Examination of the Macronuclear Replication Band in *Euplotes eurystomus* with Monoclonal Antibodies

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Abstract. A panel of eight monoclonal antibodies (MAbs) was prepared from spleen cells of mice immunized with macronuclear replication bands (RBs) isolated from *Euplotes eurystomus.* Antibodies were investigated with a solid phase radioimmunoassay (RIA) using either soluble chromatin from isolated RBs or from total macronuclei as antigen. The RIA showed that several MAbs recognized antigens present only in the RB or macronucleus, whereas others recognized antigens present in both structures. Specificity of the MAbs was also examined by indirect immunofluorescence. Antibody C10 recognized an antigen in the rear zone of the RB, whereas MAbs G6 and B2 appeared to stain both the forward and rear zones of the RB. Antibody A7 recognized an epitope distributed throughout the macronucleus except in the RB. Cytochemical studies with degradative enzymes suggested that antigens localized by immunofluorescence were composed of proteins. Immunoblots of SDS PAGE permitted identification of a few proteins that reacted with three of the RB-specific MAbs. Monoclonal antibodies that identify the presence or absence of reactivity of specific proteins in the RB could prove useful in the study of chromatin structure and the mechanism of chromatin replication.

Euplotes eurystomus, like other hypotrichous ciliated protozoa, possess two types of nuclei during vegetative growth: a transcriptionally inactive micronucleus composed of high molecular weight DNA and a transcriptionally active macronucleus composed of short, gene-size DNA molecules. The micronucleus serves as the germ-line nucleus and makes little or no RNA during vegetative growth. The macronucleus is derived from micronuclear fusion products after conjugation and functions as the somatic nucleus (22).

During vegetative growth, macronuclear chromatin is replicated within a specialized structure called a replication band (RB).[1] 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 (7, 9, 14, 15, 23). In *Euplotes*, an RB forms at each end of a crescent-shaped macronucleus, and they migrate toward each other until they finally meet and disappear. Macronuclear chromatin is reorganized at the advancing border of the RB into two morphologically distinct zones: a forward zone composed of chromatin organized into 40–50-nm fibers, and a rear zone composed of a mesh of chromatin fibers ~10 nm in diameter (19). DNA synthesis has been localized in the rear zone of the RB, suggesting that the forward zone participates in the preparation of chromatin for replication (5, 9, 14, 21, 23).

The ultrastructure of the RB has been extensively studied (7, 19, 23, 24), but little is known about its molecular organization. In previous studies we demonstrated an abundance of thiol groups and silver staining proteins in the RBs of *Euplotes* and other hypotrichs (2) and described a method of isolating RBs in quantities sufficient for biochemical analysis (1). As a continuation of these studies a panel of hybridomas secreting monoclonal antibodies (MAbs) was prepared from spleen cells of mice immunized with RBs isolated from *Euplotes.* In this paper we identify and characterize MAbs that recognize antigens present only in the RB or macronucleus and MAbs that recognize antigens in both structures.

[1] *Abbreviations used in this paper:* MAb, monoclonal antibody; RB, replication band; RIA, radioimmunoassay.

Materials and Methods

Preparation of Macronuclei, Replication Bands, and Soluble Chromatin

Euplotes eurystomus (Carolina Biological Supply Co., Burlington, NC) were grown in nonsterile Pringsheim solution and collected by filtration over a 1-μm nylon filter (TETKO Inc., Elmsford, NY) as described elsewhere (2). Macronuclei and RBs were isolated according to previously published methods (1). Briefly, for preparation of macronuclei, cells were lysed in a buffer containing 1% Nonidet P-40, 10 mM $MgCl_2$, and 2 mM spermidine. After low speed centrifugation, the crude nuclear pellet was banded in metrizamide and washed before chromatin preparation. RBs were prepared by mechanically lysing cells in a buffer devoid of $MgCl_2$ and spermidine, which solubilizes the non-RB chromatin. $MgCl_2$ and spermidine were added to the lysate, and RBs were purified by sequential metrizamide and Percoll centrifugation. Protease inhibitors were used throughout. A complete description of the isolation procedures and buffer compositions can be found in a prior publication (1).

