Dinoflagellates have a eukaryotic nuclear matrix with lamin-like proteins and topoisomerase II

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SUMMARY

Unicellular Dinoflagellates represent the only eukaryotic Phylum lacking histones and nucleosomes. To investigate whether Dinoflagellates do have a nuclear matrix that would modulate the supramolecular organization of their non-nucleosomal DNA and chromosomes, cells of the free-living unarmored Dinoflagellate Amphidinium carterae were encapsulated in agarose microbeads and submitted to sequential extraction with non-ionic detergents, nucleases and 2 M NaCl. Our results demonstrate that this species has a residual nuclear matrix similar to that of vertebrates and higher plants. The cytoskeleton-nuclear matrix complex of A. carterae shows a relatively intricate polypeptide pattern. Immunoblots with different antibodies reveal several intermediate filament types of proteins, one of which is immunologically related to vertebrate laminas, confirming that these proteins are ancestral members of the IF family, which is highly conserved in eukaryotes. A topoisomerase II homologue has also been identified in the nuclear matrix, suggesting that these structures could play a role in organizing the Dinoflagellate DNA in loop domains. Taken together our results demonstrate that the nuclear matrix is an early acquisition of the eukaryotic nucleus, independent of histones and nucleosomes in such a way that the mechanisms controlling the two levels of organization in eukaryotic chromatin would be molecularly and evolutionarily independent.

Key words: nuclear matrix, Dinoflagellate, lamin, topoisomerase II

INTRODUCTION

Dinoflagellates are primitive unicellular algae that present some prokaryotic characteristics in the organization of their DNA and chromosomes, which lack histones and nucleosomes (Herzog and Soyer, 1981, 1983; Herzog et al., 1984, Soyer-Gobillard and Herzog, 1985; Taylor, 1987a; Rizzo, 1987) and contain a substantial replacement of thymidine by the rare base hydroxymethyl-uracil (Steele and Rae, 1980). They represent the only eukaryotic Phylum that lacks histones and nucleosomes. Their DNA compaction and chromosome architecture, as well as other features in which they resemble the bacterial nucleoid, have led several authors to assume a primitive status for the Phylum, which would have emerged near the bottom of the eukaryotic tree (see Loeblich, 1984).

However, recent results of comparisons of 24-28 S rRNA sequences do not confirm the latter hypothesis but indicate a rather late appearance of the Dinoflagellates, which represent a monophyletic group with close relationships to yeast and ciliates (Sala Rovira et al., 1991; Lenaers et al., 1991). Besides the molecular structure of their rDNAs, they show other eukaryotic features such as a high DNA content/cell, the presence of repeated sequences interspersed in a similar way to other eukaryotes, and also a similar G+C content (see Herzog et al., 1984). The molecular structure of the Dinoflagellate snRNAs also appears to be eukaryotic (Reddy et al., 1983).

Phylogenetic trees based on sequence data of 5 S RNA and snRNAs indicate that the Dinoflagellate lineage joins the tree at the point of plant-animal divergence, which implies that the absence of histones and nucleosomes in the eukaryotic chromatin of Dinoflagellates would be the result of a secondary loss by deletion of the histone gene cluster (Cavalier-Smith, 1981), and not an indication of a primitive state. Their unusual chromatin composition perhaps resulted in the evolution of an unusual chromosome architecture (Loeblich, 1984; Soyer-Gobillard and Géraud, 1992).

From a structural point of view, Dinoflagellates show a well organized nuclear envelope and nucleolus. At present the knowledge of the Molecular Biology of the Dinoflagellates is very rudimentary and does not allow the establishment of a precise taxonomic classification of this Phylum.

Therefore Dinoflagellates can be regarded as being very close to prokaryotes and considered as a stage in the evolution of eukaryotic chromatin, or be firmly placed within the eukaryotes, but having an alternative to nucleosomes in their packaging of huge amounts of DNA, which have only limited evolutionary potential. For this reason, the study of the higher-order organization of the nucleus and DNA in Dinoflagellates is crucial for understanding the processes of gene organization in eukaryotes.

DNA has several forms of higher-order structure in eukaryotes, which are organized in a particular pattern that results in
the expression of the proper genes in a specific tissue and the correct cell timing (see Pienta et al. 1991; Getzenberg et al., 1991, for reviews).

