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Spatial arrangement of intra-nucleolar rDNA chromatin in amplified *Xenopus* oocyte nucleoli: structural changes precede the onset of rDNA transcription

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ABSTRACT **Amphibian oocyte nucleoli are a particular suited object for research on nucleolar chromatin organization. By selective rDNA amplification each pachytene oocyte nucleus accumulates 30 pg of extrachromosomal rDNA, this amount corresponds to 2 million rDNA copies. Following the selective amplification stage, the amplified gene copies are finally distributed within more than thousand extrachromosomal nucleoli per individual oocyte nucleus. The aim of the present study was first to obtain a precise documentation of the fate of amplified rDNA during early *Xenopus* oogenesis until the final functional integration of these copies into individual oocyte nucleoli, and, second, a close correlation of the structural data with determination of rDNA transcription rates by S1 transcript analysis for the subsequent stages of oocyte differentiation. In order to investigate the structural complexity of the intranuclear rDNA translocation process in detail, a confocal laser scan microscope (CLSM) was used, equipped with an external UV-laser. This instrumentation unambiguously allowed (i) the detection of small clusters of rDNA copies and (ii) the precise spatial documentation of the intranuclear position of rDNA clusters in relation to the protein-free pre-nucleolar protein bodies, a specific characteristic of late pachytene/early diplotene amphibian oocyte nuclei. Our results indicate that the major rDNA translocation processes, e.g. the association of rDNA clusters with pre-nucleolar protein bodies, the formation of ribbon-like pre-nucleolar units sensu Van Gansen and Schramm (*J. Cell Sci. 10:* 339-367, 1972), and, finally, the translocation of fused rDNA units into the interior of pre-nucleolar protein bodies, occur – for the most part – in absence of massive rDNA transcription. As shown by the S1 transcript analysis, the onset of massive rDNA transcription starts concomitantly with an unraveling of the densely packed rDNA clusters into finely dispersed rDNA units, which were shown by CLSM analysis to be distributed throughout the entire nucleolar volume.**

KEY WORDS: *Xenopus oocyte nucleoli, rDNA transcription, confocal microscopy, electron microscopy, S1 transcript analysis*

Introduction

Since several decades, the giant nuclei (germinal vesicles GVs) of amphibian oocytes have been one of the preferred objects for structural analysis of nucleoli and chromosomes. In regard to fine structural organization of nucleolar chromatin, oocyte nucleoli have been considered as particularly suited material, since each GV contains many hundreds of amplified, extrachromosomal nucleoli. An additional, important trait is, that these nucleoli originate very early in oogenesis – by selective rDNA amplification from the chromosomal rDNA locus – as an extrachromosomal amplified rDNA mass (Gall, 1968) and only later differentiate into typical extrachromosomal nucleoli and thus persist for many months during oogenetic growth.

Previous investigations have mainly centered on structural and biochemical analyses of rDNA transcription rates during oogenetic growth (Miller and Beatty, 1969; Anderson and Smith, 1978; for review see Sommerville, 1977; Davidson, 1986; Trendelenburg and Puvion-Dutilleul, 1987). In addition to transcription analysis, a number of attempts had been made to correlate the functional data with structural observations at the level of intact nucleoli, aiming to visualize the intra-nucleolar rDNA chromatin organization. From the early studies on (Brachet, 1949; Painter and Taylor, 1942) it was observed, that rDNA chro-

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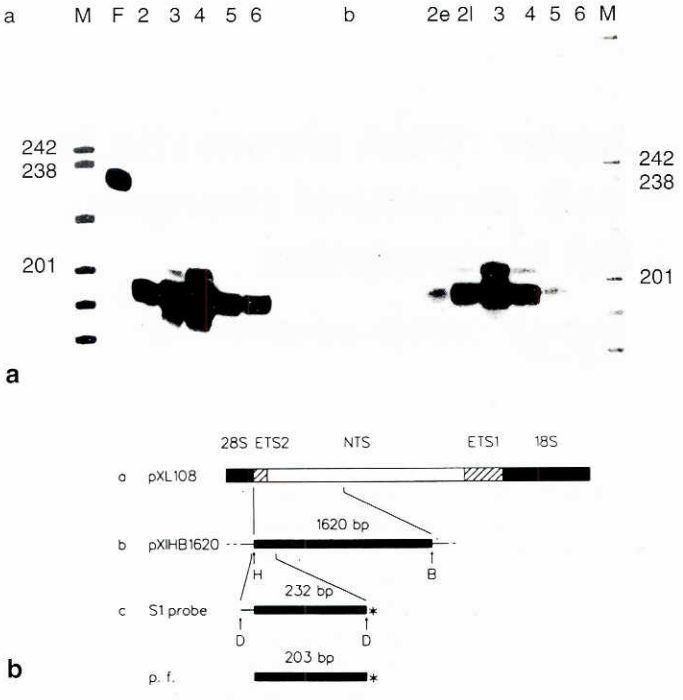

Fig. 1. Transcriptional activity during Xenopus oogenesis stages II-VI. (a) *RNA of 5 oocytes of Dumont stages II to VI was hybridized against the 232 bp S1 probe* **(b, line c)** *and digested with nuclease S1. Nuclease S1 resistant hybrids were analyzed on 6% polyacrylamide sequencing gels. M: pBR322/Hpa II marker; numbers relate to stages; 2e and 2l: early and late stage II oocytes (see text).* **(b)** *Generation of the S1 probe. Line a: Insert of pXL108: Hatched and black boxes: transcribed rDNA sequences; white box: nontranscribed spacer (NTS); ETS: external transcribed spacer; 18S and 28S: 18S and 28S rDNA; Sp Prm: spacer promoter; T1: 3' end of 28S rDNA; T2: transcription termination site. Line b: Insert of pXlHB1620: pXL108 was cut with Hind III and BamH I. The resulting 1620 bp fragment was ligated into the vector Bluescript KSM13+. Thin line: vector sequences; thick line: rDNA insert; B: BamH I; H: Hind III. Line c: S1 probe and protected fragment: pXlHB1620 was cut with Dra II (D) to generate the S1 probe. The 232 bp fragment was 5'labeled. Hybridization of the probe to oocyte rRNA leads to a 203 bp protected fragment (p. f.). Thin lines: vector sequences; thick lines: rDNA insert; asterisk: radioactive label.*

matin inside intact oocyte nucleoli could preferentially be visualized when present in a compact, highly condensed form by the cytochemical reactions applied (Thomas and Schram, 1977; Thiébaud, 1979).

