Molecular architecture of the amplified nucleoli of *Xenopus* oocytes

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SUMMARY

An understanding of the functional organization of nucleoli, the sites of ribosome biosynthesis, is limited by the present uncertainty about the topological arrangement of the transcribing rRNA genes. Since studies with 'standard' nucleoli from somatic cells produced conflicting results, we have examined the amplified nucleoli of *Xenopus* oocytes. These nucleoli are unique in that they contain high copy numbers of rRNA genes, are not attached to chromosomes, lack non-ribosomal DNA and can be examined in light microscopic spread preparations of nuclear contents. By immunostaining and confocal microscopy we show that in growing stage IV oocytes the sites of rDNA are surrounded by the dense fibrillar component. The rDNA is actively transcribed as revealed by BrUTP injection into oocytes and localization of components of the nucleolar transcription machinery (RNA polymerase I and the transcription factor UBF). At the ultrastructural level, the rDNA sites correlate with the fibrillar centers of amplified nucleoli fixed in situ. The results provide clear evidence that the transcriptionally active rRNA genes are confined to the fibrillar centers of the oocyte nucleoli and open the possibility to analyze the protein composition of almost native, transcriptionally highly active nucleolar chromatin by immunofluorescence microscopy.

Key words: Nucleolus, *Xenopus* oocyte, RNA polymerase I, UBF, fibrillarin, rDNA transcription

INTRODUCTION

A characteristic feature of amphibian oocyte nuclei is the presence of multiple nucleoli which, unlike their somatic counterparts, are not attached to the chromosomes. Due to their extraordinary size these extrachromosomal nucleoli are easily visible by light microscopy and have attracted attention of numerous early cytologists (for references see Trendelenburg et al., 1996; Wu and Gall, 1997). In the late sixties, with the advent of nucleic acid hybridization in situ, amplified rDNA has been located in the multiple nucleoli of *Xenopus laevis* and other amphibian species (reviewed by Gall, 1969; Macgregor, 1972). Quantitative measurements revealed that a single *Xenopus* oocyte nucleus contains 22-35 pg rDNA in addition to the 12 pg of chromosomal DNA (Gall, 1969; Macgregor, 1972; Thiebaud, 1979a). Since an average rDNA repeat of *Xenopus laevis* comprises 11.6 kb of DNA (for references see Gerbi et al., 1987), an oocyte nucleus contains at least 2 million extra rRNA genes which are distributed in about 1,300 nucleoli (Perkowska et al., 1968; Thiebaud, 1979a). Thus, at the average each amplified nucleolus harbors 1,500 copies of rRNA genes. The actual number depends on the size of the individual nucleolus and ranges from 500 to 11,000 rRNA genes as measured by fluorescent Feulgen-staining (Thiebaud, 1979a; see also Wu and Gall, 1997). Miller and Beatty were able to directly visualize the rRNA genes in the electron microscope after hypotonic dispersal of the nucleolar chromatin (Miller and Beatty, 1969a; Miller and Beatty, 1969b). These spectacular images of genes in action showed that during the growth phase of *Xenopus* oocytes the vast majority of the amplified rRNA genes are fully loaded with RNA polymerase I (pol I) complexes, indicative of maximum transcriptional activity (Miller and Beatty, 1969b; Martin et al., 1980).

The functionally active nucleoli of growing *Xenopus* oocytes usually appear as compact spheroidal bodies with diameters up to 15 µm. They have been described as structurally bipartite, with a granular cortex surrounding a fibrous core (Miller, 1966; Miller and Beatty, 1969a; Miller and Beatty, 1969b; Van Gansen and Schram, 1972; Macgregor, 1972; Boloukhere, 1984; Spring et al., 1996). When exposed to buffer solutions of reduced ionic strength (20-30 mM alkaline salts), the granular cortex disperses to varying degrees thus allowing visualization of the nucleolar cores which are often arranged in the form of beaded rings in such light microscopic spread preparations (Miller, 1966; Macgregor, 1972; Thiebaud, 1979b; Wu and Gall, 1997). These necklace-like structures contain chromatin as indicated by their fragmentation after DNAse digestion (Miller, 1966) and DNA fluorescent staining (Thiebaud, 1979b; Wu and Gall, 1997). Further reduction of the ionic strength completely destroys the nucleolar higher-order organization and causes unwinding of the cores into the tandemly repeated rDNA transcription units along an axial chromatin fiber (Miller and Beatty, 1969a; Miller and Beatty, 1969b; Miller, 1981).

The presence of a high copy number of rRNA genes and the absence of non-ribosomal DNA make the amplified amphibian oocyte nucleoli a valuable model system to analyze topological principles of ribosome biosynthesis and to gain insights into the mechanisms by which a separate piece of rDNA is capable

of organizing a nucleolus. However, a molecular interpretation of their architecture will be impossible without knowing the location of the transcriptionally active rRNA genes within the nucleolar body. So far it has not been possible to identify the structural correlate of the transcribing amplified rRNA genes in situ and results based on DNA fluorescent staining are contradictory (Thiebaud, 1979b; Spring et al., 1996; Trendelenburg et al., 1996; Shah et al., 1996; Wu and Gall, 1997). Here we show that the active rRNA genes are confined to a specific structural component of the amplified nucleoli which escaped detection in most previous electron microscopic studies. Our results demonstrate that the organization of the amplified *Xenopus* nucleoli is remarkably similar to that of somatic nucleoli.

MATERIALS AND METHODS

Oocytes

Small ovary pieces were removed from anesthetized *Xenopus laevis* females and placed in modified Barth's medium (Peng, 1991). For some experiments, ovary pieces were incubated in the presence of actinomycin D (Serva, Heidelberg, Germany) at a final concentration of 10 µg/ml for 4 hours.

Antibodies

Antibodies against the following antigens were used: xUBF (rabbit antiserum against recombinant full length *Xenopus* UBF; Cairns and McStay, 1995), RNA polymerase I (human autoimmune serum S18; Reimer et al., 1987a), nucleolin (mAb P7-1A4-4 raised against *Xenopus* nucleolin; Messmer and Dreyer, 1993), fibrillarin (mAb P2G3; Christensen and Banker, 1992; human autoimmune serum S4 and mAb 72B9; Reimer et al., 1987b), ribosomal protein S1 (mAb RS1-105; Hügle et al., 1985) and DNA (mAb AC 30-10, Progen, Heidelberg; Germany, for characterization see Scheer et al., 1987). The reactivity of these antibodies with *Xenopus* material has been documented (Bell et al., 1992; Bell et al., 1997; Bell and Scheer, 1997; Scheer et al., 1987; Hügle et al., 1985).

