type of nucleolus. Some of the earlier claims involved faulty interpretation of condensed chromatin or amplified nucleoli, but others were based on such careful morphological and cytochemical observations that they cannot be lightly dismissed (1–3). The discovery of the nucleolus organizer by Heitz (4) and McClintock (5) in the early 1930s and the later elucidation of the role of the nucleolus in ribosome biogenesis (6) may have led to the notion that the "problem" of the nucleolus had been resolved and that these older observations of nucleolar diversity might be ignored. However, one type of prominent nuclear organelle, the "sphere," is clearly not a typical nucleolus. It has been described from oocyte nuclei of many amphibians (refs. 7 and 8; reviewed in ref. 9), but until now its significance has remained unknown. We show here by immunofluorescence microscopy that spheres contain the Sm antigen and trimethylguanosine, both of which are indicative of small nuclear ribonucleoproteins (snRNPs). In amphibian oocytes, spheres occur both free in the nucleoplasm and attached to specific chromosome loci, the sphere organizers (SOs). We have reexamined several examples from the earlier literature in which diverse types of nucleoli were described from invertebrate oocytes. Some of these certainly involve spheres with snRNPs.

MATERIALS AND METHODS

Animals. Newts (*Notophthalmus viridescens*) were obtained from Lee's Newt Farm (Oak Ridge, TN); frogs (*Xenopus laevis*) were from Xenopus 1 (Ann Arbor, MI); crickets (*Acheta domesticus*) came from Carolina Biological Supply (Burlington, NC); and spiders (*Achaearanea tepidariorum*) were collected in Baltimore, MD.

Cytology. Lampbrush chromosome preparations were made as described (10) with the following modifications. Oocyte nuclei of *Notophthalmus* were isolated in 83 mM KCl/17 mM NaCl/10 mM Na_3PO_4/1 mM $MgCl_2$/1 mM dithiothreitol, pH 7.2; for *Xenopus* nuclei, the $MgCl_2$ and dithiothreitol were omitted. The nuclear envelope was removed with jeweler's forceps, and the nuclear contents were dispersed in 21 mM KCl/4 mM NaCl/2.5 mM Na_3PO_4/1 mM $MgCl_2$/5 μM $CaCl_2$/1 mM dithiothreitol/0.1% paraformaldehyde, pH 7.2, in the case of *Notophthalmus*, or in half this concentration without dithiothreitol, in the case of *Xenopus*. Dithiothreitol has no obvious effect on the morphology of the nuclear contents. After the preparations had been centrifuged to attach the chromosomes to the glass slides, they were fixed for 30 min in 2% paraformaldehyde in 150 mM NaCl/20 mM Na_3PO_4, pH 7.0 (PBS). They were then rinsed in PBS and processed immediately for immunofluorescence. RNase A (100 μg/ml) and RNase T1 (50 units/ml) were used together in 0.3 M NaCl/0.03 M sodium citrate, pH 7.0.

Immature oocytes from *Acheta* and *Achaearanea* were isolated in OR2 saline (11) and squashed between a microscope slide and coverslip as described (12). The slide was frozen in liquid N_2, the coverslip was pried off with a razor blade, and the preparation was postfixed in 2% paraformaldehyde in 95% (vol/vol) ethanol for 30 min. After storage in 95% ethanol, the preparations were hydrated through a descending series of ethanols and placed in PBS. In most cases, the preparations were treated with 3 M urea for 3 min before immunofluorescent staining, a procedure that often increases the intensity of stain dramatically (13).

Immunofluorescence. Indirect immunofluorescence was carried out with two primary monoclonal antibodies (mAbs): mAb Y12, specific for the Sm antigen of snRNPs (14), and mAb K121, which recognizes the trimethylguanosine cap of snRNAs (15). In both cases, ascites fluid was diluted 1:200 to 1:500 with 10% horse serum in PBS and placed on the preparation for ≈1 hr. The secondary antibody was rhodamine-labeled goat anti-mouse IgG, also used for ≈1 hr. Preparations were mounted in 50% (vol/vol) glycerol containing 1 μg of the DNA-specific dye 4′,6-diamidino-2-phenylindole per ml. Fluorescence photographs were taken with a Zeiss epifluorescence microscope using a ×40 planapochromatic objective and hypersensitized Kodak Technical Pan film (Microfluor, Stony Brook, NY).

