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## Isolation and characterization of chromatin replication bands and macronuclei from *Euplotes eurystomus*

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### *Euplotes eurystomus* — macronuclei — replication bands

A method is described for isolating replication bands (RBs) from *Euplotes eurystomus* in quantities sufficient for biochemical analysis. The method involves the disruption of whole cells in a low ionic strength buffer that maintains RB integrity while dispersing macronuclear chromatin. The RBs are then stabilized with MgCl<sub>2</sub> and spermidine phosphate and isolated by gradient centrifugation. Staining with silver nitrate and thiol-specific coumarin maleimide has been demonstrated in the RBs of *Euplotes* and other hypotrichs [1]; both of these properties were maintained in isolated RBs. A method is also described in this study for isolating highly purified macronuclei. Examination of isolated macronuclei and RBs with electron microscopy (EM) indicates that the morphology of both structures remain essentially intact during purification. We also observe with EM an increase in the number of replicating molecules in RBs compared to macronuclei. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrates a consistent but minor enrichment of a 55 kilodalton protein in RBs when compared to macronuclear proteins.

### Introduction

The vegetative cells of *Euplotes eurystomus*, like other hypotrichous ciliated protozoa, possess two types of nuclei: a transcriptionally inactive micronucleus composed of high molecular weight DNA and a transcriptionally active macronucleus containing gene-sized DNA molecules. The macronucleus is derived from micronuclear fusion products following conjugation and functions as the somatic nucleus [17].

Chromatin replication in the macronucleus of vegetative cells occurs within a specialized structure called a replication band (RB). The RB, which is visible in the light microscope, forms at a specific initiation site, and chromatin is replicated as it migrates through the macronucleus [5, 6, 8, 10, 18]. Beginning at the advancing border of the RB there is a reorganization of the chromatin into two morphologically distinct zones: a forward zone composed of chroma-

tin organized into regular 40 to 50 nm fibers and a rear zone composed of a mesh of chromatin fibers approximately 10 nm in diameter [14]. DNA synthesis has been localized in the rear zone of the RB by autoradiographic and ultrastructural studies, suggesting that the forward zone participates in the preparation of chromatin for replication [3, 6, 8, 16, 18].

The RB has considerable potential for fundamental studies into chromatin structure and replication; however, little is known about the biochemical events that occur in the RB. Salvano [19] demonstrated the accumulation of nonhistone proteins in both zones of the RB by UV microspectrographic studies. We also demonstrated, in a previous study, an abundance of thiol groups and silver staining proteins in the RBs of *Euplotes* and other hypotrichs using cytohistochemical techniques [1]. A method is described in this study for isolating RBs in quantities sufficient for biochemical analysis. This should be an important step in understanding how this unique structure functions.

### Materials and methods

#### *Cell cultivation and collection*

*Euplotes eurystomus* (Carolina Biologicals) were grown in plastic photographic trays (60 cm × 50 cm × 7 cm) containing approximately 4 to 6 liters of non-sterile Pringsheim solution [1]. The cells were fed 3 times a week with *Chlorogonium elongatum* grown under sterile conditions in a yeast extract-sodium acetate medium [1]. Cells were concentrated over a 5 μm nylon filter (Tetko, Inc.) supported by a wire screen platform 36 cm in diameter with a depth of 1.5 cm.

A sample population of cells, stained with an equal volume of acetocarmine solution, was examined under a light microscope to determine the percentage of cells with RBs. The acetocarmine stain was prepared by dissolving 10% carmine in 45% glacial acetic acid at 100 °C for 5 min and filtering.

#### *Isolation of macronuclei and RBs*

A flow diagram of the steps used in isolating macronuclei and RBs is shown in Figure 1 with a description of the buffers listed in Table I. All procedures were performed at 4 °C; however, the cells were maintained at room temperature until lysed with ice-cold buffer.

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**Tab. I.** Composition of buffers used in isolating macronuclei and RBs.

	Isolation buffer	Mac buffer	RB buffer	Stabilization buffer
MES <sup>a</sup> , pH 6.15 (mM)	10	20	15	5
NP-40 <sup>b</sup> (%)	0.5	1	0.75	0.25
MgCl <sub>2</sub> (mM)	5	10	—	10
Spermidine (mM)	1	2	—	2
TLCK <sup>c</sup> (mM)	0.1	2	2	—
PMSF <sup>d</sup> (mM)	0.1	2	2	—

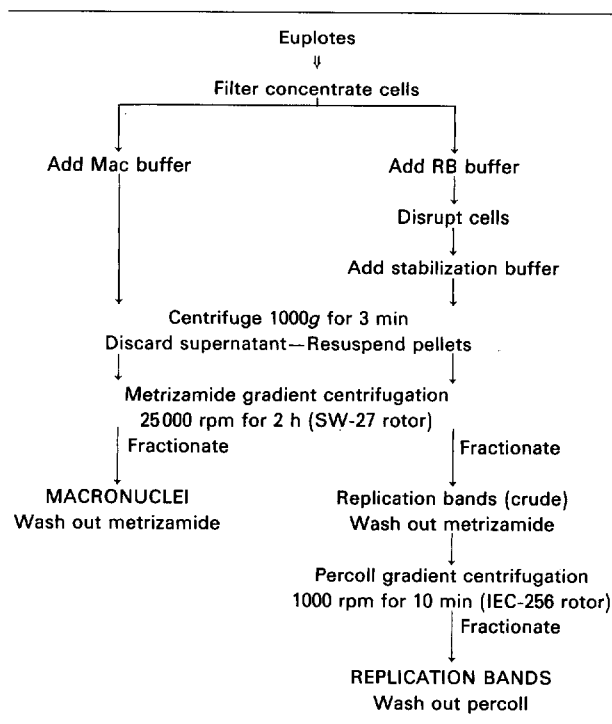
<sup>a</sup> MES (2-[N-morpholino]ethanesulfonic acid). — <sup>b</sup> NP-40 Nonidet P-40. — <sup>c</sup> TLCK Na-p-tosyl-L-lysine chloromethyl ketone (Sigma Chemical Co.). — <sup>d</sup> PMSF Phenylmethylsulfonyl fluoride (Sigma Chemical Co.); added immediately before use from a 100 mM stock solution dissolved in isopropanol.