The first-order structure of DNA is the 2 nm right-handed double helix, which consists of two antiparallel strands of purines and pyrimidines held together by hydrogen bonds and hydrophobic interactions. This B-form of DNA is in the majority in the mammalian genome, but others such as the left-handed helix of Z-DNA, or the alternative right-handed A-DNA, also exist. The physical control of this first-order organization of DNA appears to occur by methylation. The nucleosomal fibre constitutes the second order of DNA organization. The nucleosomal core is an octamer of two copies of the histones H2A, H2B, H3 and H4. The DNA winds around the nucleosome core, approximately 200 bp around each histone octamer forming the 10 nm nucleosomal chromatin fibre. The H1 histone binds to the nucleosomes and the chromatin fibre, contributing to a further packing of the chromatin fibre. Nucleosome positioning, as well as histone modifications are involved in the transcriptional regulation of genes at this level. The nucleosomal fibre may be further folded into 30 nm filaments, which provide a packing ratio of the DNA of approximately 35 to 50 within the filament. Histone H1 plays a central role in forming and stabilizing these higher-order conformations. The 30 nm filaments form topologically and functionally independent DNA loop domains of approximately 60 kb, which are topologically organized by certain nuclear matrix proteins in a sequence-controlled way, by association with the matrix-associated region (MAR) elements, and represent yet another level of the structural organization of chromatin, in such a way that the chromatin domains or loops most probably represent units of coordinate gene expression (see Pienta et al., 1991).

The DNA of Dinoflagellates is organized into two chromosomal regions: the main body, which contains genetically inactive DNA and plays a role in the maintenance of chromosome structure, involving cross-linking of DNA to the protein matrix; and the peripheral diffuse region, which contains the DNA active in transcription (Sigee, 1984). Both B- and Z-DNA have been detected in the chromosomes of Dinoflagellates by immunological methods (Soyer-Gobillard et al., 1990), and the application of spreading techniques to Dinoflagellate nuclei suggests that the DNA in them forms continuous loops (Oakley and Dodge, 1979; Dodge, 1985).

Two-dimensional electrophoresis has demonstrated low $M_f$ basic proteins in the Dinoflagellate nucleus, which are less abundant (only 10% of the DNA mass) and display significantly lower affinity for DNA than do eukaryotic core histones (Vernet et al., 1990; Sala Rovira et al., 1991; Géraud et al., 1991b). Psoralen-crosslinking reveals that only 20% of the genome presents protected regions, and these are clustered in 10-15 kilobase pairs separated by highly unprotected longer regions (Yen et al., 1978).

In higher eukaryotes, the nuclear matrix provides a three-dimensional support for the specific organization and regulation of genes (Berezney, 1991; Getzenberg et al., 1991). Therefore the investigation of a eukaryotic nuclear matrix in Dinoflagellates, which would modulate the organization of their DNA in loops corresponding to structural and functional domains, and also of the interactions between some of the proteins of this matrix and certain sequences of Dinoflagellate DNA, would be very interesting.

To begin these studies, we chose an ‘evolved’ unarmored free-living species: *Amphidinium carterae* (Dinophyceae, Gymnodiniales) (Taylor, 1987b), which presents one of the lowest DNA contents of the taxum (2.2 pg/nucleus) (Galleron and Durrand, 1978), analogous to those of other typical eukaryotes like chicken and tomato (see Marie and Brown, 1993), and a low chromosome number. All these features, and its easy growth in dense cultures in the controlled growth phase, make this species very appropriate for the analysis of the nuclear matrix.

Although there exist several studies on the nuclear ultrastructure (Babillot, 1970; Oliveira and Huyhn, 1989), cell cycle (Matthys-Rochon, 1979) and DNA organization (Galleron and Durrand 1978, 1979; Sigee, 1984) in this species, as far as we know this is the first time in which the study of the nuclear matrix of this or any other Dinoflagellate species has been undertaken.

With this aim, living cells of *A. carterae* were microencapsulated in agarose and submitted to sequential extraction to produce nuclear matrices in situ. These structures were studied by electron microscopy and their proteins separated by SDS-PAGE. The presence of two matrix proteins involved in the organization of DNA loops in higher eukaryotes (lamin and topoisomerase II) was investigated in the Dinoflagellate nuclear matrix by immunological methods.

### MATERIALS AND METHODS

Cells of *Amphidinium carterae* Hulburt, *Dinophyceae, Gymnodiniales* (Taylor, 1987b), collected from the coast of Portugal were cultured in DV medium added with AM9 antibiotic mixture (Provasoli, 1963), at 19-21°C, and 3500 lux (from cool-white fluorescent lamps) on a daily 14:10 LD cycle. Cultures from 75 ml Erlenmeyer flasks in the exponential phase of growth were transferred to 1000 ml Erlenmeyer flasks and, after approximately 25 days, dense cultures in early stationary growth phase were prepared for further study.

