In Vitro DNA Synthesis in the Macronuclear Replication Band of *Euplotes eurystomus*

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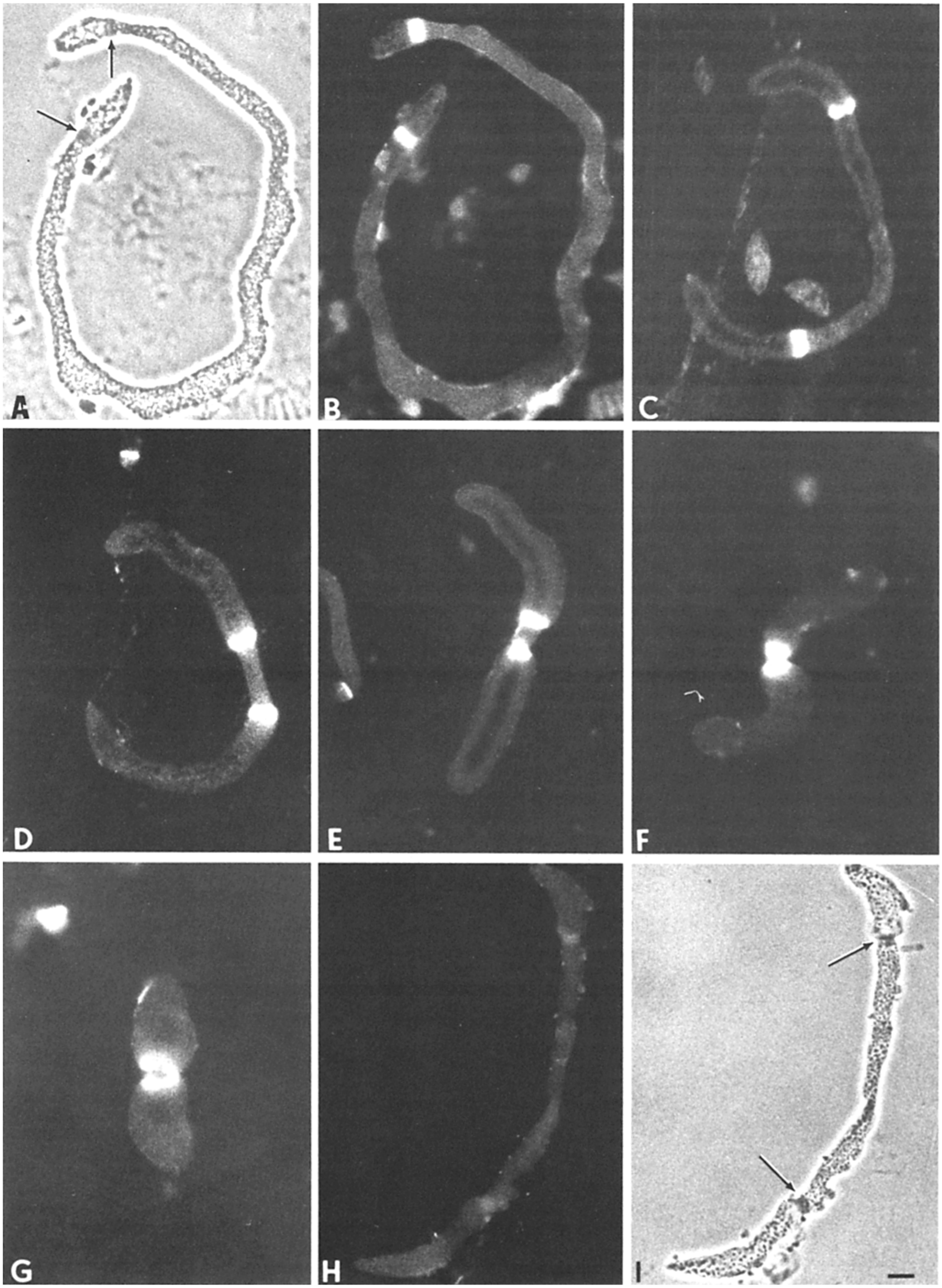
Abstract. Isolated macronuclei from the hypotrichous ciliated protozoan *Euplotes eurystomus* incorporate biotinylated dUTP specifically into the replication band (RB) as detected with immunofluorescence, using rabbit anti-biotin antibodies followed by fluorescein-conjugated goat anti-rabbit IgG. When gold-conjugated goat anti-rabbit IgG was used in a preembedded reaction, subsequent immunoelectron microscopic analysis demonstrated that the biotinylated nucleotide appeared more concentrated in the rear zone of the RB, with almost no labeling in the forward zone. It was possible to use the immunofluorescent assay to establish that incorporation of biotinylated dUTP is inhibited by simultaneous addition of *N*-ethyl maleimide or aphidicolin, and by omission of any one of the other unlabeled dNTPs. In addition, prolonged heat shock of the intact cells, before lysis and in vitro assay, yielded markedly reduced incorporation. Comparison with published data on the in vivo incorporation of [^{3}H]thymidine into *Euplotes eurystomus* RBs indicates the fidelity of the in vitro reaction.