Immunofluorescence microscopy

Nuclei were manually isolated from oocytes with diameters ranging from 0.8 to 1.0 mm (stage IV according to Dumont, 1972) and their contents spread in the 25 mM dispersal medium as described (Gall et al., 1991). After centrifugation, the microscope slides with the spread material were fixed either with 70% ethanol for 10 minutes or with 2% paraformaldehyde in PBS/1 mM MgCl$_2$ for 1 hour, transferred to PBS and processed for immunofluorescence. Both fixation protocols produced essentially identical results. Primary antibodies were applied for 30 minutes, rinsed away with PBS (2× 3 minutes) and secondary Texas-Red or FITC conjugated antibodies were added for another 30 minutes (diluted 1:75 and 1:150, respectively; Dianova, Hamburg, Germany). After several washes, the preparations were mounted in 50% glycerol (diluted with PBS) or Mowiol (Sigma, Deisenhofen, Germany). Some preparations were counterstained with the DNA-specific fluorescent dye DAPI as described (Wu and Gall, 1997). In double-labelling experiments, xUBF antibodies (diluted 1:100) were first applied followed by appropriate secondary antibodies. Then the pol I or fibrillarin specific serum (diluted 1:80) was added and detected by secondary antibodies. The preparations were examined using a Zeiss Axiophot or a Leica confocal laser scanning microscope (TCS-NT).

Microinjection of BrUTP

Oocytes were injected into the cytoplasm with 15-20 nl of a solution containing 100 mM BrUTP (Sigma), 75 mM KCl, 25 mM NaCl, 10 mM Hepes, pH 7.2. After an incubation time of 5-20 minutes, nuclei were isolated, their contents spread as described above and incubated with antibodies against BrdU (Boehringer Mannheim, Germany; diluted 1:20) followed by Texas-Red- or FITC-conjugated antibodies.

Electron microscopy

Stage IV oocytes were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for at least 3 hours in the cold, washed in the same buffer, post-fixed in 2% OsO$_4$ for 1 hour, soaked in 0.5% uranyl acetate over night, dehydrated through an ascending ethanol series and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate following standard protocols. DNA was specifically labelled on Epon ultrathin sections using the in situ terminal deoxynucleotidyl transferase (TdT) technique (Thiry et al., 1993).

For immunocytochemistry, stage IV oocytes were fixed in a mixture of 2.5% formaldehyde (freshly prepared from paraformaldehyde) and 0.5% glutaraldehyde in PBS containing 0.5% Triton X-100 for 3 hours in the cold. After several washes with PBS, the oocytes were dehydrated through an ascending ethanol series and embedded in Lowicryl K4M (Polysciences, Eppelheim, Germany) as described (Carlemalm and Villiger, 1989). The resin was UV polymerized at −25°C for 3 days and at 4°C for a further 3 days period. Ultrathin sections were mounted on nickel grids and probed with antibodies against xUBF (diluted 1:100), nucleolin (cell culture supernatant 1:10) or fibrillarin (P2G3; 4 µg/ml). All primary antibodies were diluted in PBS containing 0.1% Tween-20 and 1% BSA. Bound antibodies were visualized by incubation with appropriate secondary antibodies coupled to 12 nm gold particles (Dianova, diluted 1:20 in PBS complemented with 0.1% Tween-20 and 0.1% BSA) for 1 hour at ambient temperature. The sections were finally counterstained with uranyl acetate and lead citrate and examined in a Zeiss EM10A electron microscope.

RESULTS

Light microscopic appearance of *Xenopus* oocyte nucleoli

When nuclear contents from stage IV *Xenopus* oocytes were spread on microscope slides (Gall et al., 1991) and viewed by phase contrast microscopy, the cortex of the multiple nucleoli appeared somewhat swollen thus facilitating visualization of internal phase-dense core structures (Fig. 1A). The size and shape of the cores were quite variable and ranged from compact aggregates to extended beaded ring-like structures. The rDNA was confined to the cores as demonstrated by their fluorescence with the antibody AC 30-10 directed against DNA (Fig. 1B). Essentially the same results were obtained after staining with the DNA-specific dye DAPI (data not shown; see also Thiebaud, 1979b; Wu and Gall, 1997). When we stained the preparations with antibodies to fibrillarin, a known marker protein of the dense fibrillar component (DFC) of somatic nucleoli (Ochs et al., 1985; for further references see Reimer et al., 1987b; Shaw and Jordan, 1995; Thiry and Goessens, 1996), the cores fluoresced brightly (Fig. 1C). We conclude that the light microscopically defined cores of spread nucleoli from *Xenopus* oocytes contain nucleolar chromatin and the DFC. The cortex then must be the granular component (GC). In fact, the cortex was strongly labelled with antibodies against the ribosomal protein S1, a marker protein of the GC (Fig. 1D; for localization of S1 in nucleoli of somatic cells see Hügle et al., 1985).

Localizing the transcriptionally active rRNA genes by confocal microscopy

To analyze in more detail the topological relationship between rDNA and the DFC in the amplified nucleoli of stage IV oocytes, we performed double immunolocalization experiments using confocal microscopy. DNA was identified with the monoclonal antibody AC 30-10 directed against DNA (Scheer et al., 1987). As shown in Fig. 2A, the bulk of DNA did not colocalize with fibrillarin but was rather intimately surrounded by the DFC (for similar observations see also Shah et al., 1996; Wu and Gall, 1997).

To examine the distribution of components of the nucleolar transcription machinery, spread nucleoli were probed with antibodies against fibrillarin to label the DFC and with antibodies against the upstream binding factor (UBF), a RNA polymerase I-specific transcription factor which binds to the promotor region of rRNA genes (McStay et al., 1997; Reeder et al., 1995; Moss et al., 1998). The UBF staining was confined to the 'holes' in the DFC, i.e. the rDNA-containing regions (Fig. 2B). Antibodies to RNA polymerase I (pol I) produced essentially the same fluorescence pattern. Again, there was little overlap between the pol I and the fibrillarin fluorescence and the pol I-foci corresponded to fibrillarin-deficient roundish regions within the central part of the DFC (Fig. 2C).

