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RESEARCH ARTICLE

Marie-Odile Soyer-Gobillard
Brigitte Gillet
Marie-Line Géraud
Yvonne Bhaud

Observatoire Océanologique de Banyuls,
Laboratoire Arago, Université Paris 6,
Banyuls-sur-mer, France

Dinoflagellate chromosome behaviour during stages of replication

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Summary In most dinoflagellate species, chromosomes are characterized by an almost continuous condensation of the nucleofilaments throughout the cell cycle and the absence of longitudinal differentiation as Q, G, or C banding. Their supercoiled architecture is maintained by divalent cations and structural RNAs. Their chromatin is devoid of histones and nucleosomes and their DNA composition is distinctive: in several species, more than 60% of thymines are replaced by a rare base, hydroxymethyluracil. We report here an immunofluorescence (conventional and confocal laser scanning microscopy, CLSM) and immunogold transmission electron microscopy (TEM) analysis of some stages of the early replication process in *Prorocentrum micans* dinoflagellate cells, after long pulse incorporation (3, 6 or 9 days) with 50 µg/ml bromodeoxyuridine (BrdU) in the presence of 5-fluoro-2´-deoxyuridine (FUdR) and BrdU antibody technique (BAT) detection. The large DNA content (45 pg per nucleus) of *P. micans* cells is compacted on 100 chromosomes, 10 µm in length. In early S-phase, DNA replication sites are revealed as fluorescent domains organized in clusters, which appear in the periphery of the nucleus unlike other eukaryotes. In late S-phase, the number of labelled clusters increased; helically distributed, they did not appear synchronously in the whole chromosome. Under TEM, spherical domains of equivalent diameter appeared located all along the chromosomes after 6 days BrdU pulse. Replication occurs, but in our experimental conditions, segregation of daughter chromosomes was never observed. The blockade of the cell cycle after BrdU incorporation intervening just before the segregation of daughter chromosomes is discussed.

Key words DNA synthesis · Dinoflagellates · Chromosome replication · Confocal laser scanning microscopy (CLSM) · Electron microscopy

Correspondence to:
Marie-Odile Soyer-Gobillard. Observatoire Océanologique de Banyuls. Laboratoire Arago. F-66651 Banyuls-sur-mer. France.
Tel.: +33-468887369. Fax: +33-468887398.
E-mail: mosg@arago.obs-banyuls.fr

Introduction

In most eukaryotic cells, the cell cycle is characterized by a chromatin condensation-decondensation cycle, and DNA synthesis occurring at the end of the decondensation phase. During the early-S-phase, DNA replication sites appear, located in 100 to 300 replication foci or replication factories distributed throughout the nucleus [25, 26]. These replication sites are visualized with fluorescent probes after incorporation of thymidine analogues, such as bromodeoxyuridine (BrdU), or biotinylated dUTP, and localized with a variety of pre-replication and replication proteins such as replication protein A (RPA), cyclin A, Cdk2 or DNA polymerase [8, 50]. In mid-S-phase and late-S-phase, a clustering of replication foci occurs and replication ends at the level of nucleoli and at the nuclear periphery, where sites are associated with the nuclear matrix [9, 10, 16].

Detection of BrdU incorporation by an immunological technique (BrdU antibody technique, BAT) using BrdU antibody was settled by Vogel et al. [49]. Human lymphocyte chromosome replication was studied without synchronization and R-bands were revealed from early to late S-phase with considerable difference in intensity. Correspondence between chromosomal R-bands and clusters of replicons replicating synchronously was also demonstrated with this technique although only 1 in 200 nucleotides was replaced by BrdU [49].

The genome of dinoflagellate protists has a peculiar nature: in most species, there is a permanent condensation of the chromatin in well organized chromosomes that are surrounded by a persistent nuclear envelope (for reviews see [34, 42, 43, 45]). Chromosomes are characterized by a

total absence of longitudinal differentiation such as Q (quinacrin), G (Giemsa), or C (centromeric heterochromatin) banding [13].

Dinoflagellate chromosomal nucleofilaments are disposed in an anorthospiral arrangement with a very regular pitch as studied in the autotrophic *Prorocentrum micans* [12], with the DNA molecules being supercoiled according to 6 hierarchical degrees of organization [19]. This architecture is maintained by divalent cations [19] and structural RNAs [41]. Although numerous putative models have attempted to explain this architecture (for a review see Spector, [45]), one of them [12] was later confirmed by Oakley and Dodge [33], authors using *Prorocentrum micans* and *Amphidinium carterae* respectively as models. Light and transmission electron microscopy (TEM) of spread prepared chromosomes allowed to voice two hypotheses: first, the presence of numerous circular chromatids, evaluated at 1,300-fold for *Prorocentrum micans* (42 pg of DNA) and 700-fold for *Crypthecodinium cohnii* (6.9 pg of DNA) [12, 14], and second, in contradiction with the first—and by using measurements of viscoelastimetry retardation times—, the presence of only one to six pieces of DNA [36]. In addition to these original features, one is remarkable: Dinoflagellates are the only eukaryotes totally devoid of histones and nucleosomes [17], but their DNA is associated with specific basic nuclear proteins [35, 38, 48] localized in the periphery or in the whole chromosomes depending on the stage of the cell cycle [11]. Dinoflagellate DNA composition is also characteristic, one of its most distinctive features being the replacement of a high level of thymines (more than 60%) by a rare base, hydroxymethyluracil [18, 20]. Another typical feature is the presence of repeated sequences in their non-coding DNA [31]. The presence of a nuclear matrix and of topoisomerase II were also biochemically and immunocytochemically demonstrated in the dinoflagellate *Amphidinium carterae* [29].