In 1859 F. Stein (30), using the light microscope, described a structure of unknown function in the macronucleus of various *Euplotes* species, the "spaltformige Hohle". During the early part of the 20th century microscopists (13, 34) used stained preparations of *Euplotes* cells to visualize substructure in these regions, denoted "reconstruction bands". The function of these regions remained unknown, but since it preceded macronuclear division it was regarded as being essential for rejuvenation of the cell. In 1959, 100 years after its initial description, J. Gall (11) clearly demonstrated, using autoradiography to detect [^{3}H]thymidine incorporation, that these bands are the principal regions of DNA duplication in the macronucleus. During the 1960's and 1970's a host of publications by D. Prescott and R. Kimball and colleagues (9–11, 19, 28, 31) examined this in vivo incorporation of thymidine into the structures, now denoted "replication bands" (RB).[1] These papers collectively established: the movement of RBs from the tips of macronuclei towards the middle; the absence of RNA synthesis within RBs; the localization of DNA synthesis in the rear zone (RZ), but not the forward zone (FZ) of the RB; the timing of macronuclear S phase and RB appearance within the cell cycle; and the inhibitory effect of heat shock upon in vivo [^{3}H]thymidine incorporation. Electron microscopic autoradiography on several species of hypotrichous ciliates (10, 22, 31) has substantiated that nascent DNA appears first at the junction of FZ and RZ, extending later into the RZ. Numerous ultrastructural studies have been published (see reference 27 for a summary of older papers and for recent observations). These studies have documented the considerable conformational changes in chromatin during migration of the RB. In front of the RB (the prereplicative region) much of the chromatin is condensed into granules several micrometers in diameter. The granules coalesce and reform into very uniform cables (40–50-nm diameter) that span the entire FZ. At the junction of FZ/RZ these cables fray into 10-nm fibers, which gradually reform into condensed chromatin granules in the rear portion of the RZ.

The RB of hypotrichous ciliates has been shown to possess several unusual chemical features, namely: a high affinity for silver, and a high concentration of accessible thiol groups (1); a strong uranyl staining, unlike condensed chromatin, following the Bernhard EDTA-regressive stain (27); and RB-specific protein epitopes, recognized with monoclonal antibodies (3). In addition, RBs can be enriched from bulk chromatin by using differential lysis and isopyknic centrifugation (2). DNA from the enriched RB preparations revealed an increase in putative replicating molecules; i.e., short linear DNA molecules with single (or, rarely, double) fork regions, similar to forms observed earlier (26). The range of lengths of replicating DNA molecules did not appear to be appreciably different from that of unreplicated macronuclear DNA molecules, which average ∼2 kbp (range, 0.5–20 kbp). From the work of Prescott et al. and of Ammermann et al. (reviewed in reference 20), it is clear that these short molecules represent individual genes. Studies in our laboratory (5, 6, 17) have focused upon the properties of the native

1. *Abbreviations used in this paper*: APC, aphidicolin; bio-11-dUTP, biotinylated dUTP; bio-19-SS-dUTP, cleavable biotinylated dUTP; FZ, forward zone; NEM, *N*-ethyl-maleimide; RB, replication band; RZ, rear zone.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short macronuclear chromatin, which consists of nucleosomes with inner histones, an unusual H1, and considerable quantities of nonhistone proteins. Such studies form the basis for an eventual understanding of the chemical and morphological differences between nonreplicating chromatin and the chromatin of RBs.