The transcriptional status of the DNA was probed by microinjection of BrUTP into oocytes prior to spreading of the nuclear contents. The result was clearcut: 5 minutes after injection of BrUTP the majority of the labelled pre-rRNA transcripts were concentrated in the fibrillarin-deficient regions, i.e. the sites of the rDNA and of the transcription machinery (Fig. 2D). After 20 minutes Br-labelled RNA appeared to radiate out into the surrounding DFC regions (Fig. 2E). We interpret this staining pattern as the result of the movement of terminated pre-rRNAs away from the rDNA template during the labelling period.

To determine whether the pol I-containing foci correspond to sites of active transcription, oocytes were injected with BrUTP. After 5 minutes nuclear spreads were prepared and double-stained with antibodies to BrU and pol I. The correspondence of both fluorescence patterns clearly demonstrates that the immunocytochemically detectable pol I is engaged in transcriptional events and does not represent accumulations of inactive enzymes (Fig. 3A). Furthermore, confocal microscopy revealed an almost perfect co-localization between pol I and UBF (Fig. 3B and C). Notably, this coincident labelling pattern with both antibodies was also seen when the cores were expanded in the form of beaded rings (Fig. 3C″) or even further into loop-like structures with closely spaced tiny fluorescent dots (Fig. 3B″, arrow). Whether such linear arrays of minute pol I and UBF-containing dots reflect the tandem arrangement of individual rDNA transcription units separated by nontranscribed spacers cannot be answered at the moment. Although it is feasible to visualize individual transcriptionally active rRNA genes after complete unravelling of amplified amphibian oocyte nucleoli by immunofluorescence microscopy with antibodies to pol I or fluorescent in situ hybridization (Weisenberger and Scheer, 1995), we cannot exclude the possibility that a single dot contains a cluster of tightly packed rRNA genes.

Electron microscopic localization of the rRNA genes in situ

The above described experiments showed that the immunocytochemically detectable pol I and UBF molecules colocalized with the transcribing rDNA. Thus antibodies against these components of the nucleolar transcription machinery offered a means to trace the transcribing rRNA genes within the intact nucleolar body by employing immunogold electron microscopy. To define the ultrastructure of the amplified nucleoli, we first examined ultrathin sections
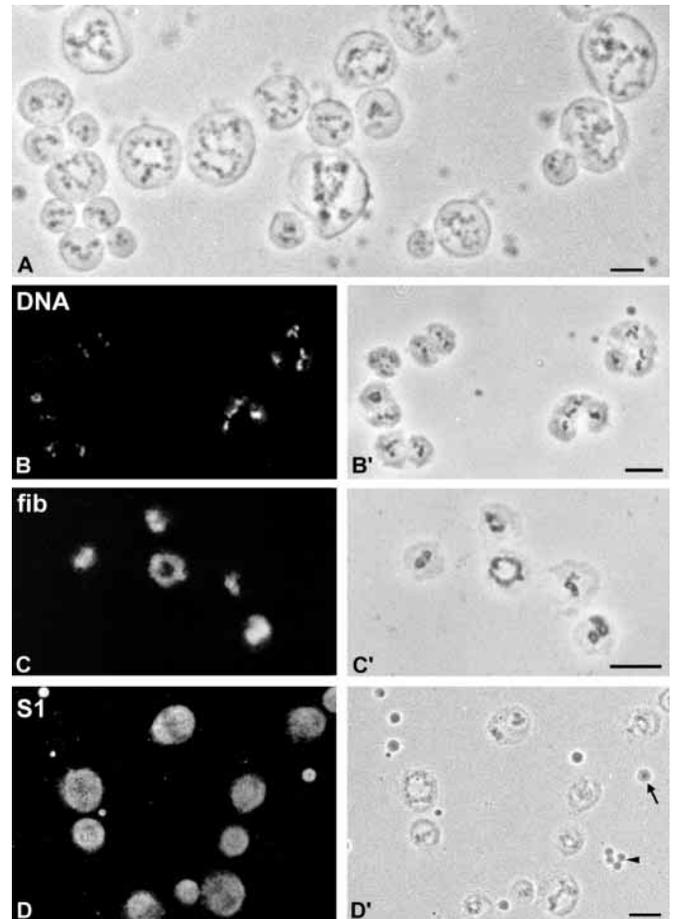