In the present study we have exclusively used DNA-specific dyes and cytochemical reactions combined with confocal laser scan microscopy (CLSM) to visualize the intra-nucleolar DNA distribution. As shown previously, CLSM light microscopy allows the visualization of very low amounts of DNA, especially when an UV-laser is used (Montag *et al.*, 1991).

Our results show, that indeed, it is possible to visualize intranucleolar chromatin, even when it is very finely dispersed, e.g. corresponding to the fully transcriptionally active state of *Xenopus* oocyte nucleoli as shown in a parallel series of experiments using nuclease S1 transcript analysis of *in vivo* labeled rRNA.

This type of nucleolar chromatin represents one of the few cases, where (i) the exclusive nucleolar chromatin component is rDNA (Gall, 1968; Miller, 1966, 1981; for review see MacGregor, 1972) and (ii) the spatial rDNA distribution and the amount of primary rRNA transcripts can be precisely correlated. Thus this type of analysis might also be of major interest to the many approaches to localize active rDNA components in nucleoli of somatic cells, where this type of correlation is far more difficult to carry out (Spring *et al.*, 1988, Rawlins and Shaw, 1990; for reviews see Hadjiolov, 1985; Fakan and Hernandez-Verdun, 1986; Sommerville, 1986; Thiry and Goessens, 1992).

Results

Time course of rDNA transcription during Xenopus oogenesis (stages II to VI)

In order to measure the activity of rDNA transcription during subsequent stages of oogenesis a nuclease S1 analysis was performed. The RNA equivalent of 5 defolliculated oocytes of stages II to VI was hybridized against a 5'labeled rDNA fragment specific for the primary 40S pre-rRNA transcript (Fig. 1b, lines a-c) and subsequently digested with nuclease S1. The intensity of the 203 bp nuclease S1 resistant hybrid signal was taken as a measure of transcriptional activity during the different stages of oogenesis.

To exclude a contribution of the rRNA synthesized in the follicle epithelium to the hybridization signal oocytes were thoroughly treated with collagenase and residual follicle epithelium of stage III to VI oocytes was removed manually. This procedure cannot be applied to stage II oocytes because of their small size. Therefore the effect of collagenase treatment of stage II oocytes was checked using the combined collagenase/RNase treatment described in materials and methods. The result of this approach enables us to exclude a contribution of follicle cell rRNA to the hybridization signal. The extensive, 4 hour collagenase treatment of the oocytes was sufficient to remove all follicle epithelium.

According to the signal intensities the following time course of rDNA transcription during oogenesis can be deduced (Fig. 1a). The transcription begins – at a very low rate – in stage II oocytes (Fig. 1a, lane 2), increases as oogenesis proceeds (Fig. 1a, lane 3), reaches its peak in stage IV and decreases slowly to a stage VI level that is comparable to the one observed in stage II oocytes (Fig. 1a, lanes 4-6). In a second experiment of the same kind using RNA of oocytes from a different frog, (Fig. 1a, lanes 2-6) stage II oocytes were differentiated into early (mean oocyte diameter 150-200 μm) and late (mean diameter 200-300 μm) stage II oocytes. The time course observed in this experiment is slightly different. Again, transcription begins at a very low level in early stage II oocytes (Fig. 1a, lane 2e). The activity increases as the oocytes grow (Fig. 1a, lane 2l). It reaches its peak in stage III oocytes and decreases again. In stage VI oocytes, primary transcript can only be detected in very low amounts.

Though the peak of activity is reached at an earlier stage in the latter experiment (stage III vs. stage IV), the tendency observed remains: During previtellogenic stage II, rDNA transcription takes place only at a very low level. In the early vitellogenic stages III and IV the activity increases dramatically, whereas in stage V and VI transcription is closing down again. In *in vitro* maturated oocytes, no transcriptional activity can be detected (data not shown).

Whether the difference observed is caused by a transcriptional machinery that is less active or by a processing machin-

