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PATRON DELIVERY PREFERENCE: Not Specified

Patron Note:

JOURNAL: European journal of cell biology

ISSN: 0171-9335

EISSN:

ARTICLE: Stereo-electron microscopy of nuclear structure and replication in ci

AUTHOR: Olins, A

YEAR: 1981

MONTH: 08

DAY:

QUARTER:

SEASON:

VOLUME: 25

ISSUE: 1

NUMBER:

PAGES: 120-130

PAGED LOCATIONS: 9will, >>9noeu<<

Staff Note: WCM nos

LOCATION: SNELL PERIODICALS STACKS

CALL NUMBER: QH1 .E6900

HOLDINGS: v.19 (1979) -v.71 (1996), v.73 (1997), v.75 (1998) -v.83 (2004)

Stereo-electron microscopy of nuclear structure and replication in ciliated protozoa (Hypotricha)

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Received December 17, 1980

Accepted March 25, 1981

Stylonychia — Oxytricha — Euplotes — replication bands

Employing stereo-electron microscopy on sections (ca. 0.1 μm thick) of the ciliated protozoa *Stylonychia*, *Oxytricha* and *Euplotes*, we have examined the ultrastructure of macronuclear replication bands (RB) and micronuclei. The macronuclear RB is composed of two zones: a "forward zone" showing a special chromatin organization consisting of regular 40 to 50 nm diameter fibers with an indication of nucleosomal substructure; and a "rear zone", the site of DNA replication, consisting of a mesh of 10 nm chromatin fibers. Micronuclei exhibit chromatin strands of 60 to 70 nm diameter. Employing the Bernhard staining procedure, we have observed that the chromatin fibers of the RB and of the micronuclei remain unbleached, whereas macronuclear condensed chromatin is bleached of stain, indicating that the replication band and the micronuclei contain chromatin in a configuration different from that of other forms of densely packed chromatin. The regularity of the chromatin fibers within the forward zone is of particular interest since it is comparable to the regularity of chromatin seen in the transcriptionally-inactive chromatin of other nuclei such as avian erythrocytes and sea urchin spermatozoa. It is likely that the forward zone chromatin fibers consist of highly ordered arrangements of nucleosomes, associated with additional nonhistone proteins.

Introduction

The hypotrichous ciliated protozoa (including *Stylonychia*, *Euplotes*, *Oxytricha*, *Urostyla*, *Gastrostyla* among others)

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possess two types of nuclei in the vegetative cells [14, 22]: micronuclei, consisting of genetically inactive chromatin composed of high molecular weight DNA packaged into nucleosomes, and macronuclei, containing transcriptionally active chromatin composed of short DNA duplex molecules ($\sim 0.4\text{--}30$ kb pairs) also packaged into nucleosomes [5, 23-27, 40, 52]. Macronuclei are derived from micronuclear fusion products following conjugation. The cytological, ultrastructural and biochemical events in macronuclear development have been well-described [1, 2, 19, 28, 29, 37, 40, 43-45, 51] and involve successive stages of chromosome polytenization, DNA cutting and elimination, and multiple waves of replication of the short DNA molecules. Macronuclear DNA replication occurs within a light microscopically-visible zone called a Replication Band (here abbreviated, RB) that begins to form at a specific initiation region and "migrates" along the macronucleus [4, 6-9, 13, 15, 16, 18, 20, 35, 37-39, 41-43, 46-50, 53, 54].

A number of ultrastructural studies of replication bands have been published [6, 7, 9, 15, 16, 20, 35, 46-49, 53, 54]. In view of the uniqueness and importance of these regions, we chose to extend these earlier studies, by employing stereo-electron microscopy of sections (about 0.1 μm thick) to permit a more realistic impression of their three-dimensional structure [31-34]. Samples were examined following conventional uranyl-lead staining or following the staining procedure of Bernhard [3, 21]. Several different organisms were examined (i. e., *Stylonychia*, *Euplotes* and *Oxytricha*) in order to explore the diversity of morphology within the RB and to establish general principles of organization of RB in hypotrichs.

