ELECTROPHORETIC CHARACTERIZATION OF NUCLEAR BASIC PROTEINS IN THE TOXIC MARINE DINOFLAGELLATE *Gymnodinium mikimotoi*

A Thesis

by

MATTHEW JAMES WARGO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2000

Major Subject: Botany

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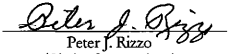

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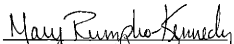

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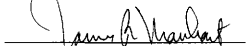

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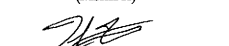

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August 2000

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ABSTRACT

Electrophoretic Characterization of Nuclear Basic Proteins
in the Toxic Marine Dinoflagellate *Gymnodinium mikimotoi*. (August 2000)
Matthew James Wargo, B.S., Susquehanna University
Chair of Advisory Committee: Dr. Peter J. Rizzo

Gymnodinium mikimotoi is a toxic unicellular marine dinoflagellate that frequently forms red tide blooms in both the northern Atlantic and northern Pacific Oceans. *G. mikimotoi* was chosen as a study organism because of its high growth rate and its relatively fragile cell wall in comparison to other dinoflagellates. Dinoflagellates do not have histones, possessing instead one to several nuclear basic proteins, which play an unknown role in the dinoflagellate nucleus. Characterization of these histone-like proteins, will further elucidate their role. Only one acid extractable protein was seen in one-dimensional SDS-PAGE, which showed a V8 protease digestion pattern similar to that of HCc. When the nuclear acid extract was run on AU-PAGE and AUT-PAGE, two and four bands were seen, respectively. It was determined that *G. mikimotoi* has four HGm variants, named $\alpha 1$, $\alpha 2$, β, and γ. Through peptide mapping, variants β and γ were seen to be similar, as were $\alpha 1$ and $\alpha 2$. Acidic native APGE showed possible oxidized protein spots, consistent with those seen previously in *Crypthecodinium cohnii*. HGm variants responded to nitrogen stress and time of day, but did not respond to other environmental variations. Therefore, while the protein was further characterized, its function remained elusive.

DEDICATION

I dedicate this work to all of the teachers who inspire students everyday.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my wife, Amy, for all of her love and support. I would also like to thank my family for allowing me to pursue a career in academia, even though it means staying in school forever.

If it wasn't for Robert Waters, my high school earth science teacher, I would not have started on the path I am today, and if it wasn't for Jack Holt, my undergraduate advisor, I'd probably be a neurobiologist or some other ghastly thing. So my thanks go out to these two.

I would also like to thank Pete Rizzo for allowing me to work in his lab, and for his guidance throughout my graduate career so far. Finally, I would like to thank my committee members for helpful suggestions for my proposal and thesis.

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INTRODUCTION

General Dinoflagellate Biology. The dinoflagellates (Dinophyta) are a phylum of unicellular eukaryotic protoctists that inhabit a wide range of aquatic environments, although most species are marine (Spector 1984). Within this phylum are approximately 130 genera and over 2000 extant species, with approximately 2000 fossil species described as well (Taylor 1987). Within the dinoflagellates there is an almost even split between autotrophic and heterotrophic species, the heterotrophs generally being holozoic or saprophytic (Spector 1984). Dinoflagellates form an important link in many marine food webs, and at times are so numerous as to change the color of the water to green, brown, or red, in spectacular but economically disastrous blooms (Spector 1984). The dinoflagellate taxonomy of Dodge (1984) describes twelve extant orders, one of which is the Gymnodiniales to which the focus organism of this study belongs. As a group, the Gymnodiniales are characterized in part by being free-living, dorsiventrally compressed motile cells, which possess thecal vesicles but no detectable thecal plates (Spector 1984).

The biggest puzzle in dinoflagellate biology is the presence of several unique nuclear features, which distinguish the group from all other eukaryotes. First, their chromosomes never visibly decondense, remaining discrete throughout the cell cycle (Rizzo 1987). Although this makes chromosome counting easier, it complicates the research of DNA replication and genome structure. Second, while dinoflagellates maintain permanently condensed chromosomes, they do so without the presence of the typical complement of eukaryotic histones (Rizzo 1987). Instead they possess one

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This thesis follows the style and format of the *Journal of Phycology.*

to three basic proteins of similar molecular mass (Vernet et al. 1990). It is these "histone-like" proteins (HLPs) that are the focus of this study. In addition to the above features, dinoflagellates also have an unusual form of mitosis, in which the nuclear envelope never breaks down (Rizzo 1987).

While the dinoflagellates do possess some odd nuclear attributes, they are not an ancient, early-branching monophyletic group, as had been originally postulated. As recent evidence indicates, dinoflagellates are most closely related to the ciliates (i.e. *Paramecium*, *Tetrahymena*, *Stentor*) and the apicomplexans (i.e. *Plasmodium*, *Toxoplasma*, *Gregarina*) (Lenaers et al. 1991). This places them in an interesting evolutionary position, especially regarding the discovery of degenerate plastids in the apicomplexans (Wolters 1991). Some authors suggest that dinoflagellates and apicomplexans had a plastid-bearing ancestor, although there are problems with this hypothesis, including the inability to resolve the species which branch near the bottom of this clade (Lang-Unnasch et al. 1998). Apart from the relationship with the apicomplexans, dinoflagellates are also evolutionarily interesting because of their "knack" for gaining and losing plastids (Schnepf 1993). This "knack" has provided some dinoflagellates, including the focus organism of this study, *G. mikimotoi*, with chloroplasts from unrelated algal phyla (Suzuki and Ishimaru 1992).

Introduction to Gymnodinium mikimotoi. *Gymnodinium mikimotoi*, (formerly *G. nagasakiense*, Takayama et Adachi), is an athecate member of the Gymnodiniales (Dinophyta)(Yamaguchi 1992). It was first observed in Omura Bay, Japan in 1965 (Ouchi et al. 1994, Hansen et al. 2000). *G. mikimotoi* is a toxic, bloom forming dinoflagellate that can have a devastating effect on the fisheries within the area of the bloom (Parrish et al. 1988, Yamaguchi 1992). During both bloom and non-bloom

periods, *G. mikimotoi* is an important part of the coastal marine food web in the northwestern Pacific Ocean (Nakamura et al. 1995). *G. mikimotoi* is photosynthetic, but rather than having typical dinoflagellate peridinin-containing chloroplasts, it has chloroplasts that are thought to be derived from a *Pelagococcus*-like ancestor (Suzuki and Ishimaru 1992). This organism and *Gyrodinium aureolum*, a closely related species (Partensky et al. 1988), have been studied in detail ecologically and toxicologically (Parrish et al. 1988, Ouchi et al. 1994, Yamaguchi 1992). *G. mikimotoi* has a tough spheroidal nucleus, a rapid growth rate for a cultured dinoflagellate, and is easy to disrupt. These traits, and its importance as a bloom forming species led to its selection as a research organism.