RESULTS

Immunofluorescent Staining of Spheres. A germinal vesicle (GV) from a medium-sized (1.0 mm) oocyte of the newt *N. viridescens* contains several dozen spheres, the largest of which are 8–10 μm in diameter. In fully mature oocytes (1.6 mm), one or two spheres may reach a diameter of 20 μm. They are less numerous than the multiple nucleoli, of which there are many hundred. As the name implies, they are nearly

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Abbreviations: GV, germinal vesicle; sn, small nuclear; RNP, ribonucleoprotein; mAb, monoclonal antibody; NO, nucleolus organizer; SO, sphere organizer.

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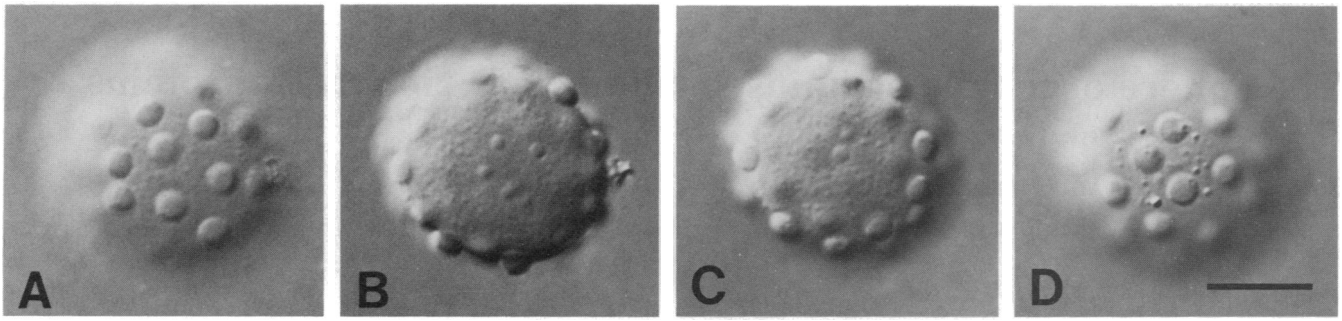

FIG. 1. A large sphere from an oocyte nucleus of *N. viridescens*, showing four successive focal planes. The surface is covered with nearly spherical protuberances (face-on in *A* and *D*); inside the sphere are numerous small granules 1–2 μm in diameter (*B* and *C*). Nomarski contrast. (Bar = 10 μm.)

spherical, and they usually have smaller spherical or subspherical protuberances on their surface (Fig. 1). In addition to the free spheres, attached spheres occur at two specific loci on chromosomes II and VI (7, 9). We stained lampbrush chromosome preparations by indirect immunofluorescence with mAb Y12, which recognizes the Sm epitope common to several major snRNPs (14). Both free and attached spheres were intensely stained, as were numerous smaller granules in the nucleoplasm (Fig. 2). In GVs from the largest oocytes there is a dramatic increase in the number of these smaller granules and in the number of large spheres. Most, but not all, lampbrush chromosome loops were strongly stained by this antibody (Fig. 2). The central chromomere axis and the nucleoli stained at background level (Fig. 2 *A* and *B*). We also stained lampbrush preparations with mAb K121 (15), which is specific for the trimethylguanosine cap found at the 5′ end of all the major snRNAs except U6. The overall staining pattern was very similar to that seen with Y12, although the chromosome loops were less intensely stained relative to the spheres (Fig. 3).