The development of an in vitro nuclear replication assay has the potential for identifying and characterizing important nuclear factors, and for working out the mechanics of DNA and chromatin assembly. The most successful in vitro eukaryotic assays have involved adenovirus, SV40, and yeast nuclei (7). Permeabilized eukaryotic cells (25, 32) and isolated nuclei (8, 33) have been shown to incorporate labeled deoxynucleotide triphosphates into presumptive native regions of replication. Microscopic localization of sites of replication have generally been conducted upon intact cells and, as with studies of isolated nuclei or permeabilized cells, have usually used ^{3}H-labeled nucleotides (see, for example, reference 23). Such autoradiographic localization is generally within nuclear regions without any distinctive ultrastructure. A more convenient method for localization of nuclear sites of DNA synthesis involves immunological assays of bromodeoxyuridine incorporation using monoclonal antibodies (4, 12, 15, 18), which can monitor replication sites in intact cells. The use of biotinylated nucleotides in combination with avidin (or streptavidin) or anti-biotin antibodies, as a marker for DNA replication, has the additional advantage of forming the basis for biochemical isolation of newly replicated chromatin regions (16, 21, 29).

In the present study we have developed an in vitro replication assay in RBs that uses the incorporation of biotinylated dUTP followed by immunofluorescence or immunoelectron microscopy. This convenient assay has similarities to in vivo replication, and furnishes the potential to examine replicating chromatin molecules in greater detail.

Materials and Methods

Cells and Reagents

Euplotes eurystomus was purchased from Carolina Biological Supply Co. (Burlington, NC) and maintained in Pringsheim medium on a diet of the alga *Chlorogonium elongatum*, as previously described (1, 5).

To ensure rapidly growing cultures with significant percentages of RBs, *Euplotes* were generally starved 3-4 d, then fed well ~18 h before experimentation. Unconsumed algae was not allowed to accumulate; cells were frequently transferred to fresh dishes. Biotinylated dUTP (bio-11-dUTP), rabbit anti-biotin, and FITC-goat anti-rabbit IgG were purchased from Enzo Biochem., Inc., (New York, NY). The cleavable biotinylated nucleotide, bio-19-SS-dUTP, was generously provided by Dr. T. M. Herman (Medical College of Wisconsin, Milwaukee, WI). Aphidicolin (APC) was kindly donated by Dr. A. H. Todd (Imperial Chemical Industries, Macclesfield, Cheshire, UK). All other chemicals were reagent grade or better.

Lysis of Cells and In Vitro Incorporation Assay

Experiments were performed on dried slides subbed with chromalum gelatin. A 1-cm diameter circle was inscribed on the slide. 20 µl of highly concentrated *Euplotes* cells was deposited within the circle, followed in rapid succession with 4 µl of metofane (methoxyflurane), and 4 µl of 6× lysis buffer (2). Metofane should be added before the lysis buffer. If metafane is omitted or allowed to volatilize before adding lysis buffer, the cells can take 30-45 min to lyse. Addition of metofane has no apparent effect upon cell morphology or behavior but shortens lysis time to a mere 2-3 min. The mixture of cells, metaphane, and lysis buffer was gently oscillated 2-3 min, while observing the slide with a Zeiss dissecting microscope equipped with darkfield illumination. As soon as the first liberated macronuclei appeared, cell lysis was completed by sucking the cells into a 15-µl Lang-Levy pipette, two to three times. Immediately afterwards, a 10.0 × 10.0 × 0.15 mm agarose coverslip was placed upon the droplet. The 2% agarose coverslips were made exactly as described by Y. Fukui (35) in TKM buffer plus DTT (10 mM Tris HCl, pH 7; 150 mM KCl; 5 mM $MgCl_2$; and 5 mM dithiothreitol). Excess fluid was removed from the edges of the coverslip with filter paper wicks. 20 µl of nucleotide mixture was pipetted on top of the agarose. The slide was placed into a humid petri dish and incubated at room temperature for 90 min, except when indicated. The total time required to induce lysis and add nucleotides was ~5 min. Generally, the nucleotide mixture contained 300 µM of dATP, dGTP, and dCTP, and 60-300 µM in biotinylated dUTP, dissolved in 50 mM Tris HCl (pH 7.0). Since the agarose coverslip volume was ~15-20 µl, the final nucleotide mixture during incorporation would be reduced to ~50% of the initial concentration.