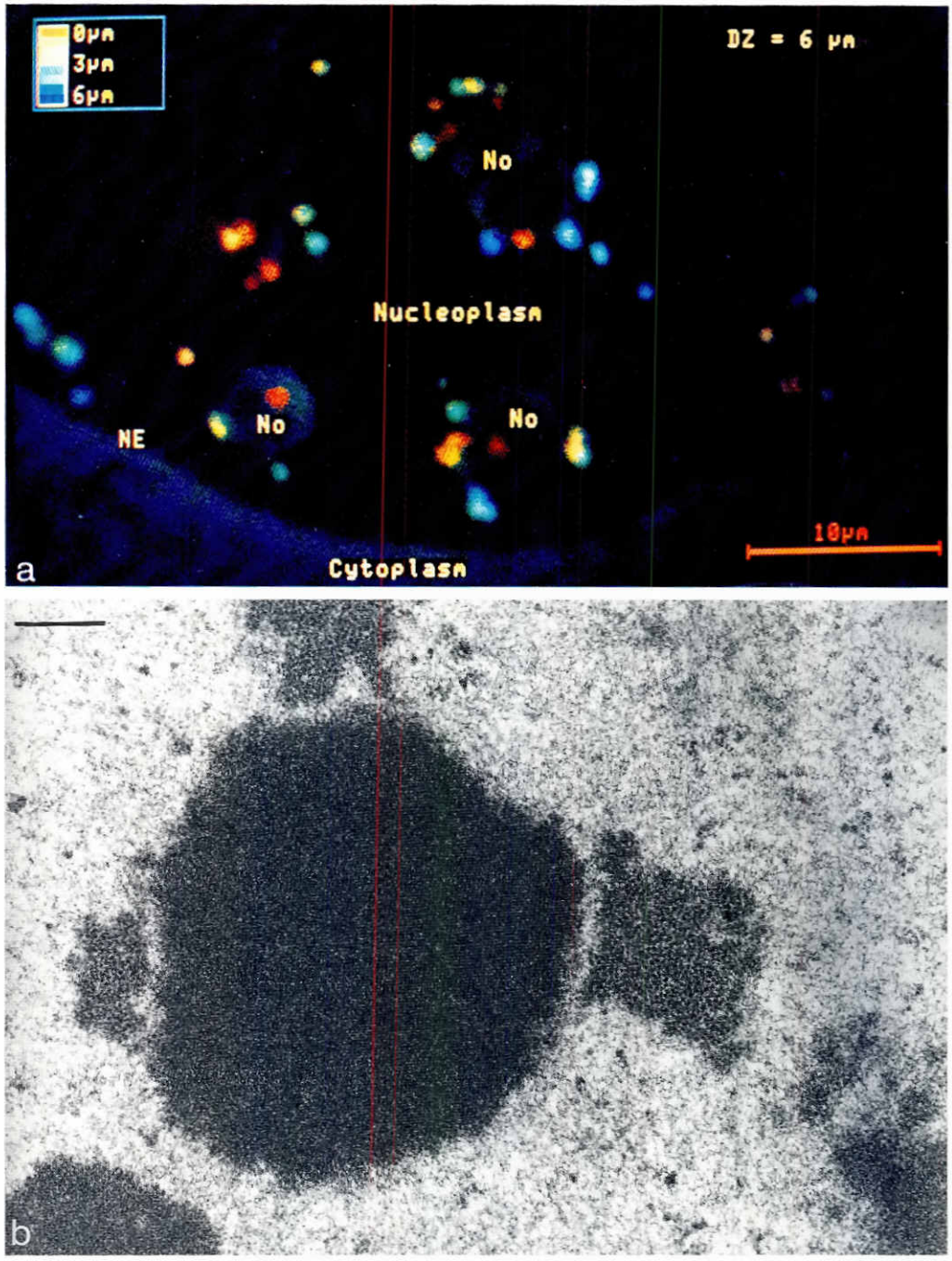

Fig. 2. rDNA distribution in pre-nucleolar bodies of stage I oocytes. *An overlay image of a series of twelve consecutive optical sections taken at an interval of 0.5 µm with the UV-laser scanning microscope is shown in* **(a)**. *Nucleolar protein bodies (No) are surrounded by several intensely stained DNA-containing particles consisting of amplified rDNA. The different (pseudo) colours of the subunits reflect their position in the stack of consecutive optical sections (see colour table in upper left corner). A corresponding electron micrograph is shown in* **(b)**. *By contrast to the very dense aspect of the compact pre-nucleolar protein masses (No) peripherally located rDNA aggregates (arrows) exhibit a more 'granular' texture. Bars: a, 10 µm; b, 1 µm.*

ery which for unknown reasons acts more rapidly in these oocytes than in others can not be discerned with the assay used. The different time courses observed indicate that the transcriptional and/or processing behavior of different females is very variable.

rDNA distribution in nucleoli of stages I to VI

In order to see whether the changes in transcriptional behavior would correspond to fine structural changes in oocyte nucleoli, oocytes of the same batches used for the molecular biological analysis were studied microscopically. For morphological observations and cytochemical experiments the biological material was stained with DNA-specific dyes like DAPI, Hoechst 33342 or with the Feulgen/Schiff reaction for DNA (see Materials and Methods). Specimens were analyzed with a confocal laser scanning microscope equipped with an additional UV laser. All staining methods applied yielded identical results.

For a direct correlation between the morphological appearance and the rDNA distribution a pair of micrographs from each nucleolar stage depicting the image in differential interference contrast (DIC) in transmitted light (left image) and the corresponding confocal fluorescence image of the same focal plane