### Macronuclei

Macronuclei were isolated by first lysing cells suspended in 100 ml of Pringsheim solution ( $1 \times 10^4$  to  $1.2 \times 10^5$  cells/ml) with an equal volume of Mac buffer. The crude macronuclear suspension was then centrifuged at 800g for 3 min in 50 ml disposable centrifuge tubes. After centrifugation, the supernatants were discarded, and the pellets were resuspended by vortexing. The resuspended pellets were pooled and layered over a linear metrizamide gradient and centrifuged for 1 h at 25000 rpm in a SW-41 rotor. A single band formed in the gradient during centrifugation at a density of approximately 1.242. The band, which contained the macronuclei, was removed and resuspended in a 5-fold volume of  $1 \times$  isolation buffer. The macronuclei were centrifuged and washed 3 times in  $1 \times$  isolation buffer at 800g for 3 min.

### Replication bands

Replication bands were isolated by adding 50 ml of cells in Pringsheim solution ( $2 \times 10^4$  to  $2.4 \times 10^5$  cells/ml) to 100 ml of RB buffer.



**Fig. 1.** Flow diagram showing the isolation of macronuclei and RBs from Euplates eurystomus.

Normally only cell preparations with greater than 35% RBs were used. The cellular suspension was then disrupted in a Tekmar tissue-sizer for 30 s at setting 30. Immediately following the disruption of the cells, 50 ml of stabilization buffer was added to maintain RB integrity. The crude RB suspension was then partially purified on a metrizamide gradient as described for isolating macronuclei. A broad band formed in the metrizamide at approximately the same density (1.242) where macronuclei sedimented. The final preparation from metrizamide contained RBs and chromatin granules released from the macronucleus during cell lysis. The RBs were separated by size from the chromatin granules by modification of a procedure described for separating micronuclei and macronuclei of Tetrahymena using percoll gradients [2]. The preparation containing RBs and chromatin granules was layered over a 50% percoll gradient and centrifuged for 5 min at 1100 rpm in an IEC-253 rotor. One-milliliter fractions were removed and monitored by phase microscopy. Fractions containing chromatin granules and RBs were pooled, respectively, in 15 ml disposable centrifuge tubes and filled with  $1 \times$  isolation buffer. The fractions were centrifuged and washed 3 times in  $1 \times$  isolation buffer at 800g for 3 min.

### Metrizamide gradients

Gradients were generated with a linear gradient maker by combining two different density solutions of metrizamide (Accurate Chemical and Scientific Corp.). The solutions were prepared from metrizamide dissolved in distilled water until a refractive index of 1.423 was obtained. The less dense solution consisted of 66% metrizamide solution, 17% distilled water and 17%  $6 \times$  isolation buffer; the more dense solution contained 83% metrizamide solution and 17%  $6 \times$  isolation buffer. Metrizamide gradients could be stored overnight at 4°C.

### Percoll gradients

Fifty percent percoll gradients were made in polyallomer centrifuge tubes (14 × 89 mm) containing a solution of 5.0 ml of percoll (Pharmacia) and 5.0 ml of  $2 \times$  isolation buffer. The polyallomer tubes were placed inside 15 ml corex tubes and centrifuged at 27000g for 15 min in a SS-34 rotor with the brake off. The gradients formed by centrifugation were removed and stored for up to 24 h at 4°C.

### Histochemistry

Staining of isolated RBs with silver nitrate and coumarin maleimide was performed as described by Allen and Olins [1]. Certain slides with RBs were pretreated with 1 mM dithiothreitol (DTT) in  $1 \times$  isolation buffer for 10 min (room temperature) prior to staining with coumarin maleimide.

### Electron microscopy

Preparations of macronuclei and RBs were embedded, sectioned and examined by EM. The samples were concentrated into pellets by centrifugation at 800g for 3 min and fixed at 4°C with 2% glutaraldehyde in  $1 \times$  isolation buffer for 1 h. The samples were then washed in  $1 \times$  isolation buffer containing 0.1 M sucrose and post-fixed for 1 h in 2% osmium tetroxide in  $1 \times$  isolation buffer. After washing in  $1 \times$  isolation buffer containing 0.1 M sucrose, the samples were dehydrated and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 102 electron microscope.

DNA was spread using a modification of the Kleinschmidt [9] procedure as described by Murti and Prescott [13]. Aliquots of macronuclei and RBs in  $1 \times$  isolation buffer containing 1% N-lauroylsarcosine were digested with proteinase K (1.5 mg/ml) at 37°C for

1 to 2 h. Samples were spread, stained with 0.1% aqueous uranyl acetate and examined with darkfield illumination in a Siemens Elmiskop 102 electron microscope operated at 80 or 100 kV at 10000 $\times$ . The magnification was calibrated using a carbon grating replica with 2160 lines/mm (E. F. Fullam Co.). Replicating DNA molecules were counted only if the lengths of the two arms differed by less than 0.063  $\mu$ m (approximately the length of 1 nucleosome) as measured from micrographs projected onto a Tektronix 4954 graphics tablet.

#### Gel electrophoresis

SDS-PAGE of proteins was performed as described by Laemmli [11]. Silver staining of gels was carried out following the procedure of Wray et al. [21]. Molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO).

