Chromosoma (1997) 105:470–480

CHROMOSOMA

Looking at Christmas trees in the nucleolus

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Received: 7 October 1996; in revised form: 30 January 1997 / Accepted: 12 February 1997

Abstract. We describe novel nucleolar structures, observed by thin section electron microscopy in oocyte nuclei of the grashopper *Locusta migratoria*, which we interpret, based on morphological and compositional criteria, as rDNA transcription units. Morphologically they resemble the condensed and foreshortened "Christmas trees" seen in Miller spreads of nucleolar chromatin prepared from the same biological material. They contain DNA and rRNA as shown by immunocytochemistry and in situ hybridization and are concentrated in several intranucleolar cavities. The presumptive rDNA transcription units extend throughout the interior of these nucleolar pockets or are selectively enriched at their outermost zones in close contact with the surrounding fibrillarin-positive dense component. We suggest that the nucleolar pockets of *Locusta* oocytes are equivalent to the fibrillar centers of somatic nucleoli and discuss possible implications for the current understanding of the functional organization of nucleoli.

Introduction

Nucleoli are the ribosome-producing machineries of eukaryotic cells. A growing mammalian cell, for example, synthesizes 20–70 ribosomes per second by a cascade of events involving transcription of the rRNA genes, processing of the pre-rRNAs and assembly of the preribosomal particles (Hadjiolov 1985). A large number of studies have located various steps of the ribosome biosynthetic pathway to morphologically distinct structures of the nucleolus indicating that ribosome biosynthesis is a vectorial process that begins in the fibrillar portion and continues into the adjacent granular component (GC) of the nucleolus (e.g., Hadjiolov 1985; Fischer et al. 1991; Puvion-Dutilleul et al. 1991; Shaw and Jordan 1995; Shaw et al. 1995; Beven et al. 1996; Thiry and Goessens 1996). The fibrillar part of the nucleolus is usually subdivided into one or several spheroidal regions of low contrast, the fibrillar centers (FC) and the surrounding dense fibrillar component (DFC; for a description of nucleolar ultrastructure see Smetana and Busch 1974; Hadjiolov 1985; Thiry and Goessens 1996). A detailed molecular interpretation of the nucleolar architecture will be impossible without a structural localization of the sites of the transcribing rRNA genes as previously stated by Hadjiolov: "The molecular structures underlying the observed 'Christmas tree' pattern are now understood in much detail. However, because the highly contorted and compact state of nucleolar chromatin prevents visualization of active transcription units in ultrathin sections, we may ask: what structures observed in the nucleolus are the counterparts of the structures seen in spread preparations?" (Hadjiolov 1985, p 117). Despite the development of refined electron microscopic (EM) localization techniques a generally accepted answer to this question has as yet not been found. At present it is still a matter of debate whether the transcribing rRNA genes are located in the FC, the surrounding DFC or the border region between the two nucleolar components (for a discussion see Wachtler and Stahl 1993; Scheer and Weisenberger 1994; Hozak 1995; Shaw and Jordan 1995; Raska et al. 1995; Thiry and Goessens 1996; Besse and Puvion-Dutilleul 1996). This is most likely because there is at present no direct way of selectively labeling the transcribing rRNA genes in situ and identifying them unequivocally. For instance, (i) rDNA sequences can be localized by in situ hybridization but this technique does not provide information as to their transcriptional status. (ii) In situ hybridization probes to the transcribed spacer

Edited by: S.A. Gerbi

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sequences of the pre-rRNAs label nascent transcripts, template-released pre-rRNAs up to the point at which the transcribed spacer is excised and also the excised RNA spacers generated by the processing reaction. Since the turnover rate and fate of such processing "waste" products is not known, it is a distinct possibility that they accumulate within the nucleolus and contribute to the observed in situ hybridization signal. (iii) Antibodies to RNA polymerase I do not discriminate between the active and inactive form of the enzyme and (iv) the metabolic incorporation of radioactive or otherwise tagged RNA precursors [e.g., bromodeoxyuridine triphosphate (BrUTP)] inevitably labels, even after short pulses, both nascent and template-released transcripts.

Clearly, identification of rDNA transcription units in situ by morphological criteria would resolve such interpretative uncertainties or, as formulated by G. Jordan "the old adage 'seeing is believing' might not be an untimely one for electron microscopists" (Jordan 1991). The only method so far available for the visualization of active rRNA genes requires the disintegration of the nucleoli by a hypotonic spreading medium ("Miller spreads"; Miller and Beatty 1969; Miller 1984). What the transcribing rRNA genes or "Christmas trees" look like in situ and where they are located within the nucleolar body is as yet largely unknown (see Shaw and Jordan 1995; Trendelenburg et al. 1996). In their native state they must be considerably foreshortened and compacted. For instance, a single mouse rDNA transcription unit (note that a nucleolus usually harbors 100 or more transcription units) with an axial length of approximately 4 μm as measured in Miller spreads has about the same length as the diameter of a nucleolus and, together with its adjacent spacer, could easily span the whole nucleus (see Scheer and Benavente 1990).

We expected that the presence of a large number of transcriptionally active rRNA genes might facilitate their identification in sectioned material and therefore decided to study the ultrastructural organization of the nucleoli in oocytes of *Locusta migratoria*. The haploid genome of this grasshopper species contains about 3,300 rRNA genes; this unusually high copy number (at least for an animal species) might explain why amplification of the rRNA genes apparently does not occur in the oocytes (Schäfer and Kunz 1987). We choose rapidly growing, mid-sized oocytes, which synthesize ribosomes at very high rates. Since *Locusta* oocytes are of the panoistic type and develop without nurse cells, it is reasonable to assume that most if not all of the approximately 13,000 rRNA genes present in a single diplotene-stage oocyte are transcriptionally fully active (Schäfer and Kunz 1987). Here we describe a novel nucleolar structure that we interpret as Christmas trees.

Materials and methods

Conventional thin section EM. Females of *L. migratoria* were obtained from a local breeding farm. Animals were anesthetized with ethyl acetate, ovarioles were dissected and immediately fixed for 4 h in an ice-cold 4% glutaraldehyde solution buffered with 0.05 M sodium cacodylate to pH 7.2. Alternatively, nuclei were manually isolated from growing oocytes in 3:1 medium (75 mM KCl, 25 mM NaCl, 10 mM TRIS-HCl, pH 7.2) and fixed in the same way. After several washes in cold cacodylate buffer, the material was postfixed in 2% OsO_4 for 2 h at 4°C, stained overnight in 0.5% aqueous uranyl acetate, dehydrated in an ethanol series, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to standard protocols.