### Nuclear matrix preparation

A total of 2×10^8 cells from 3,600 ml cultures at late log phase and early stationary phase were harvested by centrifugation at 1800 rpm and encapsulated in 0.5% agarose microbeads according to Jackson and Cook (1985). The beads were collected at low speed, washed, extracted with the mild non-ionic detergent Triton X-100 for 55 minutes to lyse the cells, and submitted to sequential extraction with 10 mM Tris-HCl, 5 mM MgCl₂, 1 mM PMSF, 1 mM DTT, pH 7.4, containing in each case: 1st, 20 µg/ml DNase I, 30 minutes at 20°C; 2nd, 0.25 mM MgCl₂, 15 minutes at 4°C; 3rd, 50 µg/ml DNase I and 50 µg/ml RNase A, 30 minutes at 20°C; 4th, 2 µM NaCl, 30 minutes at 4°C; and in some cases, 5th, 4 M urea, 30 minutes at 4°C.

### Protein analysis

After nuclear matrix preparation, the remaining pigments in the pellets, which interfere with SDS during electrophoresis, were extracted by sonication in light petroleum (b.p. 50-70°C) according to the method of Zardo et al. (1991). The extraction was repeated several times until the extract was colourless. After vacuum drying, rehydration in buffer and dialysis, samples were extracted at 100°C for 5 minutes in sample buffer and proteins run in either 10% or 8% acrylamide gels according to Laemmli (1970), as previously described (Moreno Díaz de la Espina et al., 1991), in a miniprotein II dual slab cell. Gels were stained with either Coomassie Blue or silver.
**Immunoblotting**

After electrophoresis, samples were transferred to nitrocellulose membranes in a Mini-transblot cell (Bio-Rad). After washing in 0.05% Tween-20 in PBS and blocking with a 3% solution of dried non-fat milk in distilled water, blots were incubated with the primary antibodies. Second antibodies were peroxidase labelled. The reaction was revealed by the ECL western blotting system from Amersham as previously described (Mínguez and Moreno Díaz de la Espina, 1993).

**Antibodies**

The following antibodies and dilutions were used: a rabbit polyclonal against chicken lamins (1:75) (Stick and Hausen, 1980) and two monoclonals: L74A2 (against lamin L1 from *Xenopus*) and L34B4 (against chicken lamin A; Mínguez and Moreno Díaz de la Espina, 1993); the IFA, recognizing a very conserved epitope in all types of IF proteins (undiluted) (Pruss et al., 1981), MAC 322 a monoclonal against carrot cytoskeletons recognizing a very conserved cytokeratin 8 epitope shared by plants and animals (Ross et al., 1991), and a polyclonal anti-human topoisomerase II (1:25) (Cambridge Research Biochemicals).

**Immunofluorescence**

Encapsulated cells and cytoskeleton-nuclear matrix fractions, were fixed in 0.3% paraformaldehyde (PFA) in PBS, pH 7.0, for 1 hour at 4°C, washed in the same buffer, spread on polylysine-coated slides, air dried, quickly dipped in methanol-acetone at −20°C and stored at −20°C.

After washing in PBS with 0.05% Tween, for 10 minutes at room temperature, and blocking with 2% BSA in PBS containing 0.05% Tween, the slides were incubated with the primary antibodies during two separate periods of 18 seconds under microwave irradiation according to Medina et al. (1994), and washed with the same buffer. Incubation with the FITC-conjugated anti-rabbit IgG (Sigma) at a 1:50 dilution was performed for 1 hour at room temperature. After washing, preparations were mounted with an antifading medium containing 90% glycerol and 0.01% paraphenylenediamine in PBS. Negative controls were performed by omitting the primary antibody or replacing it with purified rabbit IgG. The reaction with the antibody was observed under an epifluorescence microscope using a 450-490 nm filter.

**Electron microscopy**

Agarose beads containing either whole cells or cytoskeleton-nuclear matrix complexes, were fixed in 4% PFA in PBS, pH 7.2, for 2 hours at 4°C, dehydrated in an ethanol series and embedded in LR White acrylic resin as previously described (Mínguez and Moreno Díaz de la Espina, 1993).

**RESULTS**

**Microencapsulation of cells**

To prevent morphological alterations and redistribution of the cellular components during the preparation of nuclear matrices, living cells of *A. carterae* were encapsulated in 0.5% agarose microbeads. As observed under phase contrast, the cells of *A. carterae* inside the agarose beads are well preserved compared with those that were observed free in a buffer solution (Fig. 1A and B). The beads show an average diameter of 400±170 µm and usually contain abundant cells in their interiors. The process of encapsulation does not produce structural alterations in the cells, as observed in the light or electron microscope. In both free and encapsulated cells, the nuclei show a distal position within the cell and are less refringent than the cytoplasm, which appears brownish-yellow due to the abundant cytoplasmic pigments (not shown here). In the electron microscope, the encapsulated cells appear well preserved. The cell wall shows no shrinkage and both nucleus and cytoplasm appear well preserved without retractions, demonstrating a good penetration of the fixatives through the agarose (Fig. 2A,B). The cytoplasm of encapsulated cells is rich in abundant mitochondria, plastids,
vacuoles and starch grains. The nucleus has the typical arched chromosomes of Dinoflagellates, the nucleolus is bound to the nuclear envelope, and there is a rich fibrogranular nucleoplasm (Fig. 2B).