Prorocentrum micans, the selected biological model used for this study, presents an obvious eukaryotic DNA synthesis pattern [6] with a well defined S-phase. Investigations about molecules controlling dinoflagellate cell cycle are now starting: recently the presence of a cdc2-like protein kinase was demonstrated by using an antibody specific for the Pro-Ser-Thr-Ala-Ile-Arg-Glu (PSTAIRE) epitope in *Crypthecodinium cohnii* [37]. Salois and Morse [39] refuted it in a study performed using the same antibody but on another species (*Gonyaulax polyedra).* The presence of a cyclin B ($p56^{cdc13}$) homologue [3, 4], and a variation in histone H1-kinase activity during the cell cycle [7, 27] were demonstrated, as well as the presence of four cyclins detected in synchronized cultures of *C. cohnii* with a polyclonal antibody against the cyclin-box of sea urchin cyclin B1 [27]. Up to now, no data seem to have been published about the molecular mechanisms of DNA replication in Dinoflagellates.

The dinoflagellate peculiarities in both chromosomal architecture and DNA composition allowed us to observe some stages of the replication process after applying long pulses of bromodeoxyuridine and immunodetection with anti-BrdU antibodies.

Materials and methods

Cell cultures *Prorocentrum micans* Ehrenberg, strains from the Botany School of Cambridge University, UK, were grown in Erd-Shreiber medium under a 12/12 light (1200 lux) dark cycle at 20°C. Under these conditions, the cell cycle lasts 5.5 days with a discontinuous DNA synthesis phase [6].

BrdU treatment BrdU (5-bromo-2´-deoxyuridine from Sigma, St Louis, USA) was added (10, 20 or 50 µg/ml final concentration) and was left in the culture until sampling time (3rd, 6th, 9th day). In order to increase the BrdU incorporation, a thymine synthesis inhibitor, FUdR (5-fluoro-2´-deoxyuridine from Sigma, St Louis, USA), was added to the culture at equal final concentration [2, 24]. Fifty mg/ml BrdU were also added to other cultures without FUdR and were left in the cultures until sampling time (6th and 13rd day) in order to observe the effect of the thymine inhibitor FUdR on the cell populations growth.

Squashes for optical and confocal microscopy At the end of long pulse incubation of 3, 6 or 9 days, culture samples were collected and centrifuged at 146x *g* for 10 min. Pellets were fixed for 30 min in 3% paraformaldehyde in PBS (0.01 M PO_4HNa_2, 0.01 M PO_4H_2K, 0.15 M NaCl) pH 7.0. The pellets were washed for 10 min in PBS and conserved overnight at 4°C to weaken the cells. After centrifugation (180x *g*), cells were resuspended for 1h at 4°C in 1:1 alkali-urea solution (0.7 M NaOH + 4 M urea, pH 7.5) [15]. Cells were then squashed on glass slides, frozen for 1 h on a block of dry ice, and stored at –20°C. Slides can be processed for immunocytochemistry after a rapid wash with PBS.

Fixation and cryomicrotomy *P. micans* pellets were fixed in 3% formaldehyde in PBS buffer (pH 7.4). Pellets were washed in PBS three times during 10 min each and incubated overnight in 20% polyvinylpyrrolidone (PVP) and 1.7 M sucrose according to Tokuyasu [47]. After quick freezing in liquid nitrogen, cryosections 2 µm thick were obtained with a Cryonova ultramicrotome (LKB Instruments) and were placed on coverslips, in a drop of 2.3 M sucrose, and stored at –20°C. For chromosome staining, PBS-washed cryosections were incubated in 0.1 µg/ml DAPI (4´,6-diamidino-2-phenylindole from Sigma, St Louis, USA), or in 0.1 µg/ml PI (propidium iodide from Sigma, St Louis, USA) for 5 min, then rinsed in distilled water and mounted in Mowiol containing 5% N-propyl gallate as an anti-fading agent. Nuclei were observed with a Reichert Polyvar photomicroscope either in epifluorescence with a

490–520 nm filter for FITC (fluorescein isothiocyanate), a 372–456 nm filter for DAPI, with a 570–595 nm filter for PI or in bright field.

Light immunocytochemistry For BrdU detection, on both squashes or sections, a slight fixation (10 min with 3% formaldehyde in PBS) was applied after thawing. The biological material was preincubated at room temperature in 2 N HCl during 30 min for partial denaturation of the genomic DNA, which must be single-stranded for antibody recognition. After numerous washes in PBS the nonspecific epitopes were saturated by incubation (15 min) with a 1/30 dilution of normal goat serum in PBS buffer at room temperature. Incubations with the first anti-BrdU antibody (Amersham monoclonal antibody, used without dilution) were performed for 12 h at 4°C in a wet chamber. Then slides or sections were washed in PBS buffer. The second incubation was carried out with the anti-mouse Ig antibody, a fluorescein-linked whole antibody (from sheep) (Amersham, used diluted 1/100 in PBS). After incubation for 1 h at room temperature, slides were washed and mounted in Mowiol as it has been previously described. Control incubations were done using the second antibody only or both antibodies without BrdU incubation. Pictures were taken using a Reichert Polyvar (Leica, France) fluorescence microscope with both Kodak TMax or Fujichrome 400 ASA films.