EM in situ hybridization. Ovarioles were fixed for 2 h in ice-cold PBS containing 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.5% glutaraldehyde. After several washes in PBS, the specimens were dehydrated through an ethanol series and embedded in Lowicryl K4 M according to standard procedures (Carlemalm and Villiger 1989). The resin was UV polymerized at –30°C for 3 days and at room temperature for a further 3 days. Ultrathin sections were mounted on 200 mesh parlodion-coated nickel grids and placed successively on droplets of 2×SSC and 5×SSC. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) In situ hybridization was performed for 3 h at 65°C in 5×SSC, 0.8 mg/ml tRNA and 12 ng/μl digoxigenin-labeled antisense riboprobe (see below). Controls were performed with the corresponding sense probes. After sequential washes in 5×SSC, 2×SSC and PBS, hybridized probes were revealed with a monoclonal antibody (mAb) against digoxigenin (diluted 1:20; Boehringer Mannheim, Germany) followed by appropriate secondary antibodies coupled to 10 nm gold particles (diluted 1:10; Dianova, Hamburg, Germany). All antibodies were diluted in PBS complemented with 1% BSA and 0.1% Tween-20 and incubated for 1 h at ambient temperature. Lowicryl sections were finally counterstained with uranyl acetate and lead citrate for 4 and 1 min, respectively (a detailed protocol of the in situ hybridization procedure can be found in Fischer et al. 1996).

The hybridization probe was prepared by in vitro transcription of a cloned 4.8 kb *L. migratoria* 28S rDNA fragment inserted into Bluescript vector (Stratagene, Heidelberg, Germany). The original plasmid clone, pLm4B11, comprising most of the *Locusta* 28S rDNA sequences (Schäfer and Kunz 1985, 1987) was kindly provided by Mireille Schäfer (University of Göttingen, Germany). In vitro transcription with T3 and T7 RNA polymerase (SP6/T7 RNA labeling kit; Boehringer Mannheim) yielded digoxigenin-tagged riboprobes in sense and antisense orientation. Incorporation of digoxigenin-11-UTP was monitored with alkaline phosphatase-coupled antibodies after electrophoresis and transfer of the probe to nitrocellulose filters (see Weisenberger and Scheer 1995).

Chromatin spread preparations. Three to five oocyte nuclei each were manually isolated in 3:1 medium (see above), washed briefly in low salt buffer (0.1 mM borate buffer, pH 9.0) and transferred into a droplet of the same buffer containing 0.01%–0.02% Sarcosyl placed on a siliconized glass slide at 4°C for about 30 min. The dispersed nuclear contents were then centrifuged for 30 min at 4,000 *g* through a solution of 1% formaldehyde (prepared from paraformaldehyde in 0.1 mM borate buffer, pH 8.5–9.0) onto freshly glow-discharged carbon-coated EM grids. The EM grids were then briefly immersed in 0.4% Kodak Photo-flo solution, air-dried, stained in ethanolic 1% phosphotungstic acid, dehydrated in 100% ethanol and air dried again. Finally the preparations were rotary shadowed with platinum:palladium (80:20) at an angle of 7° (for details see Zentgraf et al. 1987; Trendelenburg and Puvion-Dutilleul 1987).

EM immunolocalization. Ultrathin Lowicryl sections (see above) were incubated with mAb P2G3 against fibrillarin (kindly provided by Mark Christensen; for a characterization of the antibody see Christensen et al. 1986). After a 1 h incubation at room temperature, the grids were rinsed in PBS and incubated for another hour with appropriate secondary antibodies coupled to 10 nm gold particles (diluted 1:10 in PBS; Dianova, Hamburg). After several washes with PBS the grids were stained as described above.

For DNA detection we followed a preembedding antibody-labeling protocol. Briefly, frozen sections of *Locusta* ovarioles were

fixed with formaldehyde (see below) and incubated with mAb AK-30/10 directed against DNA (10 μg/ml, Boehringer Mannheim; for a characterization of the antibody see Scheer et al. 1987). Incubation times, detection with secondary antibodies coupled to 10 nm gold particles (diluted 1:10 in PBS; Dianova, Hamburg) as well as fixation, dehydration and embedding in Epon of the cryostat sections were as previously described (Scheer and Rose 1984).

Terminal deoxynucleotidyl transferase (TdT) reaction. This DNA localization method was performed on ultrathin sections of glutaraldehyde/osmium tetroxide-fixed and Epon-embedded ovarioles following the protocol described by Thiry (1992, 1995). BrdUTP was obtained from Sigma (Deisenhofen, Germany), TdT and a mAb against BrdU from Boehringer Mannheim.

All micrographs were taken with a Zeiss EM10 electron microscope (Carl Zeiss, Oberkochen, Germany).

Immunofluorescence microscopy. Dissected ovarioles were shock-frozen in isopentane cooled by liquid nitrogen. Frozen sections (5 μm thick) were air-dried, fixed with 2% formaldehyde (freshly prepared from paraformaldehyde in PBS) for 5 min and washed several times in PBS before incubation for 30 min with antibodies directed against fibrillarin. Human autoimmune serum S4 (Reimer et al. 1987) and the mAb P2G3 against fibrillarin (Christensen et al. 1986) gave essentially the same results. Specimens were then incubated for another 30 min with appropriate secondary antibodies conjugated to Texas red or fluorescein isothiocyanate (FITC, Dianova, Hamburg; diluted 1:100 in PBS), washed in PBS, air-dried from ethanol and mounted in Mowiol (Hoechst, Frankfurt, Germany). Photographs were taken with a Zeiss Axiophot equipped with epifluorescence optics (Carl Zeiss, Oberkochen, Germany).

Fig. 2A–E. At higher magnification the elements within a nucleolar pocket are seen to consist of short fibers attached to axial structures (same preparation as in Fig. 1). Note the condensed endings of the lateral fibers. Occasionally the lateral fibers are arranged in length gradients (**A** *arrows*, **D, E**). The cross section (**B** *arrow*) shows lateral fibers arranged radially around a central axis. Longitudinal sections (**C–E**) reveal the close packing of the lateral fibers so that the central axis is only rarely recognized (e.g., at the *arrows* in **C**). Bars represent 0.25 μm (**A**) and 50 nm (**B–E**)

Results

Ultrastructure of Locusta *oocyte nucleoli*

The intense rRNA synthesis of growing *Locusta* oocytes is accompanied by an unraveling of the nucleoli into multiple bodies that line up like beads on a string (for light microscopic studies see Kunz 1967; Schäfer and Kunz 1987). In ultrathin sections of manually isolated oocyte nuclei the nucleolar bodies were readily recognized as electron-dense structures full of “holes” with diameters of up to 2 μm, comparable to pieces of Swiss cheese (Fig. 1; see also Bier et al. 1967). Since these holes contained structural elements not seen in the nucleoplasm, they were not simply indentations or channels penetrating from the surrounding nucleoplasm deep into the nucleoli. Rather, they represent specific nucleolar compartments in the form of intranucleolar pockets or cavities. The surrounding nucleolar material appeared as a compact and very dense structure resembling the DFC of somatic nucleoli. A typical granular component was lacking (Figs. 1, 2A).