For immunofluorescent assay, procedures followed those recommended by Enzo Biochem., Inc. After incubation with the nucleotide mixture, the slides were plunged into Carnoy's B fixative (ethanol/chloroform/acetic acid; 6:3:1) for ≥5 min. During the first few minutes of fixation the agarose coverslip was gently pryed off with a scalpel blade. The slides were washed in PBS (10 mM Na phosphate, 0.13 M NaCl, pH 7) for 5 min, blocking buffer (PBS + 0.1% triton X-100) for 2 min, and PBS for 5 min. After the addition of 50 µl of rabbit anti-biotin diluted 1:100 with antibody dilution buffer (2 mg/ml BSA in PBS), the slides were covered with a glass coverslip and incubated for 60 min in a moist chamber at room temperature. Three 5-min washes with PBS followed. Finally 100 µl of FITC-goat anti-rabbit IgG (1:100-1:200) was applied, the slide was covered with a coverslip, and incubated for 60 min at room temperature. Again, the slides were given three 5-min washes with PBS, and then a drop of 90% glycerin in PBS (pH 7) containing 25 mg/l DABCO (1,4-diazobicyclo-2,2,2-octane) was applied to each slide. Slides were observed on a Zeiss photomicroscope III equipped with a 75 W xenon lamp, interference filters, and immersion plan-NEOFLUAR objectives 25 and 40×. Photographs were collected on Kodak tri-X or Kodak Ektachrome 400. Individual RBs within macronuclei were identified by phase microscopy inside the scribed circle area, and subsequently viewed with epifluorescence to score the strength of reaction. A qualitative scale was devised: +++, brilliant fluorescence; ++, very strong; +, strong; +/−, weak or trace; 0, no detectable fluorescence. In general, +++ reaction was only observed when the highest levels of bio-11-dUTP were contained in the nucleotide mixtures. To economize on reagent (and on cost), the molar ratio of bio-11-dUTP/dATP was ~1:5 to 1:4. When the strongest reaction was desired (as with the immunoelectron microscopy), the ratio of bio-11-dUTP/dATP was 1:1.

For immunoelectron microscopy, the initial incubation was essentially as described above except that the ratio of bio-11-dUTP/dATP was 1:1, the reaction was carried out on subbed coverslips, and the incubation time was reduced to 45 min. Slides were fixed in ice-cold 0.5% glutaraldehyde, 1.5% formaldehyde, and 50 mM Na cacodylate (pH 7.35) for 30 min; agarose coverslips were removed during the first few minutes. After fixation, slides were rinsed for at least 10 min in PBS made 50 mM NH_4Cl to react with remaining free aldehyde groups. Subsequent steps in sequence were as follows: PBS, 5 min; blocking buffer, 2 min; PBS, 5 min; rabbit anti-biotin (1:100), 60 min, room temperature; three washes with PBS, 5 min each; 5 nm gold-labeled goat anti-rabbit IgG (Janssen Life Sciences Products, Beerse, Belgium) diluted 1:25 with antibody dilution buffer.

Coverslips were dehydrated in an ethanol series, propylene oxide, and embedded in epon. After incubating for 48 h in an oven at 60°C, the edges of the coverslips were removed with a jeweler's saw, the sample was placed into the oven for an additional hour at 60°C, and then plunged into liquid N_2 to separate the coverslip from the epon. Using a scribing objective individual nuclei with replication bands were marked, cut out, and mounted

Figure 1. Phase and immunofluorescent micrographs of *Euplotes* macronuclei after in vitro incubation in the presence or absence of bio-11-dUTP. *A* to *G* demonstrate bio-11-dUTP incorporation into RBs and have been arranged to show stages in RB migration until fusion. *H* and *I* demonstrate a control nucleus where bio-11-dUTP has been omitted. *A* and *I* are phase micrographs, with arrows indicating the position of RBs. *D* and *E* also show incorporation into RBs beginning at the tips of other macronuclei; *C* reveals the endogenous fluorescence of contaminating ellipsoid-shaped algae. Bar, 10 µm.