Confocal laser scanning microscopy (CLSM) Treated and untreated cells were prepared as described above (immunocytochemistry), but chromosomal DNA was stained with 0.1 µg/ml propidium iodide.

Fluorescence-labelled squashes were imaged on the confocal laser scanning microscope (Zeiss LSM 410) of the Development Biology Laboratory (University Paul Sabatier, Toulouse) with two different laser sources: argon (488 nm, blue) or helium-neon (543 nm, green). Slides were observed at 60x magnification and enlarged with a zoom lens. Optical sections of 0.4 µm were stocked on an optical filter and photographed with Kodak 100 ASA color film.

Electron microscopy procedures. High pressure freezing and freeze-substitution BrdU incubated living cells were centrifuged at 146x *g* in Eppendorf tubes, the bottoms of which were then cut off to retain only the pellet. Small cellulose capillary tubes with an inner diameter of 200 µm (type LD OC 02, Microdyn, Wuppertal, Germany) were then filled by capillarity with several hundreds of living cells and were immediately immersed in the freezing medium, 1-hexadecene (1-HD), and cut into 2-mm segments according to Hohenberg et al. [21]. Two capillary tube sets were then mounted into the cavity of a standard aluminum platelet filled with 1-HD and sandwiched by a second platelet without a cavity, taking care to eliminate the air. The sandwich was inserted into the holder of the high-pressure freezer (HPM 010, Bal-Tec Products, France), according to Moor [30], immediately frozen at liquid helium temperature at 2,000 bars, and stored in liquid nitrogen until further processing. The frozen specimens were then transferred from liquid nitrogen to the freeze substitution medium containing molecular sieves (4A 1/6 Union Carbide, France), stored at –87°C for 3 days, then rewarmed to –30°C, left 2 h at this temperature, and rewarmed at room temperature before being classically embedded in resins [32]. Some specimens were freeze-substituted with anhydrous acetone supplemented with 0.5% uranyl acetate, rewarmed, rinsed in acetone, transferred in absolute ethanol for 1 h and embedded in LR-White to be polymerized at –50°C (technique No. 1). Other specimens were freeze-substituted in 2.5% osmium tetroxide in acetone, rewarmed, rinsed in pure anhydrous acetone and embedded in Epon (technique No. 2). Ultrathin sections were contrasted with 2% aqueous uranyl acetate for 20 min then with lead citrate for 30 s before observations with a Hitachi H600 electron microscope.

Electron microscope immunocytochemistry Ultrathin sections were previously slightly etched with H_2O_2 according to Bendayan and Zollinger [5]. First and second antibodies were diluted in 1 M PBS + 1% BSA with 0.1% Tween-20. Incubations with the first anti-BrdU monoclonal antibody (Amersham Ref. RPN 202, 1/1 dilution) were performed overnight at 4°C in a wet chamber. Grids were washed by alternating PBS and 0.1% Tween-20. The second layer antibody was GAM 1/20 coupled with 10 nm colloidal gold. Preparations were washed as described for the first antibody. Sections were washed once in distilled water before staining them slightly in 2% aqueous uranyl acetate solution for 15 min.

Results

We used conventional immunofluorescence and CLSM to observe squashed dinoflagellate cells previously treated with BrdU, with or without 5-fluoro-2´-deoxyuridine (FUdR). Besides, we used transmission immuno-electron microscopy after high pressure (HP) to observe freezing preparations. This allowed us to conclude that in dinoflagellate DNA, unlike other eukaryotic cells, replication begins in the periphery of the nucleus, close to the persistent nuclear envelope. The number of newly synthesised DNA molecules, which are clustered in replicon domains, increased along the S-phase, and they appeared nonsynchronously in the whole chromosome set. In our experimental conditions, simultaneously with BrdU incorporation in dinoflagellate chromosomes, the cell cycle is blocked just before the daughter chromosomes individualize and start their segregation.

Mitosis progress The normal behaviour of the *Prorocentrum micans* chromosomes throughout the successive phases of mitosis was observed after specific staining with cresyle brilliant blue-eosin (Fig. 1, a), with DAPI (Fig. 1, b–f) on

squashed (Fig. 1, a, d–i) or cryosectioned cells (Fig. 1, b–c), with (Fig. 1, b–c) or without (Fig. 1, a, d, e–h) BrdU treatment. In order to perform a better squashing, we used a proteolytic (alkali-urea) pre-treatment (Fig. 1, d–i) to remove most of the chromosomal proteins [15].

During early prophase, enlarged chromosomes after DNA synthesis (0.6 µm in diameter) begin to divide as shown in Fig. 1, a–c, where the incomplete segregation axis is clearly visible, especially in cells treated with BrdU (Fig. 1, b–c). In late prophase of non treated cells, thin daughter chromatids (0.3 µm in diameter) begin to separate (Fig. 1, d–f), then the two chromosome sets segregate in anaphase (Fig. 1, g) without undergoing a classical metaphase plate figure. After telophase, when chromosome bridges are still visible (Fig. 1, h), daughter nuclei reconstitute.