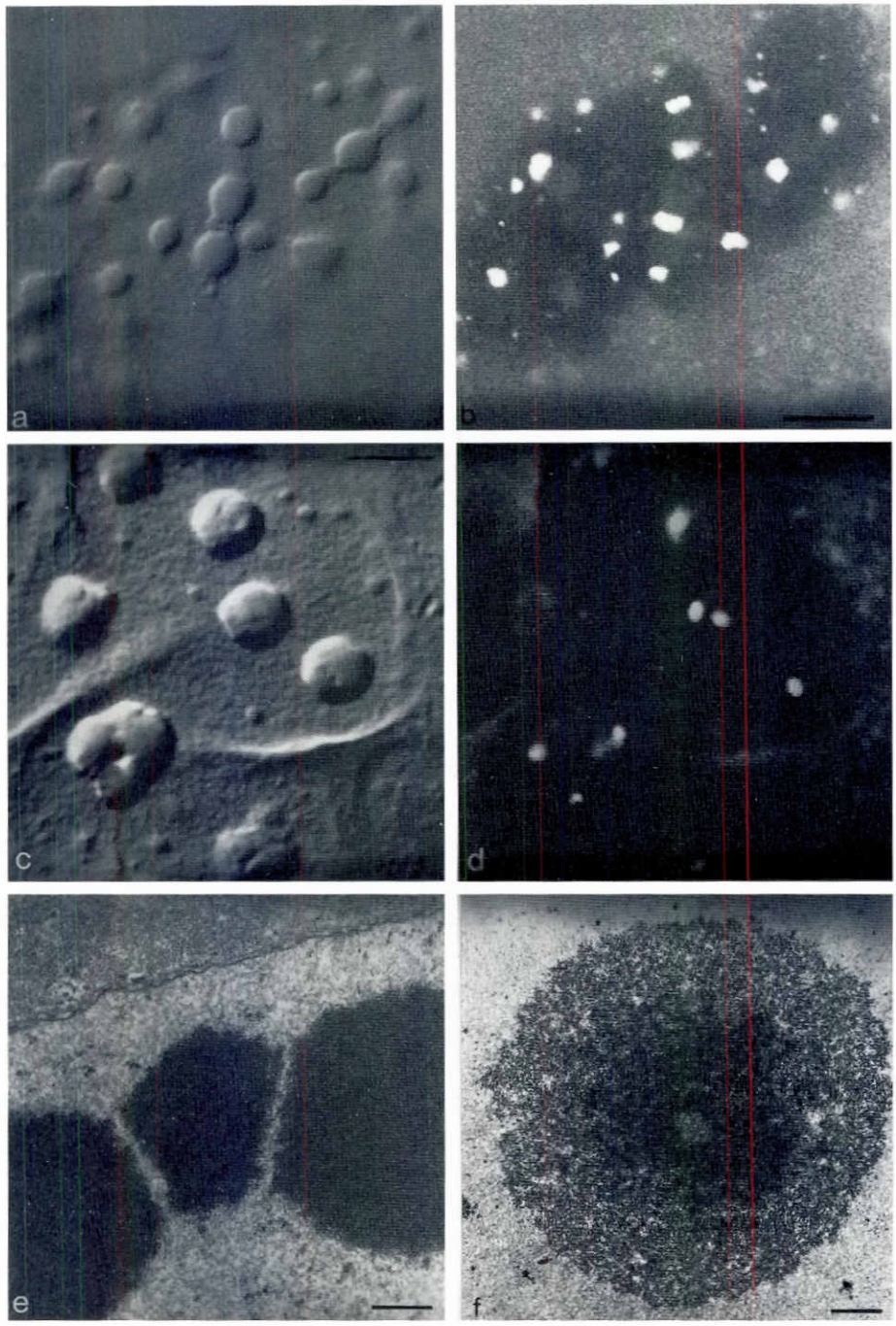

Fig. 3. rDNA distribution in pre-nucleolar bodies of stage II oocytes and in nucleoli of stage III oocytes. *Corresponding laser scan micrographs of stage II oocyte pre-nucleolar bodies are shown in differential interference contrast* **(a)** *and in confocal DNA fluorescence* **(b)**. *An electron micrograph is shown in* **(e)**. *As can be clearly seen in* **(a and e)** *pre-nucleolar bodies of stage II oocytes are directly linked to neighboring bodies in such a way that chains of two to three bodies are formed* **(a,e)**. *Interestingly, most of the small rDNA particles characteristic of stage I oocytes have apparently fused to one or several much larger complexes which interconnect the rDNA-free pre-nucleolar bodies* **(a,b and e)**. *The rDNA mass* **(b,e)** *exhibits a tighter packed organization when compared to the smaller rDNA particles characteristic for stage I oocytes (Fig. 2b). Corresponding laser scan micrographs of stage III oocyte nucleoli using differential interference contrast* **(c)** *and confocal DNA fluorescence* **(d)**. *An electron micrograph of a stage III nucleolus is shown in* **(f)**. *Micrographs* **(c and d)** *were taken from nuclei located very close to the nuclear envelope (NE). Comparison of* **(c and d)** *shows that diffuse DNA signals present in* **(b)** *can be assigned to conspicuous nuclear envelope infoldings* **(c)**, *and thus are likely to represent DNA fluorescence of cytoplasmic mitochondria. Confocal fluorescence analysis of nucleolar rDNA distribution* **(d)** *and comparison with the interference contrast micrograph* **(a)** *reveals that rDNA is now completely internally located in form of one to three intensely stained units. The electron micrograph shown in* **(f)** *shows the typical ultrastructure of an amphibian oocyte nucleolus with a dense 'core' and a much less electron dense 'cortex' zonation (position of rDNA is demarcated by arrows; c, cytoplasm; NE, nuclear envelope). Bar: a-d, 10 μm; e-f, 1 μm.*

of the specimen, respectively (right image) is presented for oocyte stages II-VI (see Figs. 3 and 4).

A survey of the spatial arrangement of pre-nucleolar components in stage I oocytes is shown in Figure 2a. In order to document this particular interesting stage of oocyte nucleolar differentiation as precisely as possible, e.g. as characterized by an almost complete absence of pre-RNA transcription (Fig. 1) we show an overlay image representing the information contained in twelve consecutive optical sections (Fig. 2a). Our results clearly show that pre-nucleolar units in stage I oocytes can be characterized as nucleolus-like spherical pre-nucleolar protein bodies (No in Fig. 2a; mean diameters 4-6 µm) which are surrounded by much smaller globular units (0.8-2.3 µm) consisting predominantly of rDNA. Note in particular, that the entire volume of the pre-nucleolar protein body (No in Fig. 2a) is free of rDNA. Note also that, on average, 2-5 rDNA units appear to be directly associated at the periphery of each individual pre-nucleolar protein body. Some rDNA units are identified as small bodies (0.8-1.2 µm in diameter), others have diameters of up to 2.2 µm and appear to be composed of two to three of these small rDNA units. As it can be clearly seen from Figure 2a, rDNA units are located around individual pre-nucleolar protein bodies without an obvious preferential orientation. For comparison at high magnification, the ultrastructure of an oocyte stage I pre-nucleolar complex is shown in Figure 2b.

Figure 3a,b shows optical grazing sections at the nuclear periphery of an previtellogenic stage II oocyte with numerous structures forming chain-like aggregates of alternating round structural components and intermittent flat layers. In the corresponding fluorescence image only these layers in between the round pre-nucleolar structures are positive for DNA indicating that the amplified rDNA is still outside in most of those structures forming the functional nucleolus in later stages of oogenesis. A weak cytoplasmic DNA background fluorescence in this and the next figures is caused by the occurrence of high amounts of mitochondrial DNA. Comparison of organization of nucleolar components in stage I versus stage II oocytes indicates, that stage II is characterized by an oriented fusion of rDNA components so that larger pre-nucleolar complexes are formed consisting of two to three spherical pre-nucleolar protein bodies which, in most cases, are held together by a flat rDNA 'bridge' (this aspect is identical to the stage described as 'ribbon stage' by van Gansen and Schram 1972; see also van Gansen *et al.*, 1976 for details).

A major change in rDNA localization is noted when nucleolar components of stage III oocytes are analysed: It is at this stage that the ribbon-like appearance of pre-nucleolar bodies characteristic for stage II (Fig. 3a,b) is no longer found, but instead, typical spherical nucleolar structures are seen. For the first time during oogenesis, the rDNA component is now exclusively located inside the nucleolar protein body (see e.g. two nucleoli in the lower half of Fig. 3c,d). Note that two to three rDNA complexes can be seen per individual nucleolar protein complex. In some nucleoli of this stage of oogenesis a central position of the rDNA component could be observed (e.g. upper half of Fig. 3c,d, see also Fig. 3f). In summary, one of the most important changes in nucleolar differentiation occurs between stages II and III, the rDNA component is transferred from an external (e.g. Fig. 3a,b and e) to an internal position of the spherical nucleolar protein component (Fig. 3c,d and f). Commonly, a structure as shown in Figure 3f can be designated as a 'true' typical amphibian oocyte nucleolus.