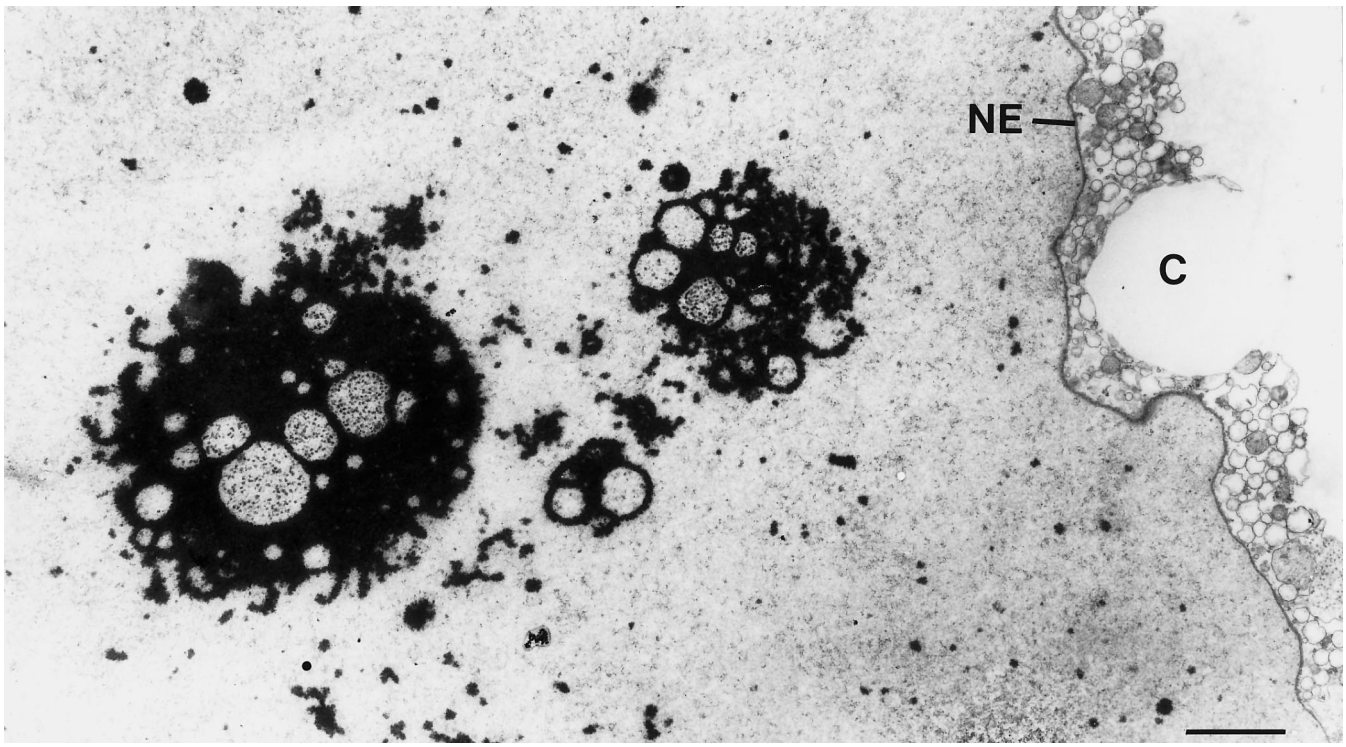
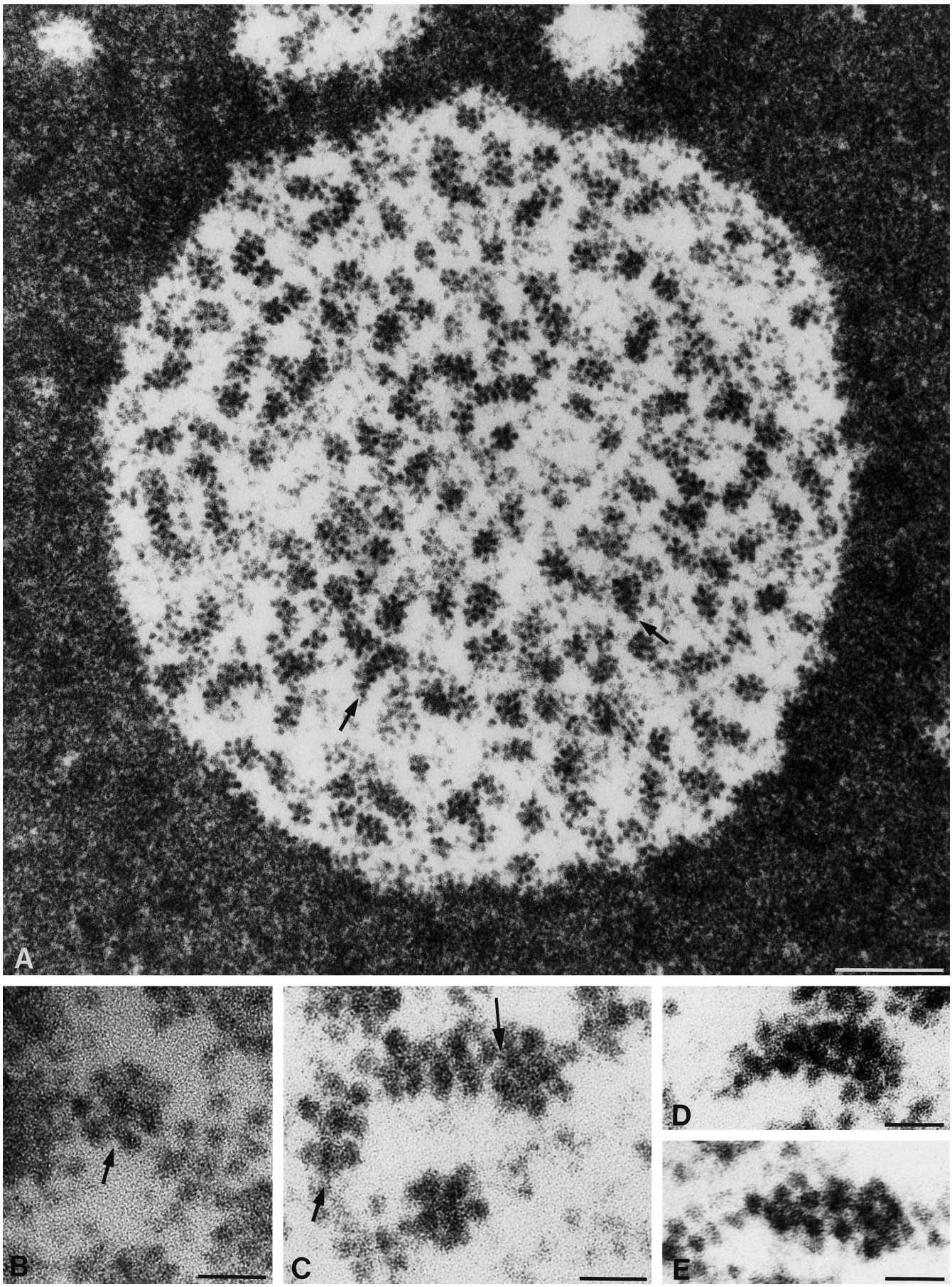


