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# Cytochemistry of the chromatin replication band in hypotrichous ciliated protozoa staining with silver and thiol-specific coumarin maleimide

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**Abstract.** A modification of the silver-staining techniques for nucleolar organizing regions (NORs) was used to stain selectively the macronuclear replication bands (RBs) and nucleoli in hypotrichous ciliated protozoa (*Euplotes, Stylonychia*, and *Oxytricha*). Silver staining of both types of structures was trypsin-sensitive and DNase I-insensitive, suggesting the involvement of proteins. Silver-staining proteins in the RB were differentially extracted with acid, without any decrease in nucleolar staining. Triton-acid-urea gel electrophoresis of an acid extract of *Euplotes* macronuclei revealed enhanced silver reaction with a single protein upon selective silver staining. An abundance of thiol groups was also demonstrated in the RBs and nucleoli by the fluorochrome 3-(4-maleimidylphenyl)-7-diethylamino-4-methyl coumarin (coumarin maleimide). Histochemical studies, including blocking thiols with *N*-ethyl maleimide (NEM), indicated that thiols were not necessary for silver staining, and that proteins in the RBs and nucleoli reacting with coumarin maleimide were not acid extractable.

## Introduction

The vegetative cells of hypotrichous ciliated protozoa possess two types of nuclei: a transcritionally active macronucleus containing gene-sized DNA molecules and a transcriptionally inactive micronucleus composed of high molecular weight DNA. The macronucleus is derived from micronuclear fusion products following conjugation (for review see Prescott 1983).

During vegetative growth the macronucleus replicates by a unique mechanism with considerable potential for fundamental studies. A structure called a replication band (RB), which is visible in the light microscope, forms at a specific initiation site and by an unknown mechanism replicates the DNA as it migrates through the macronucleus (Faure-Fremiet et al. 1957; Gall 1959; Kimball and Prescott 1962; Kluss 1962; Ringertz et al. 1967). Beginning at the advancing border of the RB there is a reorganization of the chromatin into two morphologically distinct zones: a large forward zone composed of chromatin organized into regular 40- to 50-nm fibers and a rear zone composed of a mesh of 10-nm chromatin fibers (Olins et al. 1981).

Autoradiographic and ultrastructural studies demonstrated DNA synthesis in the rear zone and suggested that the forward zone participated in chromatin reorganization in preparation for DNA synthesis (Gall 1959; Prescott and Kimball 1961; Kimball and Prescott 1962; Ringertz et al. 1967; Bonifaz and Plaut 1974). The forward zone of the RB stained more intensely than the rest of the macronucleus with DNA stains (i.e., methyl green and Feulgen) and with protein stains (i.e., bromphenol blue and fast green at low pH; however, the concentration of DNA and protein in the rear zone was too low to be demonstrated (Prescott and Kimball 1961). The presence of additional proteins in both zones of the RB has been demonstrated by UV microspectrographic studies (Salvano 1975). RNA synthesis has also been demonstrated by Prescott and Kimball (1961) in all regions of the macronucleus except the RB.

We employed silver staining and thiol-specific coumarin maleimide (Sippel 1981) to begin examination of the chemistry of the RB and to identify properties unique to the RBs. These properties should be useful in isolating and characterizing RB components.

## Materials and methods

*Cell cultivation.* The vegetative cells of *Euplotes eurystomus* (Carolina Biological Supply Company, Burlington, N. Carolina), *Stylonychia mytilus*, and *Oxytricha nova* were grown in 150 × 15 mm culture dishes in nonsterile Pringsheim solution composed of 0.02 g/l $Na_2HPO_4$ (0.140 mM); 0.02 g/l $MgSO_4 \cdot 7H_2O$ (0.166 mM); 0.2 g/l $Ca(NO_3)_2 \cdot 4H_2O$ (1.22 mM); and 0.02 g/l KCl (0.268 mM), pH 7.2. The cells were fed daily with *Chlorogonium elongatum* grown under sterile conditions in a yeast extract-sodium acetate medium consisting of 2.67 g/l yeast extract and 1.33 g/l sodium acetate, anhydrous (16.20 mM). The *Oxytricha* cell line was kindly provided by Dr. David Prescott (Boulder, Colorado) and the *Stylonychia* was a gift of Drs. Dieter Ammermann and Hans Lipps (Tübingen, W. Germany).