Soluble chromatin was prepared as described by Cadilla et al. (6). The isolated macronuclei or RBs were lysed by the addition of 10 mM EDTA, 5 mM triethanolamine-HCl (pH 7.0), 1 mM phenylmethylsulfonyl fluoride, and

1 mM *N-p*-tosyl-L-lysine chloromethyl ketone for 2 h at 4°C. Insoluble material was removed by centrifugation at 3,000 *g* for 25 min at 4°C. The soluble chromatin was then dialysed at 4°C against 0.25 mM EDTA, 5 mM triethanolamine-HCl (pH 7.0), 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM *N-p*-tosyl-L-lysine chloromethyl ketone. Protein content was determined by Coomassie Blue dye binding assay (Bio-Rad Laboratories, Richmond, CA).

Immunization and Production of Hybridomas

BALB/c mice (Oak Ridge National Laboratory colony) were immunized by intradermal injections of 200 μl of 1×10^6 RBs emulsified in Freunds complete adjuvant. Animals were boosted weekly for 3 wk by intradermal injection of 200 μl of 1×10^6 RBs emulsified in Freunds incomplete adjuvant. In the 5th wk, mice were given a single intraperitoneal injection of 200 μl of 1×10^6 RBs in phosphate-buffered saline (PBS) pH 7.6. 4 d after the final injection, spleen cells from an immunized mouse were fused with mouse myeloma SP2/0 (26) or P3-X63-Ag8 using polyethylene glycol (8). Parent myeloma cells and hybridomas were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 2 μM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 20% fetal bovine serum. Fusion products were selected from parent populations in HAT selection media (17).

Screening of Hybridomas and Preparation of Monoclonal Antibodies

Hybridomas secreting MAb were identified by RIA using 96-well microtiter plates coated with either soluble chromatin prepared from isolated RBs or total macronuclei. The RIA was performed essentially as described by Kennel (12). Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were prepared by treating the wells with 50 μl of a 100 μg/ml solution of poly-L-lysine in distilled water for 1 h at 4°C. Wells were aspirated and incubated with 50 μl of 1.0% glutaraldehyde in PBS for 5 min and washed three times in PBS. Soluble chromatin, prepared as described above and diluted to 100 μg/ml in PBS, was then added in 50-μl aliquots/well and incubated overnight at 4°C. Unreacted aldehyde groups and nonspecific binding sites were blocked before the addition of hybridoma supernatants with 100 μl of 25 mg/ml bovine serum albumin (BSA), 0.1 M glycine in PBS containing 0.1% Tween 20 (PBS-Tween) for 4 h at 4°C. The wells were aspirated and washed three times with PBS-Tween in preparation to assay for antibody activity. Hybridoma culture fluids (50 μl) were transferred to antigen-coated plates and incubated for 2 h at 4°C. The plates were washed three times in PBS-Tween, and 50 μl of ^{125}I-labeled goat anti-mouse IgG (13) was added in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.1% Tween 20. After 2 h of incubation at 4°C, the plates were washed with PBS, and the individual wells were removed and radioactivity detected in a scintillation counter.

Positive cultures were subcultured into 24-well plates and then in 25-ml flasks. Cultures that remained positive were cloned by limiting dilution. MAbs were concentrated from supernatant fluids of cloned hybridoma cell lines by precipitation with 50% saturated ammonium sulfate, and were reconstituted in 1/25 of the original volume with PBS (12).