In nucleoli of stage IV oocytes (Fig. 4a,b) the concentration of the rDNA in defined spots changes into less well defined clouds of DNA with larger diameters indicative of a dispersal of rDNA in the nucleoli concomitant with the observed maximal rate of transcription (Fig. 1). This dispersal continues up to stage V (Fig. 4c,d) the rDNA is found to be distributed throughout the entire nucleolar volume with the only exception of the nucleolar vacuoles (typical for nucleoli of stage V oocytes). Extremely large vacuoles can be seen exceptionally well in large nucleoli of late stage VI oocytes (oocyte diameter >1.2 mm; Fig. 4e,f) in which the nucleolar material surrounds a large central vacuole (Fig. 4e). The fluorescent image of this nucleolus shows again that the nucleolar vacuole is free of DNA (Fig. 4f).

In summary, in oocyte nucleoli of stages IV and V, e.g. the stages where rRNA synthesis reaches its highest levels (see Fig. 1), rDNA is found to be distributed in nearly all areas of the nucleolar volume. Concomitant to this fine dispersal of rDNA chromatin, number and size of 'vacuolated areas, as most clearly seen in differential interference contrast (Fig. 4a,c,e) increases dramatically. Interestingly, rDNA is obviously absent in the larger vacuolated areas shown in Figure 4c,d and 4e,f. In order to document the rDNA distribution in the nucleolar interior precisely, a series of nine consecutive optical sections through a stage V nucleolus is shown in Figure 5. It is evident form these images, that the rDNA component is for the first time during oocyte differentiation now distributed throughout the entire nucleolar volume, with the clear exception of the nucleolar 'vacuolated areas', a characteristic structure for amphibian oocyte nucleoli, e.g. especially for the late oogenetic stages V and VI.

By comparison with the rDNA distribution in nucleoli of early stage IV oocytes shown in Figure 4a,b; the panel of consecutive optical sections (Fig. 5) clearly demonstrates, that in most nucleolar areas, the rDNA component is found to be present in its finely dispersed state. Whereas, only in some more centrally located nucleolar areas, rDNA is seen as small, more compacted rDNA clusters. This predominance of finely dispersed rDNA during stage V of oogenesis contrasts strongly to the rDNA distribution pattern in nucleoli of stage VI oocytes where most of the nucleolar volume is occupied by a large, central, 'vacuolated area'. In nucleoli of stage VI oocytes, rDNA is seen to be arranged into a more compact form with a globular fine structure.

Discussion

Oogenesis of *Xenopus laevis* is characterized by an exceptionally high rate of rRNA synthesis. At the end of oogenesis 4-5 µg rRNA versus 80 ng mRNA (Gurdon and Wakefield, 1976) can be found within a single oocyte.

Using amphibian oocyte nucleoli, Miller and Beatty (1969) could first visualize 'ribosomal genes in action' and thus beautifully demonstrate that, indeed, transcriptionally active rDNA genes can be visualized as very long strands of tandemly arranged active genes often comprising 20 and more active genes per spread rDNA axis. In a number of investigations it could be shown, that quantification of nucleolar spread preparations from amphibian oocyte nucleoli resulted in estimations of