Nuclear Isolation and Biochemistry. One problem that has plagued dinoflagellate nuclear studies is the inability to isolate pure nuclei (Rizzo 1987). In general, dinoflagellates are not amenable to easy nuclear isolation, due to the presence of a tough theca and a relatively fragile non-spherical nucleus (Rizzo 1987). Organisms in the Gymnodiniales, however, generally do not possess a tough theca, and many are athecate. The lack of a theca makes nuclear isolation much simpler, as it removes the need for sonication, which can cause unintentional damage to the nuclei. Besides the absence of a theca, the nucleus in a Gymnodiniales cell is spherical and relatively tough in comparison with the rest of the cell, and other dinoflagellate nuclei. Although nuclear isolation procedures for numerous dinoflagellates have been successfully developed previously, a technique for the isolation of nuclei from *G. mikimotoi* is described here, which gives very pure nuclei with a high yield.

Dinoflagellates, in comparison to other eukaryotes, have a large amount of nuclear DNA. Typical values are between 35-143 pg of DNA/nucleus for members of the

Gymnodiniales, while most other dinoflagellates have a somewhat lower amount, usually between 3-40 pg DNA/nucleus (Rizzo 1987). This is in comparison to nuclear DNA contents between 0.10-0.40 pg DNA/nucleus for many other protoctist groups (Rizzo 1987). Dinoflagellates also have much less basic protein per unit DNA than do other eukaryotes. The ratio of DNA:basic protein in the dinoflagellates is generally 10:1, while in all other eukaryotes it is about 1:1 (Rizzo 1987). This high ratio is primarily due to the absence of histones in the dinoflagellate nucleus (Vernet et al. 1990). The ratio of non-basic proteins to DNA however, is comparable to that in other eukaryotes (Rizzo 1987). The ratios of DNA:basic proteins and DNA:non-basic proteins in *G. mikimotoi* are discussed below and compared to that reported for other dinoflagellates.

Dinoflagellate Histone-like Proteins (HLPs). As mentioned above, dinoflagellates do not have the normal eukaryotic complement of histones, and in fact, are the only eukaryotes that have no histones at all. They do have basic proteins called histone-like proteins (HLPs) that serve an unknown role within the dinoflagellate nucleus. The first investigation of dinoflagellate HLPs was done by Rizzo and Nooden in 1973, and information has subsequently been published in this laboratory (Rizzo 1981, Rizzo et al. 1982, Rizzo et al.1984a, Rizzo and Morris 1984b, Rizzo 1987, Rizzo 1991, Morris et al. 1993) and by the laboratory of Soyer-Gobillard (Vernet et al. 1990, Sala-Rovira et al. 1991, and Geraud et al. 1991). Despite the ecological importance of dinoflagellate bloom formation and toxin production, research on their molecular and cellular biology has been sporadic and lightly funded. As a result, very little is known about fundamental processes such as transcription in these chromosomes. Therefore, much

is to be learned by studying the HLPs of dinoflagellates, especially using new techniques and experimental conditions.

The first and most common of the electrophoretic techniques used for HLP studies is the SDS-PAGE system developed by Laemmli (1970). Using this method, dinoflagellate nuclear extracts show usually one, or sometimes two protein bands depending on the species (Vernet et al. 1990). However, when the acid extract is first subjected to acetic acid/urea/Triton X-100 electrophoresis (AUT-PAGE) as the first dimension in a two-dimensional (2D) gel, three major proteins are generally seen (Vernet et al. 1990). The molecular masses of these HLPs range from 10,000-16,000 Da (Rizzo 1987), but the absolute and relative amounts of these proteins have not been studied under varying environmental or cell cycle conditions, except in relation to growth phase (Rizzo et al. 1984).

Using immunolocalization, it has been determined that during interphase, the HLPs of *Crypthecodinium cohnii* (HCc) are localized to extra-chromosomal loops and dispersed filaments, rather than to the main body of the chromosome (Geraud et al. 1991). The extra-chromosomal loops and filaments are known to be enriched in coding sequences, thus the presence of HLPs on these actively transcribed regions points to a possible role in general transcriptional regulation (Anderson et al. 1992).

The known dinoflagellate HLP genes are part of a small gene family, two of which are known in *Crypthecodinium cohnii* (Sala-Rovira et al. 1991). One gene encodes a 113-residue protein, while the other encodes a 102-residue protein, termed HCc1 and HCc2 respectively. The two proteins possessed 82% amino acid similarity (Sala-Rovira et al. 1991). The predicted gene products from these two genes do not match the observed proteins, which may be due to post-translational modification, or to the

presence of genes that were not detected and cloned from the library (Sala-Rovira et al. 1991).

Sala-Rovira and others (1991) have predicted the secondary structure of both known HCc gene products. They confirmed the absence of any transmembrane region, although there is a relatively large hydrophobic ß-sheet region near the carboxy terminus. There is also no peptide transit signal on the protein (Sala-Rovira et al. 1991). HCc is preferentially organized into an alpha helix arrangement at the N-terminus, which could possibly be a DNA binding site (Sala-Rovira et al. 1991). The protein lacks reasonable homology with any known protein, making a structure/function argument difficult (Sala-Rovira et al. 1991), however recent BLAST searches of the NCBI database have yielded homology of other DNA binding proteins to the HLP's theoretical DNA binding site. However, the role of dinoflagellate HLPs remains a mystery.

MATERIALS AND METHODS

Culture Conditions. Gymnodinium mikimotoi (North East Pacific Culture Collection, isolate 665R, Department of Botany, University of British Columbia, 6270 University Blvd., Vancouver, B.C. Canada V6T 1Z4) was maintained in 2.8 L culture flasks of f/2 (Guillard and Ryther 1962) media, with an illumination of 70 lx on a 16L:8D photocycle at 23° C. Routinely, one liter of f/2 media was inoculated with 500 mL of early stationary phase culture, and the cells were harvested when they reached a concentration of 10^5 cells mL^{-1}. Cultures were not bubbled or shaken. Doubling time for the culture was approximately three days, and roughly 1/3 of cells divided within the hour before dawn each day. Therefore, the culture was synchronous, in that there was no division at any other time.