Electrophoresis of DNA was performed in 1.5% agarose containing 1% N-lauroylsarcosine in TBE buffer [12]. Gels were stained with 1  $\mu$ g/ml of ethidium bromide and visualized on a

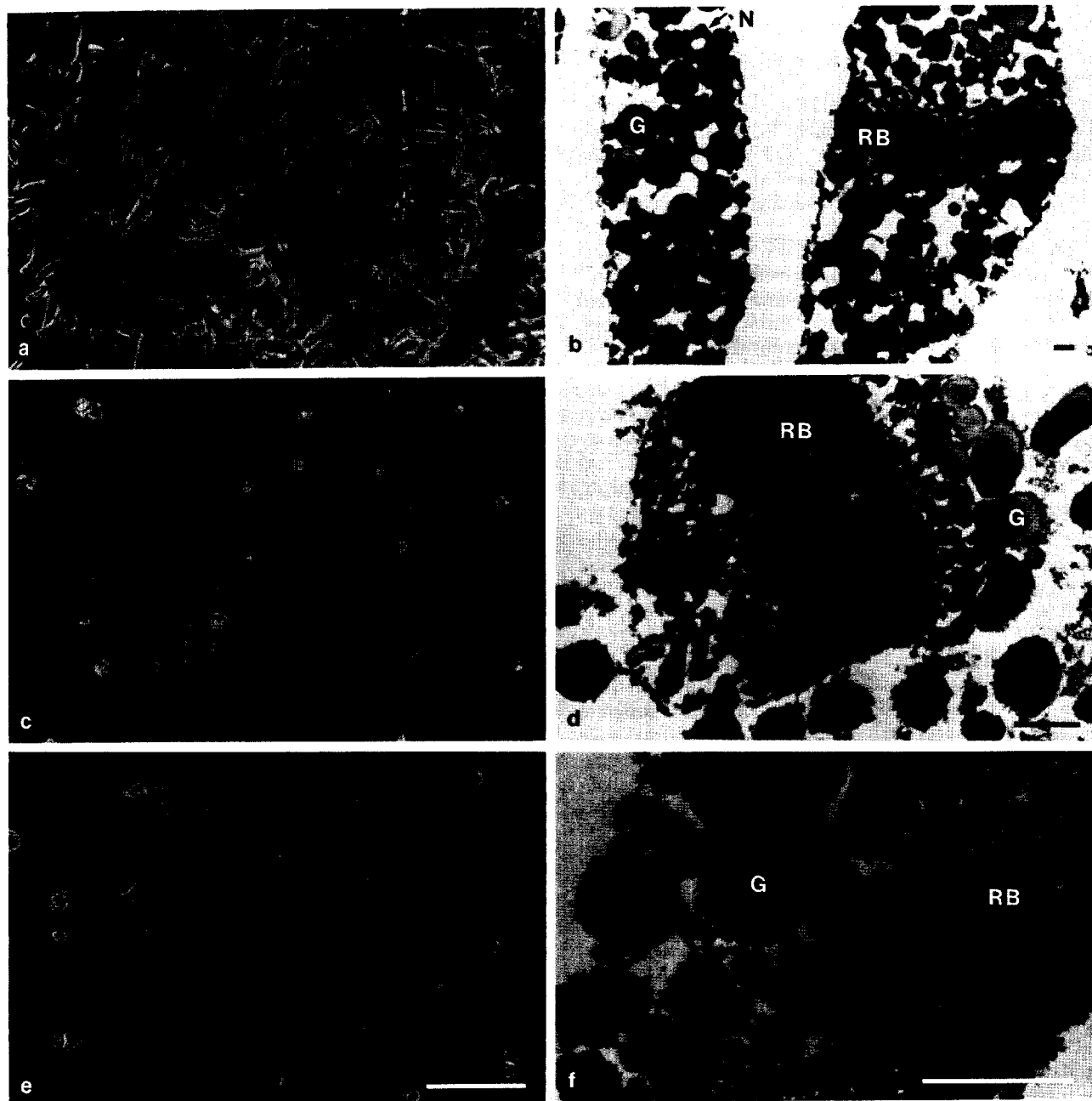


Fig. 2. Phase and electron micrographs of isolated macronuclei and RBs. — a, b. Macronuclei. — c, d. RBs after metrizamide purification. — e, f. RBs after percoll purification. — RB Replication

band. — G Chromatin granule. — N Nucleoli. — Bars 50  $\mu$ m (e), 1.0  $\mu$ m (b, d, f).