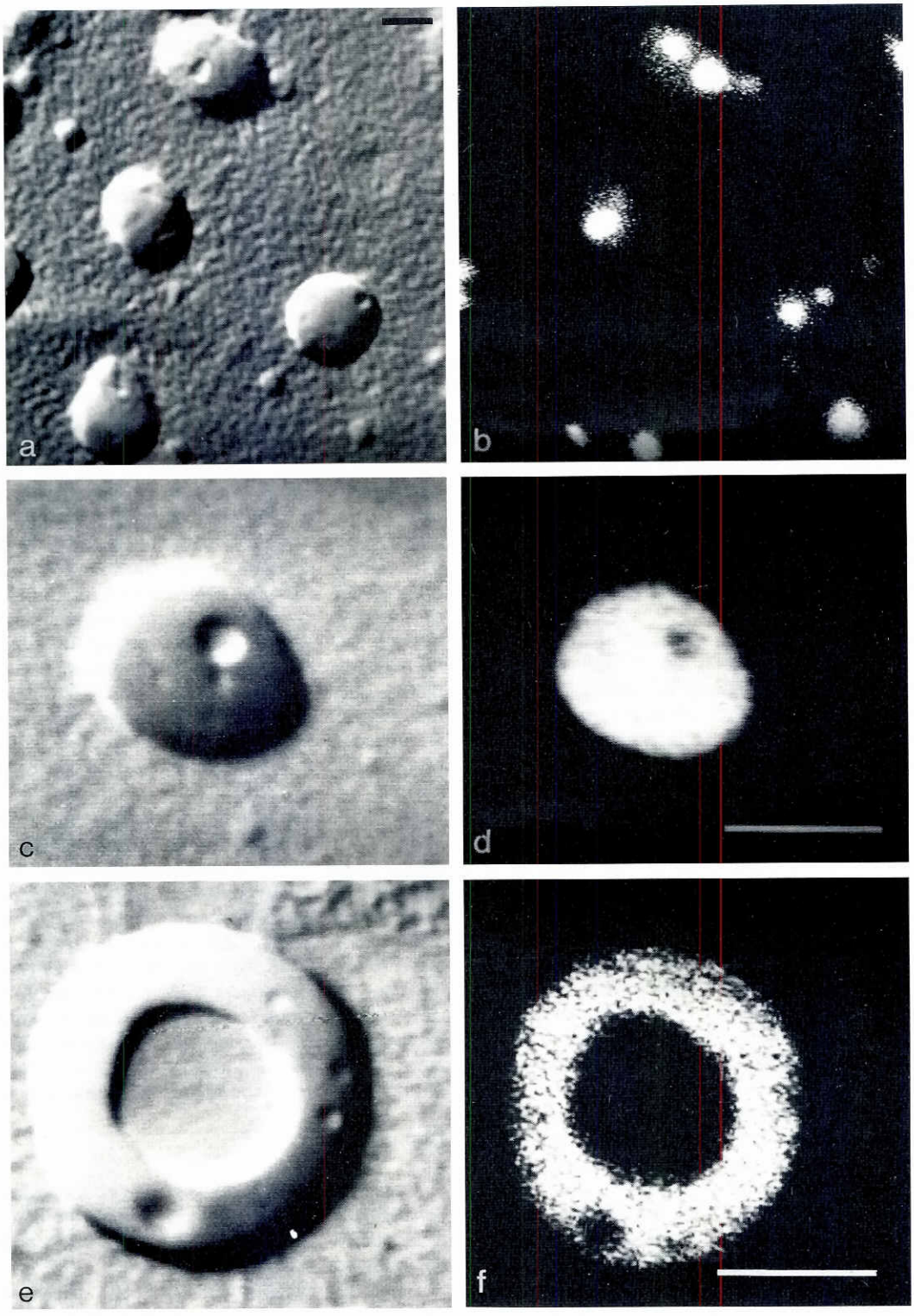

Fig. 4. rDNA distribution in nucleoli of stage IV, V, VI oocytes. *Interference contrast micrographs* **(a)**, *confocal fluorescence of DNA* **(b)** *through stage IV oocyte nucleoli reveal that rDNA is now distributed within 70-90% of the nucleolar volume. Again a large proportion of rDNA is present as a dense 'core' structure surrounded by more finely dispersed rDNA at the periphery of each core (see, e.g.* **b**). *A structural characteristic of stage V nucleoli are nucleolar caverns, conspicuous in the interference contrast micrograph* **(c)** *and confocal fluorescence micrograph* **(d).** *Note that specific DNA fluorescence in nucleoli of stage V oocytes extends through the whole nucleolar volume* **(d)**. *In many of the nucleoli of stage VI oocytes nucleolar caverns can obtain very large diameters and thus the nucleolus appears a ring in interference contrast* **(e)** *as well as in confocal fluorescence* **(f).** *Bar, 5 μm.*

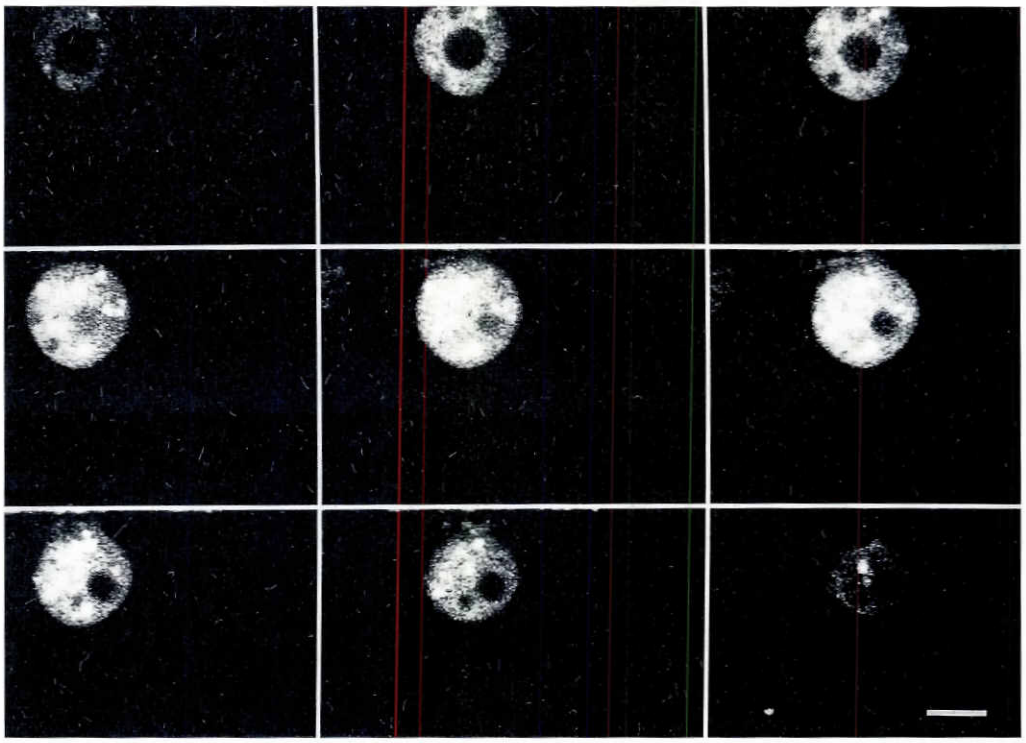

Fig. 5. Optical sections through a stage V oocyte nucleolus. *A series of 9 consecutive optical sections through a stage V oocyte nucleolus is shown using fluorescence confocal microscopy. Distances between individual optical sections are 1 μm. As it is evident from this gallery, rDNA was found to be located throughout the whole nucleolar volume, with the exception of large vacuolated areas. Bar, 5 μm.*

up to 80-90% of transcriptionally active genes per individual oocyte for stage IV and V oocytes (Miller and Beatty, 1969; Miller, 1981; Scheer *et al.,* 1976; Franke *et al.,* 1979; Trendelenburg and Puvion-Dutilleul, 1987). In addition several attempts had been made to quantify transcription rates for earlier and later stages of amphibian oogenesis, e.g. for *Triturus* (Scheer *et al.,* 1976), for *Rana pipiens* (Trendelenburg and McKinnell, 1979) and for *Xenopus laevis* (Martin *et al.,* 1980; Williams *et al.,* 1981; Mitchell and Hill, 1987). In this context a special difficulty for quantification of transcription parameters in *Xenopus* oocyte stages exists. In contrast to the strictly environmentally controlled oocyte growth period as it is the case for *Triturus* and *Rana*, laboratory maintained *Xenopus laevis* females exhibit an unusually broad variation in transcription rates within the succession of oocyte stages II to VI. It is commonly argued that transcription rates depend largely on the individual hormonal status of the specific *Xenopus* female, and – much less accentuated – on the specific size of the oocyte under study. In a previous series of experiments (Meissner *et al.,* 1991) we could show that, indeed, a particularly wide range of transcription rates exists for batches of oocytes of individual *Xenopus* females, when injected with an homologous rDNA construct, indicative of the presence of different amounts of rDNA specific transcription factors in batches of oocytes from different females. Thus one of the aims of the present study had been to correlate structural and molecular information in parallel for a given batch of oocytes.