Immunofluorescence Microscopy

Cells in 20 ml of Pringsheim solution were lysed in a 100 × 15-mm petri dish by the addition of 4 ml of lysis buffer consisting of 60 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 30 mM $MgCl_2$, 6 mM spermidine phosphate, and 3% Nonidet P-40. Nuclei released from the cells were concentrated toward the center of the dish by rotating the dish in a circular motion. Macronuclei were removed and mixed with an equal volume of 3.7% formaldehyde in 20 mM cacodylate buffer (pH 7.0). After 3 min, the nuclei were spun onto subbed slides (18) using a cytospin (Shandon Southern Instruments Inc., Sewickley, PA). Slides were washed twice for 15 min each time in PBS. The slides were then incubated for 30 min with the appropriate dilution of MAb, washed for 30 min in three changes of PBS, and incubated with a 1:80 dilution of fluorescein-coupled goat anti-mouse IgG (CooperBiomedical, Inc., Malvern, PA). After 30 min the slides were washed for 30 min in three changes of PBS, and coverslips were mounted with 90% (vol/vol) glycerol in PBS (pH 8.6) containing 25 mg/l of 1,4-diazobicyclo-(2,2,2)-octane (11).

Cytological Pretreatments

Macronuclei were fixed in formaldehyde, spun onto subbed slides, and rinsed in PBS as described above. The slides were then treated with specific enzymes to remove DNA, RNA, or proteins, washed in PBS, and immunostained. DNA was hydrolyzed with 200 μg/ml of DNase I (Worthington Biochemical Corp., Freehold, NJ) at 37°C for 30 min in 10 mM Tris (pH 7.5), 15 mM NaCl, and 5 mM $MgCl_2$. RNA was removed with 100 μg/ml of RNase A (Boehringer Mannheim Diagnostics, Inc., Houston, TX) at 37°C for 30 min in 10 mM Tris (pH 7), 15 mM NaCl, and 5 mM $MgCl_2$. Proteins were digested with 100 μg/ml of trypsin (Sigma Chemical Co., St. Louis, MO) at room temperature for 5 min in PBS. Extensive digestion with trypsin (100 μg/ml at room temperature for 5 min) was performed on slides fixed in 100% ethanol for 2 h and air dried.

Gel Electrophoresis and Immunoblotting

SDS PAGE of proteins from macronuclei and RBs was performed on 18% polyacrylamide gels essentially as described by Laemmli (16). Gels were electroblotted to nitrocellulose (28) using electrophoresis buffer without SDS. Nonspecific binding sites were blocked with 5% nonfat dried milk dissolved in PBS "Blotto" (10) for 2 h. The immunoblots were then processed essentially as described by Smith and Fisher (27) except for the substitution of Blotto for PBS-Tween. MAbs were used at a 1:50 dilution of 25× supernatant and detected using a 1:200 dilution of affinity purified goat anti-mouse IgG conjugated to alkaline phosphatase (CooperBiomedical, Inc.). Dot blots, processed as described above, were also used to detect antibody reactions to macronuclei treated with various reagents.

Results

Hybridoma cultures were screened by radioimmunoassay (RIA) using soluble chromatin prepared from isolated *Euplotes* macronuclear RBs as antigen. Of 752 SP2/0-derived hybridoma cultures, seven produced antibodies reacting with RB chromatin and retained the ability to secrete antibody upon repeated subculturing. Of 376 P3-derived hybridoma cultures, only one made antibodies reacting with RB chromatin and also maintained long-term secretion of antibody.

Supernatants from the eight hybridomas were examined by RIA using soluble chromatin from RBs or total macronuclei as antigen (Table I). Hybridomas designated G6, G11, B2, C10, and H3 all secreted antibodies reacting specifically with RB chromatin, MAb A7 bound only to macronuclear chromatin; D2 and H5 reacted with chromatin from both RBs and macronuclei. The relatively high counts observed in the RIA when RB-soluble chromatin was used as antigen probably resulted from macronuclear chromatin contaminating isolated RBs (1).