In a previous paper, we have showed [6] that the whole *P. micans* cell cycle lasts 5.5 days. DNA replication took place from 22.00 to 02.00 GMT, S-phase lasting about 4 h. The period from the end of replication to the end of mitosis (G2 + M phases) took on average 8 h. Phases S + G2 + M took less than 12 h. The G1 phase lasted at least 120 h in our experimental conditions. All these data were used for experiments with BrdU incorporation.

Conventional light immunofluorescence observations Squashes of *P. micans* whole cells were carried out after 3 (data not shown) or 6 days of 50 µg/ml BrdU incorporation. As shown in preliminary observations of Fig. 1, j–k, where cells were squashed after 6 days of BrdU incorporation and after alkali-urea treatment, BrdU substitution sites were detected with an anti-BrdU antibody as punctuated domains (Fig. 1, j–k, arrowheads), fluorescing in green-yellow whereas ancient not substituted DNA fluoresced in red-orange after propidium iodide staining. In *P. micans* cells fixed at 02.00 GMT, at the end of S-phase, numerous chromosomes were labelled (Fig. 1, j). Later, when fixation was performed at 04.00 GMT (Fig. 1, k), labelled substituted DNA was also visible as globular domains in chromosomes of the whole nucleus. Although domains were more abundant, no daughter-chromosome segregation could be seen. Moreover, substituted chromosomes were hardly sensitive to the squash pressure and to any proteolytic treatment, which made observations difficult; so, CLSM analysis was required. Nevertheless, BrdU detection after long pulse incorporation of 6 (Fig. 1, l) or 9 days (Fig. 1, m) made it possible to observe whole squashed nuclei prepared after using conventional immunofluorescence: replaced DNA was labelled in green while the complementary (old) not substituted DNA was stained in blue with DAPI (Fig. 1, l´–m´). The major observation was that the centre of the nuclei was less replaced than the periphery (arrows).

Confocal laser microscope analysis after BrdU incorporation

Fixation at 22.00 GMT (early S-phase) On the confocal optical sections of the Fig. 1, n, n´–r, t–y, ancient *P. micans* DNA, visible after staining in red with propidium iodide, and newly synthesized DNA, after BrdU substitution labelled with anti-BrdU as primary antibody and with a second FITC coupled antibody, were colocalized.

At the beginning of S-phase, anti-BrdU labelling was visible on the tip of a few chromosomes and was located only in the periphery of the nucleus, at the level of the nuclear envelope, which was not visible after this immunolabelling. On Fig. 1, n–r, newly synthesized DNA can be seen as green fluorescent spots, especially in the lowest part of the nucleus where labelling is visible in profile as a plate of the same diameter as the chromosome (Fig. 1, n, arrows); on a higher magnification of Fig. 1, n´, numerous coalescent fluorescent spots are visible. Detail of another cluster of spots located at the chromosome tip can be seen at another part of the same nucleus, shown in Fig. 1, o–r, (small arrows). In this apical view of four serial confocal sections, newly synthesized DNA can be seen as several small globular domains, cylindrically organized in a spiral manner (Fig. 1, o–r, empty arrows).

Fixation at 0.00 GMT (mid S-phase) In 5 optical sections (Fig. 1, u–y) of a series of twelve representing a *P. micans*

Fig. 1 (a–h) Course of mitosis in *Prorocentrum micans* nuclei. (a) Prophase. Dedoubling chromatids are visible (arrow) (cresyle brilliant blue). nu, nucleoli (x 4,625). (b, c) Cryosections of late prophase cells after 50 µ/ml BrdU incorporation for 9 days and where incomplete chromosome segregation axis is visible (arrows); (b) x 4,500; (c) x 5,400. (d) Late prophase with individualized daughter-chromosomes. (e–g) Anaphase. (h) Telophase. (e–h) Alkali-urea treatment. (b–h) Staining of chromosomes with DAPI. (d–h) x 2,160. (j, k) After 6 days BrdU incorporation, alkali-urea treated squashed whole nuclei reveal aligned globular domains (arrow heads) more numerous in cells fixed at 4.00 GMT (end of S-phase) (j) than at 2.00 GMT (k). No synchronization appears, ancient DNA fluoresces in red. x 5,400. (i) Control cell not submitted to BrdU treatment but treated with first and second antibody. Only DNA contrasted with propidium iodide fluoresces (× 2,160). (l, m) Whole squashed nuclei in late prophase after 6 days (l) or 9 days (m) BrdU incorporation. Substituted DNA was labelled in green by fluorescent anti-BrdU antibody, in the periphery of the nucleus, while the complementary DNA was stained by DAPI in blue (empty arrows). Observe the "curly" aspect of the non substituted DNA (l´, m´) and a non substituted nucleus in the lower part of the picture (l, l´, small arrow). Bar = 4 µm. (n–r) Cells fixed at 0.00 GMT (early S-phase). Gallery of five confocal laser optical sections (0.4 µm in thickness) of a *P. micans* nucleus showing the colocalization of ancient DNA (in red) and of 50 µg/ml BrdU substituted DNA (in green). (n) The tip of a chromosome in the periphery of the nucleus is occupied by domains organized as a plate (arrows). (n´) Enlargement of this domain formed by multiple small fluorescent spots. (o–r) In a few peripheral regions of the nucleus (small arrows), fluorescent small globular domains (empty arrows) are detectable, organized in helix (e–h, empty arrows). (n–r) Bar = 2 µm. (n´) Bar =1 µm. (u–y) Cells fixed at 2.00 GMT (mid-S-phase). Gallery of five confocal laser optical sections (0.3 µm in thickness). Regions of BrdU substituted DNA are more abundant (y) and the absence of synchronization between the chromosomes is obvious. (u–x) Detail of four sections in which three globular substituted green-yellow fluorescent domains (arrows) linked together (u, v, arrows) are visible at left (u, v) and at right (w, x) of a same chromosome (big arrow) suggesting an helicoidal organization. Bar = 5 µm. (t) Cells fixed at 6.00 GMT. BrdU substituted chromatids are labelled but not separated (arrows). Substitution was not synchronous. Bar = 5 µm. (s) Nucleus of a control cell not submitted to BrdU but to 1st and 2nd antibodies: chromosomes stained with propidium iodide only fluoresce. Bar = 5 µm