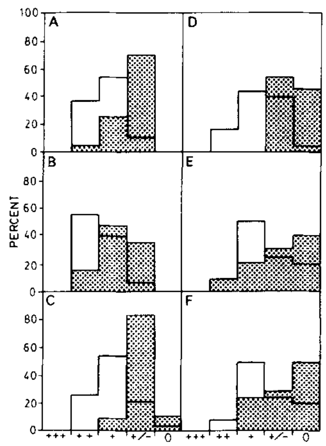


Figure 2. Histograms of fluorescent intensities of RBs after various treatments. Open bars represent the control levels of bio-11-dUTP incorporation as detected with rabbit anti-biotin and FITC-goat anti-rabbit IgG. The dotted bars represent the experimental conditions that exhibit reduced incorporation. Duplicate slides containing a total of 60–100 RBs were scored for each set of conditions. (*A*) In vitro incorporation in the presence of 2.5 mM NEM. (*B*) In vitro incorporation in the presence of 150 μM APC. (*C*) Addition of bio-11-dUTP for the time interval 0–45 min (*open bars*), 45–90 min (*dotted bars*). (*D*) In vitro incorporation after 90 min in vivo heat shock (*dotted bars*) or no heat shock (*open bars*). (*E*) In vitro incorporation with complete mixture of dNTPs (*open bars*) or minus dATP (*dotted bars*). (*F*) In vitro incorporation with complete mixture of dNTPs (*open bars*) or minus dGTP (*dotted bars*).

on epon chucks. Only the first few sections of any particular block contained nuclei. These were picked up on formvar-coated grids, stained for 10 min by immersion in a 2% uranyl acetate solution in methanol, carbon coated, and photographed in a Siemens 102 electron microscope.

Results

Immunofluorescent Detection of Biotinylated dUTP Incorporation into the Macronuclear RBs

Examination of microscope slides after the incorporation of bio-11-dUTP and subsequent immunological procedures revealed a scattered field of intact and fragmented macronuclei, partially lysed cells, and contaminating algae. A low level of autofluorescence was observed from algae and some nonspecific immunostaining was characteristic of the subbed surface. Intense and discrete immunofluorescence was observed over many of the replication bands (Fig. 1). Careful focusing and switching back and forth between fluorescence and phase indicated that the reaction was localized over the RZ of the RB. Due to variations in the strength of reaction among different RBs a qualitative grading system was devised (see Materials and Methods). Approximately 30–50 RB were scored on each slide and its duplicate, always within or immediately proximal to the scribed circle. Examination of many slides yielded the following qualitative judgements: (*a*) decreasing the ratio of bio-11-dUTP/dATP from 1:1 to 1:5 reduced the intensity of immunofluorescence in the RBs; (*b*) reaction intensity appeared to increase as the RB progressed from the macronuclear tips towards the center, being strongest just before fusion and disappearance in the middle of the nucleus; (*c*) immunofluorescence appeared to spread more into the postreplicated regions just before RB fusion.