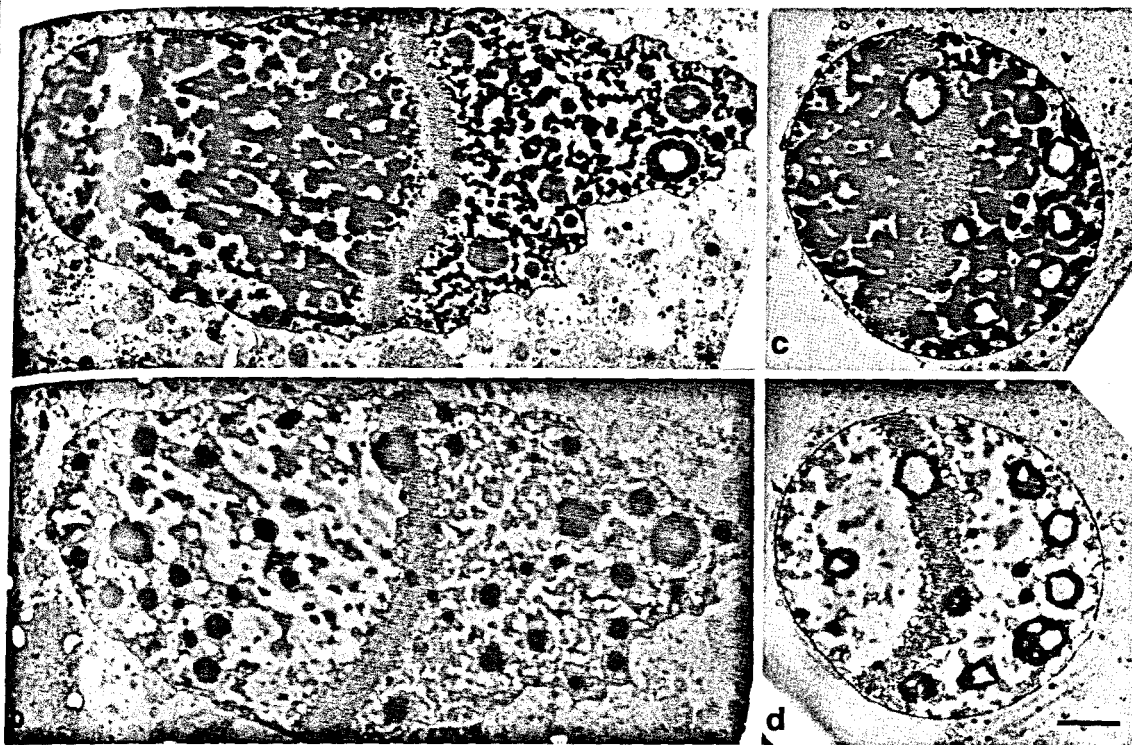


Fig. 1. Survey electron micrographs of *Stylonychia* and *Oxytricha* macronuclei with replication bands (RB). — a., b. Sequential sections of *Stylonychia* stained with uranyl and lead (a) or by the Bernhard procedure (b). — c., d., Sequential sections of *Oxytricha* stained with uranyl and lead (c) or by the Bernhard procedure (d). All micrographs are oriented so that the RB are moving from left to right; that means pre-replication DNA is at right, post-replication DNA at left. — Bar 4 μm . — 2000 \times .

Materials and methods

Stylonychia mytilus vegetative cells were grown in Pringsheim medium and fed daily with the alga, *Chlorogonium elongatum*, as described previously [2]. *Oxytricha* and *Euplotes* (both species not identified) were isolated from ponds around Giessen (Germany) and also fed with *Chlorogonium*.

Nuclei of *Stylonychia* and *Oxytricha* were obtained using published methods [2, 52] involving: lysis of rapidly growing vegetative cells in 0.05% Triton X-100, 0.01% spermidine phosphate, 10 mM Tris buffer (pH 7.0); filtration of the liberated nuclei through a 30 μm diameter nylon mesh; low speed centrifugation to pellet the nuclei; and fixation, as described below.

Concentrated suspensions of the protozoans were usually fixed with 2.5% glutaraldehyde in 50 M sodium cacodylate buffer pH 7 for 60 to 90 min. Fixed cells or nuclei were washed with 1% sucrose 50 mM cacodylate buffer pH 7 followed by embedding the pellet in 2% agarose in 50 mM cacodylate buffer. It was found that dilution of the pellet could be minimized by layering a large excess of agarose over the small pellet at the bottom of a conical centrifugation tube and stirring the pellet gently with a fine glass needle, to allow the agarose to penetrate the pellet. After the agarose solidified, it was cut into small blocks. Half of the blocks were post-fixed with 1 or 2% osmium tetroxide in 25 mM cacodylate buffer for 30 or 60 min and washed with 1% sucrose in 50 mM cacodylate. All blocks were dehydrated in an ethanol series, brought to room temperature in 100% ethanol, then processed through propylene oxide

and embedded in Epon. *Stylonychia* were found to be very sensitive to centrifugation and were usually concentrated by swirling in a Petri dish and decanting the growth medium. *Oxytricha* and *Euplotes* were gently centrifuged in the cold prior to fixation. After glutaraldehyde fixation, the cells could be centrifuged without causing significant cell breakage.