*Isolation of individual macronuclei.* Individual macronuclei were isolated as previously described (Prescott et al. 1966) with a cell lysis buffer consisting of 10 mM Hepes (pH 7.55), 5 mM $Mg(NO_3)_2$, and 1% Triton X-100. Using a dissecting microscope, nuclei were isolated with a micropipette and released onto a subbed slide in a Petri dish containing 70% ethanol (McDonald 1958). Slides were fixed in 100% ethanol for 30 min–2 h and air dried. Fixed preparations were rehydrated immediately before use through

a series of ethanol:water mixtures and equilibrated in lysis buffer without detergent (equilibration buffer) for 10 min.

*Histochemical treatments.* Replication bands were differentially stained with silver nitrate by a modification of the procedure of Likovsky and Smetana (1981). The equilibrated slides were drained and covered with 4 drops of a 25% (w/v) silver nitrate solution for 4 min followed by the same number of drops of 37% formaldehyde (Mallinckrodt) adjusted to pH 5.5 with sodium acetate. After the solutions had mixed the reaction was monitored using a dissecting microscope until the nuclei first exhibited a golden brown tint. Development was terminated by a thorough wash in distilled water; then the slides were air dried and mounted in Permount (Fisher Sci. Co.). Satisfactory results were also obtained by incubating the slides in an oven at 55° C for 3–6 min after the addition of formaldehyde.

Coumarin maleimide (Molecular Probes, Inc., Eugene, Oregon) was added to equilibrated slides and incubated for 30 min at room temperature. Coumarin maleimide, which is insoluble in water, was first dissolved as a $1.5 \times 10^{-2}$ M stock solution in 100% ethanol and later diluted as needed with cell lysis buffer to $1.5 \times 10^{-4}$ M.

*Cytological pretreatments.* Individual equilibrated slides were pretreated with specific enzymes in lysis buffer, or treated with specific chemicals to remove DNA or proteins. Pretreated slides were thoroughly washed in cell lysis buffer and stained. DNA was hydrolized with DNase I (Worthington Biochem. Corp.: 100 μg $ml^{-1}$ at 37° C for 60 min) and proteins were digested with trypsin (Worthington Biochem. Corp.: 100 μg $ml^{-1}$ at 37° C for 15 min). Basic proteins were extracted by incuating slides in 0.2 N $H_2SO_4$ or 0.1 N HCl for 20 min at 4° C. Acidic proteins (nonhistones) were extracted by incubating slides in 0.1 N NaOH for 20 min at 4° C. Sulfydryl groups were blocked with 10 mM *N*-ethyl maleimide (NEM) in equilibration buffer with or without urea at various concentrations and times.

*Mass isolation of nuclei.* The first step in nuclear isolation was a concentration of rapidly growing vegetative cells through 6-μm nylon mesh (Tetko, Inc.) followed by further concentration by centrifugation (150 *g* for 5 min). Cells (usually $12 \times 10^6$ or less) were lysed in a nuclear isolation buffer consisting of 0.05% Triton X-100, 0.01% spermidine phosphate, 10 mM Tris-HCl (pH 7.0), and 1 mM PMSF (Phenylmethylsulfonyl Fluoride). The liberated nuclei were centrifuged through 10% sucrose in nuclear isolation buffer (800 *g* for 10 min, 4° C) followed by isopycnic centrifugation in metrizamide (Accurate Chem. Co.: 36% to 46%) in nuclear isolation buffer. Metrizamide gradients were centrifuged for 1.5 h at 4° C in a SW-41 rotor at 25,000 rpm. The macronuclear band consisting of large fragments of individual macronuclei was removed and resuspended in a seven-fold dilution of nuclear isolation buffer and washed three times at 800 *g* for 3 min at 4° C.

