Cell division cycle and determination of arrest points in marine dinoflagellates

Ciclo de división celular y determinación de puntos de restricción en dinoflagelados marinos

Sonsoles González-Gil*, Angeles Aguilera, Cristina Fenoy, Soledad Andújar, Victoria López-Rodas and Eduardo Costas

Genética. Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense. 28040. Madrid, Spain.
**Present address: Instituto Español de Oceanografía. Apto. 1552, 36280 Vigo, Spain.*
Gonzalez@vi.ieo.es

ABSTRACT

Several marine dinoflagellate species, *Prorocentrum minimum*, *Prorocentrum lima*, *Prorocentrum triestinum* and *Gymnodinium catenatum*, were studied using four different methodologies, microfluorimetry, flow cytometry, spectrofluorimetry and cellular kinetics, to determine both the length of the phases of the cell cycle and the number of arrest points. Despite the technique used, all the analyzed species showed an eukaryotic model exhibiting a typical eukaryotic cell cycle with four well differentiated phases: G_1, synthesis, G_2 and mitosis. Furthermore, in order to analyze the existence of arrest points in dinoflagellates, all the species were cultured under restriction conditions, 4°C, 35°C, darkness and nutrient depletion. All species showed two restriction points, at G_1 and G2, as most of eukaryotic cells do. Depending on the restriction condition, however, some species may pause the cell cycle in G_1 while others stop in G_2.

Key words: cell division cycle, arrest points, dinoflagellates.

RESUMEN

Con el propósito de determinar la duración de las fases del ciclo celular así como el número de puntos de restricción de algunos dinoflagelados marinos, *Prorocentrum minimum*, *Prorocentrum lima*, *Prorocentrum triestinum* y *Gymnodinium catenatum*, se utilizaron cuatro técnicas: microfluorimetría, citometría de flujo, espectrofluorimetría y cinética celular. En todos los casos, sin importar la técnica empleada, los dinoflagelados mostraron un ciclo celular típicamente eucariota con cuatro fases bien diferenciadas: G1, síntesis, G2 y mitosis. Para la determinación de los puntos de restricción, se cultivaron los dinoflagelados en diferentes condiciones de restricción, 4°C, 35°C, oscuridad y falta de nutrientes, mostrando en todos los casos dos puntos de restricción, uno al inicio de la fase G1 y otro en la fase G2, como si de organismos eucariotas se tratara. Sin embargo, y dependiendo de las condiciones de cultivo, algunas especies paran su ciclo celular en G1 mientras que otras lo hacen en G2.

Palabras clave: ciclo de división celular, puntos de restricción, dinoflagelados.

INTRODUCTION

In most of the eukaryotic cells, the cell division cycle is carried out via cell division cycle-proteins (cdc proteins) that determine the entry into the two main phases of the cell cycle, the interphase and the mitosis (M phase). While interphase starts with the G_1 phase in which the biosynthetic rate of the cell reaches its highest value, the entry into mitosis is preceded by the G_2 phase. Progression from one phase to another is usually regulated by a number of controls acting on different genes and proteins implicated in the normal development of the cell cycle (Nurse 1990). Each step is to ensure that the initiation of mitosis only occurs after the completion of DNA replication (Hartwell & Weinert 1989).

One of the critical points regulating proliferation is the point called start or R point, where cells decide to commit to cycle or not to initiate it if the environmental conditions are unfavorable for growth (Pardee 1989). The main controls regulating the G_2/M boundary have been conserved throughout evolution (Nurse 1990, Norbury & Nurse 1992, Murray 1993), while controls regulating the G_1/S boundary, are less known.

One of the most controversial group of organisms with respect to nuclear organization are the dinoflagellates, since they have both prokaryotic and eukaryotic characteristics. In this sense, the chromosomes appear attached to the nuclear membrane and they lack histone proteins as the rest of the prokaryotic organisms do (Rizzo & Nooden 1974, Karentz 1982), but at the same time they contain repeated sequences of DNA like those of eukaryotes (Loeblich 1976).