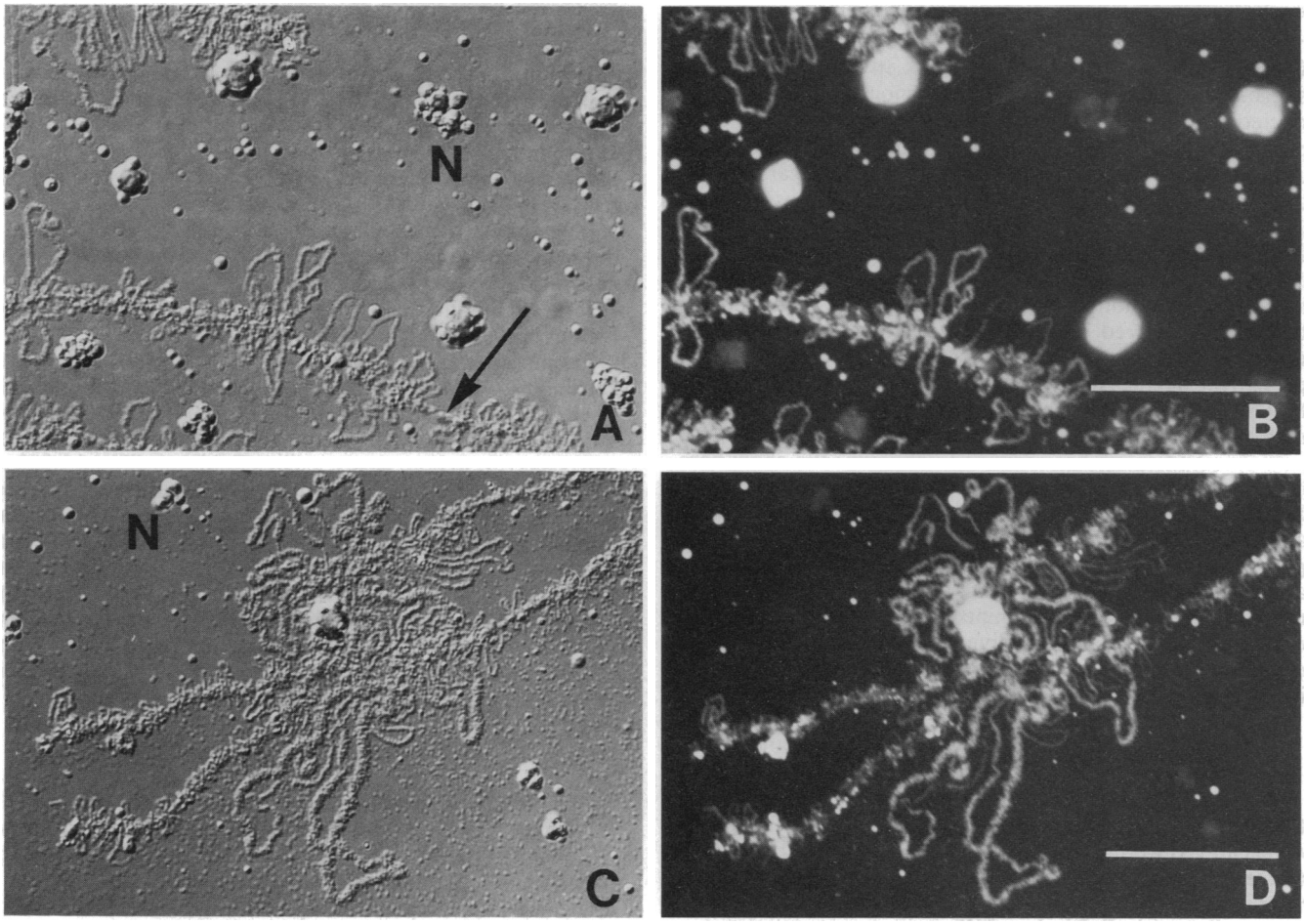

FIG. 2. (*A* and *B*) Nomarski contrast (*A*) and fluorescence (*B*) images of the same area showing portions of two lampbrush chromosomes of *N. viridescens* with nearby nucleoli, spheres, and small granules, stained with mAb Y12; mAb Y12 is specific for the Sm antigen of snRNPs. Staining is intense in the spheres and granules. The loops of the lampbrush chromosomes are stained, but not the chromomere axis (arrow). A few granules are unstained. Nucleoli (N) are near background intensity. (*C* and *D*) Nomarski contrast (*C*) and fluorescence (*D*) images of the left end of bivalent II of *N. viridescens* showing a single attached sphere connecting the two homologous chromosomes. The sphere is intensely stained, as are most of the small granules, but not the nucleoli (N). The large, well-stained loops that arise at or near the sphere locus contain histone genes (10). (Bars = 40 μm.)

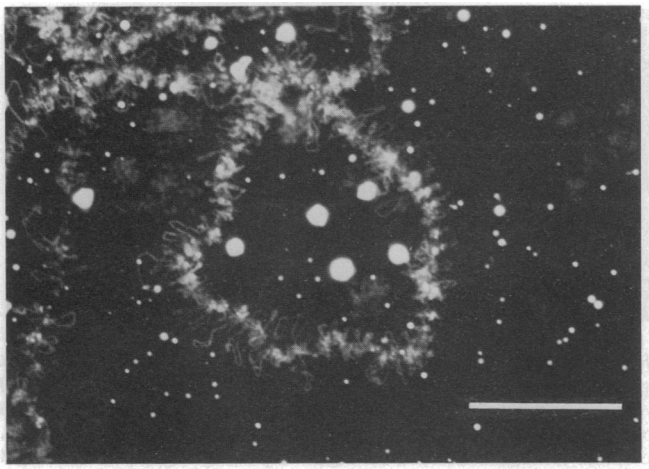

FIG. 3. Fluorescence image of part of a lampbrush chromosome of *N. viridescens* with nearby spheres, nucleoli, and small granules; stained with mAb K121, which is specific for the trimethylguanosine cap of snRNAs. The spheres and granules are strongly stained, the lampbrush chromosome loops are somewhat weaker, and the nucleoli are near background intensity. (Bar = 50 μm.)