Fig. 1. Survey electron micrograph of a manually isolated nucleus from a growing *Locusta* oocyte. The highly compact and electron-dense nucleolar bodies contain numerous vacuolar spaces in which distinct structural elements are recognized. *NE* nuclear envelope, *C* cytoplasmic side of the nuclear envelope with some adhering vesicles. Bar represents 2 μm


A
B
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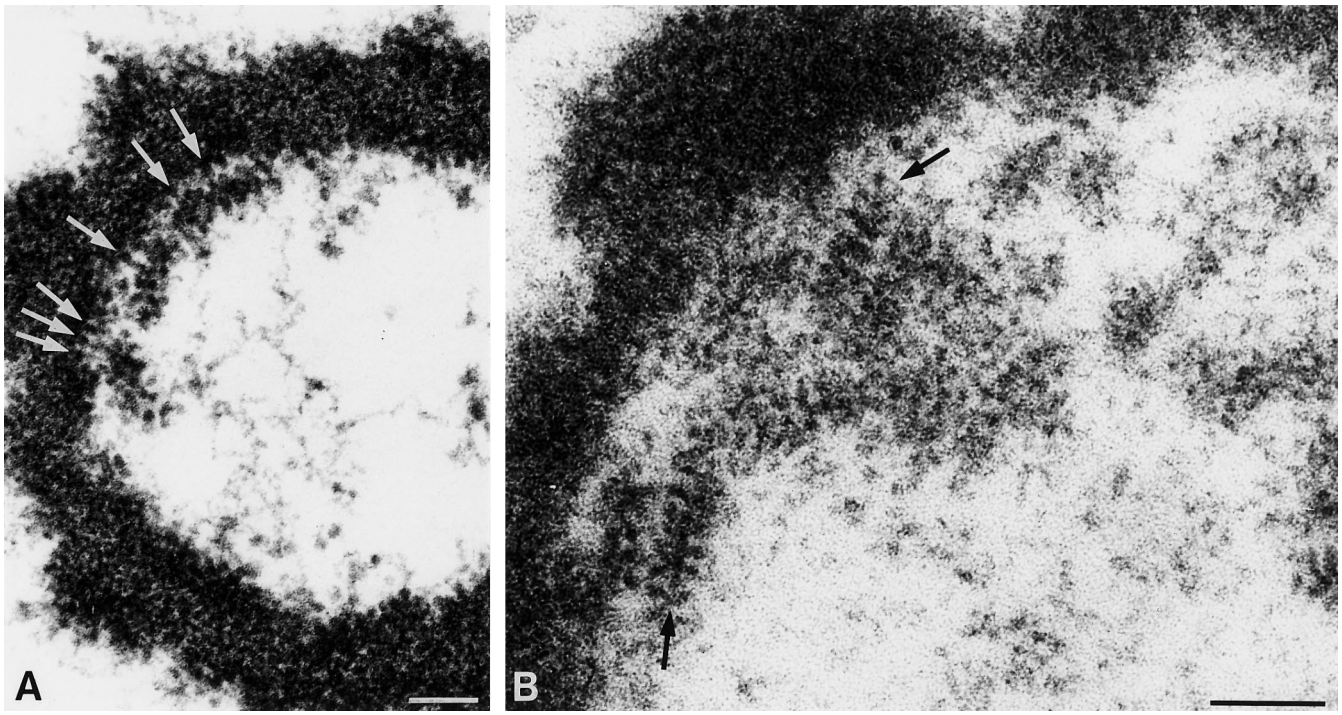

Fig. 3A, B. Nucleolar pocket with peripherally arranged "Christmas tree"-like structures (isolated oocyte nucleus). The terminal granules of the lateral fibers are in direct contact with the surrounding dense nucleolar component (**A** *arrows*). The interior of the pocket is filled with finely filamentous material (**A**). In ultrathin sections of whole oocytes the Christmas tree-like structures (**B** *arrows*) are often blurred or form a more or less homogenous fibrillogranular meshwork. Bars represent 0.1 μm

At higher magnification the structural elements inside the nucleolar pockets were seen to consist of numerous short rod-like fibers anchored to rather ill-defined central axes (Fig. 2A). The free ends of the lateral fibers were usually accentuated by a terminal thickening, giving the impression of granules attached to an axis by somewhat thinner stems. We interpret these structures as nucleolar (i.e., rDNA) transcription units or Christmas trees sectioned at random angles. The cross section shown in Fig. 2B displays a radial arrangement of lateral fibers around a central axis. The total length of a lateral fiber was approximately 30 nm and the diameter of a terminal granule ca. 14 nm. The lateral fibers were also seen as closely packed structures in longitudinal sections (Fig. 2C–E). When more distantly spaced lateral fibers left a gap, a delicate underlying axis of about 3 nm in diameter could be recognized (arrows in Fig. 2C). Occasionally, the lateral fibers were arranged in a length gradient most likely reflecting the growth of the nascent transcript fibrils at the proximal portion of an rDNA transcription unit (Fig. 2A, D, E).

The junction of the intranucleolar pockets and the surrounding dense component revealed a distinctly granular appearance (Fig. 2A). These granules may reflect the free ends of lateral fibers of Christmas trees closely apposed to or partially integrated into the dense nucleolar component. Alternatively, they may indicate the presence of preribosomal particles attached to the inner surface of the dense nucleolar component.

The density of the Christmas tree-like structures varied considerably between different pockets of a given nucleolus (Fig. 1). A nucleolar pocket with only a few Christmas tree-like structures is depicted in Fig. 3A. Interestingly, in such cases all the Christmas trees were usually located peripherally and the terminal granules of the lateral fibers appeared directly to contact the surrounding fibrillar component of the nucleolus (Fig. 3A, arrows). The internal portions of the pockets were filled with a loose network of filamentous material (Fig. 3A). When viewed at high magnification these filaments revealed a beaded structure resembling nucleosomal chains (not shown).

The Christmas tree-like structures were particularly clearly seen in ultrathin sections of manually isolated nuclei. In sections of whole ovaries they sometimes appeared blurred or even formed a more or less amorphous meshwork (Fig. 3B). We ascribe this to artifactual structural changes of the delicate transcription units resulting from a low penetration rate by the fixative into the relatively large oocytes.