Isolation of Nuclei. All steps, including cell harvest were carried out at 0-4° C. Cells were harvested in 500 mL bottles by centrifugation at 1000 x *g* for 10 min. Cells were resuspended in a total of 10 volumes of *G. mikimotoi* nuclear isolation buffer (GMNIB) which consists of 0.25 M sucrose, 10 mM Tris, 5 mM $CaCl_2$, and 0.4% Triton-X100 (v/v) at pH 7.8. The cells were allowed to equilibrate in GMNIB for 20 min, after which they were homogenized in a 40 mL Dounce-type glass homogenizer with a tight fitting glass pestle, using 5-7 up and down strokes. Following centrifugation at 2200 x *g* for 6 min, the pellet was resuspended in 10 mL of GMNIB, and the homogenization and centrifugation was repeated (without the equilibration period). The resulting pellet was resuspended in GMNIB without detergent, homogenized with 2-4 up and down strokes, and centrifuged at 3000 x *g* for 5 min. The nuclear pellet was resuspended in 10 mM Tris, 5 mM $CaCl_2$, pH 7.5 (TC) and

layered over a step gradient of 8 mL of 2.2 M and 25 mL of 1.6 M sucrose. Following centrifugation at 12,000 x *g* for 25 min, the purified nuclei were recovered in the pellet.

In some cases (nuclear isolation for environmental studies and in preparation for 2D gel electrophoresis), the sucrose spin was eliminated, as was the resuspension in GMNIB without detergent. Leaving these steps out did not greatly decrease purity and resulted in a 5-10% higher yield. Also, for preparation of these samples, thiodiglycol, dithiothreitol (DTT), and phenylmethyl sulfonyl fluoride (PMSF), were added to all solutions to a final concentration of 1% (w/v), 1 mM, and 1 mM respectively. The addition of these compounds limit oxidation and degradation of proteins. However, the addition also lowered purity to a small extent as discussed below.

Chemical Characterization of Nuclei. The nuclear pellet was resuspended in TC buffer and the nuclei were counted using a hemocytometer. The nuclear suspension was then divided into two equal portions for DNA and protein determination. DNA was determined using the diphenylamine reaction with calf thymus DNA as the standard (Burton 1956). Basic proteins were extracted by stirring with 0.3N HCl for 1h on ice. After centrifugation at 10,000 x *g* for 20 min, the acid-soluble proteins in the supernatant were precipitated overnight with 7 volumes of 7:1 acetone:ethanol. Acid soluble and acid insoluble protein concentrations were both determined by the method of Lowry et al. (1951), using calf thymus histones and bovine serum albumin as standards, respectively.

Electron Microscopy. Electron microscopy was performed as described previously by Rizzo et al. (1982). Samples were fixed for 30 min in 3% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.3) at room temperature. The pellets were postfixed in 1% osmium tetroxide after cacodylate buffer washes. Pellets were then

dehydrated in a graded ethanol series and embedded in Epon-Araldite. Sections were obtained using a Sorvall MT-2 ultra-microtome. Samples were examined with a Phillips 400T electron microscope following staining with uranyl acetate (2%) and lead citrate (4%).

Light Microscopy. Whole cells for light microscopy were fixed in a solution of f/2 media containing 4% (v/v) glutaraldehyde, pelleted at 900 x *g* in a tabletop centrifuge, and washed three times with 50% (v/v) ethanol, then stained with Lugol's iodine. Cells were then mounted in Plastic UV-mount (Polyscience Inc.). Nuclei were prepared by the above isolation procedure and fixed using the same method as for whole cells. After fixation, nuclei were stained with methyl green pyronin (Stern 1967).

SDS-PAGE and Peptide Mapping. Electrophoresis was performed in a mini-gel apparatus using a 6% stacking gel and an 18% separating gel, as described by Rizzo et al. (1988). Electrophoresis was carried out at room temperature at 175 V and the gels were stained with Coomassie Brilliant Blue (CBB). Peptide mapping was done in the presence of SDS as originally described by Cleveland et al. (1977), in which *Staphylococcus aureus* V8 protease was used to digest proteins in stained gel slices. Briefly, acid soluble proteins were first run on 0.8 mm thick SDS-PAGE gels which were then stained for 10 min with CBB and rinsed several times in destaining solution to allow visualization of bands. The bands were excised from the gel using a clean scalpel and stored in 50% (v/v) ethanol at –20° C. Prior to digestion, the excised bands were equilibrated in electrophoretic running buffer for 10 min, then placed vertically in the wells of a 1 mm gel with forceps, and covered with protease digestion buffer. This buffer contained the gel running buffer plus 10% (v/v) glycerol and 4-8 μg of V8 protease (Sigma). Electrophoresis was performed at 100 V until the dye migrated to

the lower third of the stacking gel. The power was then turned off and digestion was allowed to proceed for 30 min, after which electrophoresis was resumed at 175 V until the ion front reached the bottom of the separating gel. Visualization of peptide digest bands was done by silver-staining the gel as described in Rizzo and Morris (1984).

AUT-PAGE and AU-PAGE Analytical and Preparative Gels. Acid/urea/Triton X-100 (AUT)-PAGE and acid/urea (AU)-PAGE were performed on the minigel apparatus described above according to the techniques of Vernet et al (1990). The AUT-PAGE gels consisted of 15% acrylamide, 6.2 M urea, 6 mM Triton X-100, and 0.9 M acetic acid. AU-PAGE gels were identical to the above gels except for the omission of the Triton X-100. These gels were pre-electrophoresed for 1h at 250V, according to Morris et al. (1999). For pre-electrophoresis, cysteamine and protamine in 9 M urea and 1% mercaptoacetic acid were added to the wells, and the lower buffer chamber was made 0.1 M in thioglycolic acid. Immediately following pre-electrophoresis, the running buffer was replaced with 0.9 M acetic acid and the samples were run at 250 V until the pyronin-Y dye front reached the bottom or for 90 minutes, depending on the required separation. The gels were silver stained for 2D preparation, or Coomassie stained for 1D analysis. The Coomassie stained lanes were sliced from the gel and stored in 50% ethanol, 1 mM DTT at –20° C until run on an SDS-PAGE second dimension.

In order to isolate the HGm variants for peptide mapping, a minor modification was made in the above procedure. We utilized a stacking gel with an identical buffer system, but with only a 5% acrylamide concentration. This eliminated some smearing in the β and γ variants that made them difficult to isolate from one another in the preparative gel. Bands were excised and run on SDS-PAGE as described in the

peptide mapping section, except that the thickness of the AUT-PAGE preparative gel was 0.5 mm and the peptide mapping gel was 0.8 mm thick.