We also examined the immunofluorescent staining of lampbrush chromosome preparations after treatment with a mixture of RNase A and RNase T1. In fixed preparations of the sort we used here, RNase treatment has essentially no morphological effect. The staining of the spheres by mAb K121 was reduced but not eliminated, suggesting that not all of the snRNA caps in the spheres are accessible for digestion with RNase. Staining of the chromosome loops, which was weak in control preparations, was reduced to background level. To remove RNA completely from the spheres, we incubated fixed preparations in 0.1 M NaOH at 42°C for 4 hr. After this treatment, which had no detectable effect on morphology, mAb K121 failed to stain any structure on the slide. In contrast, pretreatment with RNase or NaOH did not reduce staining by mAb Y12.

Objects similar to the spheres of *Notophthalmus* have been described from a variety of other amphibians. GVs from medium-sized (1.0 mm) oocytes of *Xenopus* contain ≈50 free spheres. In addition, attached spheres occur at three loci on chromosomes VIII, IX, and XVI (16). The free and attached spheres of *Xenopus* stain with mAb Y12 (Fig. 4 *A* and *B*) and mAb K121 (data not shown). Again, the staining with mAb K121 is reduced by prior treatment with RNase and is eliminated by 0.1 M NaOH, but staining with mAb Y12 is unaffected.

