Molecular Cloning, Transcriptome Profiling, and Characterization of Histone Genes in the Dinoflagellate *Alexandrium pacificum*

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The nucleosomal organization of chromatin using histone proteins is a fundamental and ubiquitous feature of eukaryotic nuclei, with the major exception of dinoflagellates. Although a number of recent genomic and transcriptomic analyses have detected numerous histone genes in dinoflagellates, little is known about their expression. Here in, we aimed to investigate the expression pattern of histone genes under nutritional stress, and an attempt was made to detect histone expression at the protein level in *Alexandrium pacificum*. The presence of histones at the mRNA level was confirmed in this study by the amplification, cloning, and sequencing of 10 different genes. Relative expression profiling of these genes under different growth conditions was determined with real-time PCR and revealed considerable levels of histone transcription in nutritionally stressed cells. We were unable to detect the expression of histones at the protein level even after immunodetection and analysis using mass spectrometry, although a histone-like protein was detected as a major nuclear component. *A. pacificum* expresses multiple variants of histone, and protein sequences revealed both conservation and divergence with respect to other eukaryotes. We concluded that *A. pacificum* maintained an active transcription of histone genes within the cell, and enhanced expression of histone genes in nutritional stress strongly suggest that histones have functional significance in dinoflagellates, although expression at the protein level was below our current detection limits, which suggests a limited role of histones in DNA packaging. Finally, the plausible regulation of histone expression at the gene and protein levels in *A. pacificum* is discussed.

Keywords: Dinoflagellate, transcriptome, histone expression, immunodetection, mass spectrometry, histone-like protein

Introduction

Histone proteins package DNA in all eukaryotic organisms and play a key role in the regulation of gene expression [1]. Almost 147 DNA base pairs (bp) wrap an octamer of core histones (*i.e.*, two of each of the histones H2A, H2B, H3, and H4) to make a nucleosome [2]. In addition, about 50 bp of DNA bounded by linker histones are present between nucleosomes; these histone proteins bind to replicated DNA and are among the most stable cell proteins [3].

The exception to this nearly universal organization is dinoflagellates. Members of this group of unicellular protists

play significant roles in marine food webs and ecology [4]; the dinoflagellate *Alexandrium pacificum* [5] (previously named as *Alexandrium catenella*) [6], for example, is the primary cause of paralytic shellfish poisoning in bivalves [7]. Dinoflagellates are unusual in the possession of a number of uncommon features, including a differently organized genome in their mitochondria [8] and plastids [9]. In particular, the nuclei of dinoflagellates are very unusual; at one point, these protists were thought to be intermediate between prokaryotes and eukaryotes (mesokaryotes) [10]. The DNA of dinoflagellates is not characterized by nucleosomal packaging [11] and no pattern

of bands is seen on gels following micrococcal nuclease digestion [12]. Dinoflagellates also have very low levels of basic proteins (approx. 10% compared with DNA), whereas other eukaryotic nuclei contain up to 50% [13]. Finally, the chromosomes of dinoflagellates remain permanently condensed during the cell cycle [14].

It has been proposed that, instead of true histones, dinoflagellates possess histone-like proteins (HLPs); these have been identified in dinoflagellates by a number of studies [15–18]. Although it was long assumed that histone proteins are entirely absent from dinoflagellates, a number of recent reports have cast doubts on this idea, identifying relevant genes in a number of different species, including species of *Gambierdiscus* [19], *A. catenella* [20] and *Symbiodinium minutum* [21], species of *Lingulodinium* [22], and other species of *Symbiodinium* [23], as well as in environmental transcriptomes [24]. Another recent report identified dinoflagellate viral nucleoproteins (DVNPs), which differ markedly from histones, in dinoflagellates [40]. Although these workers argued that DVNPs function as histone substitutes, they also provided the first confirmation of expressed histone proteins in any dinoflagellate by detecting H2A peptides.