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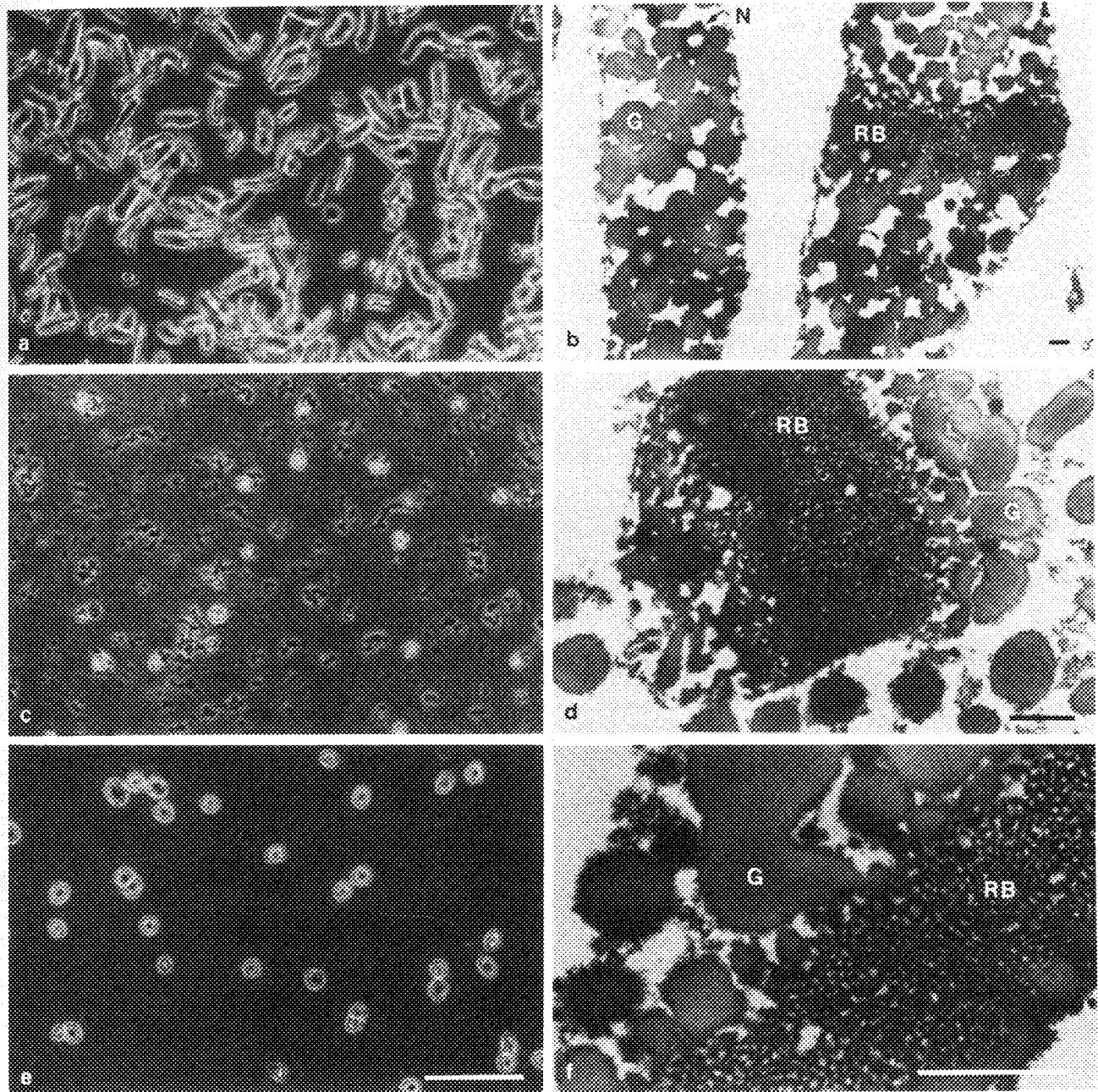


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300 nm transilluminator. Marker DNA fragments (Hind III— digested  $\lambda$  DNA) were obtained from Bethesda Research Laboratories (Bethesda, MD).

## Results

Macronuclei were relatively easy to purify by buoyant density centrifugation in metrizamide. A single band, containing macronuclei, formed in the metrizamide at a density of approximately 1.242 and could be easily removed by pipetting. The material that accumulated at the top of the gradient during centrifugation was removed prior to the removal of the macronuclei. Preparations appeared to be free of any visible cellular debris (Fig. 2a); however, most of the macronuclei were fragmented during purification. The ultrastructure of the macronucleus, when compared to the in situ configuration [14, 18], was intact after isolation except for an absence of the "interchromatin fibers" normally located between the chromatin granules and nucleoli (Fig. 2b).

The isolation of RBs involved two sequential purification steps: centrifugation through metrizamide and centrifugation through percoll. Metrizamide gradients resulted in the co-purification of RBs and "chromatin granules" released from the macronucleus during cell lysis (Fig. 2c). The results of a typical metrizamide gradient were similar to that obtained when isolating macronuclei except the band containing the RBs and chromatin granules was usually broader. The RBs were then separated from the chromatin granules by sedimentation using a 50% percoll gradient. During centrifugation the smaller chromatin granules remained near the top of the gradient while the RBs sedimented near the bottom. The chromatin granules were removed from the gradient, while the RBs contained in the remaining solution were collected. This resulted in a relatively pure preparation of RBs as demonstrated in Figure 2e.

Ultrastructural examination of isolated RBs, when compared to the in situ configuration [14, 18], demonstrated a well-defined forward zone, but a loss of normal structure in the rear zone of the RB (Figs. 2d, f). A loss of rear zone structure was also evident in the RBs of isolated macronuclei (Fig. 2b). The electron micrographs showed that a considerable amount of condensed chromatin remained attached to both ends of the RBs after purification.

In a previous study we demonstrated staining in the RBs of *Euplotes* and other hypotrichs with silver nitrate and with the thiol-specific fluorochrome, coumarin maleimide [1]. Both these properties were maintained in isolated RBs (Fig. 3). The intensity of fluorescent staining with coumarin maleimide could be increased by pretreating the RBs with DTT, a result suggesting that the thiol groups become oxidized during the isolation procedures.

Care was taken to protect proteins and DNA from degradation during the isolation procedures. In addition to performing all steps at 4°C, we optimized the buffer for inhibition of protease and nuclease activity by varying pH and the concentrations of spermidine and MgCl<sub>2</sub> [20]. Based on gel electrophoresis of proteins and DNA from

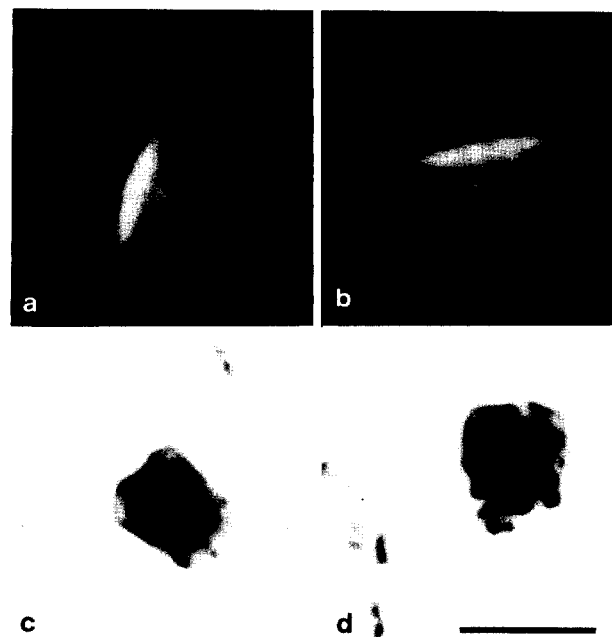


Fig. 3. Isolated RBs stained with coumarin maleimide (a, b) and silver nitrate (c, d). — Bar 10  $\mu$ m.

