Proliferating Cell Nuclear Antigen/Cyclin in the Ciliate *Euplotes eurystomus*: Localization in the Replication Band and in Micronuclei

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Abstract. Human autoimmune sera specific for proliferating cell nuclear antigen (PCNA)/cyclin (auxiliary protein for DNA polymerase δ) demonstrated the presence of epitopes within the macro- and micronuclei of the hypotrichous ciliated protozoa *Euplotes eurystomus*. Tightly bound PCNA/cyclin was localized at the site of DNA synthesis in macronuclei, the rear zone of the replication band. Starvation or heat shock, conditions that reduce macronuclear replication, resulted in a decrease of PCNA/cyclin in replication bands. Micronuclei also exhibited PCNA/cyclin localization which persisted for a large proportion of the vegetative cell cycle and exhibited significant resistance to adverse culture conditions. Immunoprecipitation of ^{35}S-labeled soluble *Euplotes* proteins with PCNA/cyclin autoimmune sera revealed a spectrum of low molecular mass proteins. PCNA/cyclin-like proteins have now been observed in the widely divergent species: human, rat, amphibian, yeast, and ciliated protozoa.

CONSIDERABLE new information has advanced our understanding of the mechanisms of eukaryotic DNA replication (Huberman, 1987; Campbell, 1988; Kornberg, 1988; Stillman, 1988; Lehman and Kaguni, 1989). It appears likely that two distinct DNA polymerases (α and δ) operate simultaneously upon the progressing replication fork. A current view (Hammond et al., 1987; Focher et al., 1988*a*; Prelich and Stillman, 1988; Stillman, 1988; Zuber et al., 1989) is that the activities of α and δ are coordinated. DNA polymerase α (which may have multiple forms, see Sabatino et al., 1988) is associated with a primase and operates on the lagging (or discontinuous) strand of the replication fork. DNA polymerase δ is suggested to operate on the leading (or continuous) strand, and is associated with a proofreading exonuclease (Sabatino and Bambara, 1988); generally, no primase has been detected associated with δ. In vitro (Prelich and Stillman, 1988), the processivity (i.e., the number of nucleotides added to a nascent strand before enzyme dissociation) for DNA polymerase δ on the leading strand is markedly increased by the addition of a protein, variously named proliferating cell nuclear antigen (PCNA),[1] cyclin, or DNA polymerase δ auxiliary protein. There remains considerable controversy with the view of a PCNA-dependent DNA polymerase δ, and resultant coordination of α and δ activities (see Lehman and Kaguni, 1989). Besides the description of a PCNA-independent DNA polymerase δ (Focher et al., 1988*b*; Syvaoja and Linn, 1989), there is a report that PCNA may act indirectly on DNA polymerase δ by inactivating an inhibitory factor (Lee et al., 1988).

PCNA was originally described as a ~36-kD nuclear protein antigen of proliferating cells, detected by the autoantibodies of a subset of human patients with systemic lupus erythematosus (Miyachi et al., 1978; Takasaki et al., 1981, 1984; Tan, 1989). Independently, Celis and co-workers (Bravo and Celis, 1980; Bravo et al., 1981) identified a nuclear protein (cyclin) by two-dimensional gel electrophoresis which correlated with the proliferative state of cultured mammalian cells. PCNA and cyclin were later shown to be identical proteins (Matthews et al., 1984). Subsequent studies presented evidence that PCNA/cyclin microscopically colocalizes with sites of DNA synthesis in the nuclei of proliferating interphase mammalian tissue-culture cells (Celis and Celis, 1985*a*; Madsen and Celis, 1985; Celis et al., 1987; Bravo and Macdonald-Bravo, 1985; Bravo, 1986). Additional enzymologic studies established that PCNA/cyclin behaves as an accessory protein to DNA polymerase δ, whose in vitro activity is inhibited by addition of antibodies to PCNA/cyclin (Tan et al., 1987) and is stimulated by addition of purified PCNA/cyclin (Prelich et al., 1987). The gene for human PCNA/cyclin was isolated after screening a cDNA expression library with specific rabbit antisera (Almendral et al., 1987), and sequenced predicting a 29-kD protein (lower than the original molecular mass measured by SDS-PAGE) with a high ratio of acidic/basic residues. The gene for rat PCNA/cyclin was also obtained from a cDNA library screened with an oligonucleotide probe specific for the amino-terminal 25 residues of the rabbit protein (Mat-

1. *Abbreviations used in this paper*: FZ, forward zone; NGS; normal goat serum; PCNA, proliferating cell nuclear antigen; RB, replication band; RZ, rear zone.

sumoto et al., 1987) and sequenced, also predicting a 29-kD protein. A yeast analogue of mammalian PCNA/cyclin has recently been identified based upon its capacity to stimulate yeast DNA polymerase III, and possessing a subunit molecular mass of 26 kD as determined by SDS-PAGE (Bauer and Burgers, 1988). Unfertilized *Xenopus* eggs also appear to possess a PCNA/cyclin protein of ~36 kD, estimated by SDS-PAGE (Zuber et al., 1989). The mechanism of PCNA/cyclin stimulation of DNA polymerase δ is still not understood, but a mode of action analogous to the β-subunit of *Escherichia coli* DNA polymerase III has been suggested (Prelich and Stillman, 1988). There remains the possibility, mentioned above (Lee et al., 1988), of an indirect effect of PCNA/cyclin on DNA polymerase δ activity.

The sites of DNA synthesis within interphase nuclei of proliferating mammalian cells can be demonstrated by autoradiographic analysis of incorporated [^{3}H]thymidine (Madsen and Celis, 1985) or by an immunofluorescent analysis of incorporated 5-bromodeoxyuridine (Nakamura et al., 1986). Under conditions of favorable resolution DNA synthesis domains are scattered in hundreds of microscopic clusters, postulated to be replicon domains (Nakamura et al., 1986). In contrast to these multiple foci of DNA synthesis, the hypotrichous ciliated protozoa (including *Euplotes, Oxytricha*, and *Stylonychia*) exhibit only a few enlarged sites of DNA synthesis within each macronucleus, the replication bands (RBs).

The hypotrichous ciliated protozoa possess several features advantageous for the study of eukaryotic nuclear structure and function (for reviews see Kraut et al., 1986; and Klobutcher and Prescott, 1986). Hypotrichs have two types of nuclei within one cytoplasm: germline micronuclei, composed of chromosomal-size DNA which undergo mitosis and meiosis and are transcriptionally inert; and macronuclei, composed of highly endoreplicated, short gene-size DNA molecules which are transcriptionally active, do not undergo mitosis or meiosis, and replicate with characteristic RBs. *Euplotes eurystomus* possesses one macro- and one micronucleus. The macronucleus is 100–150 μm long, 10–20 μm wide, and "C"-shaped within the intact cell. At the beginning of the macronucleus S-phase (~3–4 h after cell division), which lasts ~10 h in rapidly growing cells (cell doubling time, ~12 h), one RB forms at each tip of a macronucleus. During progression of S-phase the RBs migrate towards each other, fusing at the termination of S-phase. RBs are vectorial structures with a highly stratified ultrastructure (Olins et al., 1981, 1988) composed of a forward zone (FZ) with chromatin reorganization, and a rear zone (RZ), the site of DNA synthesis in vivo (Lin and Prescott, 1985) and in vitro (Olins and Olins, 1987).

In the present investigation we demonstrate that PCNA/cyclin can be recognized in *Euplotes* using human autoantisera specific for PCNA/cyclin. We demonstrate that PCNA/cyclin is concentrated within the RZ of RBs, and is reduced during starvation or heat shock (i.e., conditions that markedly inhibit DNA synthesis). PCNA/cyclin is also localized in micronuclei at defined stages of the cell cycle.

Materials and Methods

Cell Culture

Euplotes eurystomus were purchased from Carolina Biological Supply Co. (Burlington, NC) and maintained in Pringsheim medium on a diet of the alga *Chlorogonium elongatum*, as previously described (Allen and Olins, 1984; Allen et al., 1986; Cadilla et al., 1986). To achieve high levels of cells with RBs, *Euplotes* were generally starved 3–4 d, then harvested 18–24 h after feeding.

Antisera

Human sera (AK and EB) monospecific for PCNA/cyclin (Takasaki et al., 1984; Ogata et al., 1987; Tan et al., 1987) were used for all immunostaining. FITC rabbit anti-human IgG (γ-chain) was obtained from Behring Diagnostics (La Jolla, CA). For immunoelectron microscopic localization of PCNA/cyclin, 5-nm gold-conjugated goat anti-human IgG was used (Janssen Life Sciences, Olen, Belgium).