**Ultrastructural organization of the nuclear matrix**

After sequential extraction to produce the nuclear matrices in situ, the cells appear highly extracted in the light microscope in relation to the non-extracted controls. The cytoplasm appears almost colourless. Within it, the remnants of the pyrenoid and the cytoskeletal network are the most evident structures. The residual nucleus is also highly extracted and has the same relative position within the cell as it had before the extraction (Fig. 1C).

In the electron microscope the effectiveness of the extraction is very evident. The cytoplasm shows no membranous components. It appears to be composed almost exclusively of elements of the fibrogranular cytoskeleton that are connected to the cell wall and the periphery of the nuclear matrix (Fig. 3A,B). The residual pyrenoid is also connected to the cytoskeleton (results not shown here).

The nuclear matrix maintains a distal position within the cell and shows a very constant structural organization, similar to that of other eukaryotes, with a lamina, a complex network
forming the internal matrix, and a residual nucleolar matrix (Fig. 3B). The zones previously occupied by the chromosomes in the nucleus always appear as empty areas after the extraction (compare Figs 1A,C, and 2A,B with Fig. 3B).

Higher magnifications give detailed information of the ultrastructural organization of the Dinoflagellate nuclear matrix (Figs 4 and 5A).

The lamina forms a continuous thick layer at the periphery of the nucleus, and is tightly bound to the internal matrix on its inner side (Fig. 4). The fixation and embedding conditions used here are not the most adequate to display the fibrillar network of the cytoskeleton, but in preparations of cytoskeleton-nuclear matrix (CSK-NM) complexes that are eventually extracted with 4 M urea some fibres are occasionally visualized pervading the cytoskeletal network and making contact with the nuclear lamina (not shown here). The lamina is a well-developed nuclear structure in Dinoflagellates, which is evident even in unextracted cells of certain species as a dense fibrillar layer at the inner face of the nuclear envelope (Fig. 5B). The internal matrix forms an intricate network of fibrillar patches (formed by thin 5 nm fibres), corresponding to the residual elements of the interchromosomal and perichromoso-

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**Fig. 3.** Encapsulated cells of *A. carterae* after sequential extraction to produce nuclear matrices in situ. The only structures surviving the extraction are the nuclear matrix (NM) and the cytoskeleton (CSK), which is connected to both the cell wall (cw) and the nuclear matrix (NM) (A). Transverse section. (B) Longitudinal section. The nuclear matrix (NM) maintains the distal position within the cell and shows a similar organization to those of the rest of the eukaryotes. Bars, 1 µm.
mal regions of the nucleus (Fig. 5A,B). Granules are scarce in this internal matrix, but structures morphologically similar to ‘coiled bodies’, formed by twisted 10-15 nm threads, are constant components of the matrices. The nucleolar matrix presents a morphology similar to that of the unextracted nucleolus with a fibrillar structure that is very similar to those of plant and vertebrate nucleolar matrices (Fig. 4).

Protein composition of the cytoskeleton-nuclear matrix complex

Whole Amphidinium cells have a heterogeneous polypeptide pattern in 1-D SDS-PAGE gels, with many bands between 125 and 15 kDa. Their major components migrate at 120, 79, 66, 64, 60, 44 and 34 kDa. They also show abundant low molecular mass components, below 24 kDa. The CSK-NM complexes show less polypeptides than whole cells, being enriched in components at 120, 79, 51, 28 and 26 kDa. Low molecular mass components are less abundant in these preparations than in whole cells (Fig. 6).

To investigate the functions of the Dinoflagellate nuclear matrix in organizing DNA loops, we tested the presence of two proteins known to be involved in the topological organization of eukaryotic DNA: lamins and topoisomerase II.

Immunological detection of lamins

In these experiments, proteins transferred to nitrocellulose membranes were incubated with an anti-lamin polyclonal serum that recognized conserved epitopes of lamins present in birds, plants and amphibia (Mínguez and Moreno Díaz de la Espina, 1993) as well as two monoclonals against type A (chicken lamin A) and type B (L1 from Xenopus) lamins of vertebrates. As controls, antibodies recognizing very conserved epitopes of higher eukaryote intermediate filament (IF)-cytoskeletons were used: IFA, which reacts with an epitope common to all types of IF proteins, and MAC 322, which recognizes a very conserved epitope of cytokeratin 8 shared by higher plants and animals. MAC 322 shows no reaction with the proteins of the CSK-NM of Amphidinium (not shown here). The IFA recognizes three different bands. The most abundant with a molecular mass of 66 kDa and two others at 58 and 26 kDa. Low molecular mass components are less abundant in these preparations than in whole cells (Fig. 6).