Fig. 1. Nucleoli from spread preparations of *Xenopus* stage IV oocyte nuclei. When viewed in phase contrast, the amplified nucleoli exhibit a bipartite organization with a dark core surrounded by a loosely organized cortex (A). Note the highly variable shape of the cores, varying from compact aggregates to beaded strings. The nucleolar cores contain rDNA as evidenced by staining with the DNA-specific antibody AC 30-10 (B). Antibodies to fibrillarin (mAb 72B9), a marker protein of the DFC, stain the cores but not the surrounding cortex material in immunofluorescence microscopy (C). Antibodies against ribosomal protein S1 stain the nucleolar cortex (D). Since the cortex material covers the internal cores, it is difficult to visualize them as S1-negative structures by conventional immunofluorescence microscopy. The anti-S1 antibodies label also small nucleoli (e.g. arrow in D′) in contrast to similarly sized nuclear bodies (some are denoted by the arrowhead in D′; for a description and characterization of Cajal bodies and B-snurposomes see Gall et al., 1999). The corresponding phase contrast images are shown in B′-D′. Bars, 10 μm.
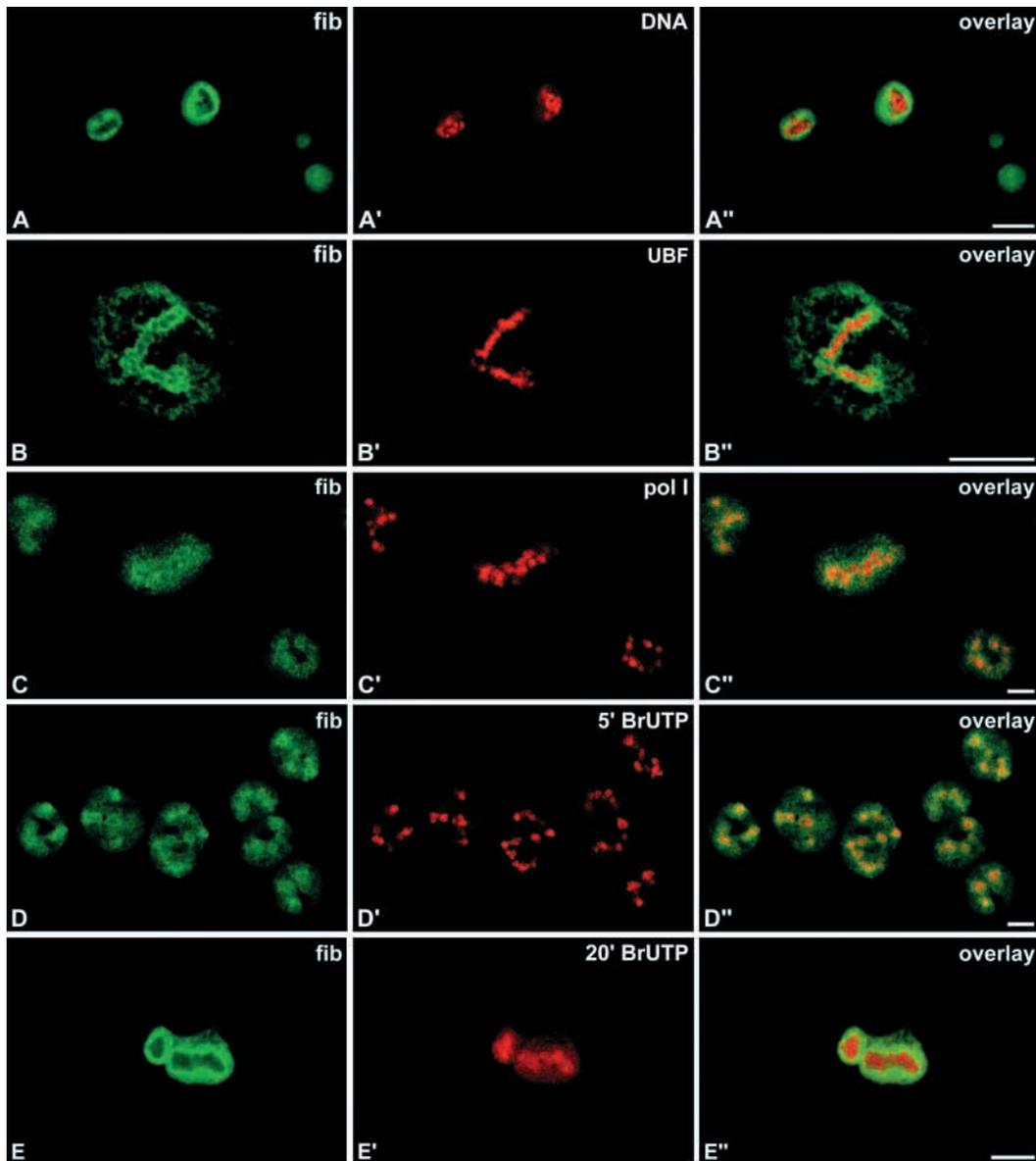
Fig. 2. Spatial relationships between nucleolar DNA, the transcription relevant proteins pol I and UBF, transcription sites and the DFC as revealed by double-label immunofluorescence microscopy. Nuclear contents of stage IV oocytes were spread and the nucleoli analyzed by confocal microscopy. Merged images are shown in A″-E″. (A) Double-localization of DNA and fibrillarin (fib). The bulk of DNA is confined to fibrillarin-deficient regions. (B) Double-localization of UBF and fibrillarin. UBF is located in the fibrillarin-deficient regions which appear here as linear arrays of 'holes' in the DFC (B). There is little overlap between fibrillarin and UBF as indicated in the overlay image (B″). The network of fibrillarin-positive strands surrounding the central DFC reflects the nucleonemal organization which is particularly pronounced in the large nucleoli. (C) Essentially the same pattern is seen after double labelling of pol I and fibrillarin. (D and E) Oocytes were injected with BrUTP prior to spreading. After 5 minutes incubation time, nascent pre-rRNA transcripts (D′) are concentrated in the fibrillarin-deficient regions (D). After 20 minutes incubation time, some transcripts localizing in the surrounding DFC may represent terminated pre-rRNAs that have moved away from their site of synthesis (E). Bars, 5 μm.