macronuclei (not shown), we found that a solution containing 5 mM MgCl<sub>2</sub>, 1 mM spermidine phosphate, 0.5% NP-40 in 10 mM MES, pH 6.15, provided maximum protection. TLCK and PMSF were added as general protease inhibitors; TLCK has been demonstrated by Cadilla et al. [4] to prevent degradation of a H1-like protein in *Euplotes* which is especially sensitive to proteolysis. A comparison of the proteins from macronuclei and RB preparations by SDS-PAGE demonstrated only minor differences. Although not readily apparent, a protein with a molecular weight of approximately 55 kilodaltons (55 kDa) was consistently observed, at varying concentrations, in RB preparations compared to macronuclear proteins (Figs. 4, 5).

Agarose gel electrophoresis of DNA from the samples is shown in Figure 6. The banding pattern corresponding to the gene-size DNA fragments was visible in all lanes without a significant difference in the migration patterns of DNA between any of the samples. Examination of the DNA molecules by EM demonstrated that the distribution of DNA lengths in macronuclei and RBs were the same,

Tab. II. The percentage of replicating molecules from macronuclei and RBs.

Preparation	% of replicating molecules (total number of molecules counted)		
	RBs	Macronuclei	RBs/Mac
A	0.44 (9969)	0.11 (12716)	4.00
B	3.77 (5724)	1.37 (3056)	2.75
C	0.65 (7046)	0.24 (12240)	2.70
D	1.22 (3033)	0.59 (3963)	2.10
E	0.68 (2350)	0.51 (1362)	1.30
F	0.53 (2264)	0.08 (2650)	6.60
G	0.65 (1530)	0.51 (1362)	1.30
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300 nm transilluminator. Marker DNA fragments (Hind III—digested  $\lambda$  DNA) were obtained from Bethesda Research Laboratories (Bethesda, MD).

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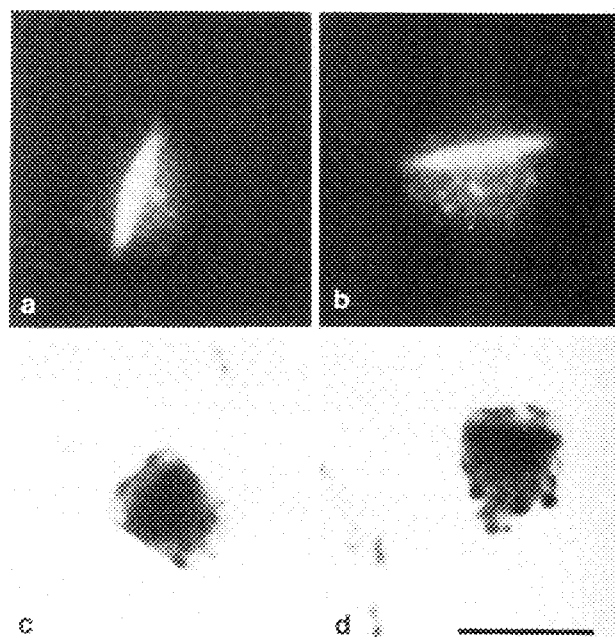


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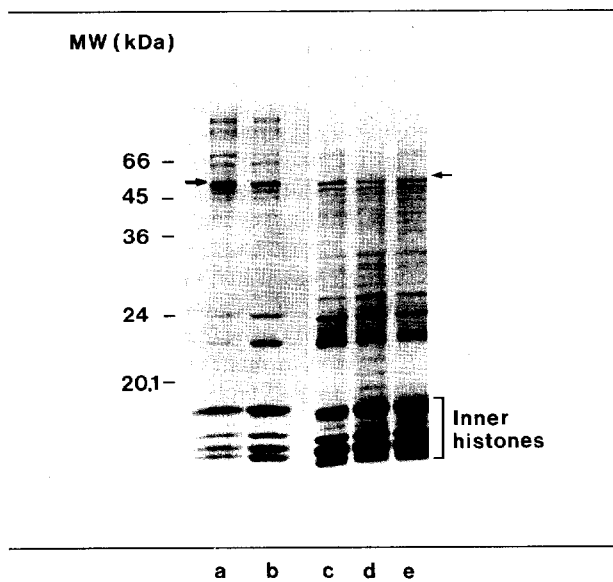


Fig. 4. SDS-PAGE analysis of proteins stained with coomassie blue (a, b) and silver (c-e). — a, e. RBs after percoll purification. — b, c. Macronuclei. — d. RBs after metrizamide purification. — Arrows in (a) and (e) indicate position of 55 kDa protein.

within errors of the technique. We also observed a modest enrichment of replicating structures in RB DNA compared to total macronuclear DNA (Tab. II). The vast majority (i.e., 376 out of 384) of replicating forms were single forks; 6 had double forks and 2 had a bubble. A montage of replicating DNA molecules from isolated RBs is shown in Figure 6.