Due to these facts, they have been considered an unique group at least among all the algal groups, even at the evolutionary level, being included as an intermediate group between prokaryotes and eukaryotes for which the term mesokaryotic has been used (Dodge 1966, Loeblich 1976, Herzog *et al.* 1984). However, more recently, and based on the molecular analysis of ribosomal RNA gene sequences, dinoflagellates have been considered as real eukaryotic organisms (Zardoya *et al.* 1995).

In this sense, the presence or absence of a true cell cycle in dinoflagellates has long been disputed, especially the relationship between cell cycle dynamics and some ecological aspects related to the bloom events they produce. Using different methodologies such as labeling of DNA precursors or total DNA measurements, both the prokaryotic pattern of continuous DNA synthesis and the eukaryotic pattern of discrete synthesis have been observed within the dinoflagellates (Dodge 1966, Filfilan & Sigee 1977).

If we look at the unicellular organisms under a more "ecological" point of view, they are generally assumed to proliferate and be limited only by the rate at which nutrients can be taken up from the medium and converted in cell materials. In particular, dinoflagellate blooms are thought to be controlled by abiotic variables (i.e.water column stratification, wind tides and currents, light, temperature and other hydrographic features) and by nutritional requirements (i.e. eutrophication, pollution, micro and macro-nutrients) (Fraga 1989, Prego 1993).

However, other mechanisms regulating mitosis rate, such as biological controls could play an important role in the autocontrol of algal proliferation. In this respect, circadian and annual rhythms, endogenous control of mitosis, and biosynthetic rates could be controlling factors in determining the proliferation of bloom-forming or toxic dinoflagellates (Fraga & Prego 1989, Margalef 1989, Taylor 1990). In this sense, several of these controls have recently been demonstrated to be present in some unicellular algae. For instance, the animal growth factor PDGF as well as the phorbol ester monoacetate 12-phorbol increased mitotic rate of marine phytoplanktonic organisms (Costas *et al.* 1993a), while some of these mitogenes are also able to germinate resting cysts of dinoflagellates (Costas *et al.* 1993b).

More recently new methodologies have been used to study the cell cycle in algal groups. Relative amounts of DNA and RNA per cell in cell populations have been measured in some phytoplankton species using flow cytometry by employing different DNA/RNA dyes (Olson *et al.* 1983, Yentsch & Mague 1983). Microfluorimetry has also been employed to study, for instance, the growth rates of phytoplankton species as well

as to measure DNA in nuclei, plastids and virus particles (Coleman *et al.* 1981). Although a lot of phytoplanktonic species had been analyzed with these methodologies, no approach has yet been made for dinoflagellate species, at least with respect to the analysis of the arrest point of their cell cycle.

Due to all the facts above mentioned, an attempt of determining both the number of the restriction points in dinoflagellates and the lengh of the different phases of their cell cycle was made. Four different techniques were used for these purposes (microfluorimetry, spectrofluorimetry, flow cytometry and cellular kinetics) in order to compare the results from the different techniques used and also to determine the best technique to use.

MATERIAL AND METHODS

CULTURES

Axenic cultures of the dinoflagellates *Prorocentrum minimum* Pavillard (Schiller), *Prorocentrum lima* Ehrenberg (Dodge), *Prorocentrum triestinum* Schiller and *Gymnodinium catenatum* Graham from the culture collection of Genetics (Veterinary Faculty, UCM, Madrid, Spain) were grown in natural seawater (from Galician coast, NW Spain) enriched with f/2 medium (sw+f/2, Guillard 1975) at 20° C and 50 µmol photons m^{-2} s^{-1} from fluorescent tubes (Phillips daylight) under 12:12 h L:D cycles as previously described (Costas 1990, Costas *et al.* 1993a). In addition, two more conditions were used with *P. minimum* such as a temperature of 15°C and an addition to the medium of 12 ng ml^{-1} of phorbol 12-monoacetate (PMA, Sigma, St. Louis, MO, USA). All the clones were maintained in exponential growth by serial transfers once every 15 days. Cultures were periodically checked using epifluorescence procedures to show the absence of bacteria (Costas 1990). All the cultures were grown in balanced growth, i.e. cells were growing desynchronized, so cells showed constant properties (Cooper 1991). For all the estimations, the procedure followed was the staining of the DNA and then:

- For the determination of the length of each phase of the cell cycle a mathematical approach was made and the following techniques were used: microfluorimetry and flow cytometry.