of conventionally fixed and Epon-embedded stage IV oocytes. Usually the multiple nucleoli displayed a bipartite organization with a dense core surrounded by a more loosely structured cortex (Fig. 4A), similar to what had been described in earlier morphological studies (Miller and Beatty, 1969a; Miller and Beatty, 1969b; Van Gansen and Schram, 1972; Boloukhere, 1984; Spring et al., 1996). In most sections the dense core appeared as a heavily and almost uniformly stained inner mass of the nucleoli without an indication of a further morphological subdivision. In contrast, in ultrathin sections of Lowicryl-embedded stage IV oocytes two substructures of the dense core were discernable so that the multiple nucleoli revealed a tripartite organization with a concentric arrangement of three morphologically distinct components (Figs 4B and 5A,B), similar to what is known from somatic nucleoli (e.g. Scheer et al., 1993; Thiry et al., 1993; Thiry and Goessens, 1996; Shaw and Jordan, 1995). A centrally located rounded or elongated zone of low contrast was surrounded by a layer consisting of
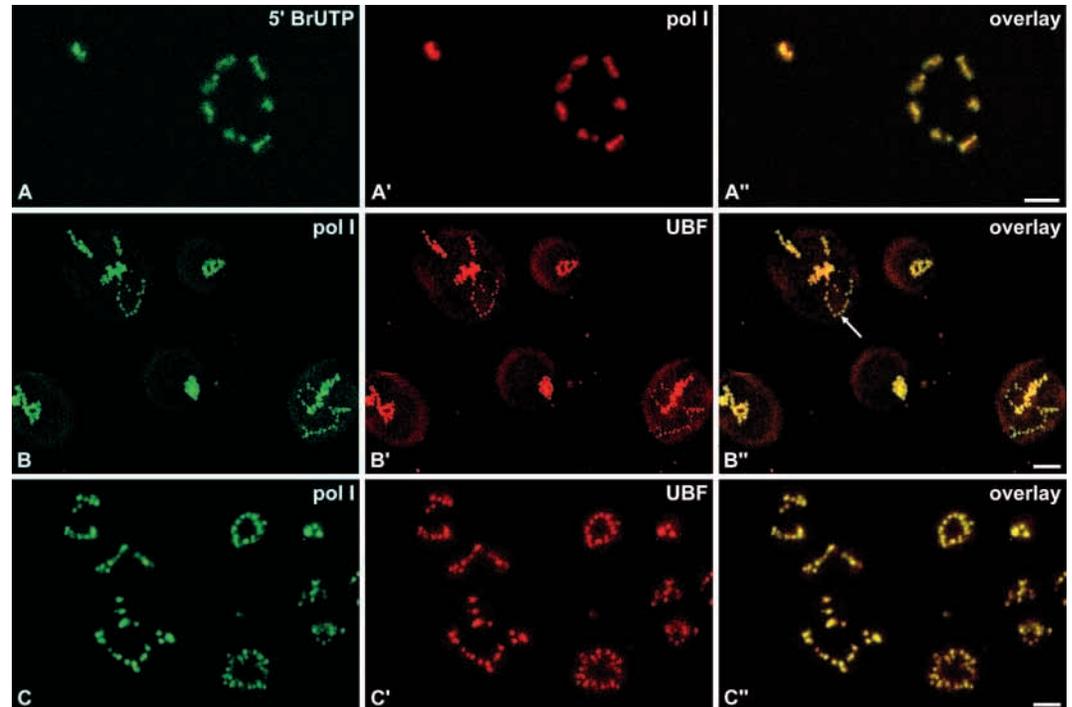
Fig. 3. Colocalization of intranucleolar transcription sites, pol I and UBF. (A) Oocytes were injected with BrUTP and 5 minutes later the nuclear contents were spread and stained with antibodies to BrdU (A) and pol I (A′). Pol I is transcriptionally active as indicated by the yellow color in the overlay (A″). (B and C) UBF and pol I colocalize at the nucleolar cores which are often expanded in the form of beaded rings (C) or loop-like structures with a finely punctate appearance (arrow in B″). Bars, 5 µm.

tightly packed and densely stained fibrils which gradually developed into the outermost cortex region with a granular appearance.

When Lowicryl ultrathin sections were reacted with antibodies to UBF, gold particles decorated specifically the central light regions of the amplified nucleoli (Fig. 4B). Quantitative measurements showed that the vast majority (93-96%) of the nucleolus-associated gold particles was concentrated in these central light regions, the remainder being scattered throughout the surrounding DFC and the cortical regions (GC) of the nucleoli. Not infrequently, several UBF-positive regions were seen in a given section suggesting that the nucleolar chromatin meanders throughout the nucleolar body. Based on the almost exclusive labelling of the central light regions of the multiple nucleoli with antibodies to UBF we conclude that these structures represent the counterparts of the UBF-positive foci seen in the confocal microscope (Fig. 3B′ and C′). Unfortunately, the pol I antibodies failed to label the amplified nucleoli after embedding in Lowicryl (similar negative results were also obtained with somatic cells).

To characterize further the structural subdomains of the amplified nucleoli and to relate them to the components of somatic nucleoli, we probed the sections with antibodies to nucleolin. Nucleolin, which is involved in the initial cleavage of pre-rRNA (reviewed by Ginisty et al., 1999) has been localized both to the DFC and GC but not the fibrillar centers (FCs) of somatic nucleoli (for references see Shaw and Jordan, 1995; Thiry and Goessens, 1996). As shown in Fig. 5A, antibodies to nucleolin labelled very strongly the dense layer surrounding the central light area and, though to a lesser extent, also the cortical region of the amplified nucleoli. Based on the morphology and the absence of nucleolin labelling we conclude that the central light regions are FCs (see also Shah et al., 1996). Their designation as FCs is further corroborated

by the fact that UBF has also been identified as a characteristic protein constituent of the FC of mammalian cells (e.g. Scheer et al., 1993; Zatsepina et al., 1993; for further references see Thiry and Goessens, 1996). Finally, antibodies against fibrillarin decorated almost exclusively the dense layer surrounding the FCs but were absent from the outermost zone of the amplified nucleoli (Fig. 5B). The fibrillarin-positive component, therefore, corresponds to the DFC and the fibrillarin-free but nucleolin-positive cortical region to the GC of somatic nucleoli.

To provide further evidence for the distribution of rDNA within the amplified nucleoli we used the TdT immunogold method which has already proven the existence of DNA in the FCs of somatic nucleoli (Thiry et al., 1993). However, the amplified nucleoli were not labelled above background levels. The same negative results were also obtained when Lowicryl sections were probed with antibodies to DNA. In contrast, when transcription was inhibited by actinomycin D, DNA could now be clearly identified in the FCs but was absent from the other nucleolar components (Fig. 6). It is well known that actinomycin D causes the condensation of nucleolar chromatin and we assume that only under such conditions the local packing density of the rDNA is sufficiently high to allow its immunocytochemical identification.

Our attempts to identify the sites of BrUTP incorporation at the electron microscopic level were frustrated by the weak signal (maximally 4 gold particles over the nucleolar area in a given section). However, the few gold particles detectable were consistently enriched in the FCs rather than the surrounding nucleolar components (data not shown).