### Discussion

A method was described in this study for isolating RBs and macronuclei from *Euplotes eurystomus* in quantities sufficient for biochemical analysis. The RBs retained the

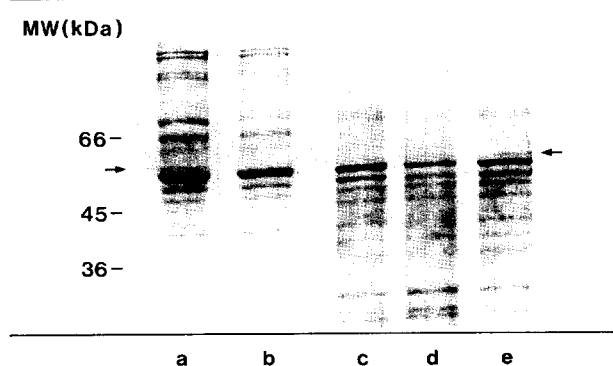


Fig. 5. Detail of Figure 4 illustrating an enrichment of the 55 kDa protein (arrows) in RBs after percoll purification (a, e). — b, c. Macronuclei. — d. RBs after metrizamide purification.

cytochemical properties of selective staining with silver nitrate and coumarin maleimide. A comparison of proteins from RBs and macronuclei by SDS-PAGE demonstrated a consistent but minor enrichment of a nonhistone protein in the RB, however, the overall spectrum of peptides was very similar. We also observed a slight increase in the number of replicating DNA molecules in RBs compared to macronuclei. The vast majority of replicating molecules consisted of single forks with very few double forks or bubbles.

Ultrastructural examination of isolated macronuclei revealed the typical array of chromatin granules and nucleoli but an absence of the interchromatin fibers normally seen in situ [14, 18]. A loss of rear-zone structure in RBs and in macronuclei with RBs was also demonstrated. Olins et al. [14] observed a similar loss of the 10 nm fibers in the rear zone of macronuclei isolated from *Stylonychia* in a buffer containing spermidine phosphate. The loss of the normal ultrastructure in these areas may simply have been due to a collapse of these fibers onto condensed chromatin by the addition of  $MgCl_2$  or spermidine contained in the isolation buffers. A loss of the chromatin could have also occurred during purification, since chromatin in these organisms exist as gene-sized fragments. Loss of chromatin would have been more likely to have occurred during the isolation of RBs as compared to macronuclei, because of their brief exposure to a low ionic strength buffer prior to buffers containing  $MgCl_2$  and spermidine. Ultrastructural studies also demonstrated an abundance of condensed macronuclear chromatin attached to both ends of the RB after purification. This suggested that RB chromatin may account for only a small fraction of the total chromatin in an RB preparation. Observing macronuclear chromatin attached to both ends of the RB with EM was also interesting, since

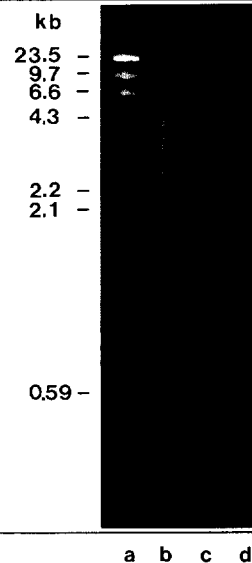


Fig. 6. Agarose gel electrophoresis of DNA. — a. HindIII digested  $\lambda$  DNA. — b. Macronuclei. — c. RBs after metrizamide purification. — d. RBs after percoll purification. — c, d. Material remaining at the origin after electrophoresis.

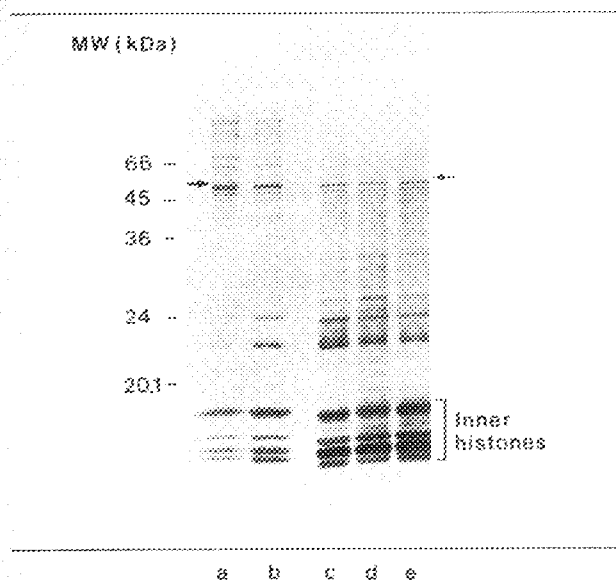


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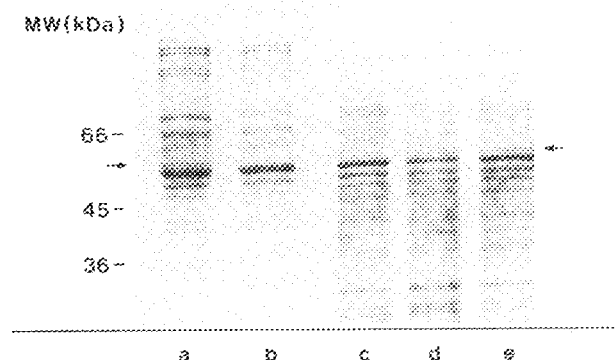


Fig. 5. Detail of Figure 4 illustrating an enrichment of the 55 kDa protein (arrow) in RBs after percoll purification (a, e). — b, c. Macronuclei. — d. RBs after metrizamide purification.

cytochemical properties of selective staining with silver nitrate and coumarin maleimide. A comparison of proteins from RBs and macronuclei by SDS-PAGE demonstrated a consistent but minor enrichment of a nonhistone protein in the RB, however, the overall spectrum of peptides was very similar. We also observed a slight increase in the number of replicating DNA molecules in RBs compared to macronuclei. The vast majority of replicating molecules consisted of single forks with very few double forks or bubbles.