The isolated macronuclei were extracted overnight in 0.2 N $H_2SO_4$ at 4° C and the acid soluble material was collected and dried under reduced pressure (Allis et al. 1979).

*Gel electrophoresis.* Triton-acid-urea gel electrophoresis in 15% polyacrylamide was performed as described (Butler et al. 1984). The gels were fixed in 100% ethanol for 2–24 h and were stained by Coomassie blue or selective silver staining. Silver staining consisted of three 10-min washes in distilled water followed by incubation in 25% (w/v) silver nitrate for 2 h. Gels were developed in a 55° C water bath by the addition of an equal volume of formaldehyde adjusted to pH 5.5 with sodium acetate and preheated to 50° C. After the desired intensity of staining was attained, the gels were thoroughly washed in distilled water.

## Results

The isolated macronuclei of *Euplotes, Stylonychia,* and *Oxytricha* stained with silver nitrate or coumarin maleimide are presented in Fig. 1. Nucleoli and RBs were selectively stained by both methods. Staining the macronuclei with coumarin maleimide or silver nitrate was relatively easy although good staining with silver depended on both temperature and time. Excessive temperatures during development with silver nitrate tended to stain the macronucleus

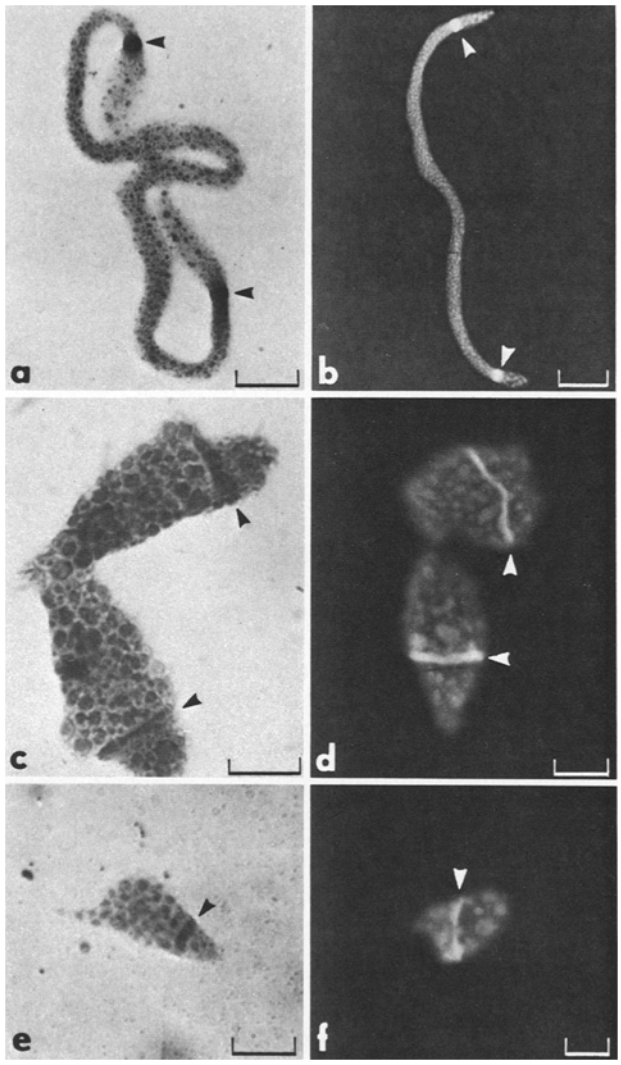


**Fig. 1 a–f.** Isolated macronuclei showing stained replication bands (*arrowheads*) and nucleoli (granular structures distributed throughout the nuclei). *Euplotes* macronucleus stained with silver nitrate (**a**) and coumarin maleimide (**b**). *Stylonychia* macronuclei stained with silver nitrate (**c**) and coumarin maleimide (**d**). *Oxytricha* macronucleus stained with silver nitrate (**e**) and coumarin maleimide (**f**). Bars in **a, b,** represent 20 μm and in **c–f,** 10 μm