Gold sections (ca. 0.1 μm thick) were stained as described previously with methanolic uranyl acetate and lead citrate [33, 34] or by the Bernhard procedure [32, 33].

A Philips 400 electron microscope, equipped with a goniometer was used to obtain the stereo-electron micrographs. For most of the sections an accelerating voltage of 100 kV was employed; some micrographs were also examined at 80 kV. No differences in objective lens current were measured for the two members of a stereo pair. Mounting and analysis of stereo pairs was carried out as previously described [31–34].

Results

Conventional thin-section electron microscopy

Sections of vegetative cells of *Stylonychia*, *Oxytricha* and *Euplotes* were stained with uranyl and lead salts or stained with uranyl acetate and bleached with EDTA prior to staining with lead [3, 21]. Macronuclei with and without replication bands (RB) were examined; micronuclei were also photographed. Low magnification micrographs of macronuclei with RB from *Stylonychia* and *Oxytricha* are presented in Figure 1; from *Euplotes*, in Figure 7b. Sequential sections of *Stylonychia* (Figs. 1a, b) and *Oxytricha* (Figs. 1c, d) were stained by both methods. Both the pre-replication and post-replication macronuclear chromatin stain intensely with uranyl and lead salts; both types of chromatin are bleached by EDTA treatment in the Bern-

Tab. I. Diameters of ciliate nuclear fibers.

Organism	Nucleus	Region ^{a)}	Stain ^{b)}	Average (nm)	Range ^{c)} (nm)
Stylonychia	macro	FZ	U	43	28-56
			U-EDTA	37	29-47
	micro	—	U	10	7-14
			U	67	56-84
			U-EDTA	63	54-72
Euplotes	macro	FZ	U	47	42-56
Oxytricha	macro	FZ	U	58	42-84

^{a)} The macronuclear regions are: FZ Forward zone; RZ Rear zone.

^{b)} The staining conditions referred to are: U Uranyl acetate and lead citrate; U-EDTA Uranyl acetate followed by EDTA bleaching and lead citrate, the Bernhard procedure.

^{c)} The range of values shown is the minimum to maximum range of diameters observed on clear fibers, that appeared not to be thinned by grazing sections or thickened by overlapping with other fibers.

hard procedure. The numerous round, sometimes "hollow", nucleoli are strongly stained by both staining methods throughout the macronucleus. The RB is also strongly stained by both methods. Figure 1a illustrates the four major ultrastructural zones of an RB: the pre-replication chromatin; the "forward zone" with arrays of electron-dense fibers; the less intensely stained "rear zone"; and post-replication chromatin. Most of the observations described in this study are of *Stylonychia mytilus*, since this organism exhibited the clearest and most ordered RB forward zone of the ciliates examined.

Stereo-electron microscopy of Stylonychia nuclei

High magnification stereo-pairs of *Stylonychia* RB after staining with uranyl and lead salts or after bleaching with EDTA are presented in Figure 2. Fibers of the forward and rear zones, nucleoli and "inter-chromatin" fibers are stained in both methods; by contrast, condensed chromatin is clearly bleached by the Bernhard procedure. Measurements of fiber diameters within the forward and rear zones are summarized in Table I. We will refer to the forward zone fibers as being 40 to 50 nm; the rear zone fibers as being 10 nm. Also shown in Table I are measurements of the diameter of micronuclear chromatin fibers (see Figs. 6a, b), which we refer to as 60 to 70 nm fibers. The larger diameter of micronuclear chromatin fibers compared to the fibers of an RB-forward zone can be readily observed in Figure 2c. This particular figure also illustrates some special features of the organization of the cytoplasm and nuclear envelope, which have been repeatedly observed in our preparations of *Stylonychia mytilus*. The nuclear envelope is very thin, appears to be associated with an about 70 nm thick cytoplasmic layer, and the cytoplasm consists of a complex system of vesicles and tubules. Similar structures of the nuclear envelope and perinuclear cytoplasm have been reported for vegetative nuclei of some chlorophycean algae such as *Acetabularia* [11, 12].