Absorption of Anti-PCNA Autoantibodies with Purified PCNA

PCNA was purified from saline extract of rabbit thymus by a multistep procedure using ammonium sulfate fractionation, DEAE-sephacel, HPLC ion-exchange chromatography, and HPLC gel-filtration chromatography (Ogata et al., 1985). The 36-kD PCNA was purified 615-fold from starting material when examined by Coomassie blue staining of SDS-PAGE (Ogata et al., 1985). This material was used in absorption of anti-PCNA from AK serum. Equal volumes of AK serum and purified PCNA (20 μg/ml) were mixed to make a final dilution of AK serum of 1:50, and incubated at room temperature for 50 min. The control consisted of a mixture of AK serum and buffered saline. Absorbed and control AK serum were tested by indirect immunofluorescence on human HEp-2 cells: AK serum + saline yielded 3+ nuclear staining; AK serum + PCNA yielded ± nuclear staining.

Cell Permeabilization and Antibody Staining

Live *E. eurystomus* were permeabilized in the detergent-microtubule-stabilizing buffer developed by Schliwa and Van Blerkom (1981). "PHEM" buffer consists of 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$ (pH 6.9). PHEM-Triton buffer contained an additional 0.5% Triton X-100.

For immunofluorescent studies, equal volumes of concentrated cells and 2× PHEM-Triton buffer were mixed. Permeabilization proceeded for 5 min at room temperature. Fresh paraformaldehyde (pH ~7) was added to a final concentration of 2% and fixation proceeded for 10 min at room temperature. Cells were centrifuged (Shandon-Elliott cyto-centrifuge; Sewickley, PA) onto chromalum-gelatin-subbed slides and washed in PBS. Human sera (AK or EB), diluted 1:40 with PBS (pH 6.9), were incubated with slides under a coverslip in a moist chamber for 30 min at 37°C. After PBS washes, the FITC-secondary antibody (diluted 1:50 with PBS) was applied for 30 min at 37°C. The PBS-washed slides were mounted in mowiol (Hoechst, A. G., Frankfurt-am-Main, FRG). Slides were examined using a Carl Zeiss Inc. (Thornwood, NY) photomicroscope III with epifluorescence. Photography was on Eastman Kodak Co. (Rochester, NY) Tri-x film. In one experiment fixation preceded permeabilization, modified from an earlier protocol (Kurki et al., 1988). Live cells were incubated with 1% formaldehyde, fixed for 2 min, centrifuged onto subbed slides, plunged into absolute methanol at −20°C for 10 min, treated briefly (1 min) in 0.5% NP-40/PBS, washed extensively with PBS, and treated with antibody as described above.

For immunogold electron microscopic localization of PCNA/cyclin, the procedure suggested by Janssen Life Sciences Products (Piscataway, NJ) was modified to our requirements. Permeabilized *Euplotes* were fixed in 1% glutaraldehyde for 25 min at room temperature, centrifuged onto subbed coverslips, washed with PBS, washed twice (10 min each) in 50 mM NH_4Cl/PBS, and again in PBS. Blocking was achieved by incubating the coverslips with 5% (preheated) normal goat serum (NGS) and 1% BSA in PBS (NGS/BSA/PBS) for 20 min at room temperature. Human serum (AK), diluted 1:50 in NGS/BSA/PBS, was applied to the coverslip and incubated at 37°C for 30 min in a moist chamber. Three washes in 0.1% BSA/PBS, 5 min each, were followed by application of the gold-conjugated second antibody diluted 1:25 in 0.1% BSA/PBS, at 37°C for 30 min. After a PBS wash and two washes in 0.1 M Sorenson's phosphate buffer, coverslips were fixed in 1% glutaraldehyde in 0.1 M Sorenson's buffer for 30 min at room temperature, washed, and postfixed in 1% OsO_4 in 0.1 M Sorenson's buffer. Dehydration, embedding in Epon, and cleavage of the plastic from the coverslip were as described previously (Olins et al., 1988). Some sections were stained both with uranyl acetate and lead citrate. Some were stained only with lead citrate, which allowed easier visualization of the gold particles, but yielded poorer contrast of nuclear substructure.

In Vitro Replication Assay on Permeabilized Cells

Detection of incorporated biotinylated dUTP into the replication bands of permeabilized *Euplotes* was based upon the previously described procedure with isolated macronuclei (Olins and Olins, 1987), with the following modification. 20 μl of *Euplotes* were pipetted onto a subbed slide, followed by 20 μl of 2× PHEM-Triton buffer. Permeabilization proceeded for 5 min at room temperature. An agarose coverslip was applied to the slide, followed by 20 μl of a nucleotide mixture containing biotin-11-dUTP. Incubation was for 90 min at 37°C in a moist chamber. Subsequent fixation and detection were as described earlier (Olins and Olins, 1987).

Immunoprecipitation Procedure

To identify the *Euplotes* soluble proteins with accessible PCNA/cyclin epitopes, immunoprecipitation procedures were used. The use of such techniques proved necessary after repeated unsuccessful attempts to obtain unequivocal immunoblots. Rapidly growing *Euplotes* (12 ml) were labeled with ^{35}S-amino acids (Tran ^{35}S-label; ICN Biomedicals, Irvine, CA) at a ^{35}S concentration of 100 μCi/ml for 18 h. Cells were harvested by low speed centrifugation in a clinical centrifuge, washed in buffer A (140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.5), and collected by low speed centrifugation. The cell pellet was lysed by incubation in 1 ml of buffer A plus 1% NP-40, 1 mM PMSF, and 1 mM TLCK for three min at room temperature, and centrifuged in an Eppendorf centrifuge (Brinkmann Instruments Co., Westbury, NY) for 10 min. The resulting supernatant was precleared by addition of 100 μl 10% protein A-Sepharose (suspended in 0.1% BSA, NET2+F buffer), followed by a brief centrifugation. NET2+F buffer consists of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.5% NP-40, 0.1% SDS, 0.02% Na-Azide, 0.5% deoxycholic acid. 10 μl serum, 100 μl protein A-Sepharose, and 500 μl NET2+F were incubated for 1 h at room temperature, centrifuged, and the pellet washed three times with NET2+F buffer. 50 μl precleared *Euplotes* extract, 10 μl 1% BSA, and 500 μl NET2+F were mixed with the washed Ig-coated protein A-Sepharose and incubated for 3 h at room temperature with continual gentle shaking. After centrifugation, the precipitate was washed five to six times with NET2+F, and finally dispersed in electrophoresis sample buffer with a final concentration of 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 62.5 mM Tris (pH 6.8), and bromophenol blue. Samples were boiled, centrifuged, and loaded on SDS-PAGE gels (15% acrylamide). After electrophoresis, the gels were stained with Coomassie blue, dehydrated with DMSO, infiltrated with 2,5-Diphenyl oxazole, dried, and exposed to x-ray film at −70°C without the use of an intensifying screen. Some of the *Euplotes* extract was used immediately; the remainder was stored at −70°C. Five human sera were tested: two (AK and EB) with well documented PCNA/cyclin specificity; and three normal sera. The normal sera were from a collection obtained at the Biology Division (Oak Ridge National Laboratory) during 1978, and stored at −70°C.

Results

Immunolocalization and Cell Cycle Distribution of PCNA/Cyclin Epitopes

In mammalian tissue-culture cells, evidence has been presented for the existence of two populations of PCNA/cyclin (Bravo and Macdonald-Bravo, 1987): an easily extracted fraction found in the nucleoplasm of both quiescent and proliferating cells; and a tightly bound fraction associated with sites of DNA synthesis within S-phase nuclei. The loosely bound fraction is readily extracted from cells by buffers containing Triton X-100; the tight fraction persists at its site during detergent extraction and remains bound at up to 0.5 M NaCl. In S-phase tissue culture cells, as much as 70–80% of the total PCNA/cyclin is removed by Triton X-100; the remaining 20–30% is believed to be part of the stable replicative complex.