Complex rearrangements of amplified rDNA clusters occur prior to intra-nucleolar rDNA localization

One of the most striking results of the present investigation is the observation that pre-nucleolar structures present in the very small stage I oocytes consist of spherical pre-nucleolar protein bodies which carry – in their outside – 2-4 small, dense rDNA patches. A major change occurs at late stage I/early stage II of oocyte differentiation: Obviously most of the small rDNA clusters fuse, but retain the location outside the pre-nucleolar bodies, thus larger rDNA complexes are formed, which are seen to connect 2, 3 or 4 pre-nucleolar protein bodies. These complexes are designated as 'ribbon-stage' (Van Gansen and Schram, 1972; Van Gansen *et al.,* 1976; Thomas and Schram, 1977). As shown by these authors as well as in the present investigation (Fig. 1a,b) there is agreement, that throughout all these rearrangements the rDNA masses remain in a fully condensed state and are not transcribed when present in this condensed form and located outside of the pre-nucleolar body.

Internalized rDNA is rapidly decondensed and immediately transcribed

Following the 'ribbon-stage' as characterized by a still exclusive 'external' localization of rDNA clusters in relation to pre-nucleolar protein bodies, during stage II of oocyte differentiation, the compact external rDNA mass disrupts again into a few smaller subunits. One or two, in a few cases up to four of these rDNA clusters are found to be located inside the pre-nucleolar protein bodies. Concomitant to this translocation, each of these dense rDNA masses analyzed was found to be surrounded by a peripheral zone of disperse rDNA material. Comparison with S1 rRNA transcript analysis shows, that, upon this translocation, transcription starts immediately, very likely at the finely dispersed rDNA zones (Fig. 3a,b).

Upon internalization into the pre-nucleolar protein body, the rDNA masses undergo a very rapid decondensation process during oocyte differentiation stages III, IV and V. Thus, in nucle-

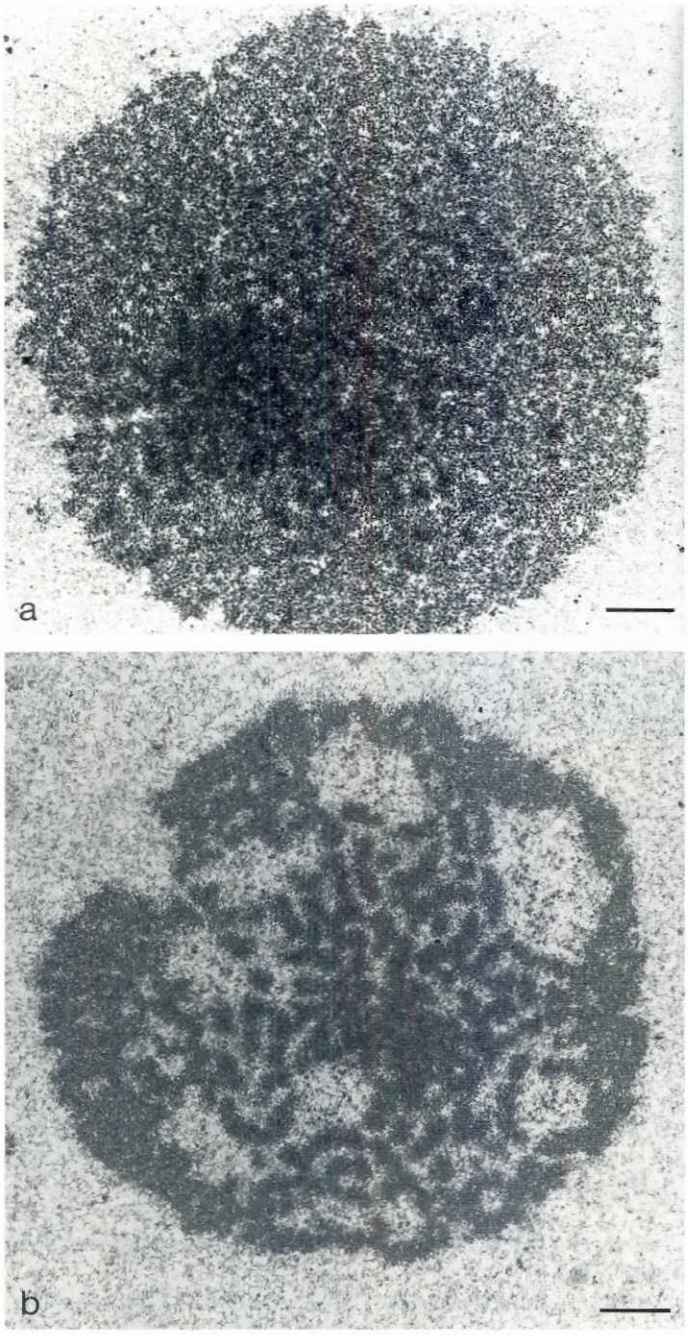

Fig. 6. Comparison of fine structural aspect of stage IV and V oocyte nucleoli. *Oocyte stages III and IV were characterized as the most active ones in pre-rRNA synthesis rates (Fig. 1a). Comparison of fluorescence confocal microscopy and electron microscopic thin section analysis indicates, that in stage IV oocyte nucleoli, the rDNA component is distributed throughout the whole volume of an individual nucleolus (Figs. 4a,b, 5a). By contrast, nucleoli of stage V oocytes are characterized by the occurrence of large 'vacuolated' areas which are essentially free of rDNA elements (Figs. 4c,d. 5, 6b).*

oli of stage IV or V oocytes the nucleolar rDNA component can be visualized in all areas of an individual nucleolus. The only exception are the so called 'vacuolated areas' a characteristic of amphibian oocyte nucleoli. First signs of small nucleolar 'vacuoles' emerge in nucleoli of stage IV oocytes (Fig. 6); the relative volume of 'vacuolated zones' raises drastically during stage V and reaches its maximum for nucleoli of stage VI oocytes. The volume increase of nucleolar 'vacuolated areas' obviously correlates with a progressive decrease of the transcription efficiency (Fig. 1). Thus an interesting conclusion can be reached. Maximal rDNA transcription is paralleled by an almost complete rDNA decondensation and fine dispersal throughout the entire nucleolar volume.