The SO. Attached spheres are not part of a lateral loop but instead connect directly to a mass of condensed chromatin that looks like an ordinary chromomere. The relationship of the sphere to the chromosome axis is best shown after staining with 4′,6-diamidino-2-phenylindole. In such preparations, one can see that the condensed chromatin follows the contour of the sphere on one side. The intimate association of the sphere with chromatin at a specific locus is similar to the association of the nucleolus with the nucleolus organizer (NO). For this reason, we refer to the chromatin at the attachment site of the sphere as the sphere organizer, SO (Figs. 2 *C* and *D* and 4). The size of attached spheres varies over the same range as that of the free spheres; a sphere may be absent, or occasionally there may be two spheres attached to a single SO (Fig. 4 *A* and *B*). In *Notophthalmus, Xenopus,* and many other amphibia, two homologous SOs may be associated with a single sphere (Fig. 2 *C* and *D*); rarely, two nonhomologous SOs may be similarly associated (7–9, 16). An interesting topological relationship exists between the SOs and the free spheres. If one examines a freshly isolated GV of *Notophthalmus* before the nuclear contents disperse, one can see that the sphere-bearing ends of chromosomes II and VI lie near each other in the nucleus and that most of the free spheres are in the same general vicinity. In some manner, therefore, the SOs appear to determine the distribution of the free spheres.

Spheres in Other Organisms. Oocyte nuclei of many invertebrates contain sphere-like organelles to which a variety of names have been given (Binnenkörper, Kugeln, pseudonucleoli, accessory nucleoli, etc.). Mancino *et al.* (17) remarked on the similarity between the spheres of amphibians and the Binnenkörper of the cricket, *A. domesticus*, described in detail by Bier *et al.* (3). We find that the Binnenkörper of *Acheta* stains intensely with both mAb Y12 and mAb K121 (Fig. 5 *A* and *B*) and must, therefore, be essentially the same organelle as the amphibian sphere. We made similar observations on the GV of the house spider, *A. tepidariorum*, which contains a single prominent nucleolus and one to several spheres (Fig. 5 *C* and *D*).

DISCUSSION

We have shown by immunofluorescent staining that the sphere organelles in oocyte nuclei of the newt *Notophthalmus* and the frog *Xenopus* react with mAb Y12, specific for the Sm antigen of snRNPs, and mAb K121, specific for the

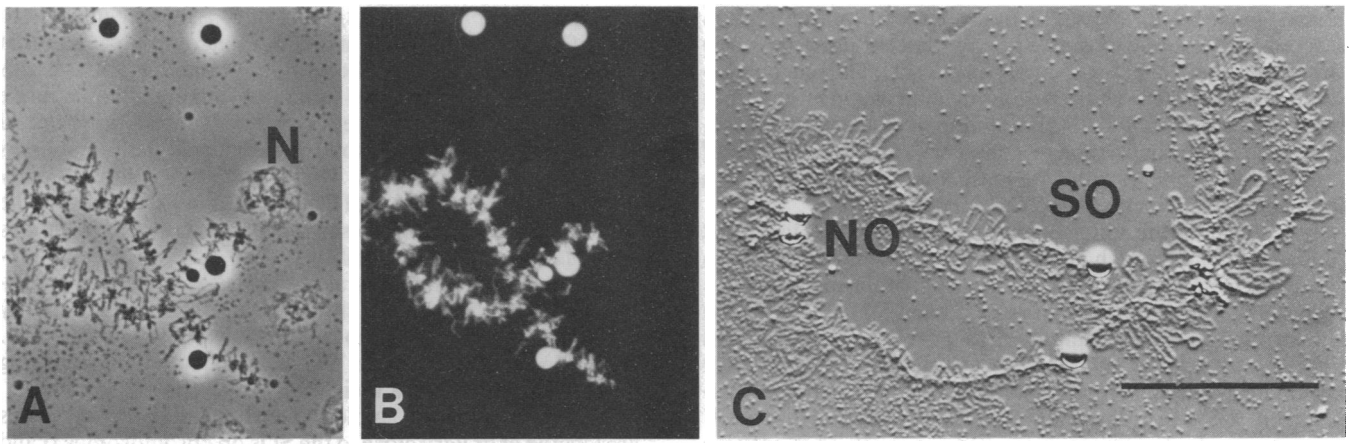

FIG. 4. Phase contrast (*A*) and fluorescence (*B*) images of bivalent IX of the frog *X. laevis* after staining with mAb Y12. The lower SO bears a single attached sphere, the upper SO bears two spheres of unequal sizes. Two free spheres and several free nucleoli (N) are visible in this field. (*C*) Bivalent 11 of the newt *Pleurodeles waltl* showing attached nucleoli at the two homologous NOs and attached spheres at the two homologous SOs. Nomarski contrast. (Bar = 50 μm.)