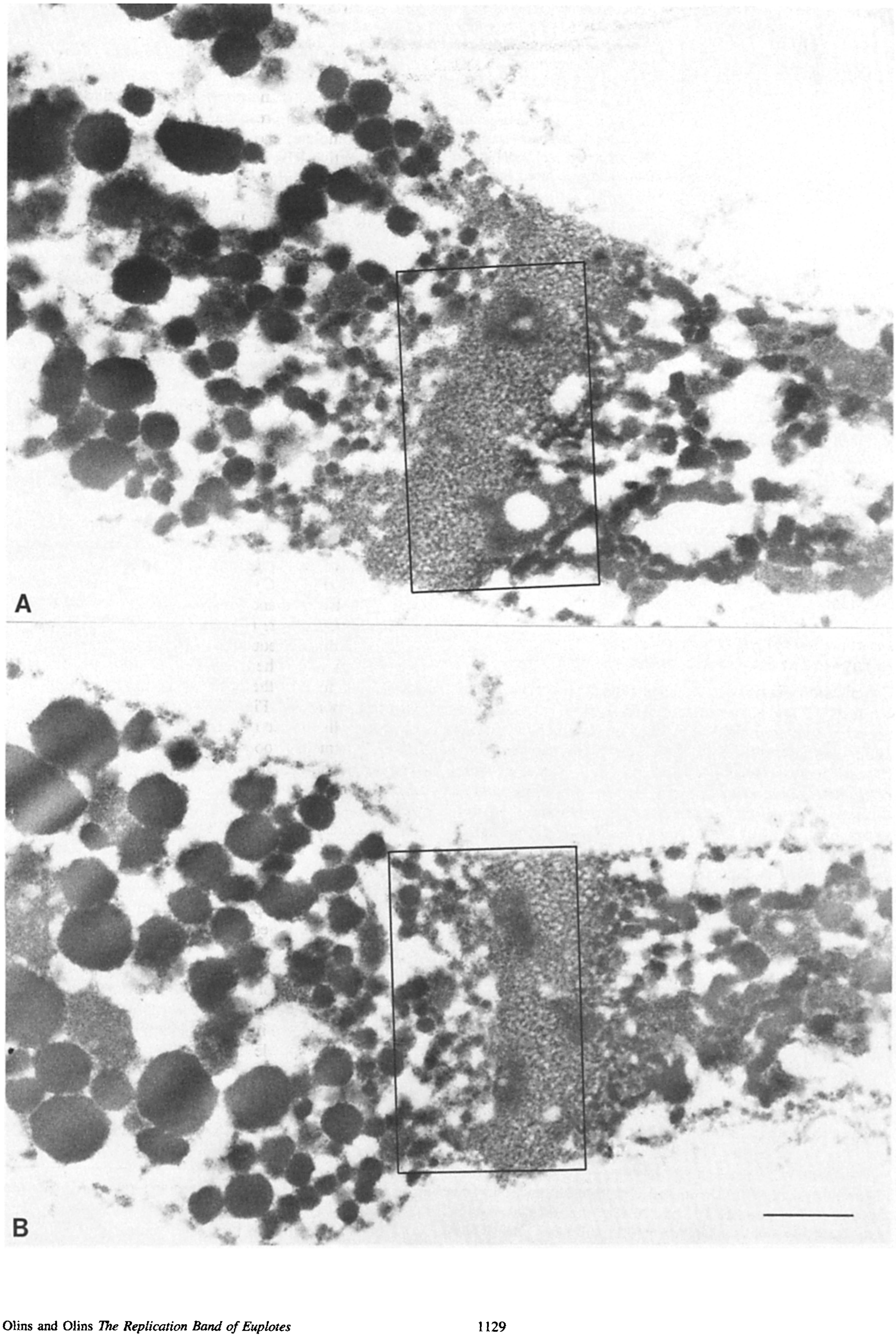
Incorporation and Inhibition of Incorporation of Bio-11-dUTP into the RB

A number of experiments were performed to examine the sensitivity of bio-11-dUTP incorporation in the presence of well-established inhibitors and nucleotide requirements (Fig. 2). Data are presented as histograms. Inhibition was seldom complete; the only circumstance that resulted in 100% negative RBs was omission of bio-11-dUTP. Experiments that included *N*-ethyl-maleimide (NEM) or aphidicolin (APC) during incorporation revealed an inhibition of immunofluorescence (Fig. 2, *A* and *B*). The levels of inhibitor required to be effective were higher than those commonly used in vitro (8, 14, 33). NEM and APC were added to the slides as soon as the cells had lysed. NEM was also present in the agarose and in the nucleotide mixture; APC was not in the agarose but present at twice the final concentration within the nucleotide mixtures. The lower sensitivity of RB incorporation to the action of inhibitors compared with in vitro solution studies (8, 14, 33) might be a consequence of intrinsic differences of the *Euplotes* DNA polymerase compared with higher eukaryotes. Equally plausible to us is the nature of the assay, which must be performed quickly and undoubtedly involves microscopic variations of reactants and inhibitors on macronuclei adsorbed to coated-glass surfaces. Although most incubations with nucleotide mixtures were allowed to progress for 90 min at room temperature, a systematic study of reaction time indicated that more incorporation occurred during the first 45 min, compared with the latter 45 min (Fig. 2 *C*).

In 1970, Everson and Prescott (10) demonstrated that heat shock of live *Euplotes* (i.e., incubation for 90 min at 36.5°C) resulted in drastic reduction of [^{3}H]thymidine incorporation into RBs in vivo. The mechanism for this inhibition is unknown but formed the basis for comparing in vivo and in vitro incorporation. Fig. 2 (*D*) demonstrates that heat shock of intact cells, before lysis and in vitro assay (both of which were performed at room temperature), resulted in a marked decrease of biotinylated nucleotide incorporation into RBs. Due to the short lifetime of the in vitro assay, we have not yet attempted to induce inhibition by in vitro heat shock. Future experiments will be directed toward extending the lifetime of the in vitro assay so that experiments involving in vitro heat shock and pulse-chase visualization of RB movement can be attempted.