DISCUSSION

Although the amplified rRNA genes of amphibian oocytes

were the first genes visualized in the electron microscope (Miller and Beatty, 1969a; Miller and Beatty, 1969b; Miller, 1981), their spatial distribution within the intact nucleolus is poorly understood. 'Miller chromatin spreads' have provided a wealth of information about the arrangement of the rRNA genes along the chromatin fiber, the molecular anatomy of transcriptional units, the structural organization of nascent transcripts and, furthermore, showed that during the growth stage of the *Xenopus* oocyte the majority of the amplified rRNA genes is heavily transcribed (Miller, 1981). An essential step of the Miller spreading technique involves hypotonic lysis of the amplified nucleoli in order to disrupt higher order
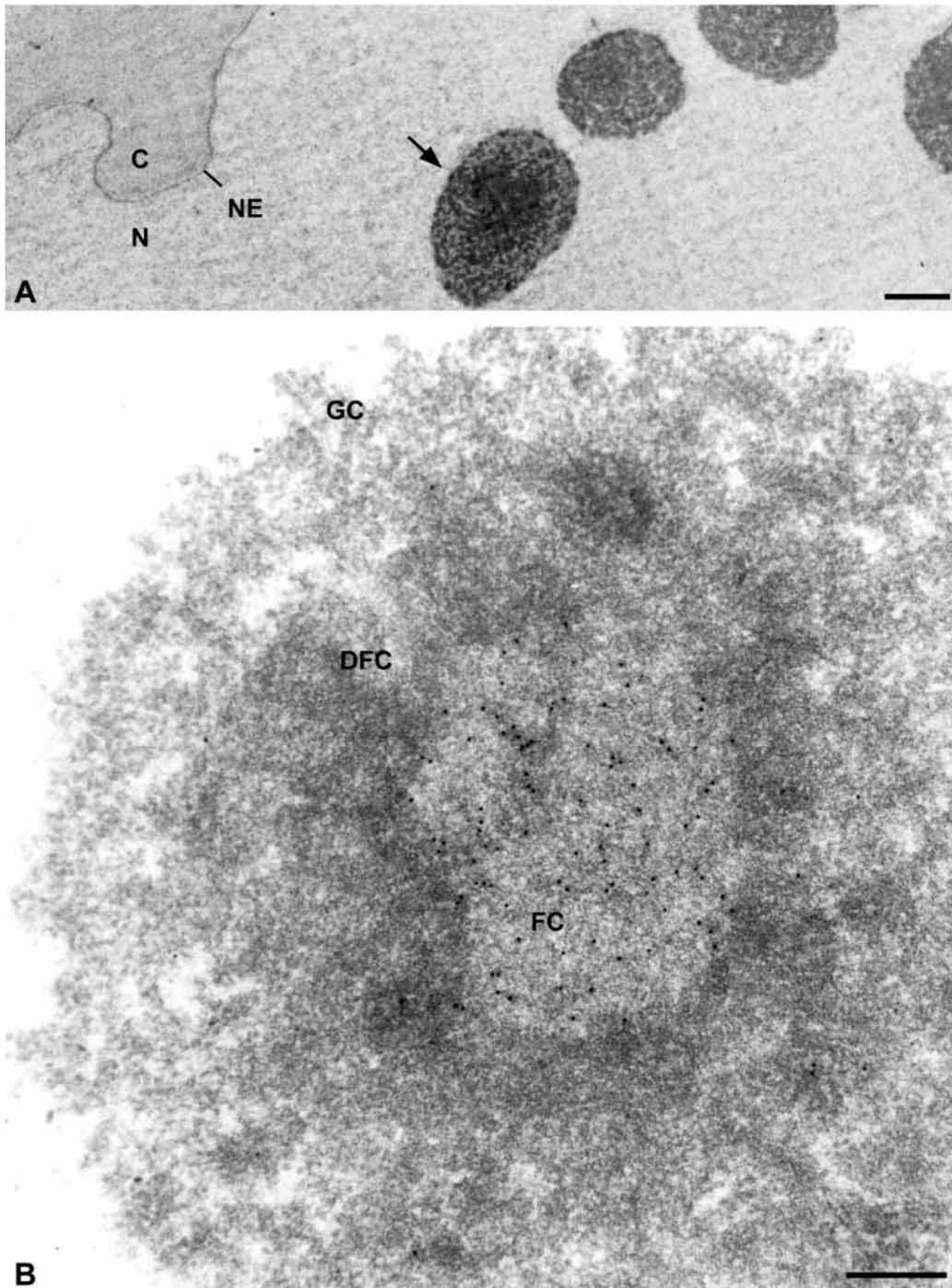

Fig. 4. (A) Low power electron micrograph of the nuclear periphery of a stage IV *Xenopus* oocyte after conventional fixation and Epon embedding. Most nucleoli display a dense core surrounded by a more loosely textured cortex (arrow). (B) In ultrathin sections of Lowicryl-embedded stage IV oocytes, however, a tripartite organization of nucleoli is evident with a central FC surrounded by the DFC and the GC. Antibodies to UBF (detected with 12 nm gold-coupled secondary antibodies) decorate the FC. NE, nuclear envelope; N, nucleoplasm; C, cytoplasm. Bars: 1 μm (A); 0.2 μm (B).