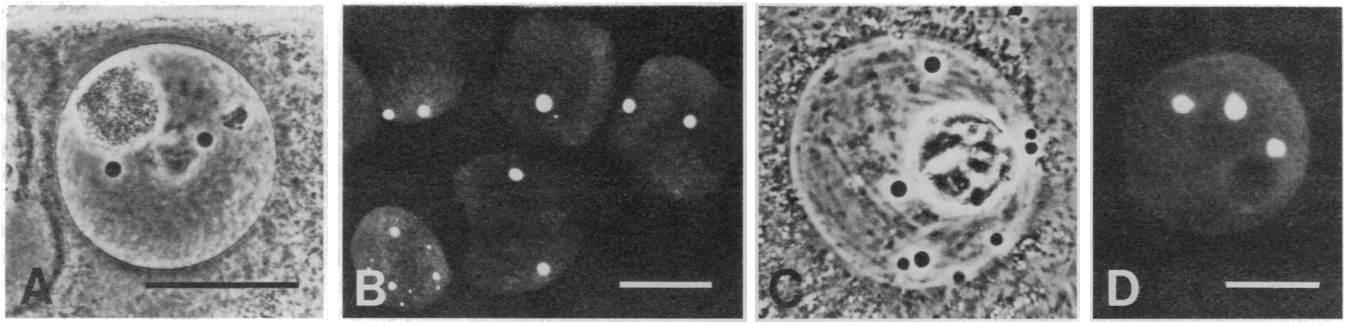

FIG. 5. (*A*) Phase contrast image of an oocyte of the cricket *A. domesticus* showing the large GV with two "Binnenkörper" (spherical black objects). The granular mass at the top left of the GV contains the amplified rRNA-encoding DNA and nucleoli (3). (Bar = 50 μm.) (*B*) Fluorescence image of a group of *Acheta* GVs squashed out of their oocytes, stained with mAb K121. The Binnenkörper stain intensely with this antibody and with mAb Y12, demonstrating their identity with the spheres of amphibian GVs. The rest of the GV is moderately stained. (Bar = 50 μm.) (*C*) Phase contrast image of a GV from an oocyte of the spider *A. tepidariorum* showing a single large nucleolus and eight sphere-like organelles. (*D*) Fluorescence image of a different GV in an oocyte of *Achaearanea* after staining with mAb Y12. Three intensely stained spheres show up against a weaker general stain in the GV. The nucleolus is conspicuously negative. (Bar = 25 μm for *C* and *D*.)

trimethylguanosine cap found on the major snRNAs (except U6). We conclude that spheres contain one or more snRNPs. Our earlier conclusion that spheres lack RNA (9) was based in part on the failure to detect [^{3}H]uridine incorporation in short-term incubation experiments. Recently, we have detected label in the spheres of *Xenopus* when oocytes were incubated in [^{3}H]uridine for 6–24 hr (data not shown). In an earlier immunofluorescent study Lacroix *et al.* (18) identified a mAb (B24) that stains spheres of several newts, including *Pleurodeles* and *Notophthalmus*. Unlike mAbs Y12 and K121, mAb B24 stains the body of the spheres without staining the small protuberances on their surface. On one-dimensional Western blots of GV proteins, mAb B24 detects a band with an apparent molecular mass of 104 kDa; on two-dimensional blots, this band is resolved into two major and two minor spots of slightly different isoelectric points. Whether these proteins are related to snRNP proteins remains to be determined.