Antigens reacting with four of the MAbs could be further characterized by indirect immunofluorescence (Table I). Antibodies C10, G6, and B2 stained the RB (Fig. 1, *a–f*). Upon close examination, MAb C10 was shown to react in a region corresponding to the rear zone of the RB (Fig. 2*a*), while MAbs G6 and B2 appeared to recognize antigens distributed in both zones of the RB (Fig. 2, *b* and *c*). Antibody A7 reacted with an epitope distributed throughout the macronucleus

Table I. Properties of Monoclonal Antibodies Prepared against Euplotes RBs

MAbs	RIA solid phase		Immunofluorescent localization		Immunoblot
Designation	RB	Mac	RB	Mac	SDS PAGE
	cpm				
G 6	4,755	871	+	−	+
G 11	4,909	455	−	−	+
B 2	6,529	400	+	−	−
C 10	5,030	1,590	+	−	−
H 3	7,344	535	−	−	+
A 7	2,494	21,813	−	+	−
D 2	9,281	9,990	−	−	−
H 5	2,956	2,038	−	−	−

Mac, macronucleus.

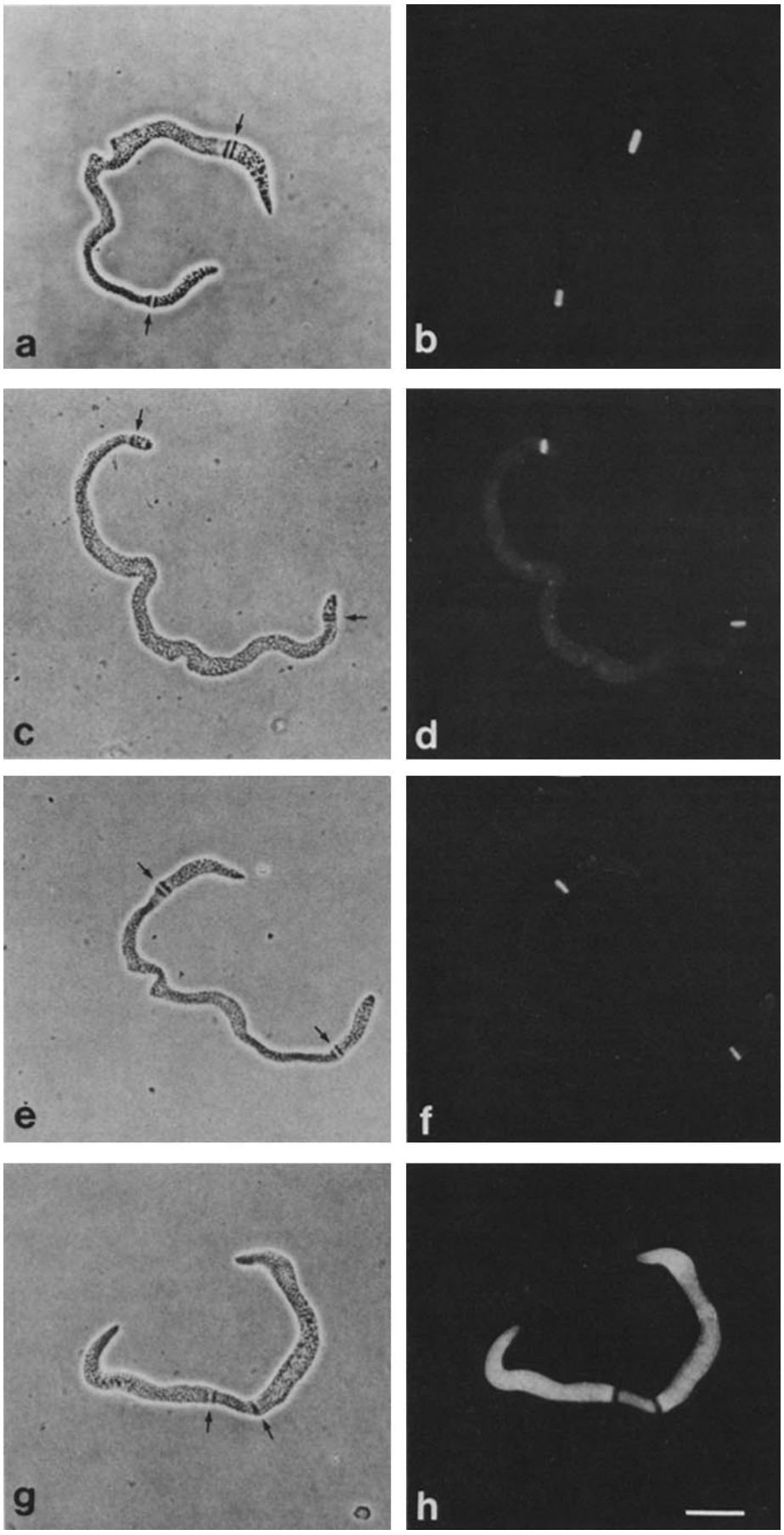


Figure 1. Staining of *Euplotes* macronuclei containing RBs (arrows) using MAbs. Phase (*a*, *c*, *e*, and *g*) and immunofluorescent (*b*, *d*, *f*, and *h*) photomicrographs after staining with (*b*) MAb C10, (*d*) MAb G6, (*f*) MAb B2, and (*h*) MAb A7. Bar, 30 μm.

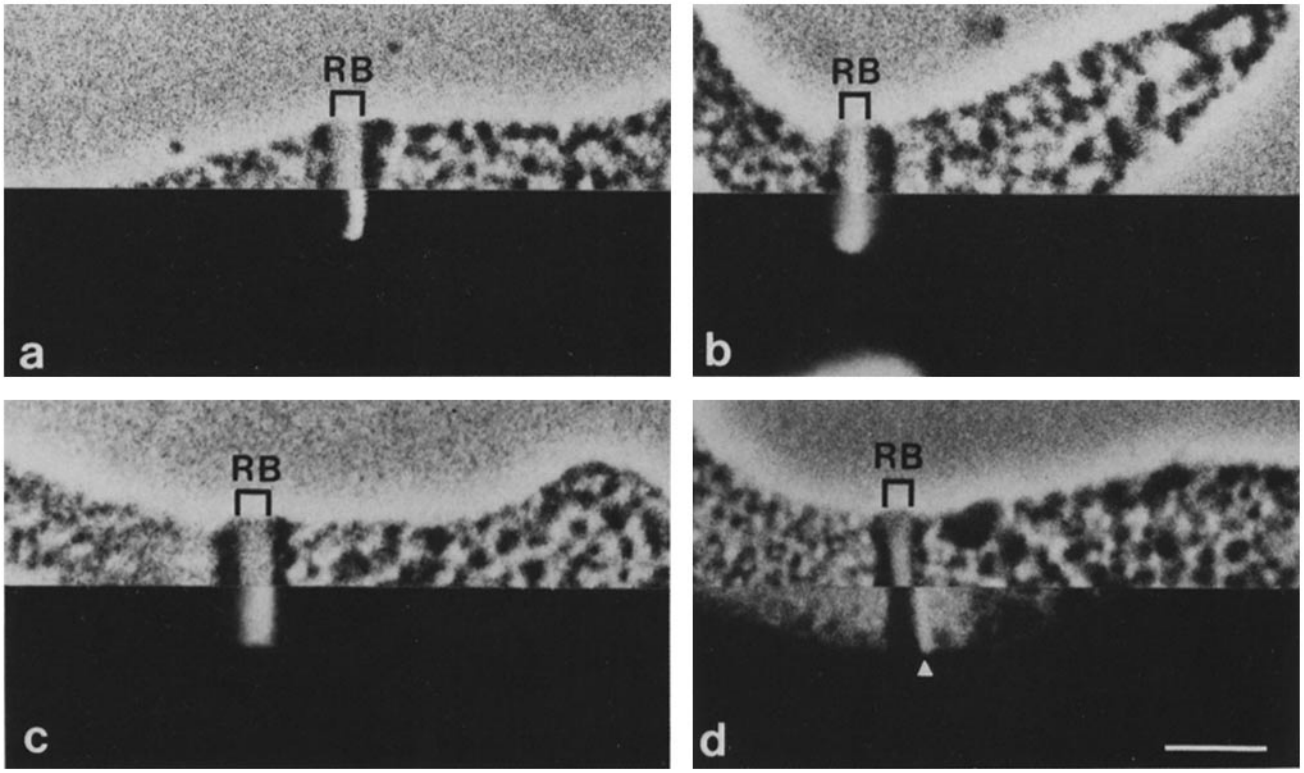
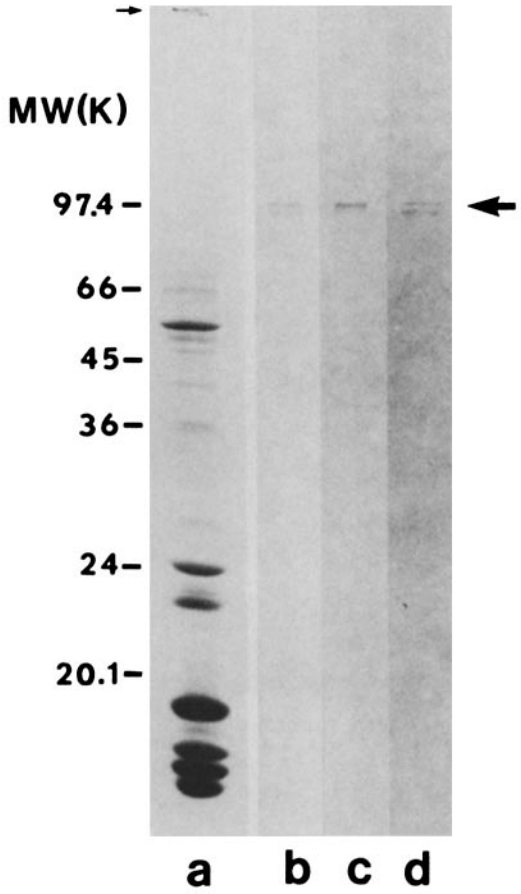