Acidic Native PAGE Analytical and Preparative Gels. Acidic native PAGE was performed according to Hames (1981), with minor modifications. The resolving gel was 15% acrylamide kept at pH 4.3 with an acetic acid buffer, and polymerized using riboflavin and high intensity visible light. The stacking gel was modified from the original method to aid in polymerization and handling. The stacking gel was 5% acrylamide kept at pH 6.8 with an acetic acid buffer, and polymerized using both riboflavin and ammonium persulfate (AMPS). The running buffer was a solution of 0.35 M β-alanine and 0.14 M acetic acid at pH 4.5. The samples were taken up in a sample buffer comprised of 20% (v/v) glycerol, 1.7 M acetic acid, and 0.04 M β-alanine, brought to pH 4.0 with KOH. Pyronin Y was added as a tracking dye. The gels were run at 30 mA until the dye front had reached the bottom of the gel. For 2D gels, entire lanes were excised for electrophoresis on SDS-PAGE.

HLP Protein Levels Under Varying Environmental and Growth Conditions. G. mikimotoi was subjected to varying environmental and growth conditions to determine if HLP protein levels changed during such conditions. HLP levels were determined for the following conditions: (1) continuous light / continuous darkness; (2) low nitrogen / standard f/2 nitrogen; (3) diel variation (4) growth phase; (5) cold stress; and (6) heat shock. Under continuous light or darkness, the only change was that of the light levels. Low nitrogen conditions were achieved by subculturing 100mL of late stationary phase cells into 1.5 L of nitrogen-free f/2 media. Sampling for diel variation was done at 4 h intervals starting at subjective noon (3:00 PM in our growth chamber), for a total of 6 samples over the course of one 24 hour period. Growth phase sampling was

done during very early lag phase, exponential phase, and stationary phase. Cold stress was performed by placing the flask in an 8° C coldroom for 48 h. Based on results reported by Ouchi et al. (1994), this induces *G. mikimotoi* to undergo sexual reproduction. Heat shock was performed for 1 h at 37° C, which is sufficient to induce heat shock in unicellular eukaryotes (Gervais and Martinez de Maranon 1995). Nuclei were isolated from cells immediately after the specified time period.

RESULTS

Nuclear Structure. Light and electron microscopic examinations of *Gymnodinium mikimotoi* whole cells and nuclei were undertaken to determine nuclear size, structure, and similarity to other dinoflagellate nuclei. A previous study by Partensky et al. (1988) on *G. mikimotoi* (previously *G. nagasakiense*) highlighted nuclear position within the cell and determined the average chromosome number to be 117 ±3. Here we show a light micrograph of the organism (Fig. 1A) to emphasize nuclear structure and size in relation to the rest of the cell. The nucleus is 1/8 to 1/6 of the total cell volume, dependent on cell size. Cell size within our strain was variable, similar to the variation described by Partensky et al. (1988). However, variation in nuclear diameter was not seen to correspond to cell size variation (data not shown).

Isolated nuclei are shown in Fig. 1B, to document nuclear condition after isolation, and to further highlight visible features of the nucleus. The nuclear clumping seen in this figure was common during isolation, and is due to a loss of the nuclear membrane, allowing chromatin and nuclear matrix adhesion between adjacent nuclei. The loss of the nuclear membrane was determined by TEM examination of isolated nuclei (discussed below), and by FM4-64 fluorescent analysis (data not shown). The loss of the nuclear envelope caused some nuclei (about 10%) to swell greatly. Apart from these nuclei, however, the diameters of the isolated nuclei were between 14 and 17 μm, which was identical to the diameters of the nuclei measured in whole cells.

A TEM transverse section through the hypocone of *G. mikimotoi* (Fig. 2A) shows that the nucleus is surrounded by a typical eukaryotic nuclear membrane. Also apparent are the characteristic dinoflagellate structures, including the tight chromosome condensation and the whirled pattern of the chromosomes. The

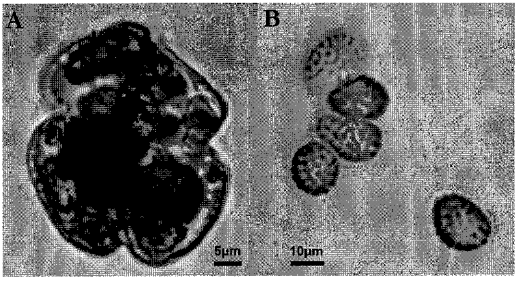

Fig. 1: Light microscopy of *Gymnodinium nikimotoi*. A) Whole cell image of *G. nikimotoi* in brightfield. The nucleus is located in the lower right of the cell. B) Isolated nuclei stained with methyl green pyronin.

nucleolus can also be seen in this figure, and was prominent in all preparations. The TEM section of an isolated nucleus (Fig. 2B) shows a loss of the nuclear envelope, as well as the clumping of adjacent nuclei due to the membrane loss. As in the whole cell TEM section, the nucleolus and the whirled chromosomal pattern were prominent in all preparations. In regard to nuclear isolation purity, no cytoplasmic organelles or membrane structures were ever observed in the TEM sections of nuclear pellets.

Nuclear Isolation. Various nuclear isolation buffers were tested to determine an optimal buffer. For example, buffers containing Dextran, ethanol, and hexylene glycol were tested at varying concentrations and in varying combinations, but none worked as well as the final formulation, termed GMNIB. GMNIB is actually a modification of an isolation buffer developed for the isolation of *Stylodinium trochoideum* nuclei as reported by Rizzo and Nooden (1973). As described in the Materials and Methods section, GMNIB is essentially a buffered sucrose solution containing a non-ionic detergent. After the general buffer system was determined, detergent concentration and sucrose concentration were optimized (Fig. 3A-B), as well as incubation time in GMNIB and the amount of mechanical disruption (Fig. 3C-D).

Optimization was measured according to (%) yield and (%) purity. Percentage yield was determined by dividing the concentration of isolated nuclei by the expected optimal yield of nuclei as measured from the whole cell population prior to isolation. The purity of the nuclear pellets was determined by counting nuclei and contaminants such as whole cells, cytoplasmic debris, and cytoplasmically tagged nuclei. Cytoplasmic components were determined by staining with methyl green pyronin (MGP), which stains cytoplasm pink and nuclei blue. The ratios of nuclei to cytoplasmic contaminants were used to generate the purity presented in Fig. 3.