Using mAbs Y12 and K121, we have demonstrated spheres in oocytes of the cricket *Acheta* and the spider *Achaearanea*. Morphologically similar organelles have been described from oocytes of many other animals (2, 3), and it is probable that some of these are spheres. By its nature, mAb K121 is not species specific, and mAb Y12 detects an epitope that is evolutionarily conserved. These two antibodies, therefore, will be useful for identifying spheres in oocytes of other organisms.

The distribution of snRNPs within somatic nuclei has been examined many times by immunofluorescence. During interphase, most nuclei exhibit a finely punctate pattern when stained with antibodies against either snRNP proteins or the trimethylguanosine cap of snRNAs (19–21). Objects comparable to the larger spheres of amphibian oocytes have not been seen in somatic cells. A somatic nucleus is, of course, many thousand times smaller than a GV, and the absence of recognizable spheres could be more a matter of scale than a fundamental difference in snRNP organization.

In addition to the spheres, mAbs Y12 and K121 stain most loops on the lampbrush chromosomes and hundreds to thousands of smaller granules in the nucleoplasm. We have recently confirmed the presence of U1 and U2 snRNAs in the spheres, granules, and lampbrush loops by *in situ* nucleic acid hybridization (data not shown). Currently, we can only speculate about the relationships among these three compartments. We presume that some of the snRNPs in the GV are organized into spliceosomes and are involved in splicing of newly transcribed RNA (22). Earlier studies showed that snRNPs are associated with sites of transcription in *Drosophila* polytene chromosomes (23) and with nascent RNP fibrils in chromatin from cultured cells (24). The association of snRNPs with the nascent RNP on lampbrush loops is further evidence that the splicing machinery is in place before transcription is complete. On the other hand, we suggest that snRNPs in the small granules and spheres are not involved directly in splicing, but instead are being assembled into spliceosomes or precursors of spliceosomes. Several studies have demonstrated that *Xenopus* oocytes contain a pool of cytoplasmic snRNP proteins (25–28). Newly synthesized snRNAs leave the GV, acquire the trimethylguanosine cap in the cytoplasm, and return to the GV in association with snRNP proteins. According to this scheme, the granules and spheres that stain with mAb K121 must contain snRNPs that have returned to the nucleus. One possibility consistent with both the biochemical and morphological data is that newly returning snRNPs appear first in the granules, which in turn aggregate into spheres, where they are assembled into spliceosomes or components thereof. However, this scheme is only one of several possibilities, and it will have to be tested by following the movements of snRNAs and snRNP proteins between the various morphological components.

No scheme of snRNP movement is complete without the answers to two additional questions: (*i*) What role is played by the SO in sphere production? (*ii*) What is the relationship between free and attached spheres? By analogy to the NO, which produces rRNA necessary for the assembly of ribosomal subunits in the nucleolus, it is not unreasonable to suppose that the SO synthesizes an RNA necessary for some step of snRNP assembly in the spheres. Whether or not such an RNA exists, we can imagine several ways in which free and attached spheres might be related. Again by analogy to the NO and the amplified nucleoli, one could postulate that free spheres contain amplified copies of the SO DNA. In this scheme, free spheres and attached spheres would arise independently because they carry separate but identical genetic information. A second possibility is that all spheres are generated at the SOs, from which they subsequently detach. The accumulation of free spheres in the vicinity of the SOs as the oocyte matures would be consistent with this hypothesis. A third possibility is that RNA molecules produced by the SO serve as assembly sites for snRNP complexes both on and off the chromosome. A fourth possibility is that free spheres move to rather than away from the SOs. According to this scheme, the SOs might be "processing points" for snRNPs on their way to the chromosomes.

In situ hybridization studies have shown that histone genes are located at or very close to the SOs on chromosomes II and VI of *Notophthalmus*; the same is true for the SOs of two other newts, *Triturus cristatus* and *Triturus alpestris* (10). The loops that transcribe the histone genes are often quite prominent (Fig. 2 *C* and *D*). It is not known whether the

histone genes are fortuitously next to the SO, are adjacent for some functional reason, or actually constitute the SO. This question can be partially addressed by looking for proximity of the histone genes to the SOs in other unrelated organisms. Ultimately, however, it will be necessary to clone the SO DNA to determine the details of its organization and its relationship to the histone genes and spheres.