topological relationships, to unfold the chromatin and to release it from its nucleolar confinement. The subsequent centrifugation step then transforms the relaxed nucleolar chromatin into a two-dimensional array. As a consequence, information concerning the spatial arrangement of the rRNA genes within the intact nucleolus is not available from such images. The only conclusion that can be drawn from chromatin spreads after a brief exposure of the nucleoli to low salt buffer is that the amplified rRNA genes often form compact aggregates which probably reflect their in vivo concentration
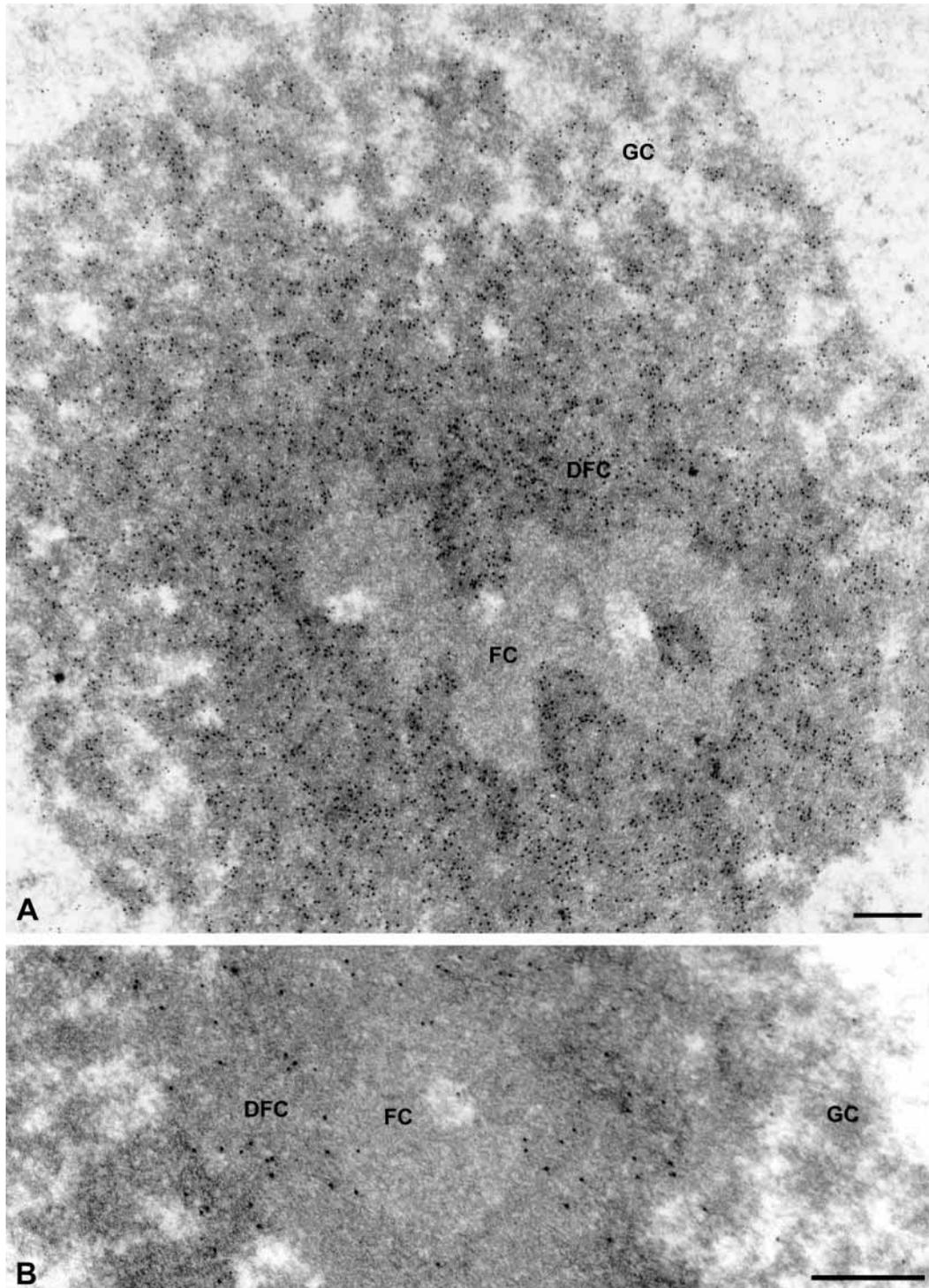

Fig. 5. Amplified nucleoli from stage IV *Xenopus* oocytes probed with antibodies to nucleolin (A) and fibrillarin (B). Postembedding immunogold electron microscopy on Lowicryl ultrathin sections. The FCs are unlabelled with both antibodies. Nucleolin is highly concentrated in the DFC but also detectable in the surrounding GC with the characteristic nucleolonema configuration (A). In contrast, antibodies to fibrillarin (mAb P2G3) label specifically the DFC (B). Bars, 0.2 μm.
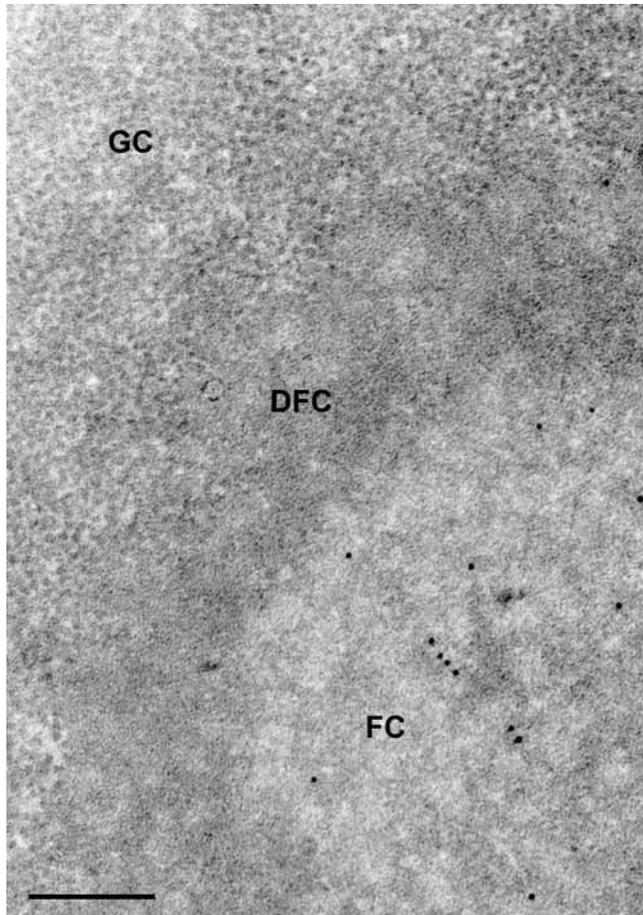
Fig. 6. Treatment of oocytes with actinomycin D leads to a clear separation of the three nucleolar components as seen in this electron micrograph after conventional fixation and Epon-embedding of the oocytes. Only the FC contains DNA as shown by the TdT-method followed by immunogold labelling. Bar, 0.2 µm.

in distinct gene clusters (e.g. Trendelenburg and McKinnell, 1979; Williams et al., 1981).