In the present study, we analyzed the expression profile of histone at the mRNA and protein levels in *A. pacificum*. Such insights can provide information about the functional role of histones in dinoflagellates. Transcriptome data of *A. pacificum* has allowed us detailed analysis of histone sequences and we identified and reported various linker, core, and histone-modifying genes. We first verified the presence of 10 different histone genes at the DNA and mRNA levels and then expression quantification of these genes was performed using real-time PCR analysis. Expression at the protein level was determined using commercially available antibodies raised against human histones or mass spectrometric (MS) analysis. The expression profile was analyzed under nutritionally stressed conditions, keeping in view that the availability of nitrogen and phosphorus is very crucial for photosynthetic dinoflagellates. Our results demonstrate that core histone gene expression was significantly increased during stress conditions, consistent with this hypothesis that histones may have a functional role in transcription regulation, especially during nutritional stress. This view is also supported by the presence of multiple histone-modifying enzymes. We were not able to detect histone expression at the protein level, and a histone-like protein was determined as a major nuclear component. In-depth analysis of histone protein sequences revealed that this species not only contains divergent types of histones

but also the conventional histones. Despite the fact of histone gene presence, their sequence conservation, and active transcription within the cell, it is clear that histones are not the major nuclear proteins in dinoflagellates, suggesting an insubstantial role in DNA compaction.

Materials and Methods

Algal Culture and Growth Conditions for Expression Analysis

The culture of *Alexandrium pacificum* (*A. tamarense* species complex Group IV) used in this study was obtained from the Key Laboratory of Marine Genetics and Breeding, Ocean University of China, Ministry of Education, China. The culture was grown at $20 \pm 1^\circ\text{C}$ in an f/2 medium under a 12-h light and 12-h dark photocycle [25] using cool white fluorescent lights with an intensity set at between 30 and $35 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ [26]. For molecular cloning and protein extraction, cells were grown in normal f/2 media and collected at the end of the ninth day following initial inoculation with a concentration of 2×10^6 cells/l. For quantitative PCR, three samples from different growth conditions were used, following initial inoculation with 2×10^6 cells/l. The first of these growth conditions was log phase (LOP), in which cells were cultured for 11 days. Nutrient depleted conditions comprised low phosphorus (LP) and low nitrogen (LN). To produce these media, $4.8 \mu\text{M/l}$ phosphorus ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) or $76.8 \mu\text{M/l}$ nitrogen (NaNO_3) was added to f/2 medium. Finally, at 12:00 on the 11th day after initial inoculation, 5×10^6 cells were collected from the LOP culture and from the two nutrient-depleted cultures. Synchronized cultures were used in all cases [27].

RNA Extraction and Complementary DNA (cDNA) Synthesis

We extracted RNA using TRIzol reagent (Invitrogen, USA), following the manufacturer's instructions, and DNA digestion of extracted RNA was achieved using DNase-I (Takara, Japan). The quality and quantity of RNA were assessed using the NanoDrop-2000 system (Thermo, USA); in all cases, $1 \mu\text{g}$ of high-quality RNA (i.e., OD 260/280, 1.9–2.1) was used for cDNA synthesis and immediately stored at -20°C for further use.

PCR Amplification, Cloning, and Sequencing of Histone Genes

Ten histone gene sequences were retrieved from the *A. pacificum* transcriptome (SRX368254); details of the genes and primers are provided in Table 1. Each unigene was then PCR-amplified, where the reaction mixture ($25 \mu\text{l}$) included $10\times$ PCR buffer ($2.5 \mu\text{l}$), MgCl_2 ($1.5 \mu\text{l}$), deoxynucleotide mix ($2.5 \mu\text{l}$), forward and reverse primer ($1 \mu\text{l}$ each), *Taq* DNA polymerase ($0.2 \mu\text{l}$), template ($2 \mu\text{l}$), and purified water ($14.3 \mu\text{l}$). The PCR amplification program was set to denaturation at 94°C for 10 min, annealing temperatures that ranged between 65°C and 68°C for 1 min, followed by extension at 72°C for 1 min. The PCR-amplified product was purified using an agarose gel purification kit. The purified products were cloned into a pMD18-T vector, and recombinants

Table 1. Genes and primers used in this study for molecular cloning and expression analysis.