In one series of experiments the unlabeled deoxyribonucleotides were omitted from the nucleotide mixture, one at a time. Results for dATP and dGTP are presented in Fig. 2, *E* and *F*; omission of dCTP gave quite similar results. Removal of any one of the unlabeled nucleotides (i.e., dATP, dGTP, or dCTP) led to a considerable reduction of bio-11-dUTP incorporation into the replication band compared with the control nucleotide mixtures. We were surprised that in all cases of depletion some reaction was observable in the RBs. We have no explanation for this phenomenon except to suggest that endogenous nucleotide phosphates may be channeled or trapped near the sites of replication (24).

Figure 3. Low magnification survey electron micrographs of preembedded reaction of gold-conjugated goat anti-rabbit IgG after in vitro incorporation of bio-11-dUTP and rabbit anti-biotin. Both macronuclei are arranged so that the RBs are migrating towards the right side of the figure. The areas of the RZs are immediately to the left of the FZs, which are recognizable as a meshwork of 40–50-nm fibers (27). The framed areas are shown as stereo-electron micrographs in Fig. 4. Bar, 1 μm.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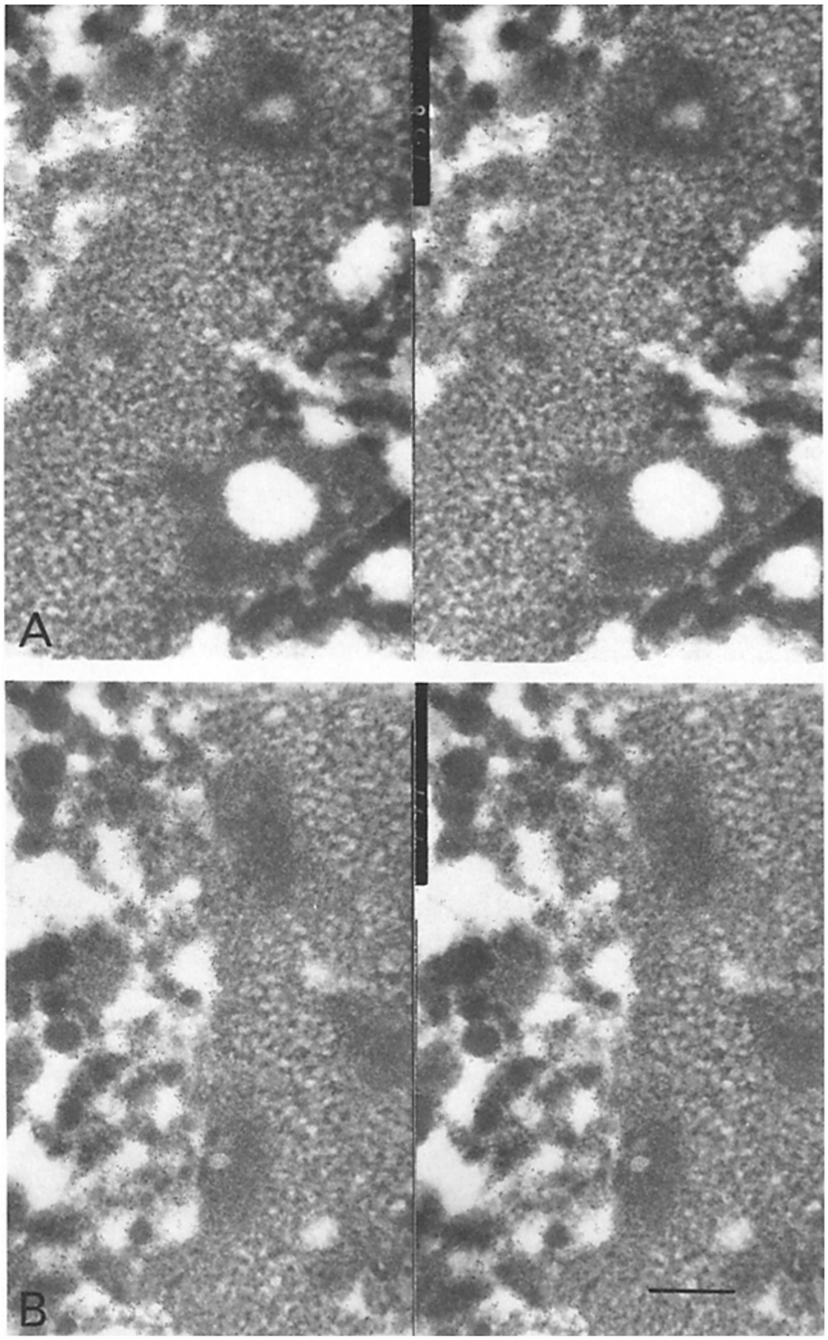