When extracted cells of A. carterae were incubated with the anti-lamin serum and observed by indirect immunofluorescence the nuclear lamina appeared decorated, standing out from the weak autofluorescence of the pigments of the Dinoflagellate cytoskeleton (Fig. 8A,C). When the IFA antibody is used, both the cytoskeleton and nuclear matrix appear strongly fluorescent, indicating the presence of abundant IF type proteins in these structures (Fig. 8B,D). In negative controls, in which incubation with the primary antibody was omitted, only a weak autofluorescence was observed (Fig. 10C,F and data not shown).

Table 1. Crossreactivity of antibodies against nuclear matrix and IF proteins with the residual proteins of Dinoflagellates

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<tr>
<th>Antibody</th>
<th>Type</th>
<th>187</th>
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<td>Cytokeratin 8 (mammalian) and nuclearoplasmic human topo II 170</td>
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<td>topo II</td>
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<td>42, 50, 55 (plants)</td>
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M, monoclonal; P, polyclonal. Intensity of reaction, strong (+++); medium (++); weak (+); no reaction (−).

Fig. 4. Nuclear matrix of A. carterae with the three domains typical of eukaryotic nuclear matrices: nucleolar matrix (Num) keeping the general morphology of the nucleolus, the internal matrix (im) corresponding to the remnants of the interchromosomal nucleoplasm, and a lamina (arrowheads) connected to the cytoskeleton (CSK). Bar, 1 µm.

Table 2. Crossreactivity of antibodies against nuclear matrix and IF proteins with the residual proteins of Dinoflagellates

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<td>42, 50, 55 (plants)</td>
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Fig. 5. (A) Higher magnification of a portion of the nuclear matrix of *A. carterae*, to show in more detail the fine organization of the lamina and internal matrix (im). The structures similar to coiled bodies (double arrowheads) form a part of the internal matrix. The 5 nm fibrils of the internal matrix are displayed individualized at the periphery of the patches of the internal matrix network, and denoted by arrowheads.

(B) Section of a *Gymnodinium splendens* whole cell labelled with an anti-DNA antibody, which marks the chromosomes. Underneath the nuclear envelope, there is a continuous dense thick well-organized lamina (arrowheads), only interrupted at the pore complexes (double small arrows). Chromosomes (Chr) are decorated by gold particles. The nucleoplasm surrounding the chromosomes is fibrillar but also shows abundant granules (arrow). Nu, nucleolus. Cyt, cytoplasm. Bars, 0.5 µm.
Immunological detection of topoisomerase II

The anti-topoisomerase II polyclonal antibody, directed against a peptide of human topoisomerase II, recognizes a band at 187 kDa in the NM-cytoskeleton of *A. carterae*, indicating that this enzyme or a related protein with common sequences is a component of the Dinoflagellate nuclear matrix (Fig. 9; Table 1).

Immunofluorescence detection of the antigen indicates that the topoisomerase II labelling is confined to the nucleus in whole cells (Fig. 10A,D), and to the nuclear matrix after sequential extraction (Fig. 10B,E), while the negative controls...
show no reaction except for some autofluorescence of the cytoskeleton (Fig. 10C,F).

DISCUSSION

In spite of the interesting features of the nuclei of Dinoflagellates (see Spector, 1984, for a review), which is the only Phylum in eukaryotes lacking histones and nucleosomes, and presenting characteristics of both prokaryotes and eukaryotes (Herzog et al., 1984; Rizzo, 1987, 1991), no studies to search for a subnuclear structure responsible for the organization of their components (Berezney, 1991; Getzenberg et al., 1991) have been undertaken.

However, the investigation of a eukaryotic nuclear matrix in the Dinoflagellates, regulating the organization of their non-nucleosomic DNA in functional and structural loop domains, is very necessary to understand fully the evolution of the two levels of chromatin organization in eukaryotes.

This situation is probably not due to a lack of interest, but rather to methodological problems in the culture and fractionation of these systems. In fact, most of the procedures used for the preparation of nuclear matrices require starting from purified isolated nuclei in relatively large amounts. The presence of a rigid cell wall and the high DNA content of most Dinoflagellate species (Rizzo, 1987) make the isolation and further handling and extraction of their nuclei very difficult.

To overcome these problems, we chose for our experiments one species (A. carterae) that presents many advantages for this kind of investigation. A. carterae is an unarmored ‘evolved’ Dinoflagellate (Taylor, 1987b) with a low DNA content of 3 pg/haploid genome (Galleron and Durrand, 1978, 1979), similar to, or even lower than, that of several higher plants from which nuclear matrices have been successfully prepared (Marie and Brown, 1993; Moreno Díaz de la Espina et al., 1991), and which grows very easily in dense cultures.