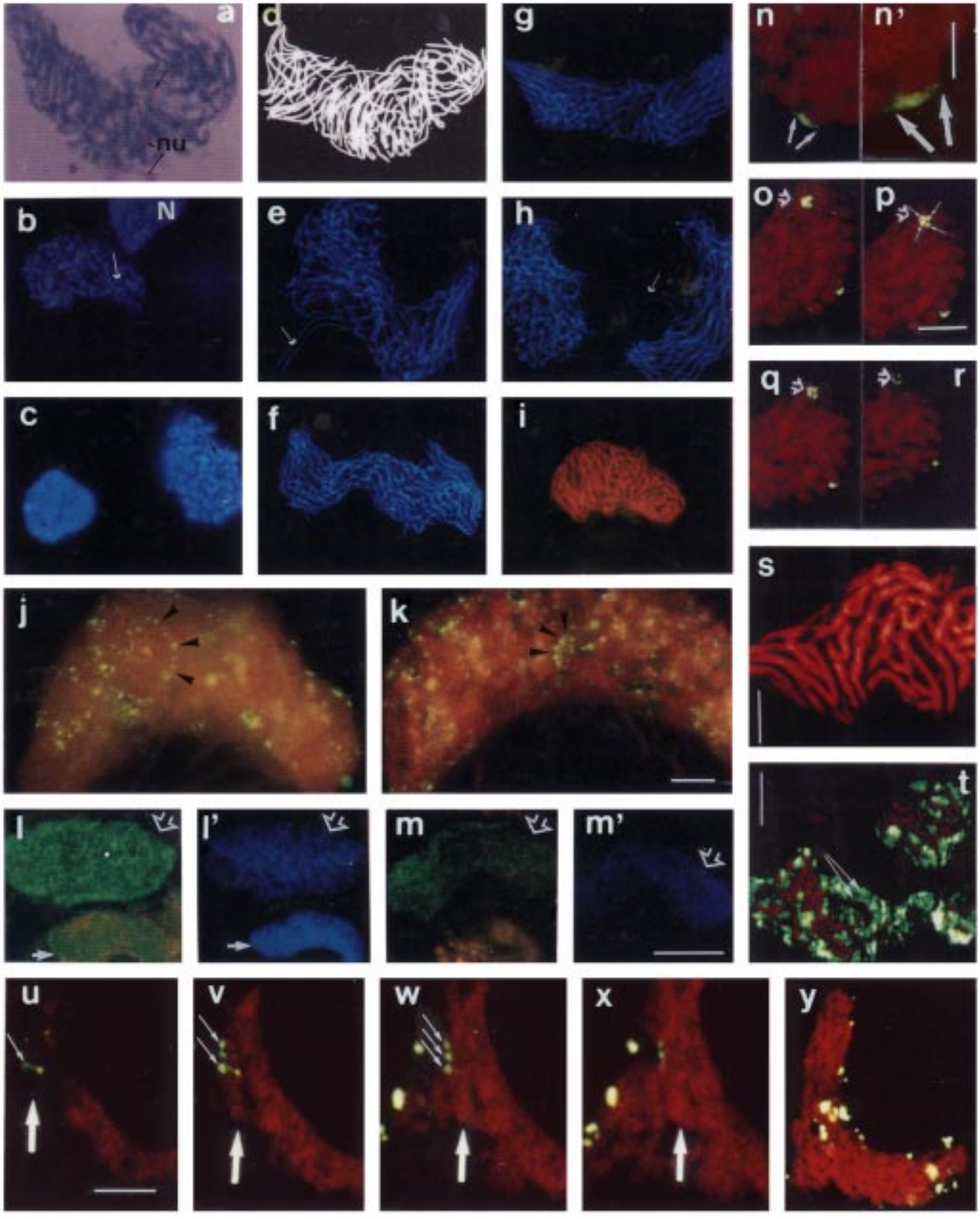

Figure 1

nucleus in mid S-phase, the labelling of the newly synthesized DNA was more abundant than in Fig. 1, n–r, but it was obvious that all the chromosomes of the same nucleus were not synchronously labelled.

Figure 1, u–x, shows three lateral and globular domains, which became visible on these four consecutive confocal laser sections. Left (Fig.1,u–v) and right (Fig. 1, w–x) aligned globular domains (about 0.20 µm diameter in average) were visible (small arrows) on the same chromosome (big arrows). Fluorescent spots were connected together (Fig. 1, u–v, small arrows) suggesting a quasi-helicoidal domain organization.