Close examination of high magnification micrographs of chromatin fibers within the RB forward zone reveals regions of distinct substructure. Striations (or lines) of stain running across the 40 to 50 nm fibers can be observed in selected regions (Figs. 3a, b). The periodicity of these striations appears to be of about 15 nm. Sometimes the striations resemble a close-packing of negatively stained spheres of less than 15 nm diameter. This substructure is best observed in micrographs of nuclei stained with uranyl

acetate and lead citrate; preparations by the Bernhard procedure do not reveal clear substructures in the forward zone fibers.

Occasionally, atypical RBs have been observed in preparations of *Stylonychia* (Figs. 4a, b, c). Such structures only reveal forward zone fibers, with no indication of any rear zone. A similar micrograph to that shown in Figure 4a has been published in a study of D₂O-treated cells [53]. The chromatin fibers of the atypical RB fall within the range of diameters observed for a normal RB forward zone. In addition, these fibers are positively stained when the Bernhard procedure is used (Fig. 4c), analogous to the fibers of a normal RB forward zone.

Macro- and micronuclei were also isolated by lysing *Stylonychia* in spermidine phosphate buffer containing 0.05% Trixon X-100, followed by low-speed centrifugation. Isolated macronuclei examined by stereo-EM revealed clear RBs (Figs. 5a, b). The forward zone fibers could be readily visualized and exhibited an average diameter of about 45 nm. The rear zone looked expanded and extracted compared to the in situ configuration; the meshwork of 10 nm fibers was largely absent. The Bernhard procedure applied to sections containing macronuclei revealed a bleaching of condensed chromatin and a positive staining of forward zone fibers and nucleoli, similar to images derived from RB fixed in situ. Micronuclear chromatin fibers remained relatively unchanged after nuclear isolation (compare Figs. 6a, c), with average diameters in the 60 to 70 nm range. Micronuclear chromatin fibers were stained by the Bernhard procedure, both in situ (Fig. 6b) and in isolated nuclei (unpublished). Internal structure (i.e., cross-striations) within the micronuclear chromatin fibers were occasionally observed (see Fig. 6a). In general the micronuclear chromatin fibers appeared of less uniform diameter than those of the macronuclear RB forward zone fiber.

Stereo-electron microscopy of Oxytricha nuclei

Low magnification pictures of *Oxytricha* macronuclei were presented in Figure 1c and d. Examination of RBs at higher magnification (Fig. 7a) revealed similar features to those observed in *Stylonychia*, except that the forward zone fibers appeared less regular, frequently looking like a mixture of fibers and clusters. The forward zone exhibited a gradient of chromatin fiber diameters ranging from the greater-than several hundred nm granules of the pre-replication region, to the about 50 nm fibers of the forward