The two preparative conditions used in this study for immunolocalization of PCNA/cyclin epitopes in *Euplotes* (i.e., fixation before permeabilization, and permeabilization before fixation) might be anticipated to reveal staining differences attributable to the existence of the two populations. Fig. 1 compares the results obtained with the different preparative conditions. When brief formaldehyde fixation preceded methanol and NP-40/PBS permeabilization, the cells (Fig. 1, *A* and *B*) were thicker and possessed considerable cytoplasmic fluorescence. Even so, RB and micronuclear fluorescence could be readily observed. Comparisons among cells incubated with a variety of human sera (data not shown) indicated that much of this cytoplasmic fluorescence is not specific. Permeabilization before fixation, generating a cytoskeletal–nuclear complex (Olins et al., 1989), yielded cells which were much flatter with very clear PCNA/cyclin localization within RBs and micronuclei, and minimal cytoplasmic fluorescence (Fig. 1, *C* and *D*). The slight macronuclear swelling and flattening, combined with a lower cytoplasmic fluorescence, may account for the apparently broadened localization of PCNA/cyclin seen in Fig. 1 *C*, compared to Fig. 1, *A* and *B*. Sera AK and EB yielded essentially identical images, although AK appeared stronger and exhibited lower background fluorescence. That the sites of localization within permeabilized cells represent epitopes shared with PCNA/cyclin, was demonstrated by an immunoabsorption experiment of serum AK with purified PCNA/cyclin (Fig. 2). Absorbed serum revealed only minimal background fluorescence with permeabilized *Euplotes* (Fig. 2, *C* and *D*); macronuclear RBs and micronuclei were devoid of reaction after absorption of AK serum. Two mouse monoclonal antibodies (19A2 and 19F4; see Ogata et al., 1987) developed against purified rabbit PCNA/cyclin were also tested on permeabilized *Euplotes*, but did not give positive immunostaining. Previous comparisons between these monoclonal antibodies and the human autoantibodies indicated that there are some differences in the spectrum of recognized epitopes (Ogata et al., 1987).

Searching the microscopic preparations of permeabilized immunostained *Euplotes* for PCNA/cyclin distribution, it became clear that cells at different stages of the vegetative cycle revealed characteristic staining patterns. Cells were photographed in both phase and fluorescence, permitting an accurate diagnosis of cell stage and PCNA/cyclin localization. Since in all cases the cells were permeabilized with PHEM-Triton buffer before formaldehyde fixation, the sites of PCNA/cyclin localization are regarded as tight-binding regions. Fig. 3 presents a montage of immunofluorescent cells arranged sequentially through the vegetative cell cycle. During rapid cell growth, as many as 80% of the cells exhibit RBs, consistent with the percentage of the cell cycle occupied by macronuclear S-phase. Fig. 3 *A* starts with a cell at the very beginning of macronuclear S-phase; one RB has just begun to show the PCNA/cyclin epitope. Fig. 3 *B*, a somewhat later cell, reveals two RBs that are staining. Progressing along Fig. 3, C–K, the RBs migrate towards one another, eventually fuse, and disappear. No RB staining is seen until after cytokinesis and the beginning of a new macronuclear S-phase. The intensity of RB staining reproducibly appears to increase throughout macronuclear S-phase. A partial explanation for this may derive from the coincident apparent shortening and fattening of macronuclei during S-phase, resulting in more apparent replication per unit length. Micronuclei exhibit a different temporal pattern of staining for PCNA/cyclin. Staining first becomes apparent during early cytokinesis (Fig. 3 *L*); micronuclear mitosis has recently been completed, and both daughter micronuclei are positive for PCNA/cyclin. Staining persists throughout

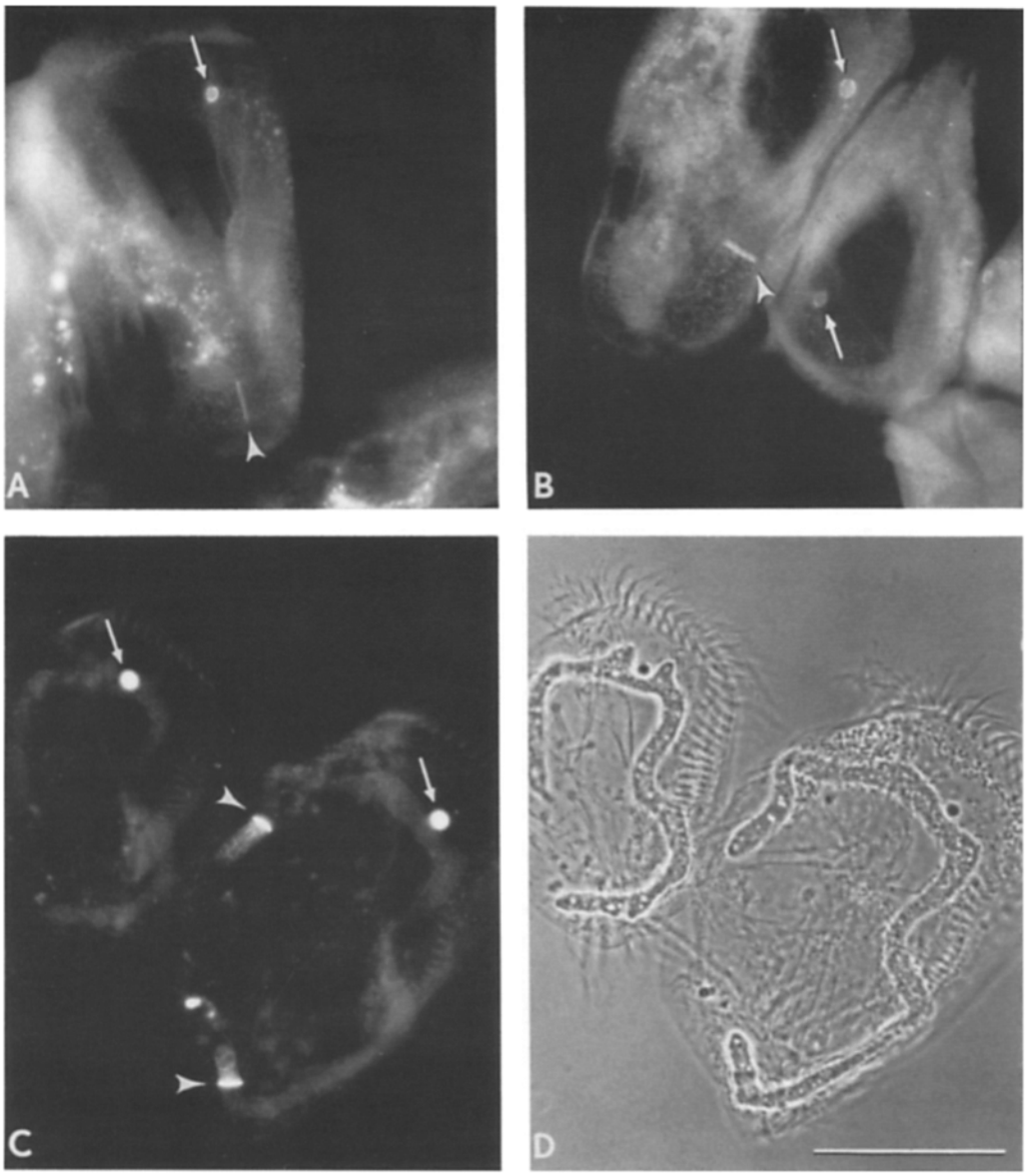

Figure 1. Immunofluorescent localization of PCNA/cyclin epitopes. (*A* and *B*) Cells fixed before permeabilization and immunostaining. (*C* and *D*) Cells permeabilized before fixation; *D* is a phase-contrast image of the field shown in *C*. *Thin arrows*, micronuclei; *arrowheads*, RBs. Bar, 50 μm.

cytokinesis (Fig. 3, *L*, *M*, and *N*), in the nascent daughter cells (Fig. 3 *O*), and until mid-macronuclear S-phase (Fig. 3, *A–F*). Micronuclear staining disappears during late macronuclear S (Fig. 3, *G–K*).

There is much less information about the timing of micronuclear events in *Euplotes* compared to macronuclear events. Using quantitative Feulgen microspectrophotometry, evidence has been presented that micronuclear S-phase occurs around the period of observable cytokinesis (Prescott et al., 1962). Morphologic studies (reviewed in Fleury, 1986; see also Ruffolo, 1976) indicate that micronuclear mitosis occurs during late macronuclear S-phase. Therefore, the present immunolocalization studies of tightly bound and exposed PCNA/cyclin epitopes generally coincides temporally and spatially with known sites of DNA replication within *Euplotes*.