Figure 2. Composite photomicrographs illustrating staining patterns of macronuclear RBs treated with MAbs. Phase (upper) and immunofluorescent (lower) images after staining with (*a*) MAb C10, (*b*) MAb G6, (*c*) MAb B2, and (*d*) MAb A7. Arrowhead indicates enhanced staining over the rear of the replication band (*RB*) with MAb A7. In all examples the RB is proceeding from right to left. Bar, 5 μm.



except in the RB (Fig. 1, *g* and *h*). A large proportion of the macronuclei reacted with antibody A7 also demonstrated an accumulation of antigen at the trailing edge of the RB as shown in Fig. 2*d*.

To elicit immunostaining reactions of isolated macronuclei or RBs with the remaining MAbs, a variety of fixatives were tried. Fixatives such as ethanol, methanol, acetone, or mercuric chloride (29) increased background fluorescence, and the morphology of the RB was partially or completely destroyed during the immunostaining reaction (not shown). The MAbs not reacting after formaldehyde fixation did not appear to reveal specific staining when any of the other fixatives were used.

Macronuclei were pretreated with DNase I, RNase A, or trypsin before immunostaining; only trypsin digestion eliminated the specific reactions. Mild digestion with trypsin (10 μg/ml for 5 min) was sufficient to destroy immunofluorescence staining with MAbs C10, G6, and B2; however, inhibition of A7 required more extensive digestion (100 μg/ml for 5 min). These results suggested that all four MAbs recognized antigens that were at least partially composed of proteins.

Figure 3. Comparison of macronuclear proteins separated by SDS PAGE and stained with MAbs, as described in Materials and Methods. (*a*) SDS PAGE of macronuclear proteins stained with Coomassie Blue. Nitrocellulose blots of a preparation identical to that in *a*, stained with (*b*) MAb G6, (*c*) MAb H3, and (*d*) MAb G11. The antibodies bind to a pair of proteins of ~98 kD (large arrow). Small arrow indicates origin of gel.