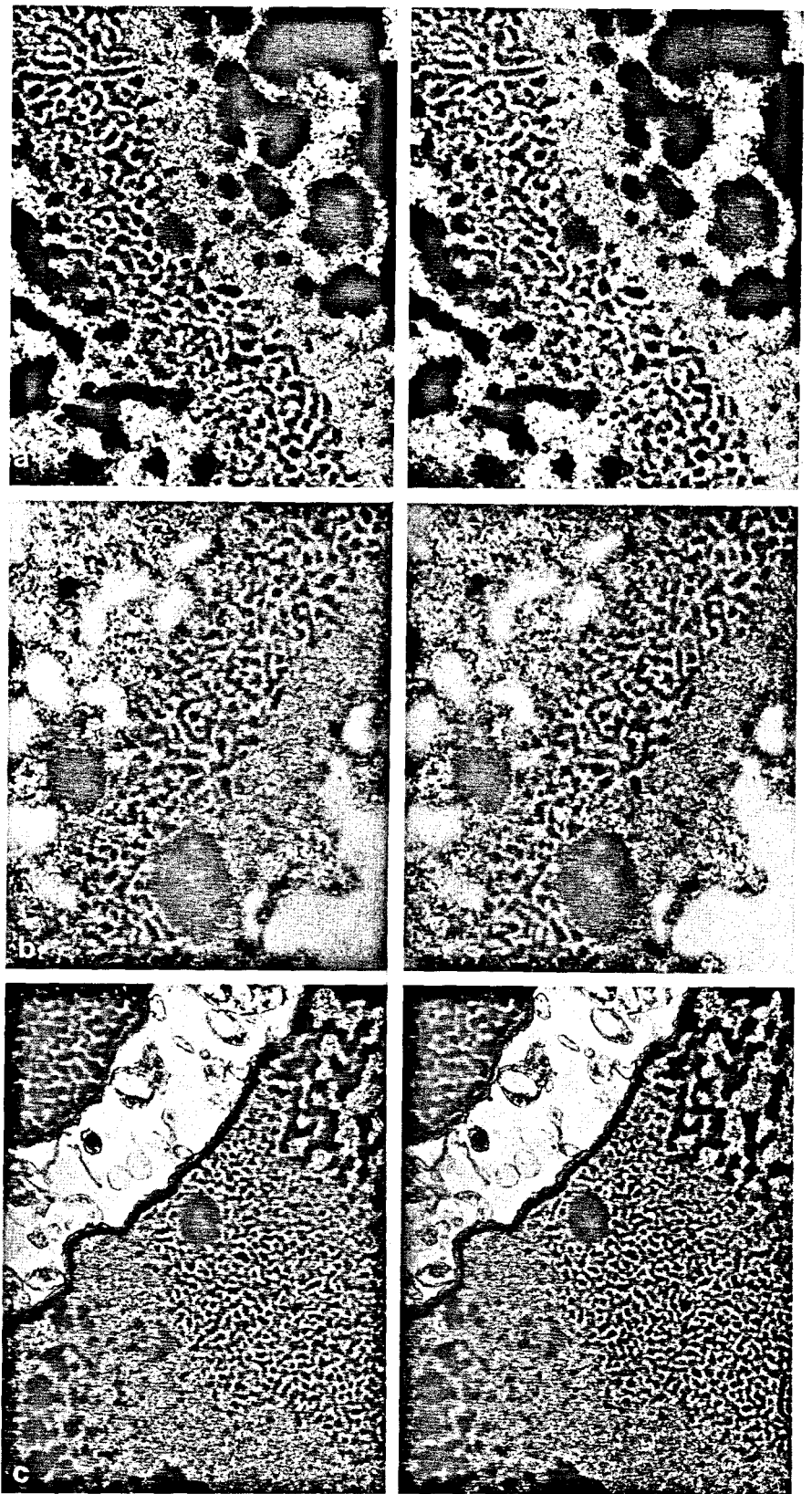


Fig. 2. Stereo-electron micrographs of replication bands in macronuclei of *Styloynchia*. — a. Stained with uranyl and lead. — Tilt angle $\pm 15^\circ$. — b. Stained by the Bernhard procedure. — Tilt angle $\pm 15^\circ$. The micrographs are oriented so that the RB are moving from right to left; that means pre-replication DNA is at the left. — a, b. 28 000 \times . — c. — Tilt angle $\pm 21^\circ$. — A portion of micronucleus is shown in the upper left corner. Stained with uranyl and lead. RB is moving from left to right. — 16 500 \times .