The availability of large amounts of starting material and the use of the microencapsulation methods to prepare nuclear matrices, avoiding the isolation of nuclei (Jackson and Cook, 1983; Jackson et al., 1988), along with the specific characteristics of these cells (lack of rigid theca and low DNA content), has allowed us to investigate the existence of a nuclear matrix in these systems.

The microencapsulation methods that have been successfully used for the preparation of in situ nuclear matrices from cultured cells (Jackson et al., 1988; Lang et al., 1993) and plasmodia (Waitz and Loidl, 1988) are shown to be very reliable for the routine preparation of nuclear matrices from Dinoflagellate species, which do not have a rigid theca. This methodology not only facilitates the handling of the cells during the extraction procedure but, what is more important, avoids the isolation of nuclei, which leads to a poor recovery of nuclear matrices. Phase-contrast as well as electron microscopy controls confirm that the cells are well preserved in the beads, and do not experience morphological distortion, breakage or shift of their components during the preparation of nuclear matrices. Extraction with the different solutions, as well as nuclease digestions proved to be very effective and homogeneous. These preparations also preserve the connections between the nuclear matrix and the cytoskeleton.

For all these reasons we think that this method constitutes the first step in routine exploration of the Dinoflagellate nuclear matrix that is applicable to other unarmored Dinoflagellate species.

In spite of the big differences in nuclear organization between Dinoflagellates and higher eukaryotes, our results reveal that they have a eukaryotic nuclear matrix, very similar to that of animals (Berezney, 1984; Verheijen et al., 1988), plants (Moreno Díaz de la Espina et al., 1991; Frederick et al., 1992) and lower eukaryotes (Wunderlich and Herlan, 1977; Waitz and Loidl, 1988; Cárdenas et al., 1990; Lang et al., 1993), but with slight ultrastructural variations corresponding...
to the peculiarities of the nuclear organization in this Phylum (Mínguez et al., unpublished data). These results demonstrate that the nuclear matrix is a very conserved structure and represents an early acquisition of the eukaryotic nucleus, independent of that of histones and nucleosomes, in such a way that the mechanisms controlling the two levels of organization of the eukaryotic chromatin would be molecularly and evolutionarily independent.

Ultrastructural organization

The Dinoflagellate nuclear matrix has three well-defined morphological components, like those of the rest of the eukaryotes studied so far: lamina, nucleolar matrix and internal matrix. The presence of a well-developed lamina in Dinoflagellates is evident, not only in the extracted matrices but also in the intact nuclei of several species that present a lamina even much more developed than is usual in higher eukaryotes. The immunological detection of lamin-related proteins in this structure confirms that the lamina is an ancestral nuclear structure in eukaryotes that should serve in essential housekeeping functions for the cell (Dessev, 1992; Gerace, 1986), and thus has been very well conserved in the eukaryotic kingdom. The detection of a lamina in other lower eukaryotes evolutionarily close to Dinoflagellates (Sogin, 1991) such as the Ciliates (Wunderlich and Herlant, 1977), yeasts (Allen and Douglas, 1989; Georgatos et al., 1989) and Myxomimycetes (Waitz and Loidl, 1988; Lang et al., 1992), confirms that this structure probably arose in the ancestral eukaryotic cell. On the other hand the identification of proteins immunologically related to lamins in Dinoflagellates confirms that these proteins are ancestral members of the IF family that have been highly conserved during eukaryotic evolution (Doring and Stick, 1990; Dodemont et al., 1990).

In higher eukaryotes the lamina provides integrity to the nuclear envelope during interphase. During mitosis it is involved in prophase nuclear disassembly, as well as in reassembly at late telophase (Benavente and Krohne, 1986; McKeon, 1991; Dessev, 1992; Moir and Goldman, 1993). In Dinoflagellates with permanently condensed chromosomes, and a 'closed' mitosis that proceeds within an intact nuclear envelope (Matthys-Rochon, 1979; Heath, 1980), the lamina, besides providing support to the envelope, probably helps to develop some special features in their unusual mitosis, such as the anchoring of chromosomes through the kinetochores, which move by the action of associated cytoplasmic microtubules that do not enter the nucleus (Oakley and Dodge, 1974; Matthys-Rochon, 1979; Heath, 1980). The existence of common nucleoplasmic domains for internal lamins and kinetochores in higher eukaryotes has already been suggested (Moir and Goldman, 1993).