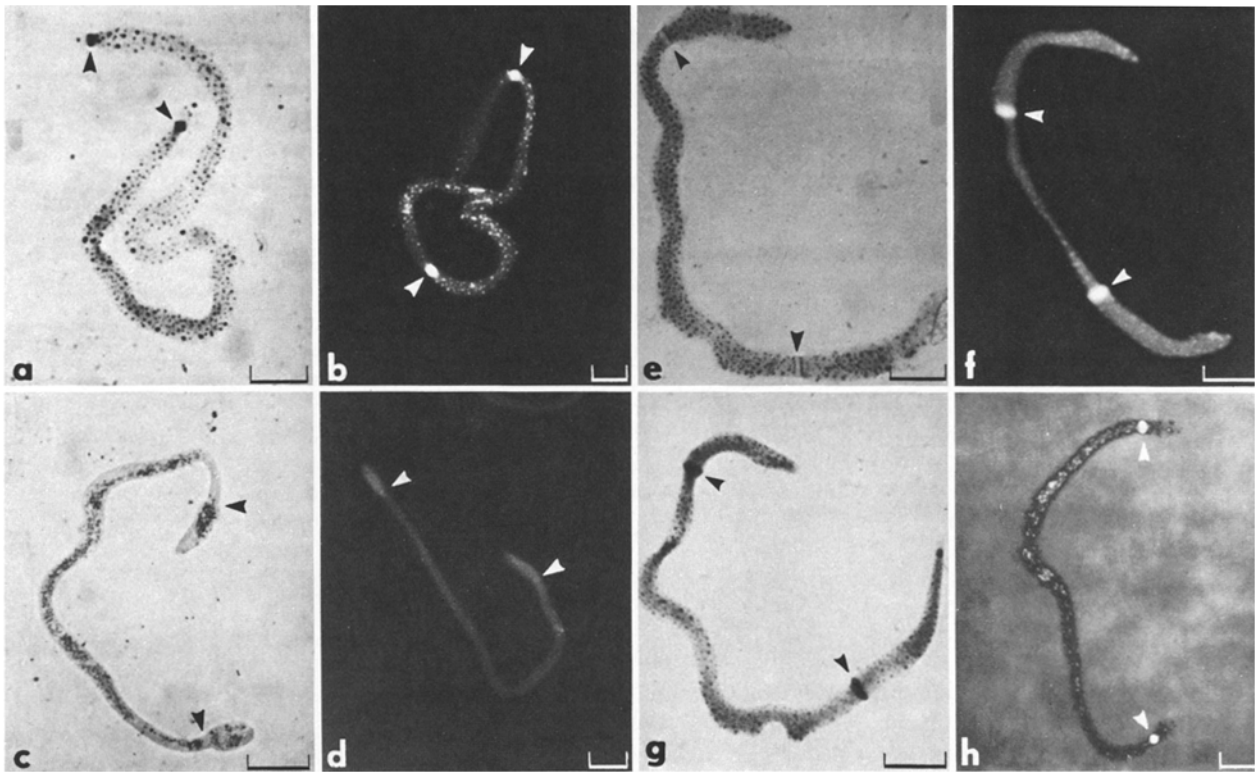


**Fig. 2a–h.** The effects of various pretreatments on isolated macronuclei of *Euplotes* showing replication bands (*arrowheads*) and nucleoli (granular structures distributed throughout the nuclei). **a** DNase I-silver stained; **b** DNase I-coumarin maleimide stained; **c** trypsin-silver stained; **d** trypsin-coumarin maleimide stained; **e** 0.1 N HCl silver- stained; **f** 0.1 N HCl-coumarin maleimide stained; **g** 0.1 N NaOH-silver stained; **h** 0.1 N NaOH-coumarin maleimide stained. Bars represent 20 μm

except for the RBs, while overstaining colored the entire macronucleus. Consistent results were obtained by developing the silver reaction without heat; however, this process required a 4–5 × longer development time.