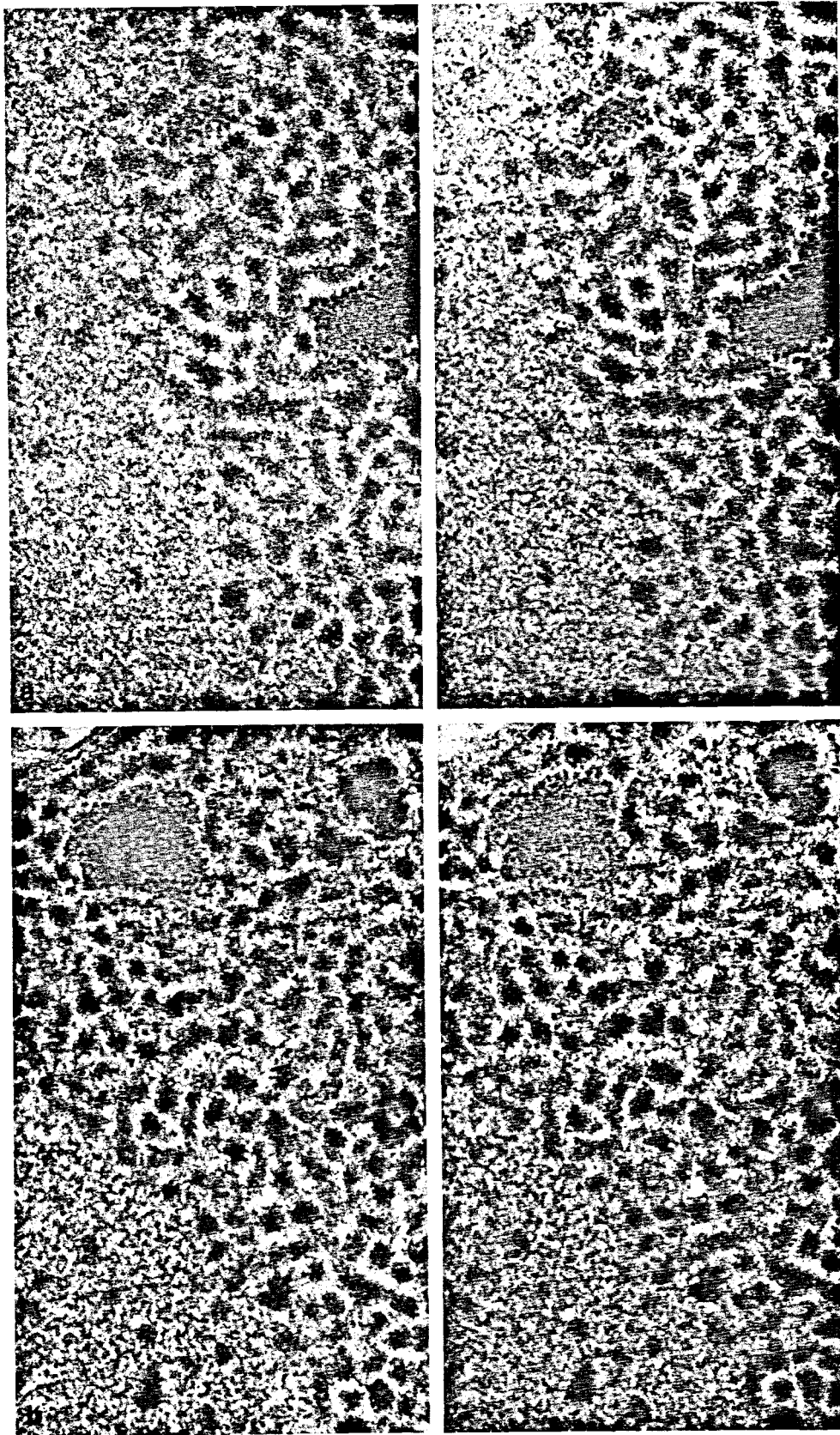


Fig. 3. Stereo-electron micrographs of replication bands in macronuclei of *Stylonychia*. — a, b. — Tilt angle $\pm 12^\circ$. — RB are moving from left to right. Portions of nucleoli with granular structure can be seen within the forward zones. — 70000 \times .