Ultrastructural examination of isolated macronuclei revealed the typical array of chromatin granules and nucleoli but an absence of the interchromatin fibers normally seen *in situ* [14, 18]. A loss of rear-zone structure in RBs and in macronuclei with RBs was also demonstrated. Ohns et al. [14] observed a similar loss of the 10 nm fibers in the rear zone of macronuclei isolated from *Stylonychia* in a buffer containing spermidine phosphate. The loss of the normal ultrastructure in these areas may simply have been due to a collapse of these fibers onto condensed chromatin by the addition of MgCl<sub>2</sub> or spermidine contained in the isolation buffers. A loss of the chromatin could have also occurred during purification, since chromatin in these organisms exist as gene-sized fragments. Loss of chromatin would have been more likely to have occurred during the isolation of RBs as compared to macronuclei, because of their brief exposure to a low ionic strength buffer prior to buffers containing MgCl<sub>2</sub> and spermidine. Ultrastructural studies also demonstrated an abundance of condensed macronuclear chromatin attached to both ends of the RB after purification. This suggested that RB chromatin may account for only a small fraction of the total chromatin in an RB preparation. Observing macronuclear chromatin attached to both ends of the RB with EM was also interesting, since

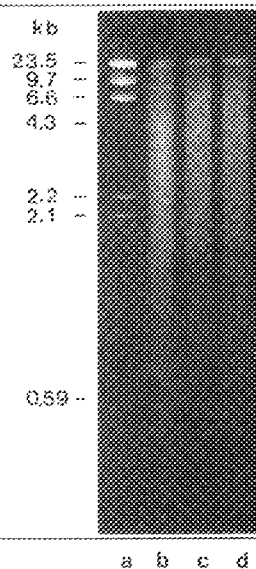


Fig. 6. Agarose gel electrophoresis of DNA. — a. HindIII digested  $\lambda$  DNA. — b. Macronuclei. — c. RBs after metrizamide purification. — d. RBs after percoll purification. — c, d. Material remaining at the origin after electrophoresis.

the boundary between the forward and rear zone was thought to be a point of mechanical weakness where macronuclei were most often broken during isolation [8].

Staining of isolated RBs with silver nitrate and coumarin maleimide demonstrated a retention of cytochemical properties previously described by us in the RBs of *Euplotes*, *Oxytricha* and *Stylonychia*. Silver nitrate was shown to react with an acid-soluble protein tentatively identified by triton-acid-urea gel electrophoresis, while coumarin maleimide reacted specifically with thiol groups [1]. It was suggested that a reduction of disulfide bonds could be a factor in promoting the chromatin decondensation within the RB. Studies are now in progress to identify thiol-rich proteins in the RB by comparing fluorescent proteins on SDS gels from isolated macronuclei and RBs that have been prelabelled with coumarin maleimide.

Only minor differences were observed in proteins from RBs and macronuclei except for a 55 kDa protein enriched in RB preparations. This protein was observed on one-dimension SDS gels which have a relatively low resolution for distinguishing individual nonhistone proteins [15]. Although the nature of the protein is unknown, it seems reasonable to assume that it would have a specific role in chromatin replication in *Euplotes* possibly as a constituent of the replication machinery or as a protein involved in generating higher order structure in the chromatin of the

RB. It is obvious from these studies, however, that to adequately characterize the proteins specifically associated with the RB other methods should be employed such as two-dimensional gel electrophoresis or isoelectric focusing along with techniques designed for fractionating nonhistone proteins.

Examination of DNA molecules by EM demonstrated a small enrichment of replicating structures in RBs compared to macronuclei. One reason for such a low enrichment factor may be a preferential loss of chromatin from the rear zone of isolated RBs, as mentioned earlier. However, a comparable enrichment factor for replicating DNA molecules has been reported in matrix preparation from HeLa cells [20]. The frequency of replicating molecules in preparations of macronuclei containing 43 to 50% RBs was roughly 8- to 14-fold higher than described by Murti and Prescott [13]. Murti and Prescott hand-isolated macronuclei with RBs and examined replicating DNA molecules by EM. The replicating structures in this study from both RBs and macronuclei were similar to those described by Murti and Prescott: the vast majority contained single forks with only a few double forks or bubbles. Based on the nucleotide sequence of the *Euplotes* actin gene, Kaine and Spear [7] suggested that a replication origin exists about 150 base pairs from one end of the molecule. This could explain why such a large number of replicating structures pos-