Cytochemistry was performed on *Euplotes* macronuclei since the RBs of this organism were most easily observed in the light microscope, compared to *Stylonychia* and *Oxytricha*. Pretreatment of *Euplotes* macronuclei with DNase I did not affect staining as shown in Figure 2a, b. When the macronuclei were pretreated with trypsin, however, staining by both methods was abolished suggesting the involvement of proteins (Fig. 2c, d). Acid pretreatment of the macronuclei with 0.1 N HCl or 0.2 N $H_2SO_4$ inhibited silver staining in the RB without affecting nucleolar staining (Fig. 2e). Nucleoli and RBs both stained with coumarin maleimide after acid extraction as shown in Figure 2f. Alkaline pretreatment of macronuclei in 0.1 N NaOH to remove acidic proteins is presented in Figure 2g, h. Little or no inhibition of either staining method was observed. Staining could be inhibited with NaOH by prolonged extraction although this reduced the macronucleus to only a shadow of the original structure. Neither the RBs nor nucleoli significantly stained after such treatment.

The completeness of extraction with 0.1 N HCl or 0.2 N $H_2SO_4$ suggested that silver-staining proteins in the RB were acid soluble. Triton-acid-urea gel electrophoresis was performed on the acid extract of *Euplotes* macronuclei to determine if a prominent acid-soluble protein or proteins could be stained with silver nitrate. Using a selective silver stain that paralleled cytochemical methods, a protein was

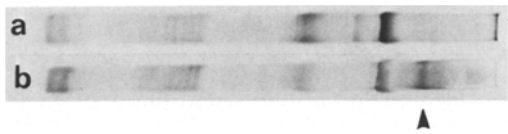


**Fig. 3a, b.** Triton-acid-urea gel of the acid soluble extract of *Euplotes* stained with Coomassie blue (**a**) and by the selective silver method (**b**). Selective silver staining displays a protein with a high affinity for silver ions (*arrowhead*)

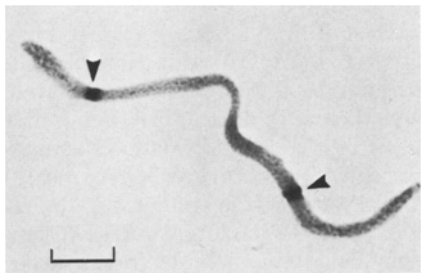

**Fig. 4.** Isolated macronucleus of *Euplotes* pretreated with 8 M urea for 24 h and stained with silver nitrate (replication bands denoted by *arrowheads*). Bar represents 20 μm

observed with a high affinity for silver, as indicated by a comparison of selective silver staining with Coomassie blue (Fig. 3).

The staining intensity of coumarin maleimide in the nucleoli and RBs was progressively decreased by preincubating the macronuclei in NEM for 1–24 h prior to staining. Under these conditions complete blocking of coumarin ma-

leimide staining with NEM was not possible although nearly complete inhibition was obtained with NEM in the presence of 1 M or 4 M urea after 1 h. Staining with coumarin maleimide could also be blocked by preincubation of the macronuclei overnight in 8 M urea alone, presumably due to oxidation of sulfhydryls. Silver staining was not inhibited in RBs or nucleoli by treatment for 24 h with 8 M urea or NEM in 8 M urea (Fig. 4). Prior staining with coumarin maleimide was also an ineffective block for silver staining.

## Discussion

The results of this study demonstrated an abundance of thiol groups and silver-staining proteins in the replication bands (RBs) of *Euplotes, Stylonchya*, and *Oxytricha*. Staining by both methods was intense within the RB although it was possible to demonstrate the involvement of different proteins and different chemical mechanisms of reactivity. The silver staining reaction was dependent on acid-extractable proteins and was not inhibited with NEM; the thiol-specific proteins were not acid extractable and depended on the availability of free, reactive sulfhydryl groups. Although by preliminary judgement silver and coumarin maleimide staining appeared to encompass both zones of the RBs, possibly reflecting chemical changes in chromatin that precede replication, additional experiments will be required. Staining in the RB by both methods coincided morphologically with chromatin decondensation; however, staining could be related to other biochemical changes necessary for replication to occur in these organisms.