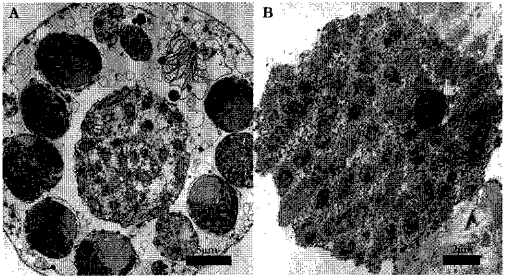

Fig. 2: Transmission Electron Microscopy (TEM) of *Gymnodinium mikimotoi*. A) Horizontal TEM section through the lower half of a *G. mikimotoi* cell. B) TEM section through an isolated nucleus.

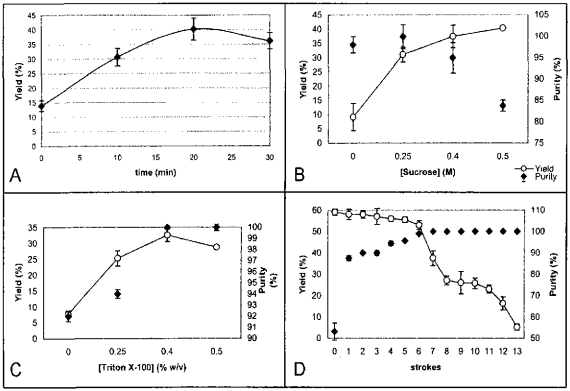

Fig. 3: Determination of *Gymnodinium mikimotoi* nuclear isolation procedure. A) Tested incubation times in GMNIB with respect to percent yield. B) Effects of varying sucrose concentrations in the isolation buffer on yield and purity. C) Effects of varying Triton X-100 concentration in the isolation buffer on yield and purity. D) Effect of stroke number on yield and purity. The legend given in 3B is the same legend for 3C-D. All error bars represent standard deviation, where an error bar cannot be seen, it is smaller than the data symbol. Closed diamonds represent purity, open circles represent yield.

Nuclear Biochemistry. The DNA content of *G. mikimotoi* (Tables 1 and 2) was found to be 47 ±4 pg per nucleus, which is close to the amount reported by Partensky et al. (1988) for *Gyrodinium* cf. *aureolum* (44 pg), and is on the same order of magnitude as that determined for *G. mikimotoi* (62 pg). The method used by Partensky et al. (1988) was flow cytometry, while we determined DNA amounts chemically, using the diphenylamine reaction as described in Materials and Methods.

The relative proportions of DNA and non-basic proteins were similar to those obtained from other dinoflagellate nuclei (Rizzo and Nooden 1973, Rizzo et al. 1982); however the ratio of DNA to basic protein in *G. mikimotoi* was twice as high as that of other dinoflagellates. Although the ratio is different, the absolute amount of basic protein in the *G. mikimotoi* nucleus is similar to that in other dinoflagellates. The error given for the measurements represent one standard deviation as calculated from nine samples run in triplicate.

HGm Characterization in 1D SDS-PAGE. In the SDS-PAGE system, *G. mikimotoi* has one major acid-extractable nuclear protein, termed HGm (Fig. 4A, lane 4). This protein cannot be seen in the whole cell protein extract (Fig. 4A, lane 1), but it is quantitatively, the major nuclear protein (Fig. 4A, lane 2). Extractability of HGm with dilute acid was not complete, and about 30 percent was left in the nucleus during all extractions. This loss is factored into the determinations of acid-soluble and acid-insoluble proteins for Table 1. However, as seen below in 2D analysis, the extractability is not dependent on protein subtype. Similar extraction problems were seen for the HLPs of *Gymnodinium breve* (Rizzo et al. 1982).

In a comparison between HGm and the HLPs of two other dinoflagellates in SDS-PAGE, HGm migrated similarly to the lower molecular weight band of *G. dorsum* (Fig.

Table 1: Chemical composition of *G. mikimotoi* isolated nuclei. Standard deviation is given after each quantity, which represents the standard deviation of 9 samples for each component.

DNA	47 ± 4 pg
Acid soluble protein	12 ± 2 pg
Acid insoluble protein	57 ± 1 pg
Acid soluble proteins:DNA	0.25
Acid insoluble protein:DNA	1.21
Total Nuclear Protein:DNA	1.47

Table 2: DNA content of typical dinoflagellates along with a representative of the chlorophytes, euglenophytes, fungi, and vascular plants.

Organism	DNA content (pg/nuc)	Reference
Gymnodinium mikimotoi	47	Present study
Gymnodinium mikimotoi	62	Partensky et al. 1988
Gyrodinium aureolum	44	Partensky et al. 1988
Crypthecodinium cohnii	6.9	Roberts et al. 1974
Gonyaulax polyhedra	200	Holm-Hansen 1969
Chlamydomonas reinhardtii	0.19	Cattolico and Gibbs 1975
Euglena gracilis	2.10	Charles 1977
Saccaromyces cerevisiae (2N)	0.046	Rizzo and Nooden 1973
Zea mays (2N)	11	Rizzo and Nooden 1973

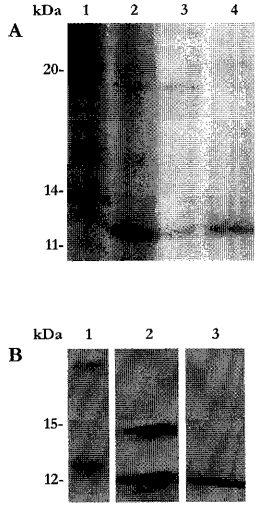

Fig. 4: SDS-PAGE of *G. mikimotoi* cellular fractions and dinoflagellate HLPs. A) SDS-PAGE of four *G. mikimotoi* protein fractions. Lane 1 shows SDS extracted whole cellular protein. Lane 2 shows SDS extracted whole nuclear protein. Lane 3 shows SDS extracted nuclear protein after acid extraction. Lane 4 shows the acid-soluble protein, HGm. B) SDS-PAGE gel of three dinoflagellate nuclear acid extracts. HCc is present in lane one, isolated from *Crypthecodinium cohnii*. The high molecular mass band in lane one is a dimer of HCc. Lane 2 shows the acid extract of *Gymnodinium dorsum* nuclei, where some degradation of the proteins is evident. Lane 3 shows the acid extractable protein from *Gymnodinium mikimotoi*, (HGm). Migration was from top to bottom.

4B). The HLP from *C. cohnii* (HCc) is larger than HGm. The primary difference between the HLPs of *G. mikimotoi* and *G. dorsum*, is that *G. dorsum* has a second, higher molecular mass protein band. The V8 protease digestion pattern of this band was not similar to any other tested HLP (Fig. 5). The V8 protease digestion experiments also showed that HGm and HCc yielded a similar pattern, producing five major fragments. The pattern of proteolytic degradation of the two *G. dorsum* HLPs differs from each other, and also from the HLPs of the other dinoflagellates tested (Fig. 5).