Figure 4. Stereo-pairs of the regions within RBs, indicated in Fig. 3. When viewed with a magnifying stereo-viewer the 5-nm gold particles can be readily observed within the RZ. Bar, 0.5 μm.

Immunoelectron Microscopy Demonstrates In Vitro Incorporation of Biotinylated dUTP into the Rear Zone of the RBs

Figs. 3 and 4 present evidence that in vitro incorporation of bio-11-dUTP can be detected by substituting gold-coupled goat anti-rabbit IgG for the FITC-conjugated second antibody. The low magnification micrographs (Fig. 3) are included to orient the reader to the direction of RB migration, even though the 5-nm gold particles can not be readily observed. Higher magnification micrographs are shown as stereo-pairs (Fig. 4) to document that the gold particles are concentrated within the RZ of the RB, and are distributed throughout the thickness of the section. Lower concentrations of gold particles could be observed elsewhere in macronuclei (primarily on chromatin surfaces), with the FZ of the RB consistently deficient in labeling. It is not possible to determine whether incorporation of bio-11-dUTP takes place throughout the RZ or at the interface of RZ/FZ from the present data. Better ultrastructural preservation and shorter incorporation times are probably necessary to make such a decision.

Discussion

The present immunofluorescent and immunoelectron microscope study of isolated macronuclei from *Euplotes eurystomus* presents evidence that incorporation of biotinylated dUTP occurs principally into the rear zone of the replication band, performed at least in part with an α-like DNA polymerase, and exhibits considerable resemblance to in vivo replication.

The assay described here has the advantage of being considerably faster than autoradiographic detection of [^{3}H]thymidine incorporation. Furthermore, the development of an in vitro assay permitted a systematic study of the effects of inhibitor (NEM or APC) addition, and of the effects of omitting unlabeled dNTPs. The in vitro incorporation for replication in its current form has two serious drawbacks that will require additional experimentation: (*a*) The immunofluorescence signal can only be interpreted semi-quantitatively; the quantitative relationship between fluorescent signal intensity and moles of incorporated nucleotide has not yet been studied. (*b*) The in vitro assay decays in its ability to incorporate labeled nucleotide; the reaction is not active enough after 90 min to be useful. The mechanism for this decay is presently unknown. It may involve phosphatases that degrade the bio-11-dUTP, or denaturation (or dissociation) of a replicative enzyme complex. Future experiments will attempt to prolong the in vitro reaction by addition of phosphatase and protease inhibitors, an ATP-generating system, varying buffer pH and divalent cation content, and adding stabilizing proteins (e.g., BSA). Prolonging the lifetime of the assay will permit experiments such as in vitro heat shock, the addition of RB-specific monoclonal antibodies (3), and enrichment of replicating DNA and chromatin.

As a step toward enrichment of replicating DNA and chromatin, in collaboration with Dr. T. Herman, we have demonstrated that the cleavable biotinylated nucleotide bio-19-SS-dUTP (16) is readily incorporated into RBs. Furthermore, after incorporation into replicating DNA, the biotin moiety can be reduced off the fixed macronuclei by treatment with 50 mM dithiotheitol in PBS. This reaction will have to be scaled up to levels sufficient to chromatograph purified DNA or chromatin on affinity columns specific for biotin. We estimate that microgram quantities of replicating molecules could be obtained from one large tray (~7–10 × 10^6) of *Euplotes* cells incubated for several minutes with bio-19-SS-dUTP. Development of the present in vitro assay that exhibits intense localization in the replication bands, as observed with in vivo studies, gives confidence that purified biotinylated replicating molecules can be derived from physiologically relevant replication sites.

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