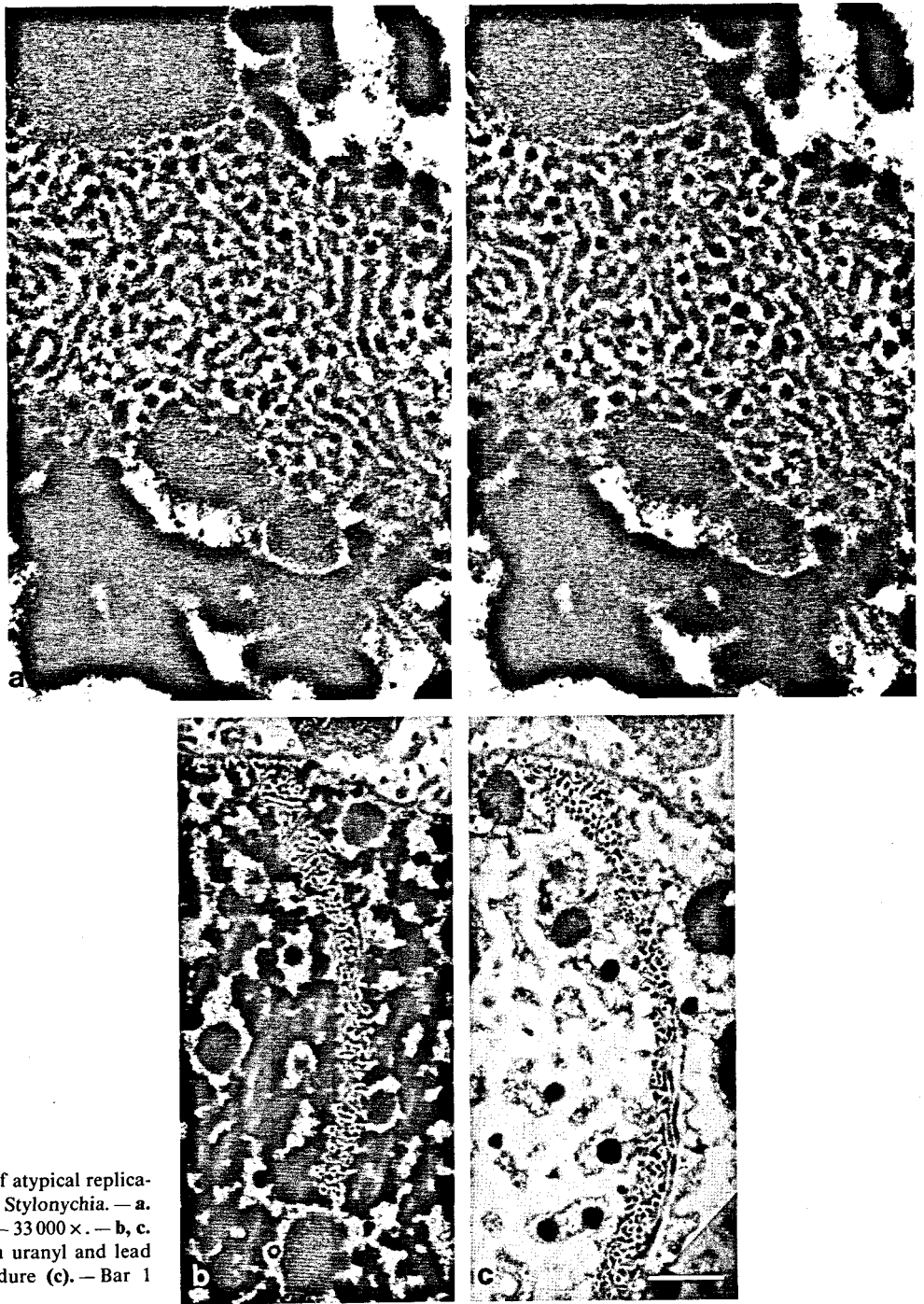


Fig. 4. Electron micrographs of atypical replication bands in macronuclei of *Stylonychia*. — a. Stereo-pair. — Tilt angle $\pm 21^\circ$. — 33 000 \times . — b, c. Sequential sections stained with uranyl and lead (b) or by the Bernhard procedure (c). — Bar 1 μm . — 10 500 \times .

zone. Isolated macronuclei with RBs exhibited a similar range of forward zone fiber diameters. The chromatin clusters within forward zone fibers of *Oxytricha* were very reminiscent of supranucleosomal clusters ("superbeads") observed in spread preparations of swollen sea urchin spermatozoa [55]. Micronuclear chromatin fibers of *Oxytricha* did not look noticeably different from those observed in *Stylonychia*.

Stereo-electron microscopy of Euplotes nuclei

The general ultrastructural characteristics of a RB in *Euplotes* macronuclei (Figs. 7b, c) are similar to those described above for *Stylonychia*. The forward zone 40 to 50 nm fibers (Fig. 7c) are more uniform in diameter than those observed in *Oxytricha*; however, they reveal an overlapping or mesh-work quality that readily distinguishes