- For the determination of the arrest points the techniques used were microfluorimetry, spectrofluorimetry and cellular kinetics.

DNA STAINING

Cells in balanced growth were collected by centrifugation at 350 x g for 10 min and then fixed with 2% buffered formaldehyde for 2 hours. Then, the samples were washed twice with methanol/acetic acid 1:3 to eliminate photosynthetic pigments. Finally, every sample were divided into two aliquots of $10^5 \pm 10^3$ cells and then one of them was treated with DAPI (4´6-diamin-dino-2-phenylindole, Sigma) and the other one with acridine orange (Sigma), both dyes at a concentration of 10 µg ml^{-1}.

MATHEMATICAL APPROACH AND LENGTH OF CELL CYCLE

The DNA staining in cultures of *Prorocentrum minimum* was measured by flow cytometry (EPIC 541, Coultronics Inc., Florida, using a specific protocol to select nuclear fluorescence from DAPI with an excitation/emission pair of 351/365 nm and 250 mV) and also by microfluorimetry (Zeiss Axiovert epifluorescence microscope using an orange acridine specific filter set of 450-490 nm excitation and 520-560 nm emission). With the remainding cultures (*Prorocentrum lima, P. triestinum* and *Gymnodinium catenatum*) the technique used was microfluorimetry. Doubling time of each

species was estimated to determine the length of each stage in the cell cycle, using the Brand *et al.* (1981) equation:

$\tau = t \ln 2 (\ln Nt\text{-}No)$

where:

τ = doubling time
t = time between Nt and No

To calculate the doubling time, three replicates of each culture were counted in a Neubauer hemocytometer chamber as well as in a fluorimeter (at an initial time No and three days later, Nt, when all the cells had replicated).

This mathematical approach is based on a graphic representation (Figure 1), with the following parts: bell curve around relative fluorescence 1 (cells in G_1 phase, with a single complement of DNA), bell curve around 2 (cells in G_2+M phase, i.e. cells that have replicated their DNA and so have a double complement) and distribution determined by the equation $2^{(1-x)}$ (cells in S phase, that are replicating their DNA and thus have intermediate amounts of DNA), where x is the percentage of cells ($^0/1$) in that phase.

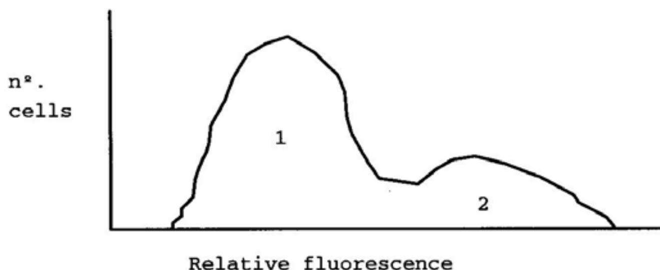

Figure 1. Plotting relative fluorescence against number of cells in each phase: normal distribution around relative fluorescence values of 1 (cells in G_1) and 2 (cells in G_2+M), while the intermediate values are distributed following a second-grade polynomial equation (cells in S).

Figura 1. Representación gráfica de fluorescencia relativa frente al número de células en cada una de las fases del ciclo celular: distribución normal en valores de fluorescencia alrededor de 1 (células en G1) y 2 (células en G2+M), mientras que los valores intermedios se distribuyen siguiendo una ecuación polinomial de segundo grado (células en S).

With these approaches is possible to count the cells in each stage, make corrections derived from equation $2^{(1-x)}$ and calculate the length of each stage in the cell cycle by applying the correction formula: doubling time x cell percent x correction factor. At last, times must be relativized, according with the doubling time. More details are given in Cooper (1991).