DNA Replication by Permeabilized Euplotes

We have previously demonstrated (Olins and Olins, 1987) that isolated macronuclei incubated in vitro with a mixture of dNTPs, including biotin-11-dUTP, incorporate the nucleotides specifically into the RB for a period of several hours, as detected with rabbit antibiotin and FITC–goat anti–rabbit IgG. Incorporation of the biotin-11-dUTP was observed to be localized in the RZ, and was inhibited by added *N*-ethylmaleimide or aphidicolin, and by prior in vivo heat shock. In the present study we have tested whether the Triton X-100–permeabilized cells are also competent to replicate macronuclear DNA in vitro. This is of particular interest since the same types of permeabilized cell preparations were shown above to possess PCNA/cyclin within RBs by immunocytochemical criteria. Fig. 4 presents representative cells at

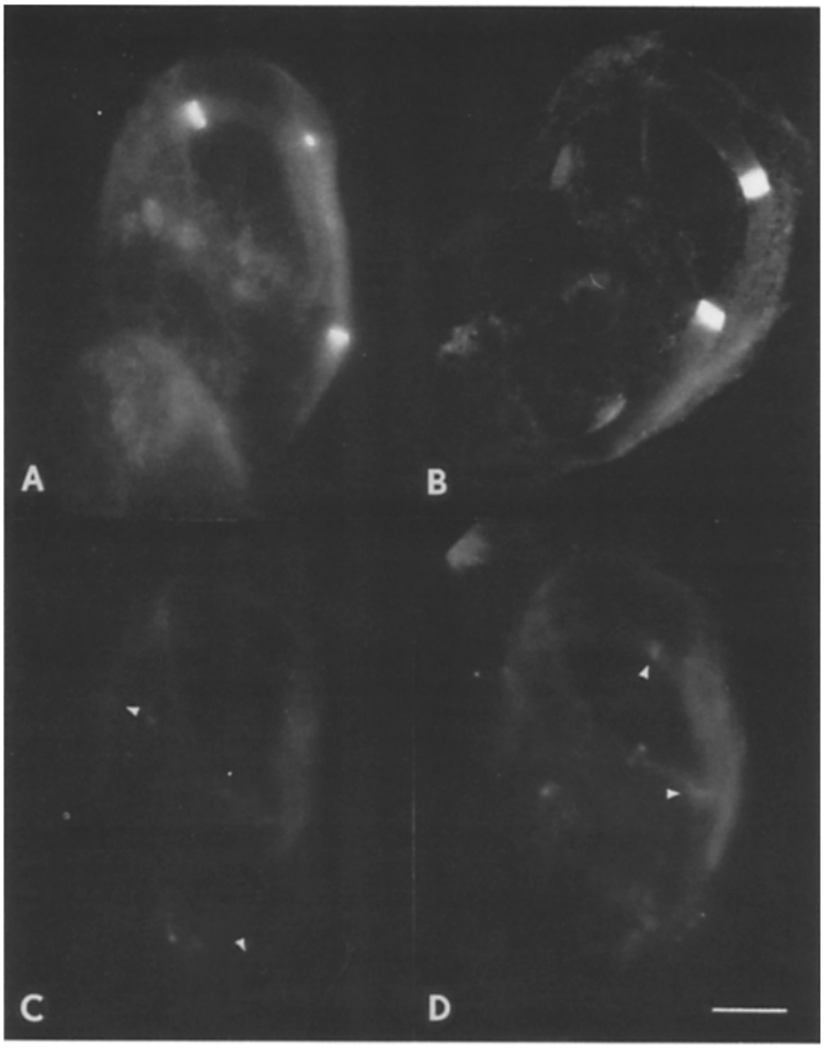

Figure 2. Effect of absorption of human autoimmune (AK) serum with purified PCNA/cyclin upon immunostaining of permeabilized *Euplotes*. (*A* and *B*) AK control (unabsorbed) serum, showing strong reactions with RBs and a micronucleus. (*C* and *D*) AK serum absorbed with PCNA/cyclin. Trace reactions with RBs are indicated by the arrowheads. We do not consider this level of reaction significant. Bar, 25 μm.

different stages of macronuclear S-phase. The results clearly demonstrated that, based upon this immunocytochemical analysis, RBs were comparably active in permeabilized cells and in isolated macronuclei from lysed cells. Several cells were observed during cytokinesis which appeared to have incorporated biotin-11-dUTP into micronuclei (data not shown); but the intensity was never as strong as that observed in RBs.

Starvation and Heat Shock Effects on PCNA/Cyclin Localization

The in vivo activity of RBs is sensitive to environmental conditions. Two conditions used in our laboratory are prolonged starvation (e.g., *Euplotes* can be starved for 8–10 d with no evident harm to the cells), or sublethal heat shock (i.e., 36.5°C for several hours). Starvation results in a gradual decline of the percentage of cells exhibiting RBs reaching a basal level (0–20% depending upon the sample); refeeding results in a rapid increase of cells with RBs, with a peak (~80–90%) between 18 and 24 h. Heat shock results in a rapid reduction of nucleotide incorporation into RBs in vivo (Evenson and Prescott, 1970) and in vitro (Olins and Olins, 1987), and a rapid collapse of the RZ with no evident changes in the FZ (Olins et al., 1988).

The results of staining for PCNA/cyclin in permeabilized *Euplotes* after starvation for 8 d are presented in Fig. 5. As anticipated, only a small percentage (19%) of cells revealed RBs. The few RBs that could be found exhibited weak (or negligible) staining for PCNA/cyclin. By contrast, virtually all (98%) micronuclei revealed intense localization of PCNA/cyclin. Heat shock (37°C for 90 min) resulted in similar, although less dramatic, staining patterns. There was a higher titer (39%) of cells with RBs, and many exhibited clear localization of PCNA/cyclin. However, for the vast majority of cells examined (86%) the relative staining intensities of RBs, compared to the micronuclei of the same cell, were considerably reduced in the heat-shocked cells compared to normal cells (data not shown). Micronuclear staining did not appear affected by heat shock (96% showed strong staining). To summarize, the presence of tightly bound PCNA/cyclin appeared to correlate with the physiological state of the RB (i.e., a reduced content correlating with reduced replication); and micronuclear localization seemed to persist under a wider range of environmental conditions.

PCNA/Cyclin Is Localized With the RZ of the RB

The stratification of the RB represents a functional as well as a structural differentiation (Olins et al., 1981, 1988) with the localization of replication being confined to the RZ (Lin