Immunolocalization of fibrillarin

Fibrillarin, an evolutionarily highly conserved protein, is a major constituent of the DFC (Ochs et al. 1985; for further references see Shaw and Jordan 1995; Thiry and Goessens 1996). When we incubated frozen sections of *Locusta* ovaries with antibodies to fibrillarin and analyzed them by immunofluorescence microscopy, the nucleolar masses within the oocyte nuclei were brightly stained except for several dot-like areas dispersed

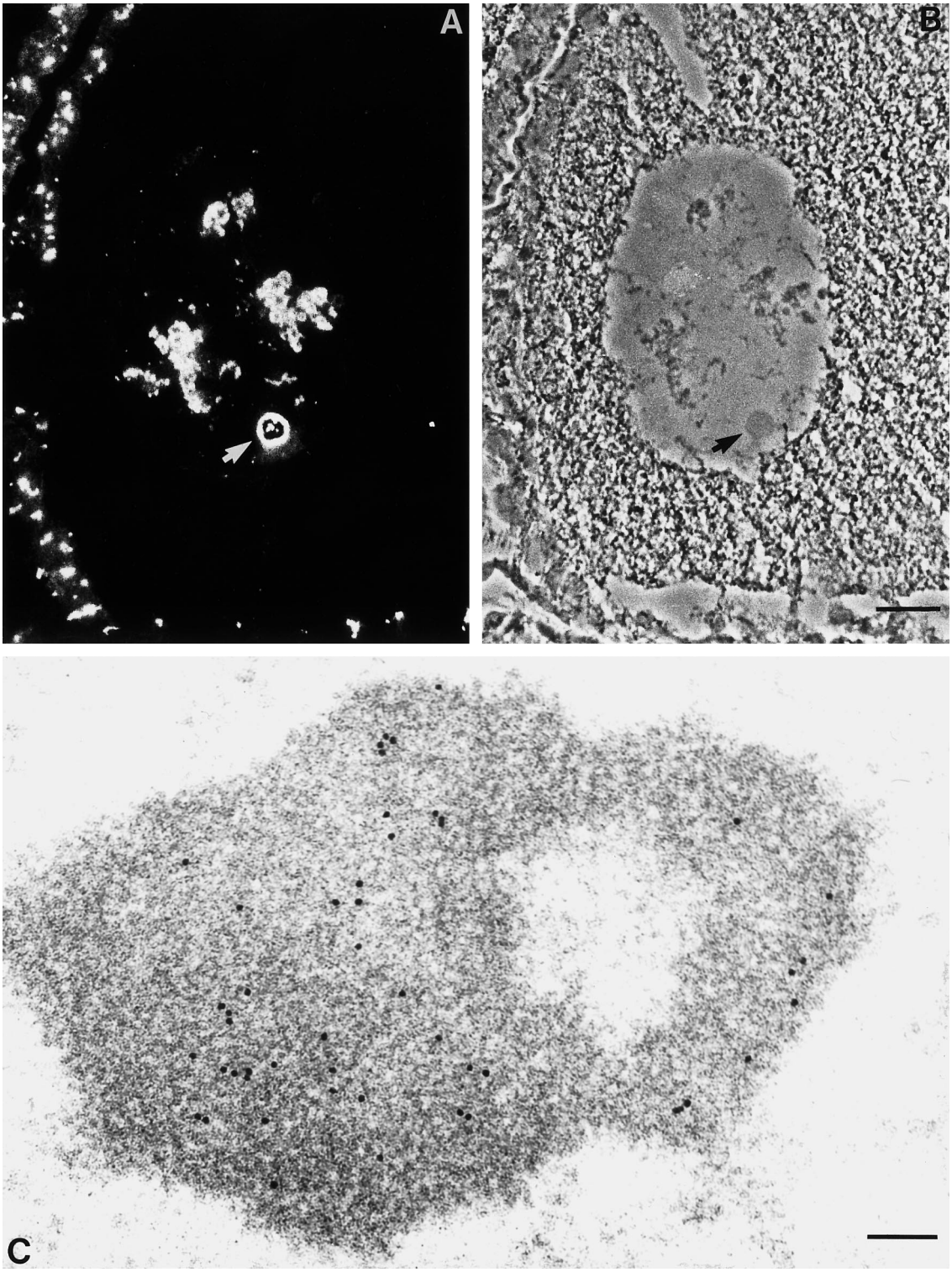

Fig. 4A–C. Immunolocalization of fibrillarin. Frozen sections of *Locusta* oocytes were incubated with autoantibodies reacting specifically with fibrillarin (human autoimmune serum S4) and processed for immunofluorescence microscopy (**A**). The nucleoli fluoresce in their entirety except for several rounded intranucleolar areas. The Binnenkörper is also stained by the fibrillarin antibodies (**A** *arrow*). Note the heterogenous distribution of fibrillarin within this conspicuous nuclear structure of insect oocytes. Strong staining also occurs in the nucleoli of the follicle cells surrounding the oocyte (**A** *left side*). The corresponding phase contrast image is shown in **B** (the *arrow* denotes the Binnenkörper). Fibrillarin localization with monoclonal antibody (mAb) P2G3 performed on an ultrathin Lowicryl section of a *Locusta* oocyte nucleolus (**C**). Gold particles are distributed throughout the nucleolar body except for the vacuolar space, which, in this particular section, reveals few internal structures. Bars represent 20 μm (**B**) and 0.1 μm (**C**)