ARREST POINTS MEASUREMENT

To study the arrest points, all the clones were grown in artificial ASPM seawater enriched with f/2 (Guillard 1975) instead of sw+f/2, in order to avoid any possible influence coming from nutrients present in the natural seawater. All of them were then cultured under especial restriction conditions such as a lacking of one of the main nutrients from the f/2 medium

(e.g. vitamins, nitrogen, phosphorus or metals), or under special physical conditions (4°C, 35°C and darkness). In this latter case the medium used was sw+f/2 instead of ASPM+f/2, since the nutrients were not as important here as in the other case.

In order to determine the arrest points of all the species, a range of techniques were used. In the case of *Prorocentrum minimum*, the DNA staining measurement was followed by three techniques: spectrofluorimetry, with a Shimazdu spectrofluorometer (Shimazdu RF 551 S), using the excitation/emission wavelengths of 325/475 nm for DAPI; microfluorimetry using a Zeiss Axiovert epifluorescence microscope with a pair of 351/365 nm for DAPI and 450-490/520-560 nm for acridine orange; and finally by a cellular kinetics system. This latter system is a theoretical approach based on the Cooper (1991) equation:

$$AP = \tau \, [\, \ln (Nt / No) / \ln 2]$$

where
AP = arrest point
τ = doubling time
Nt = number of cells at time t
No = number of cells at time 0
t = transcurred time between 0 and t

The DNA-stained measurement for the rest of the species, *Prorocentrum lima*, *P. triestinum* and *Gymnodinium catenatum* was done using spectrofluorimetry and the cellular kinetics system. In all the experiments the number of counts was estimated according to progressive means technique (Williams 1977) to obtain a confidence value of 1%.

RESULTS

LENGTH OF THE CELL CYCLE PHASES

The length of each stage of the cell cycle of *Prorocentrum minimum* in the following culture conditions: sw+f/2 at 15°C, sw+f/2 at 20°C and sw+f/2+PMA at 20°C, were determined using microfluorimetry and flow cytometry (Table 1). Both techniques gave similar values for the length of the stages G_1, S and G_2+M, with no differences between them. However, the culture conditions showed different doubling times, not between both techniques but between the different conditions. So, the lower the temperature the higher the doubling time. In the same way, the doubling time is shorter when a phorbol ester is added to the culture medium. In comparison with all the phases, G_1 is the longest and the one with more variations in time, while the remainder are relatively constant under all the different culture conditions.

The main difference between both techniques is the number of cells analyzed. This number is much lower with microfluorimetry than with flow cytometry and thus, for example, the number of counted cells for G_1 phase by microfluorimetry is 70 while by using flow cytometry is 26166.

The length of G_1, S and G_2+M stages in *Prorocentrum lima*, *P. triestinum* and *Gymnodinium catenatum* were determined using microfluorimetry and the results are shown in Table 2. As with *Prorocentrum minimum*, G_1 stage seems to be the most variable and longest stage in time for all the species, while S and G_2+M are shorter. Furthermore, the time for the G_2+M stage seems the most constant in all the species. With respect to the doubling time, *Gymnodinium catenatum* is the species that has the longest doubling time of all the species analyzed.

ARREST POINTS

The arrest points in *Prorocentrum minimum* were determined using the three techniques mentioned above, i.e. microfluorimetry, spectrofluorimetry and cellular kinetics. The results are shown in Table 3, 4 and 5 respectively. All the

techniques used show the same results, that is *P. minimum* had an arrest point at the end of G_1 phase when it was grown without nitrogen, phosphorus or metals. The second arrest point was at the end of G_2 stage when the temperature condition were 4°C and 35°C, in darkness and in vitamin restriction.

With the remainding cultures, *Prorocentrum lima*, *P. triestinum* and *Gymnodinium catenatum*, the techniques used to determined the arrest point were spectrofluorometry (Table 4) and cellular kinetics (Table 5).