The morphological and ultrastructural characteristics of the residual nucleolus or nucleolar matrix in Dinoflagellates are very similar to those of higher eukaryotes: vertebrates (Verheijen et al., 1984; Berezney, 1984), plants (Mínguez and Moreno Díaz de la Espina, unpublished data; Moreno Díaz de la Espina et al., 1991), or of lower eukaryotes (Waitz and Loidl, 1988; Cárdenas et al., 1990). This result is not surprising if we consider that the existence of a nucleolus for rRNA processing and ribosome subunit assembly is one of the universal characters of eukaryotes (Cavalier-Smith, 1981), and also constitutes one of the best-conserved eukaryotic nuclear features of Dinoflagellates (Spector, 1984; Salamin-Michel et al., 1990; Geraud et al., 1991a; Soyer-Gobillard and Geraud, 1992). Along these lines, the substructure responsible for the coordination and spatial organization of all these processes should also be preserved during evolution.

The structural organization of the internal matrix is basically similar to that described in higher (Verheijen et al., 1988; Moreno Díaz de la Espina et al., 1991) and lower (Wunderlich and Herlant, 1977; Mitchelson et al., 1979; Waitz and Loidl, 1988; Allen and Douglas, 1989) eukaryotes. It consists mainly of basic 5-10 nm fibres and scarce 20-25 nm granules, and corresponds to the residual elements of the nucleoplasm in whole cells. One characteristic of the internal matrix in Dinoflagellates is the presence in them of abundant structures morphologically similar to coiled bodies, but which share some features with interchromatin granules (IG), like the content of highly phosphorylated proteins, the presence in the nuclear matrix and some common epitopes, but whose function in the intact nuclei is not yet clearly understood (Mínguez et al., unpublished data).

Protein composition

Electrophoretic analysis shows that the polypeptide pattern of the network formed by the nuclear matrix and the cytoskeleton in Dinoflagellates is complex and differs from that of the whole cells. Residual proteins with variable molecular mass values appear constantly enriched in these residual structures.

The immunological analysis performed in parallel in blots and immunofluorescence staining of whole cells and residual structures has allowed us to identify some of the components of these residual structures.

The IFA antibody recognizes the consensus sequence at the carboxy-terminal end of the rod domain of IF proteins, which is a very conserved epitope in nearly all the IF-type proteins described so far (Pruss et al., 1981). It reveals three different IF type proteins, demonstrating that IF are ancestral proteins much more widely expressed in lower eukaryotes than currently thought. The IF antigens of Dinoflagellates do not crossreact with another type known to be shared by plant and animal cytoskeletons (Ross et al., 1991). As the phylogenetic studies point to lamins as the most ancestral members of the IF family of proteins (Doring and Stick, 1990; Dodemont et al., 1990), one could expect that the bands recognized by IFA would correspond to different lamins. But IFA immunofluorescence reveals that this is not the case, implying the existence of cytoplasmic IFA epitopes in single cell eukaryotes.

The crossreactivity of the residual IF proteins of Dinoflagellates with different anti-lamin antibodies indicates that the two bands at 62 and 58 kDa, recognized by antibodies against the two types of vertebrate lamins, correspond to Dinoflagellate lamins, while the non-reactive band at 66 kDa, which is the most abundant in these preparations, could be responsible for the IFA immunofluorescence of the cytoskeleton. Immunofluorescence after incubation with these antibodies shows that lamins are components of the well-developed lamina present in Dinoflagellates.

The band at 50 kDa, which crossreacts with all the anti-lamins but not with IFA could also correspond to a lamin or a related IF protein, due to the low reliability of this antibody in detecting IF proteins in lower eukaryotes, in which the IFA epitope is not always conserved in IF proteins, while a positive reaction to IFA has always led to the molecular characteriza-
ation of true IF proteins (Riemer et al., 1991). Interestingly, a 50 kDa lamin-like protein has also been detected in Physarum nuclear matrices (Lang et al., 1992). The molecular mass values of the bands that show crossreactivity with the anti-lamin antibodies are slightly lower than those reported for lamins in higher eukaryotes, either animal (Lehner et al., 1986; Krohne and Benavente, 1986) or plant (Mínguez and Moreno Díaz de la Espina, 1993), but are close to the values reported for Physarum lamins (Lang et al., 1992). Although further experiments would be necessary to determine the biochemical and structural characteristics of Dinoflagellate lamins, our results confirm for the first time the presence of proteins immunologically related to eukaryote lamins in the lamina of the Dinoflagellate nucleus.

The presence in Dinoflagellates of a homologue of topoisomerase II, immunologically related to the eukaryotic enzyme and with conserved molecular mass values, which presents a eukaryotic DNA in its sequence organization, with large amounts of repetitive DNA although lacking histones and nucleosomes, like the prokaryotes (Cavalier-Smith, 1981; Herzog et al., 1984), reinforces the idea of a eukaryotic origin for this Phylum. The experimental procedure used to obtain the matrices, using low temperatures and reducing conditions, excludes the possibility of the stabilization of the topoisomerase II in the matrix by heat or disulphide bond formation (Kaufmann and Shaper, 1991).