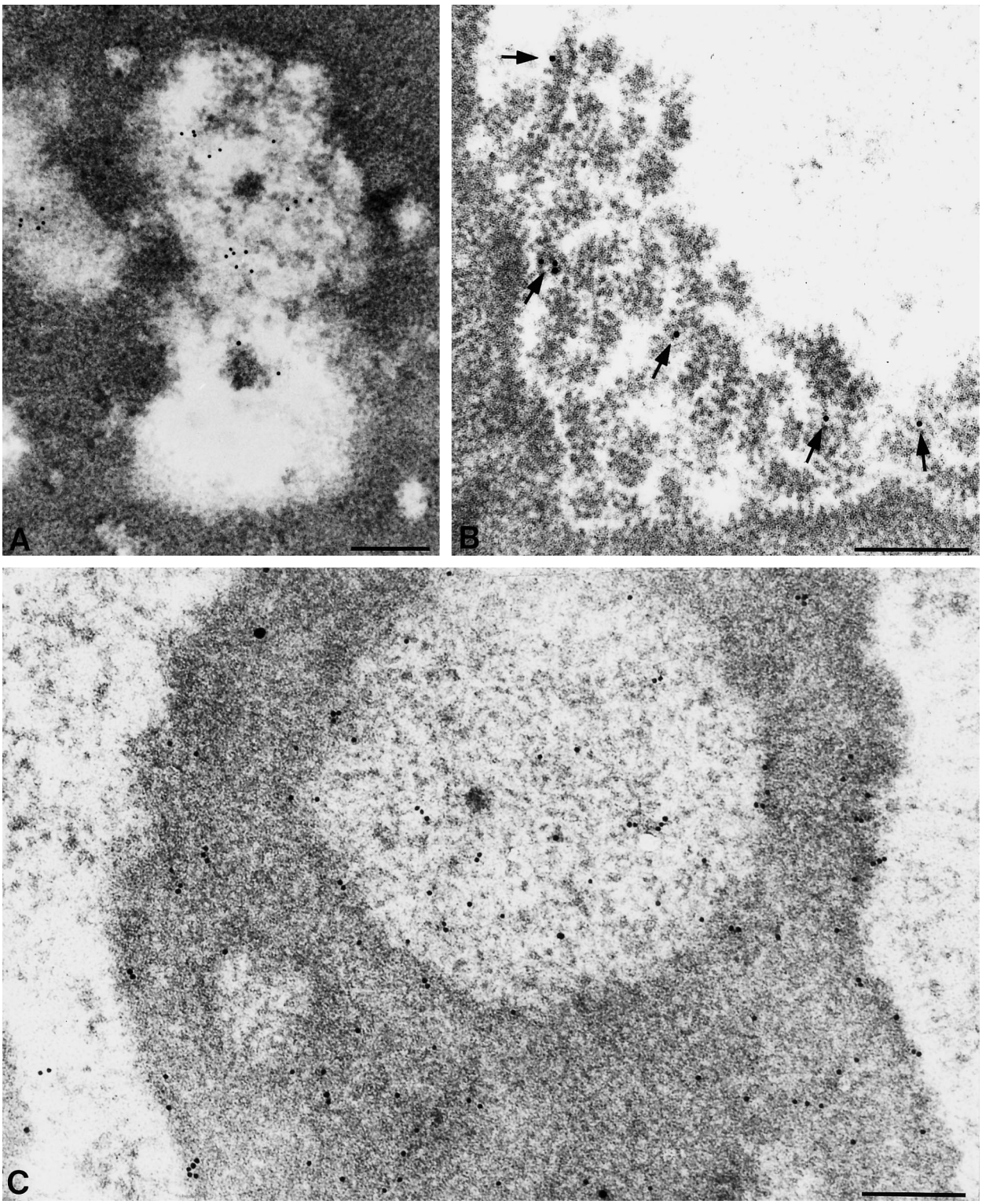

Fig. 5. A–C. Localization of DNA in nucleoli of *Locusta* oocytes. **A** Immunogold electron microscopy following a preembedding protocol. **A** A 5 µm frozen section of *Locusta* ovary was incubated with mAb AK-30/10 against DNA followed by secondary gold-coupled antibodies and processed for thin section electron microscopy. Gold particles decorate specifically the internal structures of the intranucleolar pockets. **B** Visualization of intranucleolar DNA by the terminal deoxynucleotidyl transferase-postembedding technique. Gold particles label the Christmas tree-like structures (*arrows*). **C** In situ hybridization of a digoxigenin-labeled RNA probe complementary to *Locusta* 28S rRNA performed on an ultrathin Lowicryl section of *Locusta* ovary. Gold particles are seen over the dense portion of the nucleolus as well as in the vacuolar space. Bars represent 0.2 µm

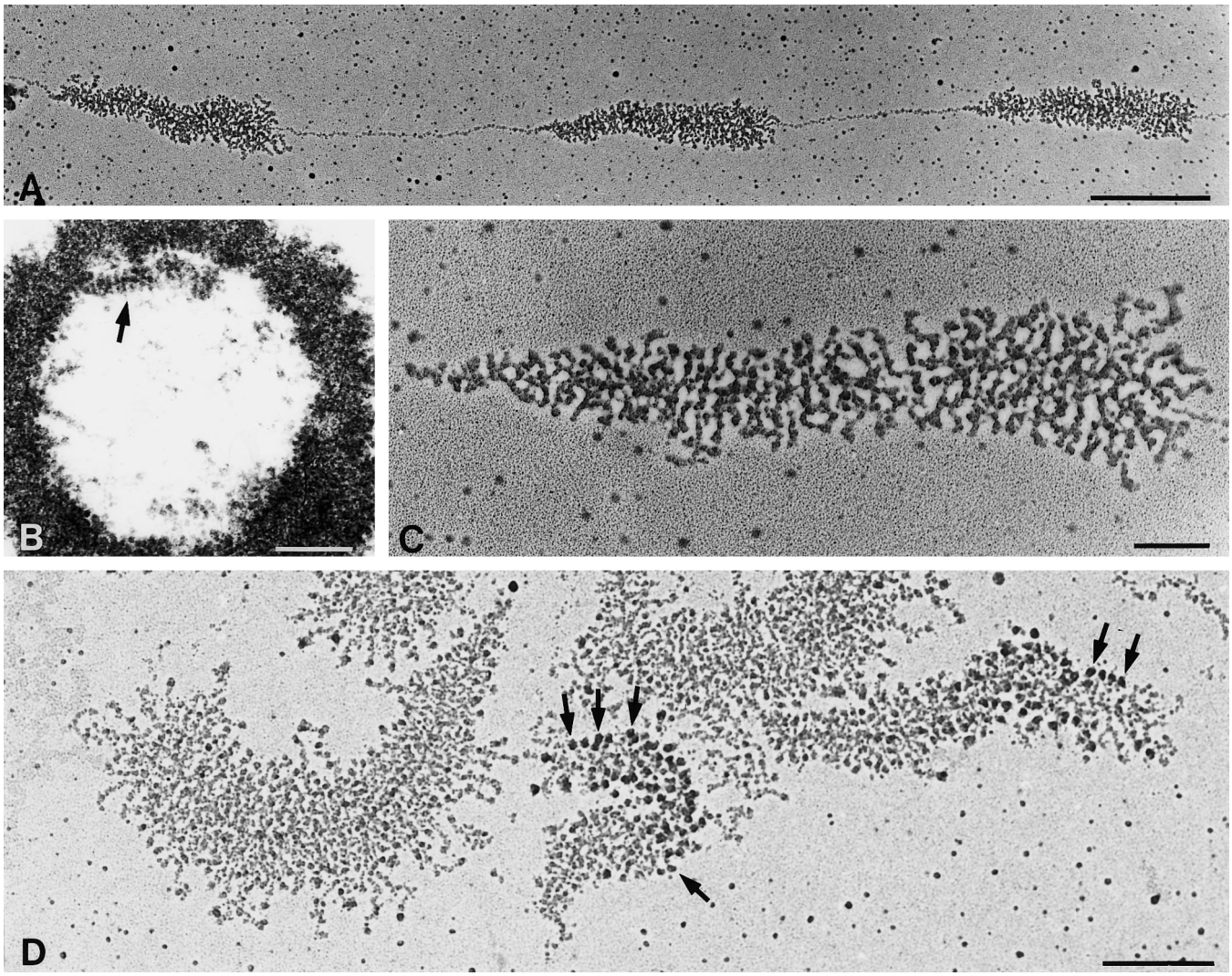

Fig. 6A–D. Miller spreads of transcriptionally active rRNA genes of *Locusta* oocytes (**A, C, D**). Note the tandem arrangement of the genes (**A**) and the close spacing of the nascent transcripts (**C**). Compare the sectioned (**B** *arrow*) and spread (**C**) rDNA transcription unit at the same magnification. In Miller spreads, incompletely unfolded lateral ribonucleoprotein fibrils have the appearance of stalked granules (**D** *arrows*). Bars represent 1 μm (**A**), 0.5 μm (**D**) and 0.2 μm (**B, C**)