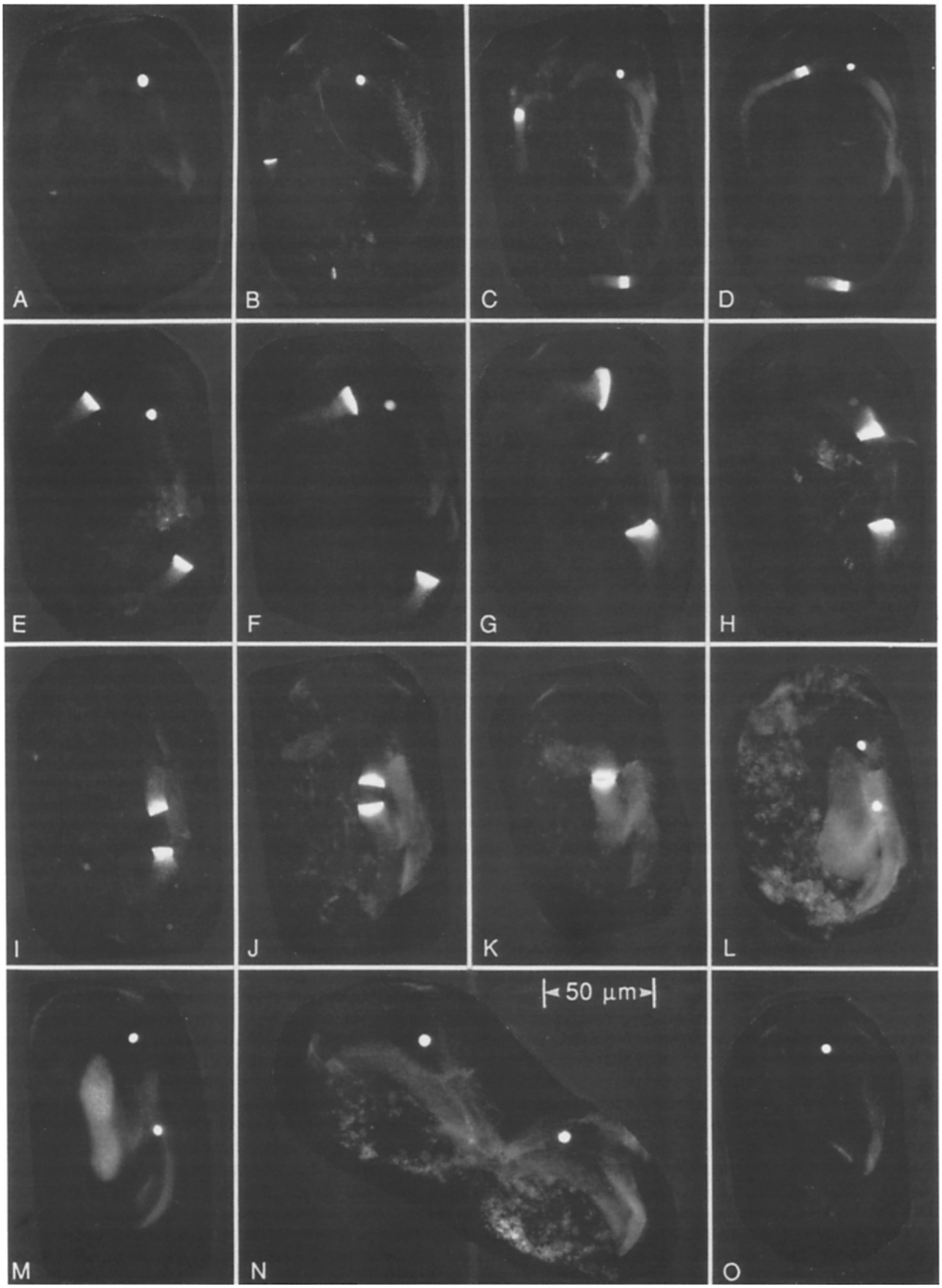

Figure 3. Localization of tightly bound PCNA/cyclin within RBs and micronuclei arranged in a temporal sequence around the cell cycle. With the exception of *N*, all cells are arranged with the anterior end pointed up, similar to the small cell of Fig. 1 *D*. The macronucleus is curved into a backward "C" until S-phase (*I*, *J*, and *K*), cytokinesis and amitosis are shown (*L*, *M*, and *N*). Bar, 50 μm.

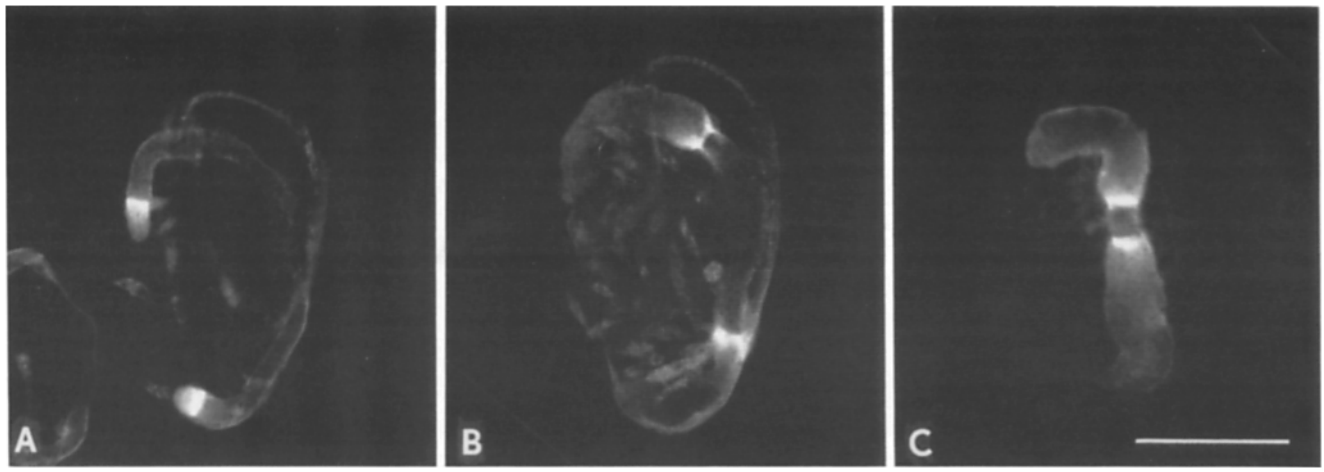

Figure 4. In vitro incorporation of biotinylated dUTP into the RBs of permeabilized cells. Three stages of macronuclear S-phase are represented, with the cells oriented in the same direction as in Fig. 1 *D*, with the anterior end up. Bar, 50 μm.

and Prescott, 1985). Although distinguishing between FZ and RZ is best accomplished by electron microscopy, under appropriate conditions the light microscope can achieve adequate resolution. Under such conditions, using phase optics, the FZ appears highly refractile (or phase dense) and the RZ appears empty (or phase light). Fig. 6 presents three RBs from permeabilized cells fixed with 1% glutaraldehyde before immune staining, where the phase images are aligned with the corresponding fluorescent localization of PCNA/cyclin. In all cases the maximum fluorescence superimposes with the phase light region; i.e., the RZ of the RB. This can be best seen on the microscope by varying the intensity of the phase image while observing the fluorescent localization.

Immunoelectron microscopy using 5-nm gold-conjugated anti-human IgG substantiated the phase/fluorescent observations described above. Fig. 7 reveals that gold particles were primarily localized within the RZ, compared to the FZ or macronuclear regions outside the RBs. In the environment of an RB, we frequently observed a decreasing gradient of gold particles moving laterally from the periphery of the macronucleus towards the center, suggesting a possible penetration problem (recall that the macronucleus and RB is 10–20 μm in lateral thickness). Away from the RB, macronuclear surfaces exhibited occasional gold particles; although, far fewer than at the periphery of RBs. In sum, both high resolution immunofluorescent microscopy and preembedded immunogold electron microscopy indicated that tightly bound PCNA/cyclin is principally concentrated within the RZ of RBs, the established region of macronuclear DNA synthesis.

Immunoprecipitation of PCNA/Cyclin Proteins

Buffer-extractable ^{35}S-labeled proteins prepared from rapidly growing *Euplotes* were reacted with human IgG–coated protein A-Sepharose beads. Five human sera were examined: the PCNA/cyclin-specific sera AK and EB, and three normal sera. Autoradiograms of SDS-PAGE of ^{35}S-immunoprecipitated *Euplotes* proteins revealed multiple bands (Fig. 8). Three bands (~23, 24, and 27 kD) were precipitated by AK and EB sera, but not by normal human sera. Several bands (~35 and 51–52 kD) were observed with PCNA/cyclin-specific and normal human sera. We tentatively identify the cluster of lower molecular mass bands with the extractable *Euplotes* PCNA/cyclin-like proteins on the basis of their apparent molecular masses and their reactivity with the PCNA/cyclin-specific sera.

Discussion

Several major observations develop from this study: (*a*) epitopes of PCNA/cyclin (auxiliary protein for DNA polymerase δ), originally recognized in mammalian cells, are observed in the distantly related ciliated protozoa, *Euplotes eurystomus*; (*b*) tightly bound PCNA/cyclin is localized within the rear zone of macronuclear RBs, where its presence correlates with replicational activity; (*c*) micronuclear PCNA/cyclin localization occurs over a wide interval of the vegetative cell cycle, encompassing a period greater than micronuclear S-phase, and apparently independent of inhibitory effects upon macronuclear DNA synthesis; and (*d*) immunoprecipitation of soluble *Euplotes* proteins tentatively identified multiple PCNA/cyclin-like proteins with molecular masses of ~23–27 kD.