Fixation at 2.00 GMT (late S-phase) At the end of S-phase, almost all the chromosomes had been replaced; they presented sometimes BrdU labelling on their two chromatid bundles (Fig.1, t, arrows). After the prophase, no daughter chromosomes were observed in spite of the many nuclei which could be seen. So, we conclude that chromosomes treated with 50 µg/ml BrdU did not segregate.

Control nuclei Cells not submitted to BrdU incorporation but treated later with anti-BrdU antibody and second FITC coupled antibody did not present any fluorescence, as shown in Fig. 1, i, and Fig. 1, s, where chromosomes appeared only contrasted with propidium iodide. Cells submitted to BrdU incorporation and treated only with the second FITC coupled antibody did not present any fluorescence except for DNA propidium iodide staining (data not shown).

Transmission electron microscope observations On ultrathin sections of 50 µg/ml BrdU 6 days incorporation of *P. micans* high pressure (HP) fast freeze fixed (FFF) cells and cryosubstituted by OsO_4 (Fig. 2), non-dividing nucleus presented chromosomes with compacted nucleofilaments with short apparent periodicity of the supercoiling (Fig. 2A).

At the beginning of the S-phase, the periodic structure of the chromosomes disorganized, and several hernia appeared along the chromosomes as shown in Fig. 2B (arrowheads). During late S-phase, numerous hernia appeared as spherical domains of 0.2 µm diameter all along the whole chromosomes (Fig. 2C, arrows). These domains were individualized by distinct linear breaking regions (Fig. 2C, arrowheads). Several spherical domains of the same diameter were visible in the nucleoplasm; they corresponded, however, to a tangential section plane of chromosome attached domains.

The ultrathin localization of the replicating DNA was presented in the late S-phase nucleus of Fig. 3. Using anti-BrdU as first antibody and conjugated as a second antibody with 10 nm colloidal gold particles, detectable but not intense labelling was visible on the ultrathin chromosome sections (Fig. 3A, big arrows). Chromosomes were not denatured beforehand but were treated with an inhibitor of thymine biosynthesis fluorodeoxyuridine (FdUrd) on cells fixed at 08.00 GMT. Although the fixation time corresponded to the end of mitosis, only partial segregation was visible among daughter chromatids as an axis (ax, long arrows). Spherical domains of about 0.2 µm in diameter were present in the chromosomes, some of which had been labelled with gold particles (big arrows), the others being only slightly labelled (empty arrows). Chromosomes presenting a partial segregation axis had a large diameter of about 2 µm (Fig. 3). Control cells after using only the second antibody were negative (data not shown).

Discussion

Choice of pulse time and BrdU concentration In an asynchronous culture of *P. micans*, 17% of the cells are naturally dividing synchronously, the population doubling every 5.5 days [6]. So, a 6-day pulse of BrdU was the best way to cover all possible cell cycle phases, and in certain cases, the pulse had been doubled (2 x 6 days). We used a 50 µg/ml BrdU concentration in the culture medium because lower doses tested did not produce adequate labelling, especially for light microscopy. Moreover, the small size of *P. micans* cells (30 µm length), and the presence of both a thick cell wall and a permanent nuclear envelope ruled out the possibility of microinjecting BrdU solutions as with other well-studied eukaryotic organisms. Therefore, it was not possible to determine the precise quantity of BrdU incorporated into the cells. For example, the average amount of BrdU penetrating into mouse cells has been estimated to range from about 55% to 90% as a function of exposure time [28].

Non segregation of the two chromatids Among Dinoflagellate species studied so far, *Prorocentrum micans* shows one of the highest (62.8%) levels of thymine substitution by the rare base hydroxymethyluracil [18]. This striking and unusual feature of Dinoflagellates is shared by bacteriophage SP8, in which the substitution rate is 100% [23]. Taking into account this rare property of Dinoflagellate DNA, we have used the thymine inhibitor FUdR, firstly, to be certain that the BrdU incorporation was maximal in the 37.2% remaining thymines, and secondly, to avoid too much incorporation into the second DNA synthesis cycle [45]. In the case of two *P. micans* cultures which had both undergone BrdU incorporation, one of them had been treated with FUdR whereas the second one had not. This latter population was seen to double in 5.5 days as in a normal culture; therefore it could be inferred that the presence of FUdR is involved in the non-segregation of the daughter-chromatids. Moreover, fluorescent replication regions consist of sequences of relaxed DNA mixed with substituted DNA sequences. After BrdU incorporation, the relaxed DNA cannot revert to its original state due to steric hindrance, because of the presence of either the rare base, hydroxymethyluracil, or Z and/or B DNA sequences [41], and topoisomerase II [29] cannot act. So, the decatenation of DNA molecules is abrogated. This represents an artificial blocking which could be used as a tool.