throughout their interior, which appeared as dark holes (Fig. 4A; human autoimmune serum S4 and mAb P2G3 gave identical results). These fibrillarin-less structures most likely represent the larger nucleolar pockets described above in EM sections (the smaller ones escaped detection owing to the very strong fluorescent signal). It should be noted that in the frozen sections the unfolded state of the oocyte nucleoli was no longer maintained owing to the tendency of the multiple nucleolar entities to collapse into larger structures in the course of the experimental manipulations. Besides the nucleoli a prominent spherical nuclear body known as the Binnenkörper was also strongly labeled with the fibrillarin antibodies (for a detailed analysis of the insect Binnenkörper and the related sphere organelles of amphibian oocyte nuclei and coiled bodies of somatic nuclei see Gall et al. 1995). In addition, the antibodies stained the nucleoli of the follicle cells surrounding the oocytes (Fig. 4A). When we probed ultrathin Lowicryl sections of *Locusta* ovarioles with mAb P2G3 to fibrillarin, nucleoli were labeled throughout except for the intranucleolar pockets (Fig. 4C). The Christmas tree-like structures seen in conventional EM sections were hardly visible in the Lowicryl-embedded material and the pockets appeared more or less empty except for a finely filamentous meshwork (Fig. 4C). Since fibrillarin is a marker protein for the DFC (Ochs et al. 1985; Shaw and Jordan 1995; Thiry and Goessens 1996) we conclude that the dense component of *Locusta* oocyte nucleoli corresponds to the DFC of somatic nucleoli. Whether fibrillarin is absent from the nucleolar pockets or present at concentrations below the detection limit of our immunocytochemical approach is not known.

Distribution of intranucleolar DNA

For the detection of DNA we used antibodies to DNA and the terminal TdT method, i.e., the most sensitive approaches presently available (for details and examples see Scheer et al. 1987; Thiry 1992, 1995; Raska et al.

1995; Thiry and Goessens 1996). When a preembedding protocol was employed, antibodies to DNA labeled the fibrillar content of the nucleolar pockets but not the surrounding DFC (Fig. 5A). Since the structural preservation of the gold-labeled material was quite poor, we also located DNA on ultrathin sections of glutaraldehyde/osmium tetroxide-fixed and Epon-embedded oocytes by using the TdT method (Thiry 1992, 1995). Under these conditions it became clear that the gold particles were on the Christmas tree-like structure (Fig. 5B). The relatively low density of the gold particles indicates a low DNA concentration within the nucleolar pockets and is fully compatible with the dispersed state of the transcribing rDNA chromatin.

Detection of rRNA in the nucleolar pockets

To obtain further evidence for the presence of transcribing rRNA genes in the nucleolar pockets we employed in situ hybridization with a digoxigenin-labeled RNA probe complementary to the *Locusta* 28S rRNA sequence. The riboprobe was hybridized to ultrathin sections of Lowicryl-embedded oocytes and detected by immunocytochemistry. Besides the DFC, the fibrillar material within the nucleolar pockets was clearly labeled (Fig. 5C). When we used the corresponding sense probes as controls, no gold labeling was detectable (data not shown). These results demonstrate the presence of rRNAs not only in the DFC (which is a well established fact; see Hadjiolov 1985; Puvion-Dutilleul et al. 1991; Shaw and Jordan 1995; Thiry and Goessens 1996) but also in the nucleolar pockets.

Miller spreads of active rRNA genes from Locusta oocytes

To obtain information on the molecular arrangement and dimensions of the transcriptionally active rRNA genes of *Locusta* oocytes, we prepared Miller spreads. The survey view (Fig. 6A) reveals tandemly arranged rDNA transcription units with the characteristic Christmas tree pattern of lateral ribonucleoprotein (RNP) fibrils. The transcription units have an average axial length of 1.64 μm (n=28) and are separated by nontranscribed spacers that are, on average, 1.84 μm long (n=26). A direct comparison between a sectioned and spread rDNA transcription unit at the same magnification shows that both the chromatin axis and the nascent RNP fibrils are considerably condensed and foreshortened in situ (Fig. 6B, C). A rough estimation of the length of the transcription unit length in situ may be derived from Fig. 6B. The longitudinally sectioned Christmas tree (indicated by the arrow) has a length of approximately 0.4 μm. Since its beginning cannot be accurately determined, this is likely to be an underestimate of the total unit length. Usually the lateral RNP fibrils are extended by the low salt spreading procedure by a factor of 8–10 as compared with the situation in situ (Fig. 6B, C). Occasionally, however, we observed in spread preparations incompletely unraveled nascent RNP fibrils that ended in striking, approximately 25 nm, large terminal knobs (Fig. 6D).

Discussion

We interpret the intranucleolar structures of *Locusta* oocytes described here as rDNA transcription units or Christmas trees based on the following criteria. (i) Morphologically they appear as close-packed short fibers that extend from a central axis. The free ends of the fibers are usually condensed into a knob-like structure. Cross-sectional views display a radial arrangement of the lateral fibers around a central axis, most likely in a helical array. (ii) A length gradient of the lateral fibers can be seen in favorable longitudinal sections, probably reflecting the growth of the pre-rRNA chains. The length increment is restricted to the proximal portion of a transcription unit, similar to the situation seen in Miller spreads (Fig. 6A). (iii) They contain DNA as shown by the TdT method, which is based on the enzymatic in situ elongation of free ends of DNA molecules exposed at the surface of ultrathin sections (Thiry 1992, 1995). (iv) They probably contain 28S rRNA sequences, as demonstrated by in situ hybridization. (v) When nascent RNP fibrils of *Locusta* rRNA genes are only partially unfolded in Miller spread preparations, they have the appearance of stalked particles and resemble the lateral fibers in situ (compare Figs. 2 and 6D, arrows; the somewhat larger size of the terminal knobs seen in Miller spreads as compared with sectioned material might be a consequence of the different preparation conditions).

It is interesting to note that structures similar to the ones described here have been observed in the outer zone of the large nucleolus of *Chironomus tentans* salivary gland cells (Olins et al. 1980). However, from morphological criteria alone the authors could not clarify whether these nucleolar strands represent transcriptional structures or rRNP storage particles.