The function of PCNA/cyclin within replication bands and micronuclei of *Euplotes* is not known; but, by analogy with studies in higher eukaryotic systems (e.g., Tan et al., 1987; Prelich et al., 1987; Prelich and Stillman, 1988; Stillman, 1988) and its localization at sites of DNA replication (i.e., the RZ of RBs), we suspect an interaction with a *Euplotes* DNA polymerase δ. Our previous study of in vitro incorporation into RBs (Olins and Olins, 1987) did not attempt to distinguish between putative α and δ DNA polymerases. We demonstrated inhibition by *N*-ethylmaleimide and by aphidicolin, both of which are effective against DNA polymerases α and δ (Hammond et al., 1987). The development of a more quantitative in vitro assay, and the use of inhibitors with greater specificity towards α (Hammond et al., 1987) could furnish strong evidence for the existence of a *Euplotes* DNA polymerase δ. Another approach being attempted in our laboratory is that of screening a *Euplotes* macronuclear

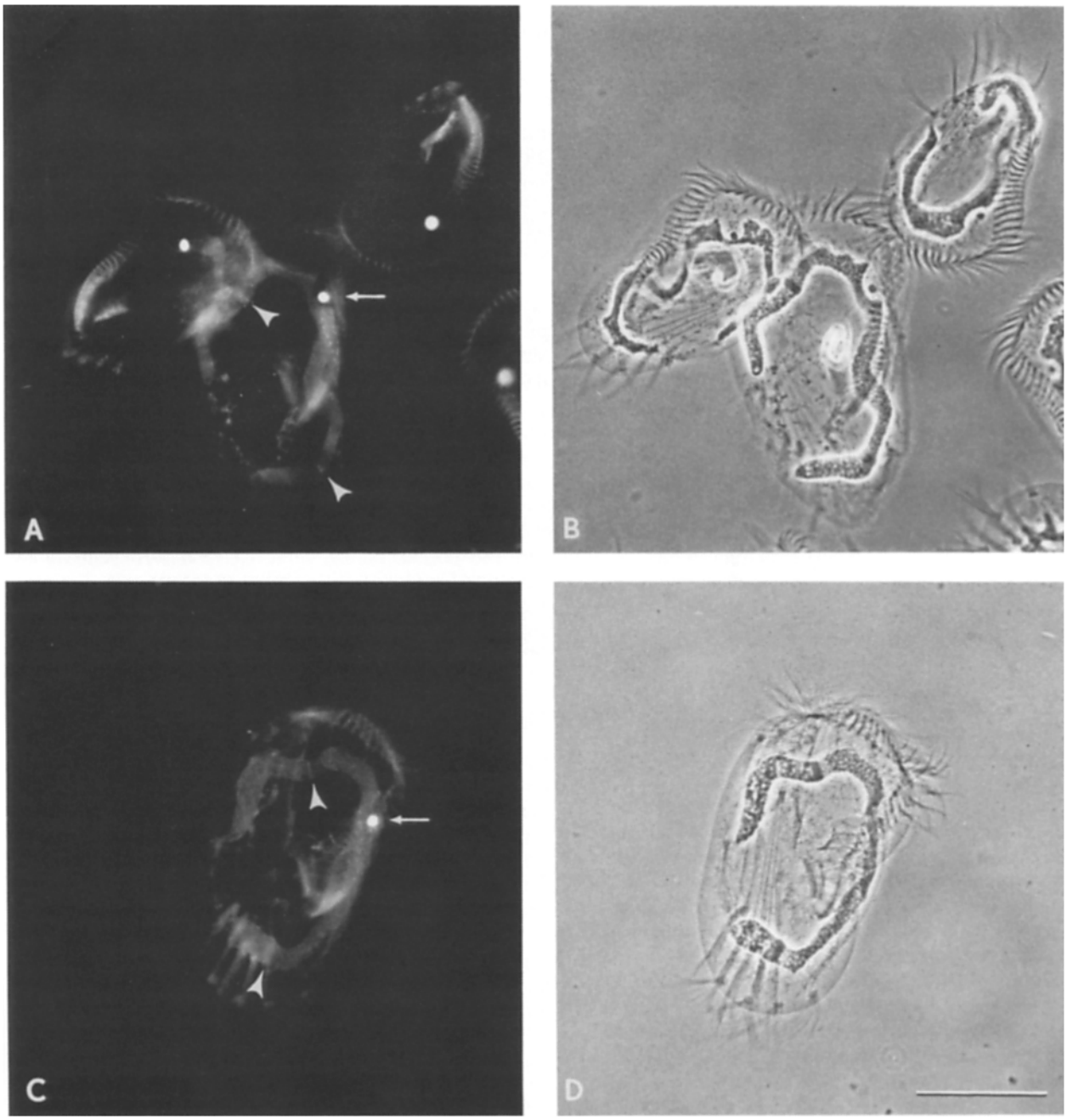

Figure 5. The influence of prolonged starvation upon the location of tightly bound PCNA/cyclin within permeabilized *Euplotes*. *A* and *C* are immunofluorescent images; *B* and *D*, phase-contrast images. *Thin arrows*, micronuclei; *arrowheads*, RBs. The apparent increased fluorescence of membranelles is a photographic artifact, arising from the use of automatic exposure on the microscope, where the exposure time was determined by the fluorescence of the tiny micronuclei. Bar, 50 μm.

DNA library with defined heterologous probes to conserved domains of DNA polymerase.

The apparent persistence of PCNA/cyclin staining in micronuclei beyond micronuclear S-phase is somewhat puzzling. It is possible that there is a fraction of late replicating DNA that was not detected in the earlier quantitative Feulgen cytophotometric study (Prescott et al., 1962). An alternative explanation is that accumulation of PCNA/cyclin can occur in the absence of DNA synthesis. If tissue-culture cells are blocked at the G1/S boundary, no DNA synthesis takes place, but there is the usual accumulation of PCNA/cyclin (Bravo and Macdonald-Bravo, 1985; Bravo, 1986). The observation that two different types of nuclei contained within one cytoplasm can reveal independent timing of PCNA/cyclin binding (and DNA synthesis) has an interesting parallel to experiments with multinucleated tissue-culture cells (Celis and Celis, 1985*b*). Polyethylene glycol–induced fusion of transformed human amniotic cells resulted in the production

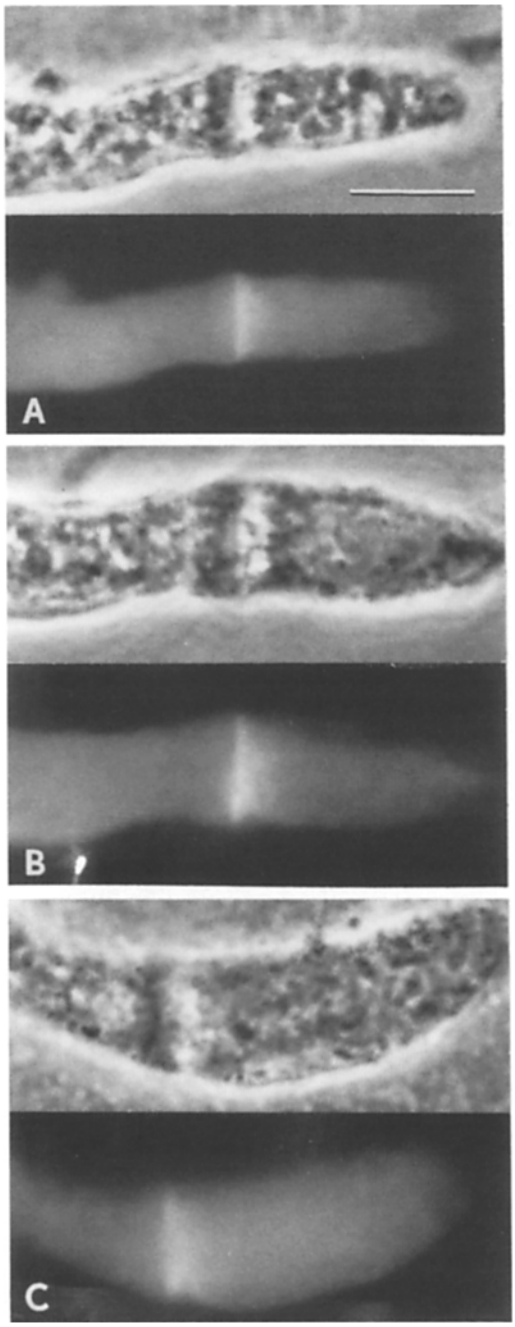

Figure 6. High resolution immunofluorescent localization of tightly bound PCNA/cyclin within macronuclear RBs of permeabilized *Euplotes*. Three pairs of images are presented; the top panel of each is the phase-contrast image, the bottom is the fluorescent image. RBs are oriented so that movement is from right to left. Bar, 10 μm.

of some multinucleated cells where the timing of PCNA/cyclin binding and coincident DNA synthesis differed among the different nuclei within one cytoplasm. We were also surprised that PCNA/cyclin epitopes persist within micronuclei under conditions of environmental stress that might be expected to inhibit replication. It should be noted, however, that starvation of *Euplotes* appears to stimulate conjugation. Whereas the macronucleus only synthesizes DNA during the vegetative cycle, the micronucleus must be triggered to synthesize DNA before mitosis or meiosis.