Table 1. Length in hours of the different phases (G_1, S and G_2+M) of *Prorocentrum minimum* in the following culture conditions: sw+f/2 at 15°C, sw+f/2 at 20°C and sw+f/2+PMA at 20°C, using microfluorimetry and flow cytometry. τ= doubling time; PMA= monoacetate 12-phorbol; sw+f/2= natural seawater enriched with f/2.

Tabla 1. Duración en horas de las diferentes fases del ciclo celular (G1, S y G2+M) de *Prorocentrum minimum* en las siguientes condiciones de cultivo: sw+f/2 a 15°C, sw+f/2 a 20°C y sw+f/2+PMA a 20°C, usando microfluorimetría y citometría de flujo. τ = tiempo de duplicación; PMA= monoacetato 12 de forbol; sw+f/2= agua de mar natural enriquecida con f/2.

	Microfluorimetry			Flow cytometry		
	sw+f/2 15°C	sw+f/2 20°C	sw+f/2+PMA 20°C	sw+f/2 15°C	sw+f/2 20°C	sw+f/2+PMA 20°C
τ	31.70	29.10	21.60	31.7	29.1	21.6
G_1	24.97	22.29	15.54	25.8	23.9	13.7
S	2.99	3.27	2.83	2.9	2.4	2.4
G_2+M	3.66	3.50	3.22	3.0	2.8	2.5

Table 2. Length in hours of the different phases (G_1, S and G_2+M) in *Prorocentrum lima*, *P. triestinum* and *Gymnodinium catenatum* estimated by microfluorimetry. τ = doubling time.

Tabla 2. Duración en horas de las diferentes fases (G_1, S y G_2+M) de *Prorocentrum lima*, *P. triestinum* y *Gymnodinium catenatum* usando microfluorimetría. τ = tiempo de duplicación.

	P. lima	*P. triestinum*	*G. catenatum*
τ	34.00	25.60	38.20
G_1	22.36	14.99	24.70
S	3.89	2.99	5.49
G_2 + M	7.75	7.62	7.92

Table 3. Arrest points (DNA contents) of *Prorocentrum minimum* estimated by microfluorimetry. sw+f/2= natural seawater enriched with f/2; ASPM+f/2= artificial seawater ASPM enriched with f/2.

Tabla 3. Puntos de restricción (cantidad de DNA) de *Prorocentrum minimum* usando microfluorimetría. sw+f/2= agua de mar natural enriquecida con f/2; ASPM+f/2= agua de mar artificial ASPM enriquecida con f/2.

Condition	DNA	SD
sw+f/2, darkness	214	10
sw+f/2, 4°C	206	12
sw+f/2, 35°C	214	13
ASPM+f/2, without vitamins	215	6
ASPM+f/2, without metals	107	10
ASPM+f/2, without phosphorus	104	6
ASPM+f/2, without nitrates	103	8

Table 4. Arrest points of *Prorocentrum minimum*, *P. lima*, *P. triestinum* and *Gymnodinium catenatum*, using the spectrofluorometry technique. Numbers represent the time (in hours) before mitosis in which the arrest point is. sw+f/2= natural seawater enriched with f/2; ASPM+f/2= artificial seawater ASPM enriched with f/2.

Tabla 4. Puntos de restricción de *Prorocentrum minimum*, *P. lima*, *P. triestinum* y *Gymnodinium catenatum*, usando espectrofluorimetría. Los números representan el tiempo (en horas) antes de la mitosis, en el que el punto de restricción aparece. sw+f/2= agua de mar natural enriquecida con f/2; ASPM+f/2= agua de mar artificial ASPM enriquecida con f/2.

	P. minimum	*P. triestinum*	*P. lima*	*G. catenatum*
sw+f/2, darknes	2.18	1.03	1.98	1.02
sw+f/2, 4°C	2.1	1.99	2.09	1.05
sw+f/2, 35°C	2.18	1.94	0.97	1.06
ASPM+f/2, without vitamins	2.19	1.94	1.98	2.03
ASPM+f/2, without metals	1.09	1.97	0.96	0.96
ASPM+f/2, without phosphorus	1.06	0.97	0.97	1.08
ASPM+f/2, without nitrates	1.05	2.06	0.95	1.04

Table 5. Arrest points of *Prorocentrum minimum*, *P. lima*, *P. triestinum* and *Gymnodinium catenatum*, using the cellular kinetics technique. Numbers represent the time (in hours) at which the arrest point appears before the mitosis occurs. sw+f/2= natural seawater enriched with f/2.