Gene ID	Gene name in the <i>A. pacificum</i> transcriptome (SRX368254)	Purpose	Primers used, forward/reverse (5'-3')	Product (bp)
H2A.a	Algae_064-1_Unigene_BMK.10533	Cloning	GAAGGGCAGCCCGAAGAAGAAGC GGCGAGCACGATCATGTGAGACA	715
		RT-PCR	CGTGCTGCCGAACATCCACACA TGCTCCATTCCGTCTCCTTTGC	124
H2A.b	Algae_064-3_Unigene_BMK.9977	Cloning	TCATGGGAGACGGCCTCACGATC CGTCAGCAGGGACTTGTGCGATGTG	440
		RT-PCR	GTCGCAAGGACCTGAAGACCAAG TCAGCAGGGACTTGTGCGATGTGG	142
H2B.a	Algae_064-3_Unigene_BMK.33389	Cloning	ACCAGAGCCCCATCCTCATTTAGT CTCTTGGCAAGCACAGTCACCCTC	448
		RT-PCR	CCAAAGCGGACAAAGAAGAGGA CGGTGACACAGGACTCCATGAC	124
H2B.b	Algae_064-2_Unigene_BMK.13294	Cloning	AAAAGAGGCGATGATTGCAGACT GAGCGGCATCTTGGCGTACTTG	427
		RT-PCR	CAGATTACCCAGAGTGTGGCATT TCCGTCTCTTCATGGTCTTAACA	124
H3.a	Algae_064-1_Unigene_BMK.42014	Cloning	TCGCTCATCATCCGCTGTCCCTTA ATCGTGACCCGCTTGGCATGTATC	428
		RT-PCR	CACGGACCTTCTCATTGCGAAAC CAAGCACAGCCTGGGATTGGAAC	101
H3.b	Algae_064-1_Unigene_BMK.10241	Cloning	AGATCATGCACTACCGCAAGACCA ATACATATACACAGCAGCTTCGCC	526
		RT-PCR	CTTCGAGGACGCCAACCTCTGC TGCGACGACACCCCTTTACCC	125
H4.a	Algae_0641_Unigene_BMK.40313	Cloning	AACGGCTGGGCCAACCATGTCTT ATCCACAAACCCCCATCCCTCG	439
		RT-PCR	CGCAGGAAGACGGTCACGGCACT GTCACCTACCCGAGCCCTCCAC	135
H4.b	Algae_064-2_Unigene_BMK.7539	Cloning	TCCAAGGGCGACAAGTTCAGCAAG CAGCAGCCGAAAGTTAAACGATG	407
		RT-PCR	AGGGCGACAAGTTCAGCAAGGAG CAGGCTTGGTGATGCCCGAGAT	108
H1.a	Algae_064-1_Unigene_BMK.29547	Cloning	AGGGAGGAGTTAGTGGACAAGTGC CTGGAGATCCGTGAGCGAGTAGG	390
		RT-PCR	GCTCAAGGACCCTGCCATCTTT ACTACCGCTTTGCTTTCCCTTTA	159
H1.b	Algae_064-3_Unigene_BMK.36228	Cloning	TCCACGCCTGCTGTTTGGTCTC GCTTCCTTGGTCGGTGTGCTG	403
		RT-PCR	GACGGTGACTCCCTGGTGATTG TTCAAGTGCGGCTGCACCTTTT	152
Control	GAPDH ^a	RT-PCR	TCGCGTTGTATTCTTGTGTAGCC TCGTGCGCGATGTAAGTGTCT	197

^aReference primer sequences were previously validated by our group (unpublished data).

with inserted genes were tested using colony PCR. All sequencing was completed at Beijing Genomic Institute, China; the generated

sequences were then analyzed by alignment with a corresponding sequence from the transcriptome data.