In summary, the intra-nucleolar organization of transcriptionally active rDNA in *Xenopus* oocytes is remarkably different to that of somatic nucleoli. In most types of somatic nucleoli studied, a clear zonation of nucleolar areas exists, e.g. fibrillar centers, dense fibrillar regions, pars granulosa. Nucleolar regions exhibiting a comparable type of ultrastructure could only rarely be identified in oocyte nucleoli. (Moreno Diaz de la Espina *et al.*, 1982). In the majority of cases, the ultrastructural aspect is the one shown in Figs. (3d, 6a) for stage III oocytes and the aspect shown in Fig. 6b) for a stage IV/V oocyte nucleolus. In both of these cases, the typical structural zonation as characteristic for somatic nucleoli (see above) cannot be detected. Among the reasons for this major difference are (i) the unusually high transcription efficiency of oocyte nucleoli (ii) the entirely different cytogenetic situation: Extrachromosomal oocyte nucleoli are one of the characteristics of the diplotene stage of oocyte differentiation, e.g. a cell stage arrested for many months in prophase of meiosis, and thus primed for a long range extremely high transcriptional activity, in order to supply the egg cell with 10^{12} ribosomes required for the early phase of embryonic differentiation.

Materials and Methods

Isolation of oocytes, collagenase treatment and RNA extraction

Oocytes of *Xenopus laevis* females were isolated and treated with collagenase to remove the follicle epithelium as described (Colman, 1984). Remnants of adhering follicle epithelium were removed manually. Individual oocytes were frozen and RNA was extracted by the proteinase K method, as described previously (Steinbeißer *et al.*, 1989). Staging of oocytes was done according to Dumont (1972).

Collagenase and RNase treatment of stage II oocytes

Batches of five stage II oocytes were treated with collagenase for 4 h and additionally incubated with RNase A for either 0 or 30 min. This additional RNAse treatment was used to degrade the follicle epithelium rRNA. The incubation was stopped by freezing of the oocytes and subsequent RNA extraction by the proteinase K method.

Generation and labeling of the S1 probes

The experimental procedures were done as described (Maniatis *et al.*, 1982). A 1620 bp Hind III/BamH I fragment of the clone pXL108 (Boseley *et al.*, 1979; Fig. 1b, line a) containing non transcribed spacer sequences downstream of the 3' end of 28S rDNA (T1; Labhart and Reeder, 1986; Meissner *et al.*, 1991) and upstream of the first spacer promoter was ligated into Bluescript KSM13+ yielding pXlHB1620 (Fig. 1b, line b). To obtain a fragment specific for the primary transcript pXlHB1620 was cut with the restriction enzyme Dra II. The resulting 232 bp fragment contains 29 bp of vector DNA and 203 bp of rDNA sequences between T1 and the transcription termination site (T2; Fig. 1b, line c; Meissner *et al.*, 1991). Hybridization of this fragment to oocyte rRNA leads to a 203 bp protected fragment.

S1 nuclease mapping

For the S1 nuclease mapping experiments an RNA equivalent of 5 defolliculated oocytes was hybridized overnight at 65°C against 5000-10000 cpm labeled DNA fragment in 20 µl 80% deionized formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), I mM EDTA. Samples were then digested with 60 U S I nuclease for 1 h in 300 µl of 200 mM NaCl, 5 mM $ZnSO_4$, 30 mM NaOAc, pH 6.4 at 37°C. The reaction was stopped by addition of 60 µl 1 M NaOAc pH 9.5, 330 mM Tris-HCl, 67 mM EDTA and the nuclease S1 resistant fragments were precipitated with ethanol. The hybrids were analyzed on 6% polyacrylamide sequencing gels. Gels were then fixed in 10% CH_3COOH, dried for 40 min at 80°C and exposed at -70°C with a LGY intensifying screen (Dr. Goos, Heidelberg, Germany).

Microscopy

Ovary fragments containing oocytes of stages I to III as well as whole oocytes (see above) and isolated germinal vesicles from oocyte stages IV and VI were stained directly with DAPI or Hoechst 33342 dissolved in 5:1 medium (Callan 1986) according to the conventional procedures. Alternatively, the material was slightly fixed with 2% formaldehyde in the same buffer prior to the staining step and the observation in the microscope. In a third procedure we fixed the oocytes overnight in 2% formaldehyde and processed the material according to the method of Feulgen with Schiff's reagent for specific DNA-fluorescence made up with pararosaniline (Spring *et al.*, 1988, Montag *et al.*, 1991). After embedding in resin the oocytes could be observed either directly as whole mount preparations (ovarian fragments with oocytes of stages I-III) or after preparation of 5-20 µm thick sections with a microtome (oocyte stages IV-VI).

Light microscopy was performed with confocal laser scanning microscopes (LSM 10 UV as well as inverted type LSM 410 UV; Carl Zeiss, Oberkochen and Jena, Germany) with 543 nm HeNe-lasers (used for transmitted light differential interference contrast (DIC) according to Nomarski), 488 nm argon ion lasers (used with the Feulgen-stained material in fluorescence) as well as additional 364 nm argon lasers for UV confocal fluorescence with DAPI or Hoechst 33342 (for details of instrumentation, see Montag *et al.*, 1991). For electron microscopic observations, the material was routinely fixed with 2.5% glutaraldehyde made up in 5:1 medium for 4 hours. After postfixation in 2% osmium tetroxide overnight the cells were dehydrated in graded steps of ethanol and embedded in epon 812. Ultrathin sections were stained with uranylacetate and lead citrate according to the conventional techniques. We used a Zeiss 10 CR or a Siemens Elmiskop 102 for electron microscopic observations.

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