The estimated molecular masses of the *Euplotes* PCNA/cyclin-like proteins are in reasonable agreement with those of yeast (~26 kD; Bauer and Burgers, 1988), and rat and human (~29 kD; Almendral et al., 1987; Matsumoto et al., 1987). The multiplicity of PCNA/cyclin-like proteins, observed in this study, has some precedent. Matsumoto et al. (1987) report Southern hybridization analyses of restriction enzyme-treated human and rat genomic DNA with cDNA probes. Humans appear to possess a single gene for PCNA/cyclin; rats appear to possess a family of PCNA/cyclin-related genes. It is conceivable that the multiple nuclei of *Euplotes* require different types of PCNA/cyclin. It is also possible that some of the PCNA/cyclin-like proteins represent degradation products. In the present investigation no attempt was made to extract tightly bound PCNA/cyclin for immunoprecipitation analysis. In mouse tissue-culture cells the bound PCNA/cyclin remains unextracted in 0.5 M NaCl (Bravo and Macdonald-Bravo, 1987). The extraction buffer used in this study did not exceed 0.14 M NaCl. Future studies will be directed towards characterizing the tightly bound form(s) of PCNA/cyclin, and using the PCNA/cyclin epitope for immunoaffinity enrichment of replicating macronuclear DNA and chromatin.

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References

Allen, R. L., and D. E. Olins. 1984. Cytochemistry of the replication band in hypotrichous ciliated protozoa staining with silver and thiol-specific courmarin maleimide. *Chromosoma (Berl.).* 91:82–86.

Allen, R. L., S. J. Kennel, L. Cacheiro, A. L. Olins, and D. E. Olins. 1986. Examination of the macronuclear replication band in *Euplotes eurystomus* with monoclonal antibodies. *J. Cell Biol.* 102:131–136.

Almendral, J. M., D. Huebsch, P. A. Blundell, H. Macdonald-Bravo, and R. Bravo. 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. *Proc. Natl. Acad. Sci. USA.* 84: 1575–1579.

Bauer, G. A., and P. M. J. Burgers. 1988. The yeast analog of mammalian cyclin/proliferating-cell nuclear antigen interacts with mammalina DNA polymerase δ. *Proc. Natl. Acad. Sci. USA.* 85:7506–7510.

Bravo, R. 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. *Exp. Cell Res.* 163:287–293.

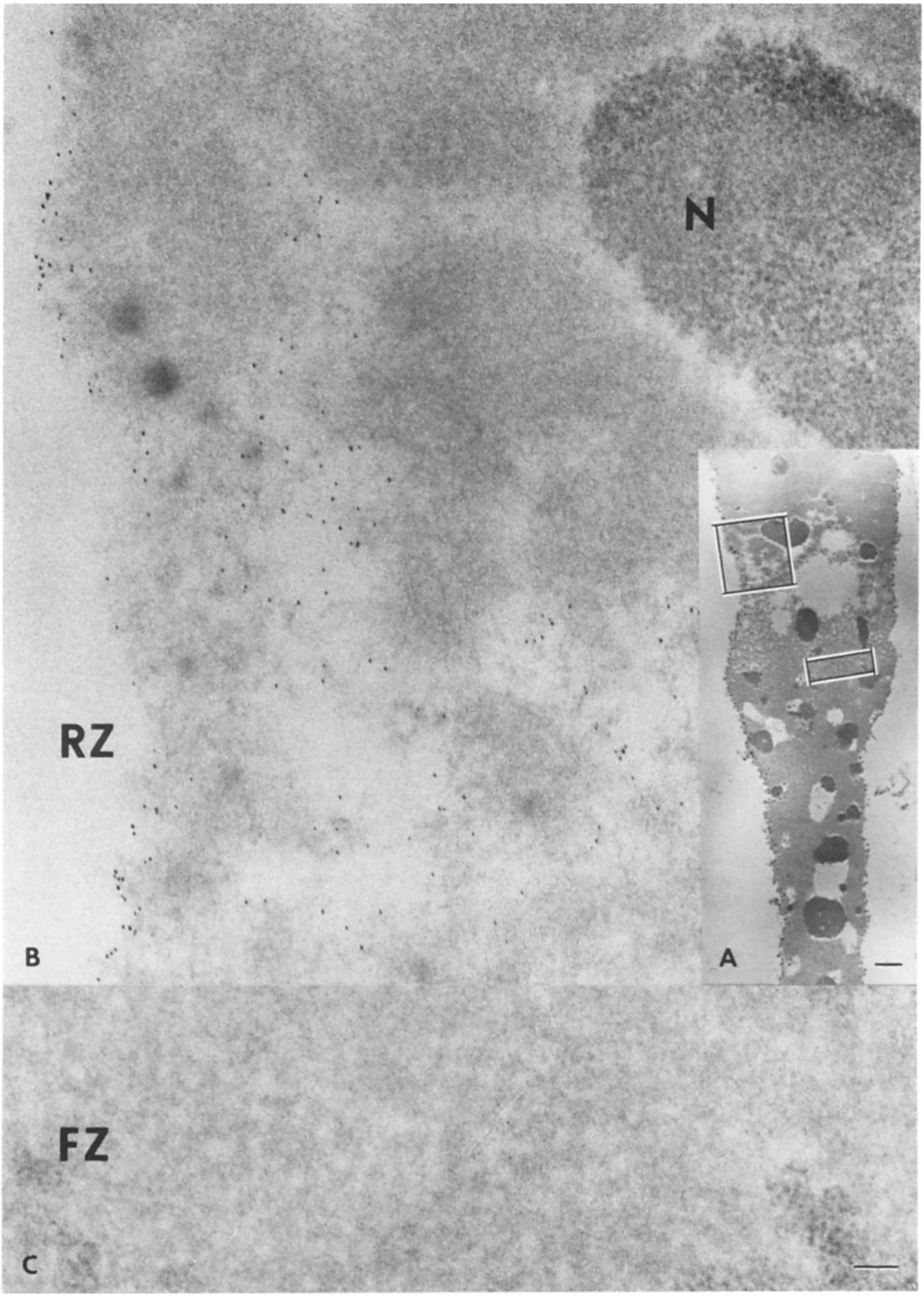
Bravo, R., and J. E. Celis. 1980. A search for differential polypeptide synthesis throughout the cell cycle of Hela cells. *J. Cell Biol.* 84:795–802.

Bravo, R., and H. Macdonald-Bravo. 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:655–661.

Bravo, R., and H. Macdonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549–1554.

Bravo, R., S. J. Fey, J. Bellatin, P. M. Larsen, J. Arevalo, and J. E. Celis. 1981. Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. *Exp. Cell Res.* 136:311–319.

Cadilla, C. L., J. Harp, J. M. Flanagan, A. L. Olins, and D. E. Olins. 1986. Preparation and characterization of soluble macronuclear chromatin from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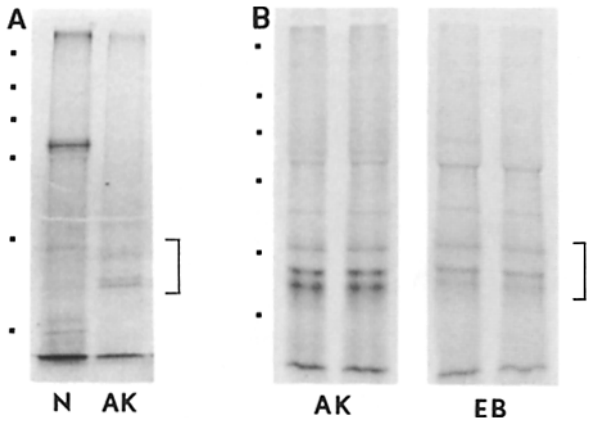

Figure 8. SDS-PAGE autoradiograms of immunoprecipitates of soluble PCNA/cyclin-like proteins from ^{35}S-labeled *Euplotes*. (*A*) Comparison of precipitates using one normal serum (*N*) with those of a PCNA/cyclin-specific serum (*AK*). The other two normal sera gave essentially identical autoradiograms compared to *N*. (*B*) Duplicate lanes of two PCNA/cyclin-specific sera (*AK* and *EB*). Dots aligned adjacent to autoradiograms indicate the positions of prestained molecular mass markers, in descending order 220, 100, 68, 43, 27, and 18 kD. The brackets denote the region of putative PCNA/cyclin proteins. Bands of 35 and 51–52 kD seen with AK and EB sera were also observed with normal human sera. It is not clear with what *Euplotes* proteins normal sera react.

hypotrich *Euplotes eurystomus*. *Nucleic Acids Res.* 14:823–841.

Campbell, J. 1988. Eukaryotic DNA replication: yeast bares its ARSs. *Trends Biochem. Sci.* 13:212–217.