The longest Christmas tree we could trace in favorable longitudinal sections had a length of ca. 0.4 μm. Accepting this as the minimal length of a rDNA transcription unit in situ, we can calculate the in situ compaction ratio of the transcribed rDNA. The transcribed *Locusta* rDNA comprises 7 kb (Schäfer and Kunz 1985) or 2.38 μm B-DNA, i.e., the axis of active rRNA genes is foreshortened in situ by a factor of about 6. This is a maximal figure since the transcription unit length is likely to be underestimated to some extent. For comparison, in Miller spreads the transcribed rDNA is extended close to the length of the B-DNA (compaction ratio about 1.4). Since the axes of the rDNA transcription units are densely covered by RNA polymerases (see Fig. 6D), the apparent foreshortening in situ cannot be caused by a nucleosomal arrangement of the rDNA, since there is simply no space left, but must occur at another level, e.g., by coiling of the chromatin axis. Direct observation of the low salt-induced dispersal of nucleolar chromatin from oocytes of the house cricket, *Acheta domesticus*, by a combination of thin section EM and video-enhanced light microsopy, indicated a compaction of “native” rDNA transcription units by a factor of 3–4 and more

than 10, depending on the type of rDNA analyzed (chromosomal vs amplified; Tröster et al. 1985; Trendelenburg et al. 1996).

So far the only other genes that have been identified in situ and in Miller spreads are the Balbiani ring genes of the salivary glands of *C. tentans* (for review see Daneholt et al. 1982). A comparison of the number of lateral RNP fibrils per unit length of chromatin axis in sections and in Miller spreads indicated a 3 to 8-fold compaction of the Balbiani ring DNA in situ (Andersson et al. 1980; Olins et al. 1980, 1982, 1983; Daneholt et al. 1982). Thus transcriptionally active protein-coding and rRNA genes appear to be foreshortened in situ to about the same degree.

Our results have several implications for the current understanding of the functional architecture of nucleoli. First of all they indicate that transcriptionally active rRNA genes can generate a structural entity on their own. In *Locusta* oocytes, the structures we interpret as rDNA transcription units form loose spheroidal aggregates within lightly stained pockets of the dense component of the nucleolus. Since nucleoli are the morphological manifestation of the highly conserved process of ribosome biogenesis, it is reasonable to assume that their basic organization is similar in all eukaryotic cells. In fact, nucleoli of plant and animal cells have a comparable organization in that the DFC surrounds one or several areas of low contrast, the FCs, which have been shown to contain not only rDNA but also components of the rDNA transcription machinery such as RNA polymerase I, DNA topoisomerase I and the transcription initiation factor UBF (for references see Scheer et al. 1993; Scheer and Weisenberger 1994; Raska et al. 1995; Shaw and Jordan 1995; Thiry and Goessens 1996). Our interpretation of the novel nucleolar structures of *Locusta* oocytes as rDNA transcription units harmonizes with these localization studies and, furthermore, provides a strong argument that the rDNA present in FCs is indeed engaged in transcription (see also Thiry and Goessens 1996). Our data do not support the view that FCs serve as storage sites for inactive RNA polymerase I molecules and rDNA (e.g., Hozak et al. 1994; Hozak 1995; Shaw and Jordan 1995).

Nascent pre-rRNA transcripts assemble at their leading ends a 5′-processing complex that is visualized in Miller spreads as a terminal granule (Mougey et al. 1993a). A functionally important component of this processing complex is U3 small nuclear RNP (snRNP) (Mougey et al. 1993b). Fibrillarin is known to be associated with several nucleolar snRNAs including U3 (for review see Maxwell and Fournier 1995) and has been directly identified at the 5′ ends of nascent ribosomal transcript fibrils in Miller spreads (Scheer and Benavente 1990). Thus, besides being a structural component of the DFC, fibrillarin should be also present in the nucleolar pockets of *Locusta* oocytes. We assume that our postembedding labeling protocol was not sensitive enough to detect the relatively few fibrillarin molecules associated with the transcribing rRNA genes.

At first sight our conclusion that the transcriptionally active rRNA genes are distributed throughout the FCs is difficult to reconcile with recent reports describing a selective labeling of the border region between fibrillar centers and the surrounding DFC after BrUTP incorporation into permeabilized HeLa cells (Dundr and Raska 1993; Hozak et al. 1994; Raska et al. 1995). We propose that the spatial arrangement of the rDNA transcription units analyzed in these studies conforms to the situation we have observed in nucleolar pockets of *Locusta* oocytes containing only a few transcription units. In such cases the transcription units were intimately apposed to or even partially integrated into the surrounding DFC, whereas the interior of the pockets was filled with a loose filamentous meshwork, most likely representing inactive nucleolar chromatin. Our finding offers an explanation for the observed physical interaction between sites of rRNA synthesis and fibrillarin-containing structures upon mechanical stretching of nucleoli (Garcia-Blanco et al. 1995). Taken together we conclude that transcriptionally active rRNA genes are located in the fibrillar centers of nucleoli, where they are either distributed more or less uniformly or selectively enriched in the outermost layer abutting the DFC. The specific mode of arrangement may depend on the cell type, the transcriptional status and/or the number of rRNA genes per fibrillar center. In addition our results emphasize the importance of the boundary zone between a fibrillar center and the surrounding DFC, since here the transcription units as well as structural components of the DFC are intimately intermingled, thus making a distinction between the two nucleolar components almost impossible.

It is unclear why the rDNA transcription units of "standard" nucleoli have so far escaped detection by thin section EM. One possible explanation might be their lability toward conventional fixation methods. When we examined sections of whole *Locusta* ovarioles rather than isolated nuclei, the Christmas trees were often quite indistinct or even aggregated into a more or less homogenous fibrillogranular network (Fig. 3B) reminiscent of the FC material of somatic nucleoli (for examples see Smetana and Busch 1974; Hadjiolov 1985; Thiry and Goessens 1996). We take this as an indication that they had undergone substantial structural rearrangements or even lost their structural integrity during the immersion fixation of whole oocytes, which prevents rapid access of the fixative to the nucleolar structures. We are confident that with the development of refined procedures for nucleolar isolation and ultrastructural preservation, rDNA transcription units will eventually also be identified within nucleoli of somatic cells.

Acknowledgements. We thank Mireille Schäfer for the *Locusta* rDNA clone, Mark Christensen for mAb P2G3, Georg Reimer for autoimmune serum S4 and Marc Thiry for his help with the TdT method. We further thank Silke Hofbauer for excellent photographic work and preparation of the figures and Hanswalter Zentgraf for preliminary tomographical analyses of the sectioned Christmas trees. This work received financial support from the Deutsche Forschungsgemeinschaft (Sche 157/10–1).

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