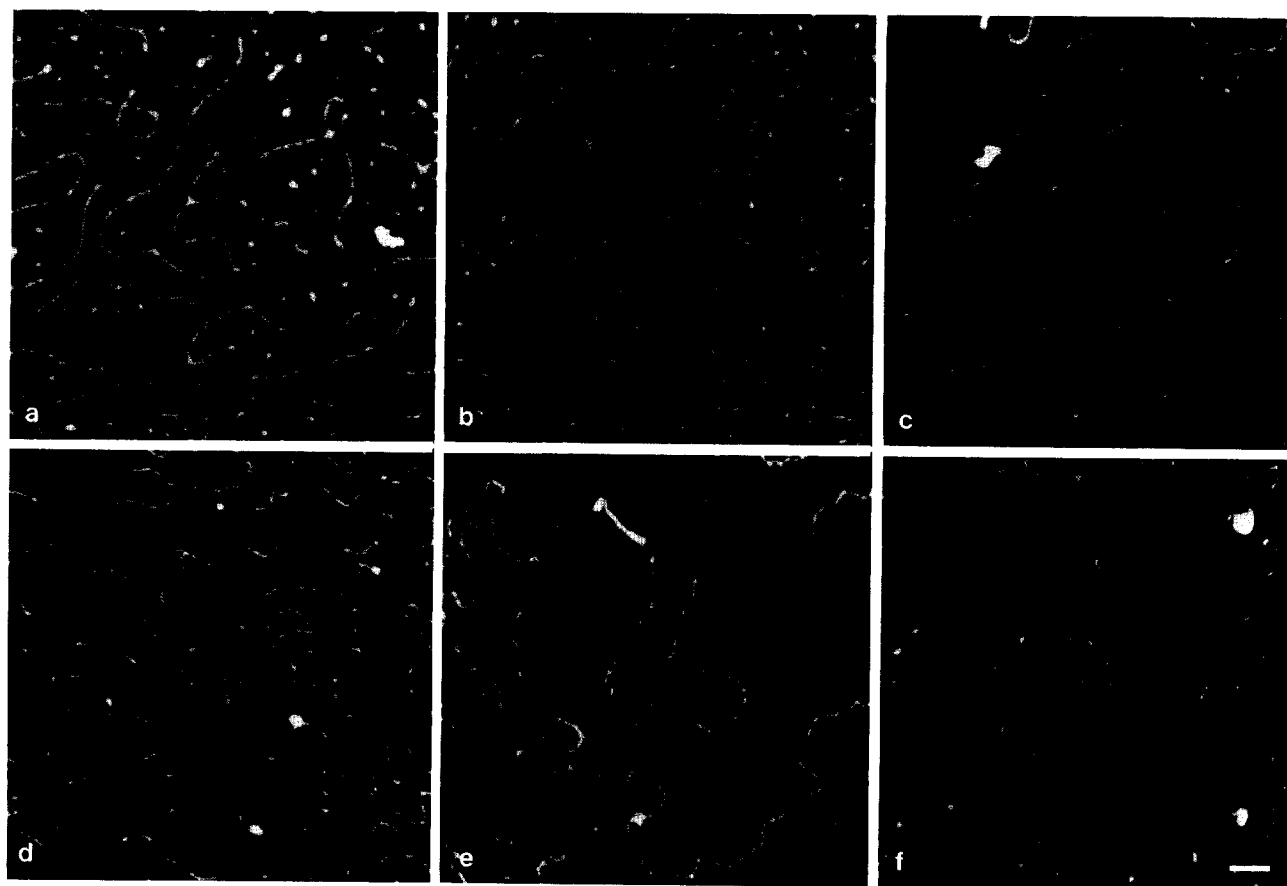


Fig. 7. Replicating DNA molecules from RB preparations. — Bar 0.1  $\mu$ m.

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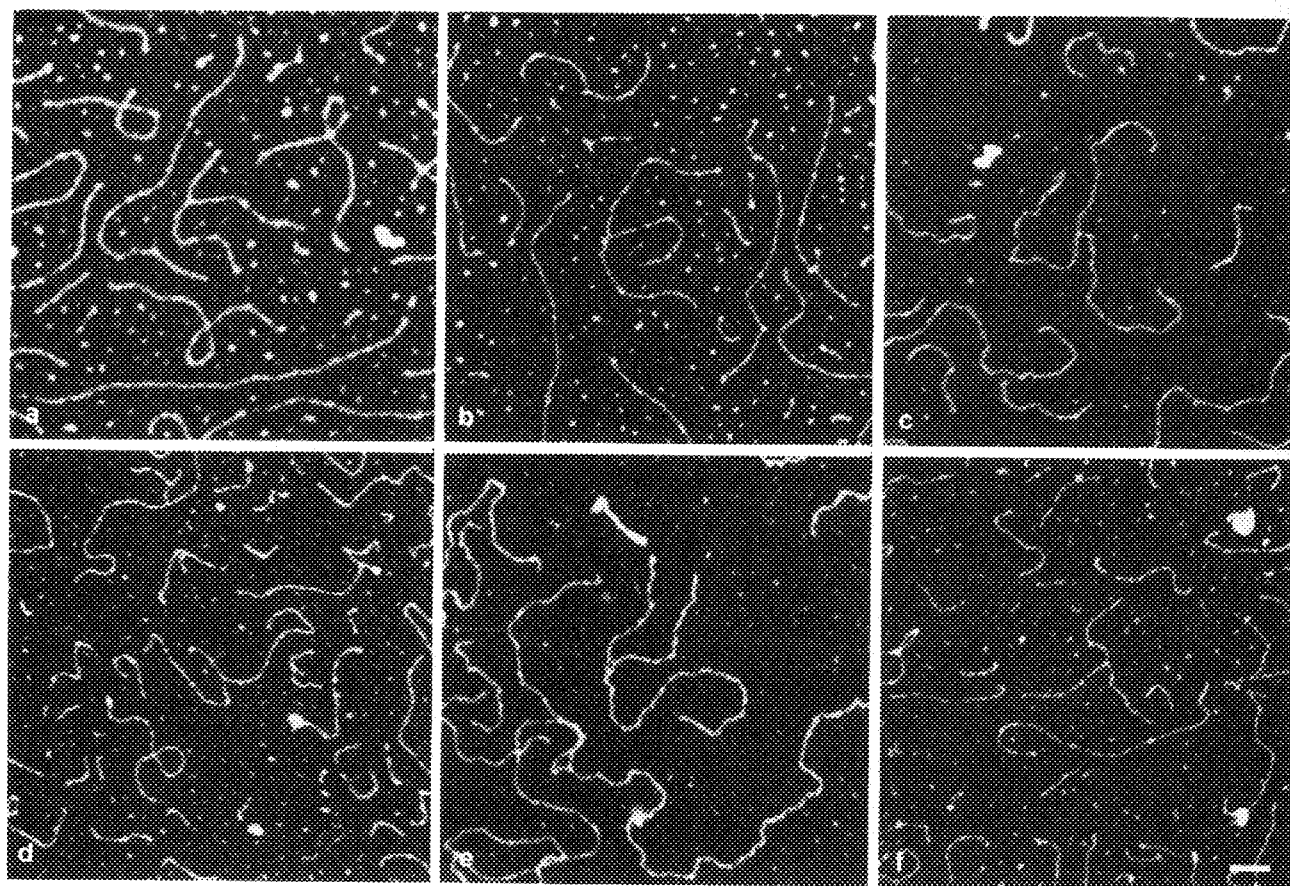


Fig. 7. Replicating DNA molecules from RB preparations. — Bar 0.1  $\mu$ m.

sessed only one fork, since one of the forks (assuming replication was bidirectional) would quickly run off the molecule.

Isolation of the RB should be very useful in future studies to identify proteins and protein modifications involved in the preparation of chromatin for replication and the replication of chromatin. Studies are now in progress to characterize a panel of monoclonal antibodies made to isolated RBs that recognize antigens present only in the RB or macronucleus and that recognize antigens in both structures.

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