Celis, J. E., and A. Celis. 1985*a*. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin in cultured cells. *Proc. Natl. Acad. Sci. USA.* 82:3262–3266.

Celis, J. E., and A. Celis. 1985*b*. Individual nuclei in polykaryons can control cyclin distribution and DNA synthesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1187–1192.

Celis, J. E., P. Madsen, A. Celis, H. V. Nielsen, and B. Gesser. 1987. Cyclin (PCNA, auxiliary protein of DNA polymerase δ) is a central component of the pathway(s) leading to DNA replication and cell division. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 220:1–7.

Evenson, D. P., and D. M. Prescott. 1970. Disruption of DNA synthesis in *Euplotes* by heat shock. *Exp. Cell Res.* 63:245–252.

Fleury, A. 1986. Morphologie et dynamique morphogenetique chez les cilies hypotriches: implications phylogenetiques et biologiques. Ph.D. thesis. University of Paris-South, Orsay, France. 280 pp.

Focher, F., E. Ferrari, S. Spadari, and U. Hubscher. 1988*a*. Do DNA polymerases δ and α act coordinately as leading and lagging strand replicases? *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 229:6–10.

Focher, F., S. Spadari, B. Ginelli, M. Hottiger, M. Gassmann, and U. Hubscher. 1988*b*. Calf thymus DNA polymerase δ: purification, biochemical and functional properties of the enzyme after its separation from DNA polymerase α, a DNA dependent ATPase and proliferating cell nuclear antigen. *Nucleic Acids Res.* 16:6279–6295.

Hammond, R. A., J. J. Byrnes, and M. R. Miller. 1987. Identification of DNA polymerase δ in CV-1 cells: studies implicating both DNA polymerase δ and DNA polymerase α in DNA replication. *Biochemistry.* 26:6817–6824.

Huberman, J. A. 1987. Eukaryotic DNA replication: a complex picture partially clarified. *Cell.* 48:7–8.

Klobutcher, L. A., and D. M. Prescott. 1986. The special case of the hypotrichs. *In* The molecular Biology of Ciliated Protozoa. J. G. Gall, editor. Academic Press, Inc., Orlando, FL. 111–154.

Kornberg, A. 1988. DNA replication. *J. Biol. Chem.* 263:1–4.

Kraut, H., H. J. Lipps, and D. M. Prescott. 1986. The genome of hypotrichous ciliates. *Int. Rev. Cytol.* 99:1–28.

Kurki, P., K. Ogata, and E. M. Tan. 1988. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA) cyclin as probes for proliferating cells in immunofluorescence microscopy and flow cytometry. *J. Immunol. Methods.* 109:49–59.

Lee, S.-H., Y. Ishimi, M. K. Kenny, P. Bullock, F. B. Dean, and J. Hurwitz. 1988. An inhibitor of the in vitro elongation reaction of simian virus 40 DNA replication is overcome by proliferating-cell nuclear antigen. *Proc. Natl. Acad. Sci. USA.* 85:9469–9473.

Lehman, I. R., and L. S. Kaguni. 1989. DNA polymerase α. *J. Biol. Chem.* 264:4265–4268.

Lin, M., and D. M. Prescott. 1985. Electron microscope autoradiography of DNA synthesis in the replication of two hypotrichous ciliates. *J. Protozool.* 32:144–149.

Madsen, P., and J. E. Celis. 1985. S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 193:5–11.

Matsumoto, K., T. Moriuchi, T. Koji, and P. K. Nakane. 1987. Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:637–642.

Matthews, M. B., R. M. Bernstein, B. R. Franza, Jr., and J. I. Garrels. 1984. Identity of the proliferating cell nuclear antigen and cyclin. *Nature (Lond.).* 309:374–376.

Miyachi, K., M. J. Fritzler, and E. M. Tan. 1978. An autoantibody to a nuclear antigen in proliferating cells. *J. Immunol.* 121:2228–2234.

Nakamura, H., T. Morita, and C. Sato. 1986. Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* 165:291–297.

Ogata, K., P. Kurki, J. E. Celis, R. M. Nakamura, and E. M. Tan. 1987. Monoclonal antibodies to a nuclear protein (PCNA/cyclin) associated with DNA replication. *Exp. Cell Res.* 168:475–486.

Ogata, K., Y. Ogata, R. M. Nakamura, and E. M. Tan. 1985. Purification and N-terminal amino acid sequence of proliferating cell nuclear antigen (PCNA)/cyclin and development of ELISA for anti-PCNA antibodies. *J. Immunol.* 135:2623–2627.

Olins, A. L., D. E. Olins, M. Derenzini, D. Hernandez-Verdun, P. Gounon, M. Robert-Nicoud, and T. M. Jovin. 1988. Replication bands and nucleoli in the macronucleus of *Euplotes eurystomus*: an ultrastructural and cytochemical study. *Biol. Cell.* 62:83–93.

Olins, A. L., D. E. Olins, W. W. Franke, H. J. Lipps, and D. M. Prescott. 1981. Stereo-electron microscopy of nuclear structure and replication in ciliated protozoa (*Hypotricha*). *Eur. J. Cell Biol.* 25:120–130.

Olins, D. E., and A. L. Olins. 1987. In vitro DNA synthesis in the macronuclear replication band of *Euplotes eurystomus*. *J. Cell Biol.* 104:1125–1132.

Olins, D. E., A. L. Olins, M. Robert-Nicoud, T. M. Jovin, J. Wehland, and K. Weber. 1989. Differential distribution of α-tubulin isotypes in *Euplotes eurystomus* determined by confocal immunofluorescence microscopy. *Biol. Cell.* In press.

Prelich, G., and B. Stillman. 1988. Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. *Cell.* 53:117–126.

Prelich, G., C.-K. Tan, M. Kostura, M. B. Matthews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature (Lond.).* 236:517–520.

Prescott, D. M., R. F. Kimball, and R. F. Carrier. 1962. Comparison between the timing of micronuclear and macronuclear DNA synthesis in *Euplotes eurystomus*. *J. Cell Biol.* 13:175–176.

Ruffolo, J. J., Jr. 1976. Fine structures of the dorsal bristle complex and pellicle of *Euplotes*. *J. Morphol.* 148:469–488.

Sabatino, R. D., and R. A. Bambara. 1988. Properties of the 3′ to 5′ exonuclease associated with calf DNA polymerase δ. *Biochemistry.* 27:2266–2271.

Sabatino, R. D., T. W. Myers, R. A. Bambara, O. Kwon-Shin, R. L. Marraccino, and P. H. Frickey. 1988. Calf thymus DNA polymerases α and δ are capable of highly processive DNA synthesis. *Biochemistry.* 27:2998–3004.

Schliwa, M., and J. Van Blerkom. 1981. Structural interaction of cytoskeletal components. *J. Cell Biol.* 90:222–235.

Stillman, B. 1988. Initiation of eukaryotic DNA replication in vitro. *Bioessays.* 9:56–60.

Syvaoja, J., and S. Linn. 1989. Characterization of a large form of DNA polymerase δ from HeLa cells that is insensitive to proliferating cell nuclear antigen. *J. Biol. Chem.* 264:2489–2497.

Takasaki, J., J. S. Deu, and E. M. Tan. 1981. A nuclear antigen associated with cell proliferation and blast transformation. Its distribution in syn-

Figure 7. Immunoelectron microscopic localization of tightly bound PCNA/cyclin with 5-nm gold-conjugated anti-human IgG. (*A*) Low magnification view of a single RB, moving from top to bottom. The areas indicated with boxes are enlarged in *B* (*RZ*, rear zone; *N*, nucleolus and postreplicated chromatin) and *C* (*FZ*, forward zone). This section has been stained with lead citrate only. Bars: (*A*) 1 μm; (*B* and *C*) 0.2 μm.

chronized cells. *J. Exp. Med.* 156:1899–1909.
Takasaki, J., D. Fischwild, and E. M. Tan. 1984. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus area. *J. Exp. Med.* 159:981–992.
Tan, C.-K., K. Sullivan, X. Li, E. M. Tan, K. M. Downey, and A. G. So. 1987. Autoantibody to the proliferating cell nuclear antigen neutralizes the activity of the auxiliary protein for DNA polymerase delta. *Nucleic Acids Res.* 15:9299–9308.
Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* 44:93–151.
Zuber, M., E. M. Tan, and M. Ryoji. 1989. Involvement of proliferating cell nuclear antigen (cyclin) in DNA replication in living cells. *Mol. Cell. Biol.* 9:57–66.