Tabla 5. Puntos de restricción de *Prorocentrum minimum*, *P. lima*, *P. triestinum* y *Gymnodinium catenatum*, usando la técnica de cinética celular. Los números representan el tiempo en horas en el que el punto de restricción aparece antes de que ocurra la mitosis. sw+f/2= agua de mar natural enriquecida con f/2.

	P. minimum	*P. triestinum*	*P. lima*	*G. catenatum*
sw+f/2, darkness	3.57	15.8	7.04	24.7
sw+f/2, 4°C	3.07	2.98	7.16	26.1
sw+f/2, 35°C	3.96	3.16	29.9	23.8

These results showed a high variability between the different species studied when they were grown under the same culture conditions. In this sense, when the darkness was the specific condition, *Prorocentrum minimum* and *P. lima* showed arrest points in G_2 while *P. triestinum* and *Gymnodinium catenatum* do it in G_1. With 4°C as temperature condition, *G. catenatum* showed an arrest point at G_1, and the remainding species at G_2. With a temperature of 35°C, *Prorocentrum minimum* and *P. triestinum* arrested at G_2, and *P. lima* and *Gymnodinium catenatum* at G_1.

When the culture restrictions are metals or nitrogen, the *Prorocentrum lima* arrest point is at G_2, and for the remainding species it is at G_1. In cultures without phosphorus all species studied showed the arrest point at G_1, in cultures without vitamins all species studied arrested at G_2.

DISCUSSION

The four species of dinoflagellates analyzed represent the two evolutionary levels of this group, *Prorocentrum minimum* being the most primitive and *Gymnodinium catenatum* the most evolved. Although some works related to the cell cycle of dinoflagellates have showed that species such as *Prorocentrum minimum* or *P. lima* belonging to more ancient group, the prorocentroids, tend to have a similar cell cycle to that of prokaryotic organisms (Loeblich 1976,

Filfilan & Sigee 1977, Galleron & Durrand 1979), the results obtained here show that these species have a typical eukaryotic cell cycle, at least in terms of cell cycle analysis.

Studies of the cell cycle have usually been carried out in synchronized cultures (Cheng & Carpenter 1988, Cetta & Anderson 1990) and not in balanced growth cultures, where the cells grow asynchronically. When the cells grow without · synchrony, the experiments can be repeated time after time without apparently any change (Cooper 1991), and no matter what methodology is used for the measurement of the length of the cell cycle phases. Also, these facts are extendible to the characteristics of a natural population, in which the cells have no synchrony at all.

Flow cytometry and microfluorimetry are probably the most useful techniques to measure the fluorescence intensity of the DAPI- or acridine orange-DNA complex of each cell (Coleman *et al.* 1981, Yentsch & Mague 1983). Flow cytometry allows the investigator to measure rapidly and with great accuracy a variety of cell constituents, of which DNA is the most commonly measured. The best characteristic of flow cytometry is the capacity of quantitative measurements on a large number of cells (typically 50 000 cell min^{-1} for a suspension of 10^6 cell ml^{-1}) (Olson *et al.* 1983). However, the main problem with respect to flow cytometry is the use of species as *Gymnodinium catenatum* which is chain forming, or *Prorocentrum lima* which grows in colonies on the bottom of the flasks. These facts make the measurement of the individual cells more difficult than in other alternative techniques, such microfluorimetry.

The results obtained by microfluorimetry are in agreement with those proposed by Dean & Jett (1974) for mammalian cells, that is cells in asynchronic cultures which are in G_1 phase of their cell cycle have an arbitrary unit of DNA while the cells in G_2+M have two units and cells in S phase have an intermediate value between 1 and 2. So, plotting relative fluorescence against number of cells in each phase give us a normal distribution around fluorescence values of 1 and 2, while the intermediate values are distributed following a second-grade polynomial equation (see Figure 1). G_1 phase is the longest in time for all the species analyzed as also occurs with mammalian cells. It is in this phase when the cells may pause their cell cycle in a quiescent state if the environmental conditions are unfavorable for growth (Alberts *et al.* 1983).