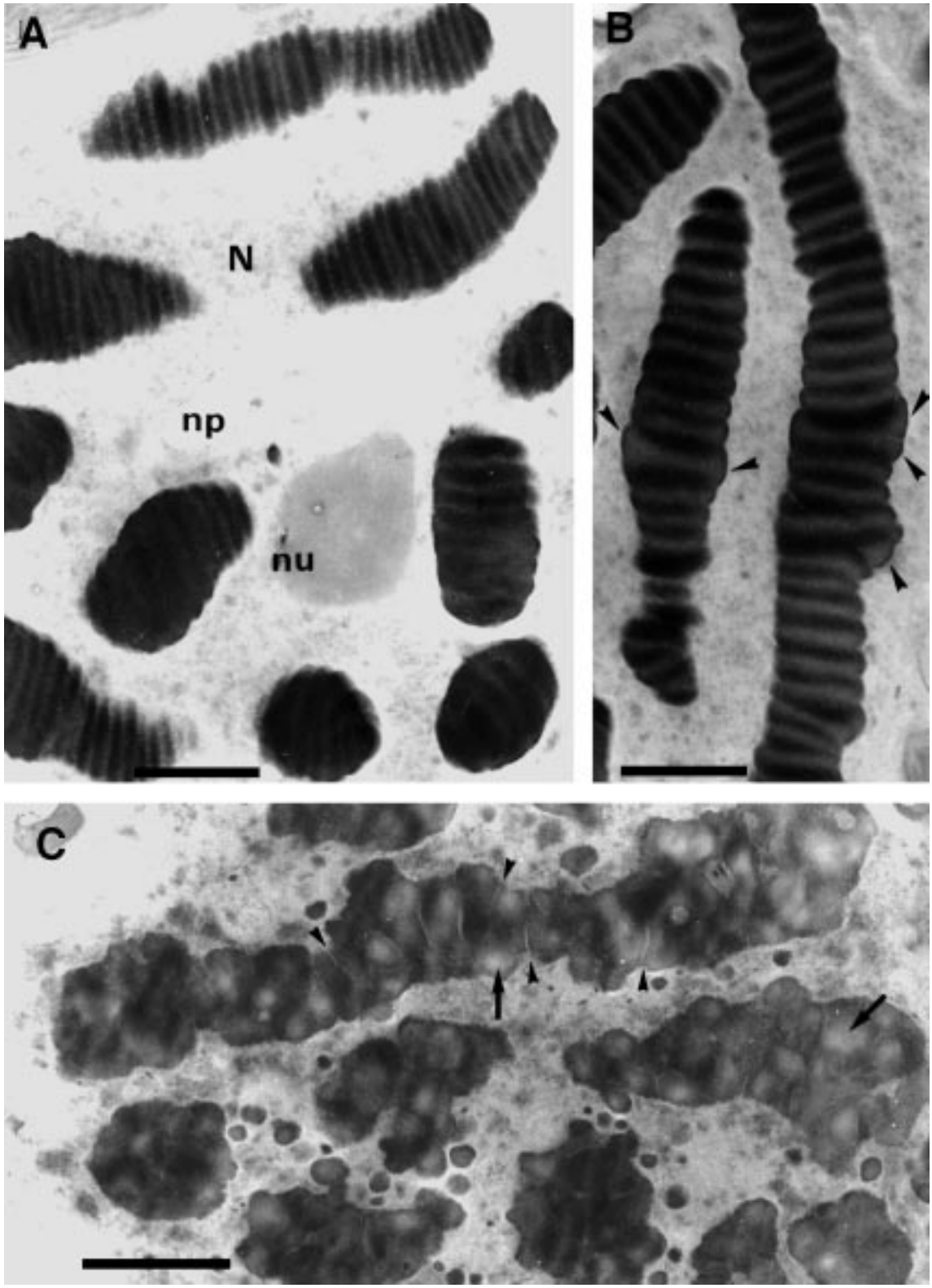

Fig. 2 Ultrathin sections of *Prorocentrum micans* nuclei after 6 days of 50 μg/ml BrdU incorporation and HP cryofixation followed by a OsO_4 cryosubstitution. (A) G1-phase nucleus (N). Nucleolus (nu) is present and chromosomes are compacted. (B) Early S-phase in which the periodic organization of the chromosomal nucleofilaments begins to disorganize (arrowheads). (C) Late S-phase in which all the chromosomes present spherical hernia (arrows) individualized by crack regions (arrowheads). Bars = 1 μm