HGm Characterization Using 2D Gel Systems. Using one-dimensional SDS-PAGE, as described above, *G. mikimotoi* is seen to have only one major basic nuclear protein, termed HGm. However, when nuclear acid extracts are run on AU (Acid/Urea)-PAGE, two protein bands are seen (Fig. 6A). The first band accounts for 75% of the HGm protein, the second accounts for 25%. When the same acid extract was run on AUT (Acid/Urea/Triton)-PAGE, four proteins bands were seen (Fig. 6B), suggesting differences in hydrophobicity in the HGm protein family.

The 2D AU-PAGE showed only two major bands (Fig. 6C), similar to the basic proteins of *Crypthecodinium cohnii* (Vernet et al. 1990). However, the higher AU-PAGE mobility spot in *C. cohnii* shown by Vernet et al. (1990), represents a much higher molecular weight group of proteins (16-17 kDa), while in *G. mikimotoi* they are only slightly (300-500 Da) larger than the major HGm component. The 2D AUT-PAGE showed four proteins (Fig. 6D & 6E), which, based on their migrations in AUT-PAGE and SDS-PAGE, are termed $\alpha 1$, $\alpha 2$, β, and γ (Fig. 6E). The highest AUT-PAGE mobility protein was termed $\alpha 1$ and the lowest AUT-PAGE mobility protein was termed γ. HGm$\alpha 1$ and $\alpha 2$ both have an apparent molecular mass of 12,200 Da, and HGmβ and γ both have an apparent molecular mass of 12,000 Da. The

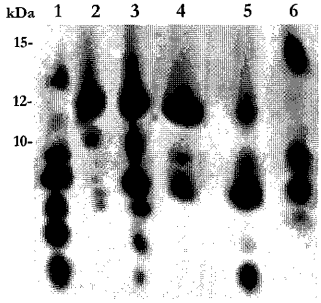

Fig. 5: V8 protease digestion of dinoflagellate HLPs. The highest molecular mass spot in each lane is undigested protein. Lane 1 shows digested HCc. Lane 2 shows HGm with no enzyme added. Lane 3 shows the digestion pattern of HGm. Note the pattern similarity to the digested HCc, each possessing 5 major digestion fragments. The lower molecular mass band from *G. dorsum* is shown in lane four with no enzyme added. Lane 5 shows the low molecular mass band from *G. dorsum* with protease added. Lane 6 shows the higher molecular mass band from *G. dorsum* digested with protease. Migration was from top to bottom.

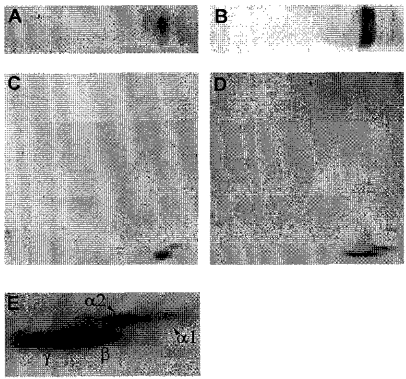

Fig. 6: One and Two-dimensional gel electrophoresis of acid extractable proteins from *Gymnodinium mikimotoi*. A) AU-PAGE of HGm proteins. B) AUT-PAGE of HGm proteins. C and D) 2nd dimension SDS-PAGE gels of A and B respectively. E) The proteins are labeled according to the naming system of Vernet et al. (1990), as seen in an AUT/SDS 2D PAGE system. Gels A and B run from left to right. Gels C, D, and E show first dimensional migration from left to right and second dimensional migration from top to bottom.

nomenclature is based on the system of Vernet et al. (1990). The proteins described in *C. cohnii* by Vernet et al. (1990), termed p16, p16.5, and p17, were not seen on any gel of *G. mikimotoi* nuclear acid extracts. This suggests either their absence in *G. mikimotoi* or a difference in their acid solubility so as to preclude their extraction with acid. Also, *C. cohnii* has three HCc proteins, *G. mikimotoi* has four HGm proteins.

Peptide Mapping of HGm Variants. The four HGm variants were excised from an AUT-PAGE gel for peptide mapping (Fig. 7). It can be seen that γ and β were similar and that $\alpha 1$ and $\alpha 2$ were similar, but that these two variant groups differed from each other in peptide maps.

HGm Characterization in Acidic Native PAGE. When an acid extract of *G. mikimotoi* was solublized in an acidic native sample buffer and run under native conditions at low pH, four primary protein bands were seen (Fig. 8A). When a 2D gel was run with acidic native PAGE as the first dimension and SDS-PAGE as the second dimension, five primary protein spots could be seen (Fig. 8B). By comparing our gel with a non-reducing AU-PAGE gel of a *C. cohnii* acid extract (Vernet et al. 1990), the faster migrating group in the acidic native PAGE gel was hypothesized to be the oxidized protein.

Environmental Effects on HGm Levels. Environmental effects on HGm levels were analyzed using one-dimensional AUT-PAGE because all four proteins were easily resolved, while in the second dimension the proteins tended to smear together. The first environmental change tested was continuous darkness. Proteins were extracted from isolated nuclei of control cells grown in 16L:8D, and from nuclei of cells grown in continuous darkness for 72 h. The samples were normalized as to the number of nuclei extracted, and run on AUT-PAGE (Fig. 9). Compared to control

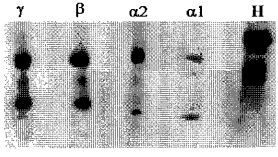

Fig. 7: Peptide mapping of HGm variants with V8 protease. The variant maps are shown along with the calf histone standard (H).

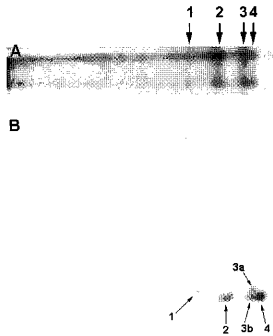

Fig. 8: Acidic native PAGE of *G. mikimotoi* nuclear acid extract. A) 1D acidic native page. B) 2D of HGm using acidic native PAGE as the first dimension, SDS-PAGE as the second dimension. Migration in A is from left to right. Migration in B is from left to right for the first dimension and from top to bottom for the second dimension.