Besides its catalytic function, topoisomerase II also performs a structural role in the maintenance of the attachment of the eukaryotic DNA loop domains to the nuclear matrix (Razin et al., 1991; Hsieh, 1992; Razin and Vassetzky, 1992). Thus the detection of topoisomerase II in the Dinoflagellate nuclear matrix strongly suggests that this structure is involved not only in the described eukaryotic functions of their nuclei, like those related to RNA synthesis and further processing (Reddy et al., 1983; Liu et al., 1984; Franca, 1980), but also in maintaining their unusual DNA in loops, which have been already visualized in spreading preparations of Dinoflagellate chromosomes (Dodge, 1985).

The presence of lamins and topoisomerase II, both involved in the binding of MAR sequences to the nuclear matrix (Ludérus et al., 1992; Razin et al., 1992), as well as the preliminary results of the experiments showing the association of Drosophila MAR sequences with the Dinoflagellate matrix (data not shown here), confirm that the Dinoflagellate nuclear matrix plays a role in organizing the DNA in functional loops (Bodnar, 1988), and therefore that the mechanisms controlling the two levels of organization of eukaryotic chromatin, i.e. nucleosomes and loops, are evolutionarily independent.

Implications for the evolutionary position of Dinoflagellates

The transition between prokaryotes and eukaryotes is a problem of cellular evolution involving structures with basic properties, which can be studied in living species. The fact that Dinoflagellates posses all 22 universal characteristics defined for eukaryotes (Cavalier-Smith, 1981), but only a few of those typical of prokaryotes, makes it very unlikely that the ancestor of Dinoflagellates was a prokaryote.

The only confirmed prokaryotic features of this Phylum are the lack of histones and nucleosomes, the arch-shaped structure of their chromosomes, and the attachment of the dividing chromosomes to membranes (the nuclear envelope in the case of Dinoflagellates). On the contrary, they show many characteristics of eukaryotic cells. Their cytoplasmic organization is as complex as that of any other eukaryotes (Dodge and Greuet, 1987), and they have similar meiosis.

Dinoflagellate DNA is fully eukaryotic in its sequence organization with large amounts of repetitive DNA (Rizzo, 1987, 1991). Our results demonstrate that Dinoflagellates have a topoisomerase II that is immunologically related to the eukaryotic enzyme to negatively supercoil their DNA, which could also play a role in organizing DNA loops. As this enzyme appears attached to the Dinoflagellate nuclear matrix, it would allow independent uncoiling of different chromatin domains for gene expression as in eukaryotes.

Dinoflagellates also have a discrete S period of DNA synthesis in the cell cycle and tandemly repeated rRNA sequences (Herzog et al., 1984). The presence of snRNPs involved in the processing of the 3′ end of mRNA also represents a eukaryotic feature of Dinoflagellates. Sequence data of rRNAs, 5 S RNA and snRNAs have confirmed the eukaryotic position of Dinoflagellates (Lenaers et al., 1984; Liu et al., 1984).

All the above findings confirm the eukaryotic origin of this Phylum.

The fact that the most primitive extant Dinoflagellates, the parasitic species and Oxyrrhis marina, have histones and chromosomes that lack the arched whorls of chromatin, suggests that their absence in the free-living species is due to a secondary loss (Loeblich, 1984). Along these lines, if the common ancestor of Dinoflagellates lost their nucleosomes by deletion of the clustered histone genes, which appears to be the most probable mechanism in view of the molecular data available (Loeblich, 1984; Cavalier-Smith, 1988), the regulatory system of loop domains (Bodnar, 1988) was able to control gene expression in the absence of nucleosomes.

On the other hand, their unusual mechanism of closed mitosis (shared with the parasitic Dinoflagellates and Parabasalian flagellates, both of which have histones), probably helped to protect their non-nucleosomal DNA against nucleases during mitosis, avoiding shearing, and probably allowed them to survive the chromosomal deletion that eliminated the clustered histone genes (Cavalier-Smith, 1981).

An interesting question that remains open is which developed first in evolution: DNA loop domains or nucleosomes.

Experiments in progress on the association of conserved MAR sequences with the proteins of the Dinoflagellate nuclear matrix and also with this structure in situ, should definitively clarify the functions of this nuclear matrix in the organization and control of loop domains in the unusual Dinoflagellate chromatin. They should also allow evaluation of the conservation of the MAR sequences and the corresponding binding proteins during the evolution of eukaryotes.

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Dinoflagellate nuclear matrix 2873


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