Previous attempts to locate the rRNA genes in the amplified *Xenopus* nucleoli have led to conflicting results. Furthermore, these earlier light microscopic studies were based exclusively on the use of fluorescent DNA-specific dyes and hence could not discriminate between transcriptionally active and inactive DNA. Using a confocal laser scanning microscope equipped with an UV-laser, it was reported (Spring et al., 1996) that in stages of high transcriptional activity (stage IV-V oocytes) the rDNA disperses throughout the entire nucleolar volume and cannot be related to a specific nucleolar component. In contrast, other authors localized the DNA to the cores of spread nucleoli (Thiebaud, 1979b; Shah et al., 1996; Wu and Gall, 1997). Counterstaining with antibodies to fibrillarin and nucleolin further suggested that the DNA-containing structures were intimately surrounded by the DFC (Shah et al., 1996; Wu and Gall, 1997). Our double-label localization experiments with antibodies against DNA and fibrillarin in conjunction with confocal microscopy revealed a similar organizational principle also in the transcriptionally highly active nucleoli of growing (stage IV) oocytes. The monoclonal DNA-antibody we have used is a highly sensitive reagent for the

immunocytochemical detection of DNA and has already been employed for the visualization of DNA in the amplified nucleoli of cryosectioned *Xenopus* oocytes (Scheer et al., 1987). However, due to the relatively poor structural preservation of the nuclei of frozen-sectioned oocytes, these earlier studies did not allow to assign the rDNA to a specific nucleolar component. Our present results clearly show that the bulk of fibrillarin does not colocalize with DNA but rather forms a shell around the rDNA compartment. We note that the central regions occupied by rDNA were not completely negative with the fibrillarin antibodies but fluoresced slightly, indicating that some fibrillarin might be – directly or indirectly – associated with nucleolar chromatin. Furthermore, from the results of the BrUTP incorporation experiments and the localization of UBF and pol I we can now conclude that at least a certain fraction of the fibrillarin-ensheated rDNA is actively engaged in transcription. Whether the nascent transcripts, before being released from the template, are also confined to the rDNA-compartment or extend into the surrounding DFC, cannot be answered from our present study. In this context it would be extremely important to learn more about the specific conformation of the nascent pre-rRNA transcripts in vivo (for estimations based on different experimental approaches see, e.g. Lazdins et al., 1997; Scheer et al., 1997). Taken together, our results provide clear evidence that, despite a marked structural variability, the light microscopically defined nucleolar cores consist of a central element with the transcriptionally active nucleolar chromatin and the surrounding DFC.

To correlate the sites of rDNA transcription with specific nucleolar substructures we have tried to extend the BrUTP incorporation studies to the electron microscopic level. Unfortunately, after 5 minutes of BrUTP incorporation the immunogold signal was hardly above background levels and, although the few gold particles were predominantly found in the FC, deemed us little convincing. Since our light microscopic studies have revealed a perfect colocalization of the BrUTP incorporation sites with the rDNA-specific transcription factor UBF (as well as pol I), we conclude that the distribution of UBF is equally well suited to monitor the spatial arrangement of the transcriptionally active rRNA genes. In fact, UBF is likely to be a more precise localization probe since its distribution mirrors the topology of the transcribing rDNA chromatin rather than that of the nascent transcripts.

From the electron microscopic localization of UBF we infer that the transcriptionally active rRNA genes are confined to the FCs. Although FCs are characteristic structural elements of somatic nucleoli, their functional significance remained as yet enigmatic and it is still a matter of debate whether they represent storage sites of inactive pol I and other transcription relevant proteins, accumulations of transcriptionally inactive rDNA with stalled polymerase complexes, skeletal elements for the attachment of pol I complexes or the sites of the active rRNA genes (for references see Shaw and Jordan, 1995; Thiry and Goessens, 1996; Raska et al., 1995; De Carcer and Medina, 1999). Our results using the amplified nucleoli as a model system clearly support the view that the FC is the nucleolar compartment harboring the active rRNA genes. Moreover, the uniform distribution of UBF throughout the FCs as seen by immunogold electron microscopy (Fig. 4B) appears to rule out that the rRNA genes lie exclusively in the boundary zone of

FC and DFC as this has been proposed for nucleoli of mammalian and plant cells (Shaw and Jordan, 1995; Raska et al., 1995; De Carcer and Medina, 1999). It is interesting to note that FCs have not been identified in earlier thin-section electron microscopic studies of conventionally fixed and Epon-embedded oocytes (e.g. Miller and Beatty, 1969a; Miller and Beatty, 1969b; Macgregor, 1972; Miller, 1981; Van Gansen and Schram, 1972; Boloukhere, 1984; Spring et al., 1996; Trendelenburg et al., 1996). Despite a careful analysis we also failed to see FCs in most ultrathin sections of stage IV *Xenopus* oocytes prepared in the same way. Instead, the central portion of the nucleoli appeared as a largely homogenous dense aggregate. A clear distinction between FCs and the surrounding DFC, however, was readily apparent after fixation of the oocytes for immunocytochemical purposes and embedding in Lowicryl. In our hands the best structural preservation of the oocytes was achieved by fixation in a mixture of formaldehyde/glutaraldehyde in the presence of 0.5% Triton X-100 to facilitate penetration of the fixative. We can exclude that visualization of the FC was caused by detergent extraction of nucleolar proteins since the tripartite nucleolar organization was also seen when Triton X-100 was omitted from the fixative. In fact, FCs have been described recently also in stage II-III and VI *Xenopus* oocytes after fixation in formaldehyde (without Triton X-100) followed by embedding in LR White which, like Lowicryl, is a methacrylate resin with hydrophilic properties (Shah et al., 1996). From a comparative analysis of the ultrastructural appearance of the multiple nucleoli after various fixation conditions and embedding in epoxid and Lowicryl resins we gained the impression that the primary determinants for the tripartite nucleolar structure are the specific properties of Lowicryl along with the low temperature dehydration and polymerization of the oocytes. Since we have seen FCs also in conventionally fixed and Epon-embedded full-grown oocytes (unpublished observations), AMD-treated oocytes (Fig. 6) and isolated germinal vesicles (Moreno Diaz de la Espina et al., 1982) we conclude that amplified nucleoli, like their somatic counterparts, contain FCs as integral elements which, however, require adequate preservation methods to become manifest. A similar conclusion has also been reached from studies of yeast nucleoli where FCs were only recently identified by using refined electron microscopic preparative methods (Leger-Silvestre et al., 1997; Leger-Silvestre et al., 1999). In our opinion it is the presence of the highly dynamic and delicate transcriptional structures of the rRNA genes which contribute to the remarkable lability of the FCs towards ionic conditions and conventional preparative methods (see also Vandelaer et al., 1996). Now that we have localized the transcribing rRNA genes in the FC, the next step will be the development of adequate methods for optimal preservation of the FC material and direct visualization of the 'Christmas trees' in their native state (for a description of putative rDNA transcription units in ultrathin sections of an insect oocyte see Scheer et al., 1997). Furthermore, the observations in this study provide a basis for further investigations of the protein composition of transcriptionally active nucleolar chromatin by immunofluorescent staining.

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