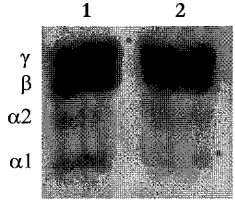

Fig. 9: Changes in HGm protein levels due to 72h of continuous darkness. AUT-PAGE of HGm proteins extracted from control cells (Lane 1) and from dark treated cells (Lane 2). Protein nomenclature for this and all similar figures based on Fig. 6E. Migration was from top to bottom.

cells, there are less of all HGm variants in total darkness, but their quantities relative to each other remained constant. The drop in total HGm protein amount of 9% ±1.1%, is statistically equal to the whole nuclei protein drop of 11% ±2.3%. The drop in amount of protein is most likely due to cell starvation in total darkness.

Under nitrogen stress (conditions of very low nitrogen), it was found that HGm protein levels also dropped (Fig. 10). However, unlike changes induced by continuous darkness, the amount of change induced by nitrogen stress is not the same in all HGm variants. The total drop in HGm protein is 20% ±3%, and the total drop in whole nuclear protein is 22% ±5.1%. The drop in protein levels for HGmα1 and HGmγ mirror this total drop in protein levels (Table 3). Protein levels for HGmα2 and HGmβ, however, show independent changes in protein levels: HGmβ drops only slightly compared to its level in control cells; HGmα2, on the other hand, drops over 30% (Table 3). These results may reflect a differential production of HGm protein types in response to nitrogen stress, or differential degredation of proteins.

According to Ouchi et al. (1994), one method to induce sexual reproduction in *G. mikimotoi* is to drop the temperature below the normal growth temperature. However no noticeable increase in zygote formation in *G. mikimotoi* was seen using light microscopy at 8°, 10°, or 15°C (data not shown). While the initial intent was to examine the effects of sexual reproduction on HGm protein levels, cold treatment was instead examined. It was found, however, that there was no significant change in HGm levels resulting from the cold treatment described above. There was however, a slight drop in total protein of whole cells during most cold treatments (Fig. 11).

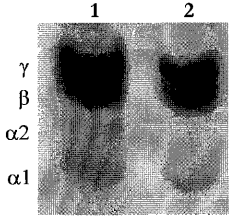

Fig. 10: Changes in HGm protein levels under nitrogen stress. AUT-PAGE of HGm proteins extracted from cells grown in standard f/2 media (Lane 1), and from cells grown in f/2 media without the addition of nitrogen (Lane 2). Migration was from top to bottom.

Table 3: Percent changes in HGm protein levels under nitrogen stress as compared to control HGm levels.

HGm type	% drop (±SD)
α1	19% ±1.4%
α2	32% ±2.1%
β	9% ±4.0%
γ	22% ±3.6%

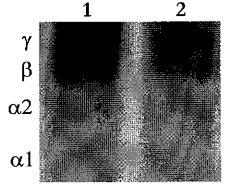

Fig. 11: HGm protein levels during cold adaptation. AUT-PAGE of HGm proteins extracted from control cells grown at 23° C (Lane 1), and from cells grown at 8° C for 48h (Lane 2). Migration was from top to bottom.

The occurrence of changes in HGm protein levels due to heat-shock, were also tested. As shown in Fig. 12, heat-shock, like cold treatment, had no observable effect on HGm protein levels.

G. mikimotoi nuclei were acid extracted during lag phase, exponential phase, and stationary phase to determine if there were differences in HGm variant levels over the culture phases (Fig. 13). During lag and exponential phases, HGm levels were roughly equal (Fig. 13, lane 1 & 2). When cells are in stationary phase, HGm levels were lower (Fig. 13, lane 3). While this could be a real effect, it is thought that intact but dead cells affected the calculation of the loading correction factor. This would have caused the loading of less extract per live cell when compared to lag and exponential phase. However, viability testing was not done to examine this hypothesis.

The amount of HGm per nucleus was also examined to determine if there was a diel cycle in protein abundance, with sampling every four hours. Although most diel variability in HGm amount was irreproducible, the dip in total HGm protein level at subjective midnight was reproducible (Fig. 14, lane 4). This dip (19% $\pm$ 3%) in HGm amount is seen both when the protein loading is based on nuclear counting and when it is based on DNA quantification. The gel pictured here was loaded according to nuclear counting.

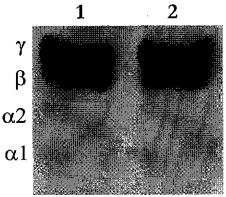

Fig. 12: HGm protein levels during heat-shock. AUT-PAGE of HGm proteins extracted from control cells grown at 23° C (Lane 1), and from cells grown at 37° C for 1h (Lane 2). Migration was from top to bottom.

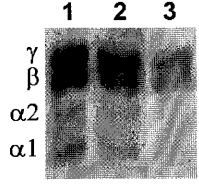

Fig. 13: Variation in HGm levels at different cell culture phases. Lane 1 is lag phase, lane 2 is exponential phase, and lane 3 is stationary phase. Migration was from top to bottom.

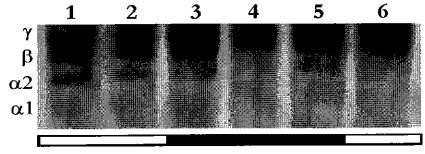

Fig. 14: Diel variations of HGm in the nucleus of *G. mikimotoi*. Lane 1 is subjective noon, and lane 4 is subjective midnight. The other lanes are spaced at four hour intervals, with the bar at the bottom showing rough sample time. Migration was from top to bottom.

DISCUSSION AND CONCLUSION

Dinoflagellates are of interest because of their taxonomic position, their unusual chromatin and chromosome structure, and their importance in the phytoplankton communities in both freshwater and marine habitats. Because *G. mikimotoi* has no cell wall and can be grown to high cell concentrations, it is an ideal organism for the study of dinoflagellate nuclear proteins and nuclear genes. *G. mikimotoi* also belongs to the Gymnodiniales, a polyphyletic group of somewhat uncertain position within the dinoflagellate clade (Taylor 1999, Hansen et al. 2000). This makes it a taxonomically strategic organism for investigations of dinoflagellate evolution. Finally, *G. mikimotoi* produces toxins (Parrish et al 1998) and forms economically harmful red tides (Ouchi et al 1994, Yamaguchi 1992), making it not only ideal as a model dinoflagellate system, but also of interest to a wide variety of cell, molecular and toxicological researchers.