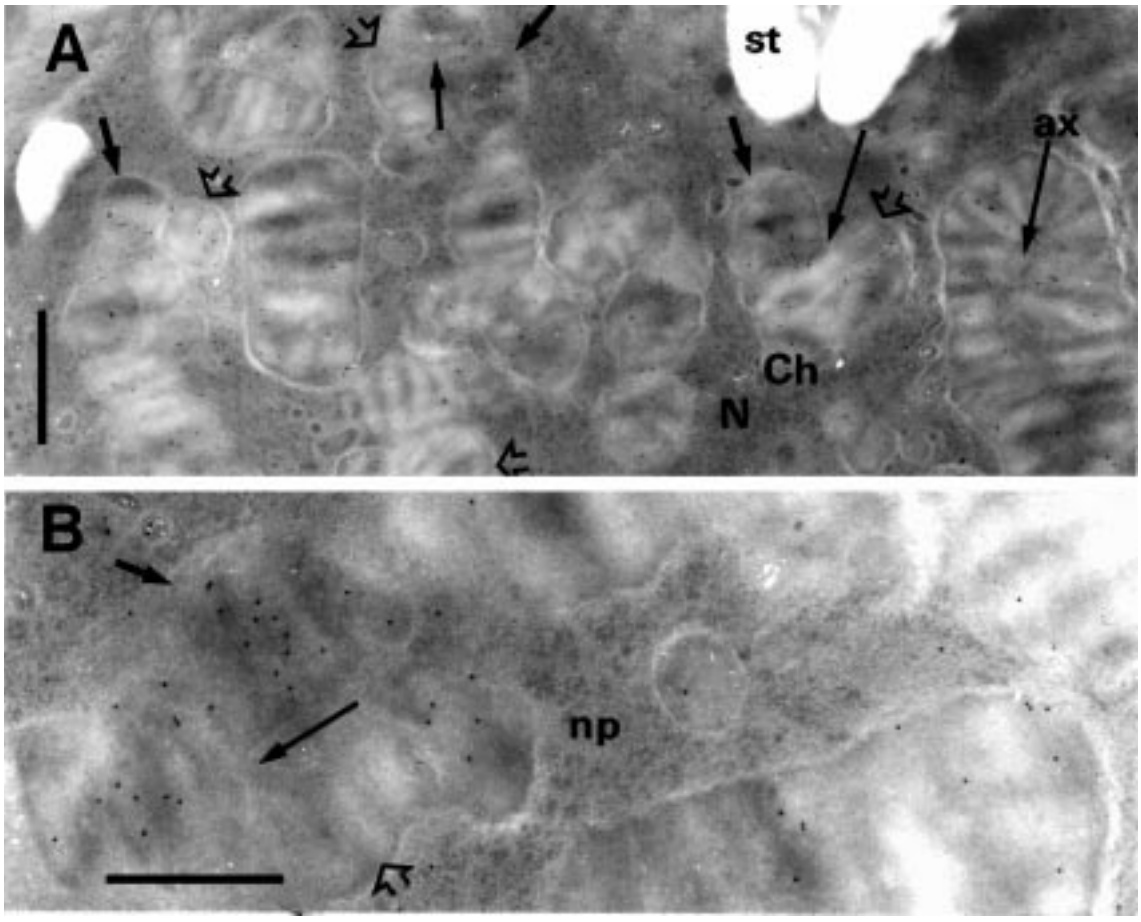

Fig. 3 Fine localization of the DNA replicating sites on ultrathin sections of a late S-phase nucleus after 6 days BrdU incorporation. Chromatids are incompletely labelled with antiBrdU antibody conjugated with 10 nm gold particles. (A) Spherical hernia are visible (arrows) but some chromatids incompletely separated are more labelled (thick arrows) than others (empty arrows). Almost no gold particles are visible in the nucleoplasm (np). Some segregation axes (ax) are visible (thin arrows). (B) Detail of a segregating chromosome in which one chromatid is apparently more labelled (big arrow) than the other (empty arrow). st: starch. Bars = 1 µm

Correspondence between light and electron microscopy Thymines replaced by analogue molecules (BrdU) and detected with anti-BrdU antibodies were located on 0.2 µm diameter domains (mean of about 15 optical sections) visible with the same apparent diameter at light and ultrastructural levels. As S-phase progressed, these clusters organized helically along the ancient chromosome. Pictures suggested that new DNA replication domains were organized as a helix. Actually, *P. micans* whole chromosome spread on water and observed under TEM showed its nucleofilaments organized in double right-hand helix [12]. This architecture, maintained by divalent cations [19] and structural RNAs [41], seems to continue to control the distribution of the new replication domains. Moreover, replication seems to begin at the chromosome periphery, where the active part of the chromatin was believed to be located [1], and to continue toward the chromosome axis. Later, clustered domains occupied the whole chromosome volume, and the helix henceforth became invisible.

Asynchronous early replication Origins of replication appeared asynchronously and replication gradually continued all along the chromosome. This lead to an asynchrony, which is clearly obvious, among the hundreds of chromosomes present within the nucleus of *P. micans*.

Early replication: comparison between the dinoflagellate model and other eukaryotes The initial replication foci, grouped in clusters, were located in the tip of chromosomes at the beginning of S-phase in the vicinity of the nuclear envelope, contrary to what occurs in other eukaryotic cells, where replication begins in the centre of the nucleus and ends in its periphery [16, 26]. The lamin, as a continuous thick layer of the nuclear matrix, is located in the periphery of the dinoflagellate nucleus, as demonstrated in *A. carterae* by Minguez et al. [29]. Study of interactions between dinoflagellate chromosome tips and the lamin could bring information about this original behaviour. Note also that the nuclear envelope of Dinoflagellates persists over the whole cell cycle, and that this contact between chromosome and nuclear envelope is necessary for the envelope to participate in the chromosome segregation [40].

Dinoflagellates, a new model to study replication Elucidation of the relations between dinoflagellate replication foci and the nuclear matrix is essential, especially in lamins and the homologue of chromatin assembly factor 1 (CAF-1). In fact, Spann et al. [44] demonstrated that, in *Xenopus laevis* egg extracts, the disruption of nuclear lamin organization alters the distribution of the replication factors and inhibits DNA synthesis. Lamins are necessary for nuclear functions and especially for the DNA

replication machinery (for review see [22]). In dinoflagellates, there is little information so far on lamins. In *Amphidinium carterae*, however, several types of intermediate filaments have been described, one of which is immunologically related to vertebrate lamins [29]. The implications of the presence of both these proteins and topoisomerase II on the replication of permanently compacted chromosomes should be investigated. The investigation of dinoflagellate DNA synthesis is at an early stage, this present study on the immunolocalization of early replication foci being the first step for more extensive analyses.

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