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1. Maréchal, J. (1907) *Cellule* **24,** 5–239.
2. Jörgensen, M. (1913) *Arch. Zellforsch.* **10,** 1–126.
3. Bier, K., Kunz, W. & Ribbert, D. (1967) *Chromosoma* **23,** 214–254.
4. Heitz, E. (1931) *Planta* **15,** 495–505.
5. McClintock, B. (1934) *Z. Zellforsch. Mikrosk. Anat.* **21,** 294–328.
6. Vincent, W. S. & Miller, O. L., eds. (1966) *International Symposium on the Nucleolus: Its Structure and Function,* National Cancer Institute Monograph 23 (National Cancer Institute, Bethesda, MD).
7. Gall, J. G. (1954) *J. Morphol.* **94,** 283–352.
8. Callan, H. G. & Lloyd, L. (1960) *Philos. Trans. R. Soc. London Ser. B.* **243,** 135–219.
9. Callan, H. G. (1986) *Lampbrush Chromosomes* (Springer, Berlin), pp. 1–254.
10. Gall, J. G., Stephenson, E. C., Erba, H. P., Diaz, M. O. & Barsacchi-Pilone, G. (1981) *Chromosoma* **84,** 159–171.
11. Wallace, R. A., Jared, D. W., Dumont, J. N. & Sega, M. W. (1973) *J. Exp. Zool.* **184,** 321–333.
12. Hulsebos, T. J. M., Hackstein, J. H. P. & Hennig, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3404–3408.
13. Hausen, P. & Dreyer, C. (1982) *Stain Technol.* **57,** 321–324.
14. Lerner, E. A., Lerner, M. R., Janeway, C. A. & Steitz, J. A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2737–2741.
15. Krainer, A. (1988) *Nucleic Acids Res.* **16,** 9415–9429.
16. Callan, H. G., Gall, J. G. & Berg, C. A. (1987) *Chromosoma* **95,** 236–250.
17. Mancino, G., Barsacchi, G. & Nardi, I. (1969) *Chromosoma* **26,** 365–387.
18. Lacroix, J. C., Azzouz, R., Boucher, D., Abbadie, C., Pyne, C. K. & Charlemagne, J. (1985) *Chromosoma* **92,** 69–80.
19. Lerner, M. R. & Steitz, J. A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5495–5499.
20. Reuter, R., Appel, B., Bringmann, P., Rinke, J. & Lührmann, R. (1984) *Exp. Cell Res.* **154,** 548–560.
21. Spector, D. L. & Smith, H. C. (1986) *Exp. Cell Res.* **163,** 87–94.
22. Steitz, J. A., Black, D. L., Gerke, V., Parker, K. A., Krämer, A., Frendewey, D. & Keller, W. (1988) in *Structure and Function of Major and Minor Small Nuclear Ribonucleoproteins,* ed. Birnstiel, M. L. (Springer, Berlin), pp. 115–154.
23. Sass, H. & Pederson, T. (1984) *J. Mol. Biol.* **180,** 911–926.
24. Fakan, S., Leser, G. & Martin, T. E. (1986) *J. Cell Biol.* **103,** 1153–1157.
25. De Robertis, E. M., Lienhard, S. & Parisot, R. F. (1982) *Nature (London)* **295,** 572–577.
26. Zeller, R., Nyffenegger, T. & De Robertis, E. M. (1983) *Cell* **32,** 425–434.
27. Mattaj, I. W. & De Robertis, E. (1985) *Cell* **40,** 111–118.
28. Mattaj, I. W. (1988) in *Structure and Function of Major and Minor Small Nuclear Ribonucleoproteins,* ed. Birnstiel, M. L. (Springer, Berlin), pp. 100–114.