The nuclei of *G. mikimotoi* can be isolated with much greater yield and purity than any other dinoflagellate previously examined. Examination of the nuclear pellets under light microscopy using MGP staining revealed no cytoplasmic contamination. Similarly, thin sections of nuclear pellets examined under TEM revealed no cytoplasmic organelles or membranes. The ability to isolate pure nuclei renders *G. mikimotoi* very useful as a model system for dinoflagellate nuclear studies. The genus *Gymnodinium* contains many important bloom-forming species, of which *G. mikimotoi* is one. *G. mikimotoi* may thus serve as a model for dinoflagellate nuclear studies in general, and also for the bloom forming dinoflagellates within the genus.

The ultrastructural and biochemical properties of nuclei isolated from *G. mikimotoi* are similar to those of other dinoflagellates (Rizzo 1987). *G. mikimotoi*, and all dinoflagellates so far investigated, lack histones and nucleosomes. Even in cases where

dinoflagellate nuclei exhibit a positive alkaline fast green reaction, usually indicative of the presence of histones, biochemical analysis reveals one major histone-like protein (see Rizzo 1991 for a discussion).

G. mikimotoi nuclei contain one major HLP as evidenced in SDS gels with an estimated molecular mass of 12,000 Da, which we have called HGm. In AUT-PAGE, HGm was resolved into four components. AUT-PAGE separates proteins based on size, net charge and hydrophobicity, while AU-PAGE separates proteins based on size and net charge. The difference between AU-PAGE and AUT-PAGE gels suggests that $\alpha 1$ and $\alpha 2$ differ only in their hydrophobicity. Likewise β and γ differ only in their hydrophobicity. The hydrophobicity difference suggests either differential post-translational modification, or a slight difference in amino acid content within the HLP gene family.

As in *G. mikimotoi*, Vernet et al. (1990) showed four proteins in AUT-PAGE for *C. cohnii*; however, the two bands of highest mobility were shown to be proteins with masses of 16, 16.5, and 17 kDa when run on a 2D gel. These weights are 2 – 3 kDa larger than the molecular mass for HCc. In contrast, the two highly mobile bands in AUT-PAGE from *G. mikimotoi* are only slightly larger than the two less mobile bands. This leads us to include these proteins as variants of HGm rather than as part of the "p16-like" protein group (Vernet et al. 1990). Unfortunately, antibodies are not available to determine the cross-reactivity of these variants.

Peptide mapping of the HGm variants shows that differences exist between the $\alpha 1/\alpha 2$ group and the γ/β group. Although the differences are significant, a difference in function is not necessarily implied, as V8 peptide mapping shows only the position of Glx residues (glutamic acid and glutamine). The position or numeric difference of

these two amino acids may not play a role in governing HLP function. Future work will be done to determine which fragment(s) contains the DNA binding domain, and if this domain is protected from proteolytic degradation when it is bound to DNA. The search for HGm cDNAs should eventually be undertaken in order to determine the extent of gene duplication and functional overlap within the HGm gene family.

The pattern of protein spots seen in acidic native PAGE in relation to the spots in non-reducing AU-PAGE gels run by Vernet et al. (1990) suggests that urea does little to change the migration of the proteins relative to each other. This may be because HGm is such a small protein, with very little calculated secondary structure (Vernet et al. 1990), that there is very little to be denatured by urea. Future work will include a reducing acidic native gel to determine the validity of comparing HGm in acidic native PAGE to HCc in non-reducing AU-PAGE. Eventually the immunolocalization of individual variants within the nucleus should be undertaken, as well as a determination of oxidation level of the proteins in vivo.

The examination of HGm variant levels under varying environmental conditions did not reveal significant changes, with the exception of a shift in the relative proportions of the variants during nitrogen stress. The changes in HGmβ and HGmα2 levels during nitrogen stress may indicate a role for HGm in gearing down for encystment, or it may indicate the variable loss of HGm components due to selective protein degradation under nitrogen stress.

The reason for the drop in total HGm levels at subjective midnight is not known, although many photosynthetic organisms show diel variations in protein abundance. One interesting hypothesis involves the observation that HLP localization in the nucleus changes in relation to cell cycle changes (Geraud et al. 1991). Perhaps HGm is

recycled immediately prior to division (which occurs about 1 h before dawn) in preparation to undergo mitosis. However, this hypothesis has no supporting evidence, and the variability in HGm levels at other times precludes us from hypothesizing further.

If HLPs are involved in general transcription (Geraud et al. 1991), then a decrease in HLPs may indeed be the method by which the cell inhibits transcription. Although a defined role for the dinoflagellate HLPs remains elusive, it seems likely that they play a role in transcription, based on their DNA binding properties and localization to transcriptionally active DNA strands in the nucleus. Mutagenic studies, while currently unfeasible in dinoflagellates, may become so in the future. If this is not the case, perhaps anti-sense RNA technology could be used to lower the levels of translated HGm proteins, which could provide a method for determination of the role of HGm in dinoflagellates. Another method for determination of the function of HLPs may be to transform a heterologous system with an HLP gene. *Saccharomyces cerevisiae*, *Chlamydomonas reinhardtii*, and *Arabidopsis thaliana* would all be good heterologous systems in which to determine HLP function because of their tractable genetics.

This is only the fourth gymnodinoid dinoflagellate so far examined for nuclear HLP content, and it is already apparent that differences in the HLPs of these organisms exist. Further study of the gene structure and putative function of these proteins will offer a means of better understanding evolution in the Gymnodiniales, and in other dinoflagellates as well. Because *G. mikimotoi* is ideally suited for nuclear studies, we feel that it will be useful as a model system for the study of dinoflagellate nuclear function and gene regulation.

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Education

- 1994-1998 Susquehanna University, Department of Biology, B.S. in Biology. Summa Cum Laude and Departmental Honors.
- 1998-2000 Texas A&M University, Department of Biology, pursuing M.S. in Botany. Thesis Title: Electrophoretic analysis of nuclear basic proteins in the toxic marine dinoflagellate *Gymnodinium mikimotoi*.

Teaching Experience

- 1998 (fall) – 2000 (spring) Texas A&M Univ. BIOL 423 Laboratory Instructor. 10 sections, 124 total lectures/lab, 179 students.

Publications: Papers

Wargo, M.J. and Rizzo, P.J. 2000. Characterization of *Gymnodinium mikimotoi* (Dinophyta) nuclei and identification of the major histone-like protein, HGm. (in print).

Wargo, M.J. and Holt, J.R. 1998. Determination of stream reach in a ridge and valley creek using diatom periphyton communities. Journal of Freshwater Ecology v13 n4, pp447-456.

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- Development of a TA manual to complement the lab manual for Cell Biology. June 2000.
- Biology Department Award for Teaching Excellence, MS level. Spring 2000.
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