Relative Quantification of Gene Expression Using Quantitative RT-PCR

We selected nine histone genes for RT-qPCR using a Roche LightCycler 480 system; details of each gene and primer are presented in Table 1. All the selected genes were cloned using a pMD18-T vector, and 10-fold serial dilutions were prepared in each case in order to get a standard curve. The reaction mixture (20 µl) included SYBR Premix (10 µl), forward and reverse primer (0.5 µl each), purified H₂O (7.2 µl), and 2 µl of cDNA. The RT-qPCR amplification program was set to denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 60°C for 1 min.

We performed expression analyses for the three growth conditions (*i.e.*, LOP, LP and LN). The glyceraldehyde-3-phosphate dehydrogenase and actin genes were used as references/internal control, while the LOP was used as the control condition. We used the comparative threshold ($2^{-\Delta\Delta C_t}$) method to calculate fold change [28], and the standard error (SE) and mean Ct values were calculated for each condition and gene; a change in gene expression was considered significant when the expression was increased more than 2-fold (*i.e.*, upregulated) or decreased to less than 0.5-fold (*i.e.*, downregulated) [29].

Acid Extraction of Histone Proteins and Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) Analysis

The trichloroacetic acid (TCA) precipitation method was used to isolate histone proteins from the *A. pacificum* culture and from log phase of *Saccharomyces cerevisiae* (control) as described previously [22] but incorporating a few modifications. Cells were collected and suspended in 1 ml of ice-cold acid extraction buffer (*i.e.*, 1.5 mM MgCl₂, 10 mM HEPES at pH 7.9, 0.5 mM DTT, 10 mM KCl, 1.5 mM phenylmethylsulfonyl fluoride, and 0.25 M HCl), disrupted using a bead beater, and incubated with stirring for 1 h on ice. The resultant cell lysate was then centrifuged at 11,000 ×g for 10 min at 4°C, the supernatant was collected, and 100% TCA was added to obtain a final concentration of 33% (w/v) TCA. This solution was then incubated at 4°C for at least 3 h and acid-soluble proteins (ASPs) were obtained by centrifugation at 16,000 ×g for 15 min at 4°C. The pellet was retained, washed twice with cold acetone, and dissolved in 100 µl of double-distilled H₂O. Quantification was then performed using a Bradford assay [30].

Extracted ASPs were resolved using 15% SDS-PAGE analysis as described previously [31].

Immunodetection of Core Histones

We performed western blot analysis using commercial rabbit polyclonal antibodies against human histones (*i.e.*, H3-D124055 and H4-D222482; Sangon, China). We ran this test simultaneously for *A. pacificum* and *S. cerevisiae* ASPs; proteins from the gels were transferred to a polyvinyl difluoride membrane using a semi-dry electrophoretic transfer cell (Bio-Rad, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Following the addition of chemiluminescent

substrate (ECL-C510043; Sangon, China), the membrane was exposed to a chemiluminescent imaging system (MiniChem, China).

MS Analysis

We resolved 30 µl of *A. pacificum* total acid-extracted protein on a 15% gel and the most prominent band (approx. 15 kDa) was excised from the gel and analyzed using matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS). Destaining of the band, tryptic digestion, and MS analyses were all performed at Sangon, China.