It was not surprising that nucleoli of the macronucleus stained with silver nitrate since we used a modification of the silver staining technique for nucleolar organizing regions (NORs) to stain the RB. However, we were able to show a cytochemical difference between silver staining of the RB and nucleoli by acid extraction, which inhibited staining in the RB without affecting nucleolar staining. The acid-soluble nature of the silver-staining proteins in the RB were unique since nearly all silver-binding proteins have been described as very acidic (Goodpasture and Bloom 1975; Howell 1977; Schwarzacher et al. 1978; Lischwe et al. 1979; Olert et al. 1979).

The acid solubility of the RB silver-staining proteins provided as possible method for enriching and identifying RB proteins by gel electrophoresis. Two basic problems with this approach should be mentioned: (1) almost all proteins and nucleic acids can bind silver under appropriate conditions (Clavaguera et al. 1983) and (2) the cytochemical basis for silver staining is unknown and may result from the interactions of silver ions with protein-protein or protein-nucleic acid complexes rather than a specific protein (Clavaguera et al. 1983; Medina et al. 1983). However, by using a staining protocol essentially the same as the cytochemical method, an RB protein from the acid extract of macronuclei was identified in triton-acid-urea gel electrophoresis by its high affinity for silver.

Acid-soluble nuclear proteins consist largely of histones and some nonhistone proteins including the high mobility group proteins (HMGs). Histones were probably not involved in selective silver staining because of their ubiquitous nature and low affinity for silver ions (Clavaguera et al. 1983). A function of the silver-binding proteins in the RBs may be in helix destabilization since they have a predisposition for binding DNA by their basic amino acid residues. These proteins, as characteristic of nearly all silver-staining proteins studied, may also contain acidic regions, which provide the high affinity binding sites for silver ions. These argyrophilic regions could further destabilize DNA by interacting with the histones as has been postulated for silver-staining nucleolar proteins (Mamrack et al. 1979; Olson et al. 1983). The localization of HMGs in the RB would be attractive since HMG contain almost an equal molar amount of acidic amino acids (see Johns 1982) and it has been shown that certain HMGs can destabilize DNA helical structure (Javaherian et al. 1978; Isackson et al. 1979).

Our observations suggested that sulfhydryl groups were not responsible for silver staining in the nucleoli or RB even though they coincided in location. These observations included a selective blocking of thiol groups with NEM and coumarin maleimide without affecting silver staining. We were also able to selectively extract the silver-binding proteins in the RB from the thiol proteins.

The occurrence of thiol groups in the RB could have resulted from an influx of thiol proteins, the exposure of previously buried thiol groups, or the reduction of disulfide bonds. Histone H3 in most species contains a cysteine residue with a free sulfhydryl group; however, staining with coumarin maleimide was not inhibited after the removal of the histones by acid extraction. The most obvious thiol-containing protein involved in replication would be DNA polymerase-$\alpha$ which can be strongly inhibited with NEM. The localization of DNA polymerase-$\alpha$ on the RB would be consistent with studies demonstrating that only a small amount of the enzyme was transported into the nucleus from the cytoplasm during replication (Brown et al. 1981). Another attractive possibility consists of a redox equilibrium between sulfhydryl and disulfide bonds at the advancing border of the forward zone, which would provide a mechanism for the decondensation of macronuclear chromatin as the RB progresses. Oxidation of the sulfhydryl groups could then occur as a normal process of chromatin maturation. The specific decondensation of mammalian sperm nuclei has been known to occur in vitro after treatment with disulfide reducing agents (Marushige and Marushige 1978; Perreault and Zirkin 1982).

Thiol groups have also been involved in binding of metals and coenzymes and in the stabilization of protein conformation. The specific role of thiol groups or silver-binding proteins in the RB must await further biochemical studies that are now in progress.

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