The molecular specificities of the MAbs were examined on immunoblots using proteins from either macronuclei or RBs transferred to nitrocellulose from SDS PAGE. Of the eight MAbs tested, G6, G11, and H3 gave positive results (Fig. 3). All three MAbs appeared to react with the same pair of proteins migrating at ~98 kD. Myeloma culture supernatant from P3 cells did not react with any proteins on the SDS PAGE immunoblots. None of the eight MAbs reacted with acid-soluble proteins of RBs or macronuclei transferred to nitrocellulose from Triton-acid-urea gels (data not shown). We were unable to identify the protein recognized by A7 on immunoblots transferred from SDS PAGE or Triton-acid-urea gels; however, we did obtain a reaction using dot blots of total macronuclei. The dot blot analysis demonstrated the reaction to be particularly sensitive to SDS and weak acids. Dot blot reactions were enhanced by treating the macronuclei with 8 M urea, 5% 2-mercaptoethanol, and heat (100°C for 15 min), suggesting that A7 was not recognizing a specific tertiary or quaternary protein structure.

Discussion

The macronuclear RB of *Euplotes* offers a unique opportunity to study the changes in chromatin that occur during chromatin replication. In this study a panel of eight MAbs was prepared from spleen cells of mice immunized with RBs. The MAbs were first investigated by RIA according to their reactivity with RB or macronuclear soluble chromatin and subsequently characterized by indirect immunofluorescence. Of the eight antibodies, four gave positive immunofluorescent staining. Three of these (C10, G6, and B2) recognized antigens in the RB, while antibody A7 recognized an antigen in the macronucleus absent from the RB. All the MAbs reactions demonstrated by immunofluorescence were sensitive to trypsin, suggesting the involvement of proteins.

Antibody C10 stained only the rear of the RB, possibly recognizing a protein directly involved in DNA synthesis or chromatin maturation (i.e., nucleosome assembly). The immunostaining reaction of MAb C10 was also very intense, which suggested the presence of a large concentration of epitope. Antibodies G56 and B2 recognized proteins in the forward and rear zones of the RB, probably reflecting changes in chromatin that precede replication. We were confident that localization of antigens in the RB was not a result of variations in antigen accessibility, since immunofluorescent staining correlated with the results obtained with the RIA (i.e., MAb C10 stained only the RB and reacted with only RB-soluble chromatin). However, a more definitive localization of antigens within the RB will certainly require other studies including immunoelectron microscopy.

Of the MAbs localized by immunofluorescent staining only antibody G6 gave positive results by immunoblot. The MAb recognized a pair of proteins migrating at ~98 kD on SDS PAGE. Two other MAbs (G11 and H3) specific for the RB by RIA also recognized the same pair of proteins, but were negative by immunofluorescence, indicating that a different epitope was recognized by MAb G6.

Antibody A7 recognized a protein abundant in the macronucleus except for the RB as shown by RIA and indirect immunofluorescent staining. Besides staining the macronucleus, an accumulation of A7 antigen was also observed over the trailing edge of the RB. This suggested that the protein recognized by A7 might be removed from the macronucleus as the forward zone of the RB formed and could only reassociate after replication. Degradation or displacement of a macronuclear protein resulting in decondensation of chromatin would be an attractive model for the formation of the forward zone. The initiation of sperm nuclear decondensation and DNA synthesis in the fertilized egg apparently results from a selective degradation of sperm protamine initiated by a reduction of protamine disulfide bonds (20). Previous studies have also demonstrated an abundance of reactive thiol groups in the RB of *Euplotes* and several other hypotrichous ciliated protozoa (2). Examples of physiological and developmentally regulated proteolytic processing of histone H1 and H3 have recently been reported in tetrahymena micronuclei (3, 4). Alternatively, MAb A7 could have recognized a protein involved in the synthesis of RNA such as RNA polymerase, since RNA synthesis has been shown to occur throughout the macronucleus except in the RB (21). The monoclonal antibodies prepared in this study demonstrate specific changes in the composition of chromatin that precede replication and that occur during replication. In future studies these MAbs will be used to purify specific proteins or chromatin fractions by immunoaffinity chromatography or immunoprecipitation techniques.

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