These results may also be seen in *Prorocentrum minimum* when its doubling time under the different culture conditions was analyzed, as well as in the analysis of the length of each phase. Thus, when PMA is added to the culture medium of *P. minimum* its doubling time is shorter than the doubling time in the other conditions (15°C or 20°C). The PMA is a potent mitogen in mammalian cells that acts in the inositol pathway activating the mitosis by stimulation of protein kinase C (Parker 1986, Lamph *et al.* 1988). Several studies had been previously carried out successfully on many dinoflagellates and other algae to prove the importance of these mitogenes and growth factors in algae (Costas *et al.* 1993a). The action of PMA on the lenght of G_1 phase, could be the reason why the length of the G_1 phase is shorter when this phorbol ester is added to the culture than at other temperature conditions. The optimal temperature for *P. minimum* to grow is 20°C, so if we modify this condition to another of 15°C, the period for the doubling time is lower.

Little is known of the origin and ecological implications of PMA-like mitogenes or growth factors in marine ecosystems. Like other eukaryotic cells, dinoflagellates may obtain growth factors

mainly via endogenous production. However, growth factors may also be originated through bacterial activity. Recently, the production and secretion of diacylglicerol, an activator of protein kinase C for cell division, has been reported in bacteria (Morotomi *et al.* 1990). Moreover, some evidence suggests that dinoflagellates are also able to secrete telemediators, which act paracrinely on the near cells. In this respect, studies on rhythmicity show that dinoflagellates are able to intercellular cross-talk by telemediators (Cornelissen *et al.* 1986), and also inhibit diatom growth by means of telemediators (Arzul & Gentien 1990).

Although several works suggest that some dinoflagellates have a restriction point at the end of G_1 while others has it at the end of G_2 (Costas *et al.* 1988, Cetta & Anderson 1990), the species analyzed here show both restriction points, i.e. at G_1 and G2 phases, as has been shown when they were grown under restrictive culture conditions. Depending on the restriction condition, some species may pause their cell cycle in G_1 or in G_2, and thus when the culture medium is lacking phosphates, all the species stop the cell cycle in G_1, since phosphorus is a vital component for the synthesis of the DNA, the next step in the cell cycle (S phase). In contrast, when the medium has no vitamins, all the species pause their cell cycle in G_2, because vitamins are needed in the achromatic tubuline polymerization (Alberts *et al.* 1983).

However, when nitrogen lacked, *Prorocentrum triestinum* was the only species that pause its cell cycle in G_2, while the others did it in G_1. Nitrogen is the main structural component of proteins and enzymes, so it is necessary for the completion of a correct S phase. Some species can store more nitrogen in the cytoplasm than others, so maybe this could be the reason why *P. triestinum* have an arrest point in G_2 instead of in G_1.

In order to estimate the importance of the temperature and light in the duration of the different phases of the cell cycle, we used cellular kinetics approaches, and once again all the mathematical and theoretical approximations agree with the existence of two arrest points, sometimes at G_1 and in some others at G_2, in the same way as eukaryotic cells.

All the methodologies and approximations, point to the existence of a real eukaryotic cell cycle for dinoflagellates, with the same arrest points as the rest of the eukaryotic organisms. More studies related to the molecular basis of these arrest points could be very helpful in the study of the phylogenetic position of these controversial organisms.

ACKNOWLEDGEMENTS

This work was supported by the Spanish DGICYT Grant PB93-O433. SGG and AA are supported respectivelly by Ministerio de Educación y Ciencia (Spain) fellowships PF94-7224875 and AP94-51384765. Thanks are due to Marta Castillejo for technical assistance with laboratory cultures.

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Manuscrito recibido en diciembre de 1997 y aceptado en mayo de 1998