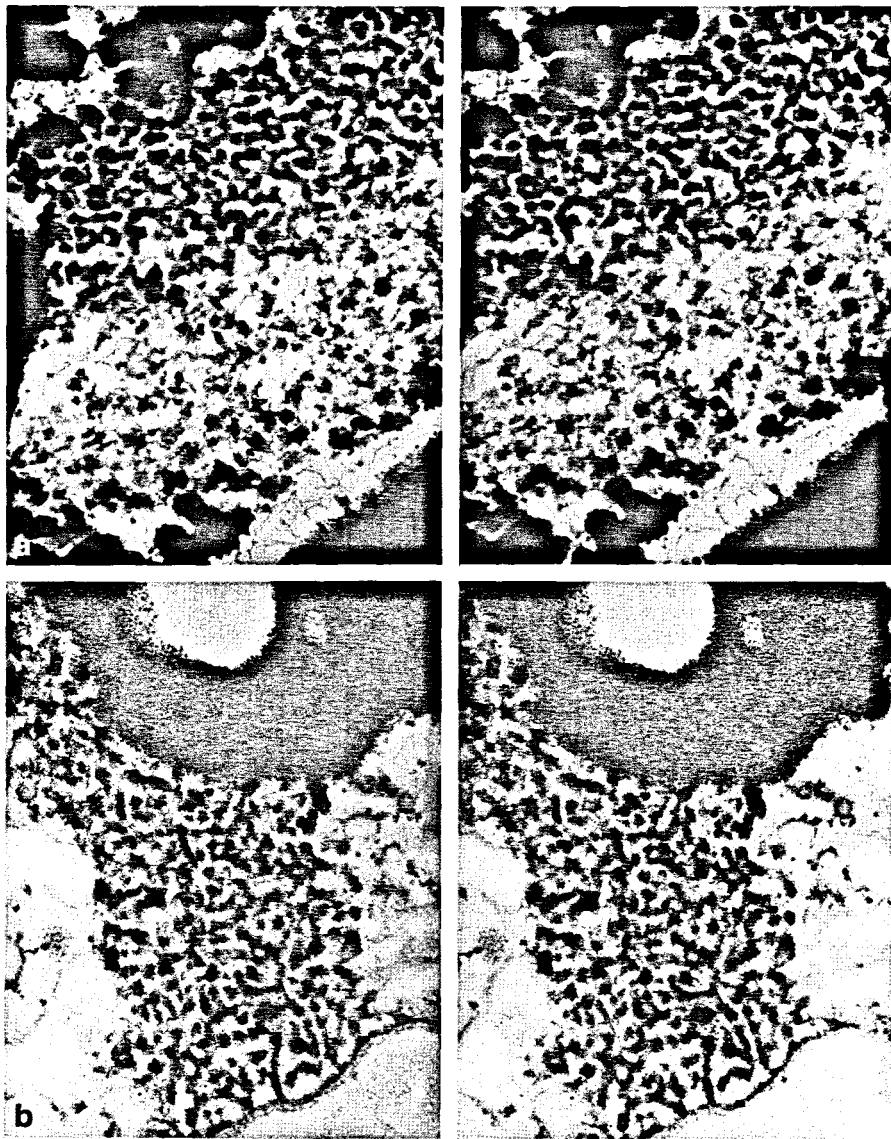


Fig. 5. Stereo-electron micrographs of isolated macronuclei from *Stylyonchia*. — a. Normal RB oriented with forward zone in the upper half of the micrograph; rear zone in the low half. — Tilt angle $\pm 15^\circ$. — 28 000 \times . — b. Atypical RB stained by the Bernhard procedure. A prominent "hollow" nucleolus is shown at the top of the photograph. The condensed chromatin is bleached; the 40 to 50 nm fibers are strongly stained. — Tilt angle $\pm 15^\circ$. — 28 000 \times .

them from similar regions in *Stylyonchia*. Cross-striations (ca. 15 nm separation) are quite readily observed in the forward zone fibers of *Euplotes*. In other respects (i. e., micronuclear structure, and staining by the Bernhard procedure), the properties of *Euplotes* nuclei are very similar to *Stylyonchia* nuclei.

Discussion

The principal features of the RB's of the macronuclei studied are: (1) the striking regularity of the chromatin fibers in the forward zone of a macronuclear replication band (RB); and (2) the strong staining of the forward and rear zones of RB, following the Bernhard procedure, even when adjacent macronuclear chromatin is very clearly bleached. Other observations of interest pertain to micronuclear structure: (1) micronuclear chromatin fibers exhibit a greater diameter than the corresponding macronuclear RB forward zone fibers; and (2) micronuclear chromatin fibers

are also positively stained following the Bernhard procedure.

Ultrastructural and autoradiographic studies have convincingly demonstrated that DNA synthesis occurs in the rear zone of the replication band of the macronuclei of hypotrichous ciliates [4, 7, 13, 18, 36, 38, 39, 41, 46]. The forward zone appears to be a region of chromatin reorganization, preparatory to DNA synthesis. Older ultrastructural studies of the RB forward zone have yielded a wide range of estimated fiber diameters, from 14 nm [46] to 50 to 60 nm [6]; more recent studies have presented clear images, but no measurements have been made [49, 53]. The forward zone fibers of *Stylyonchia* and *Euplotes* look especially regular, exhibiting a fiber diameter of 40 to 50 nm and frequently exhibiting cross-striations of about 15 nm periodicity. *Oxytricha* looks somewhat less regular, often revealing a discontinuous fiber structure.

The forward zone fibers are among the most regular chromatin fibers observed, certainly comparable to the