Sequence Analysis

Histone gene sequences retrieved from *A. pacificum* transcriptome data were confirmed using a combination of BLAST searches on NCBI and Swissprot. A number of sequences were downloaded for alignment purposes, and multiple alignments were carried out using the software MUSCLE [32], visualized using JalView [33]. The physiochemical properties of the proteins were determined using resources at <http://web.expasy.org/protparam> [34], and conserved domains were predicted using resources at <https://www.ebi.ac.uk/interpro> [35] and <http://pfam.xfam.org> [36]. Secondary structures of core histones were determined using an online resource (Phyr2) by putting the whole-gene-deduced protein sequence into the search box following the default settings (<http://www.sbg.bio.ic.ac.uk/phyre2>) [37].

Results

Genes for Linker Histones, Core Histones, and Histone-Modifying Enzymes Are Present in *A. pacificum*

We identified gene sequences for all core and linker histones in the *A. pacificum* transcriptome (SRX368254) (Table 2). Although we identified 31 unigenes for histone H1, none was a complete gene sequence; among core histones, we identified three unigenes for H2A (two full gene sequences), two unigenes for H2B (both comprised full gene sequences), 15 unigenes for H3 (two full genes sequences), and six unigenes for H4 (three full gene sequences).

A number of genes responsible for histone modification, chromatin formation, and remodeling are also present in *A. pacificum*, including histone acetyltransferases and deacetylases, methylases and demethylases, reader domains,

Table 2. Histone sequences detected in *A. pacificum* transcriptome data (SRX368254).

Total genes retrieved from transcriptome data					Complete gene sequences in the dataset				
H1	H2A	H2B	H3	H4	H1	H2A	H2B	H3	H4
31	3	2	15	6	0	2	2	2	3

Table 3. Identification of *A. pacificum* sequences responsible for histone modification, chromatin formation, and remodeling.

Category	Family/Domain	Unigenes	Biological role	Reference
Histone acetyltransferases	GNAT	85	Acetylates conserved lysine amino acids linked to transcriptional activation	[64]
	MYST	0		
	Nuclear receptor coactivator	1		
Histone deacetylases	Histone deacetylase	13	Removes acetylation marks	[65]
	Sir2	21		
Histone methyltransferases (HMT)	SET	16	Transfers methyl groups; methylated histones can repress or activate transcription	[66]
	Dot1	26		
(lysine specific)	Unclassified	5		
HMT				
(arginine specific)	PRMT5	4		
Histone demethylase	Lysin specific	3	Removes methyl group from histone and regulates gene expression	[67]
	Arginine specific	4		
Reader domain	Bromodomain	13	Proteins that recognize specific marks on histone proteins	[68]
	Chromodomain	17		
	PhD	11		
	TUDOR	29		
	PWWP	1		
	WD40	543		
Chromatin remodeling	SNF2	120	Allows regulatory transcription machinery proteins to access condensed DNA	[69]
	SLIDE	0		
	SnAC	0		
	DBINO	0		
	SWIB	1		
	SWIRM	0		
Facilitates chromatin transcription	FACT-spt-16	1	Role in transcription through chromatinized DNA	[70]
	SPT 16	1		
	SSRP1	1		
Histone chaperones	ASF1	1	Binds histones and regulates nucleosome assembly	[71]
	CAF-1	0		
	Hira	10		
	NAP	3		

Families and domains listed above were selected from Marinov and Lynch [45].

FACT (facilitates chromatin transcription) complex, and histone chaperones (Table 3). Histone acetyltransferases (HATs) have multiple families including the GNAT-type HAT family, MYST, nuclear receptor coactivator, and others. Results showed that *A. pacificum* expresses more numbers of sequences for the GNAT family (85) compared with other families within this group. Indeed, of the two classes of histone lysine methyltransferases (HMTs) identified, the majority belonged to the DOT1 domain class (26) as opposed to the SET class (16). Four classes of histone

deacetylases (HDACs) are known [38]; classes I, II, and IV are characterized by a histone deacetylase domain and class III is characterized by the presence of a SIR2 domain. Twenty-one unigenes identified as having a SIR2 domain and 13 unigenes belonged to other classes. WD40 was the most abundant of the reader domains; this binds to trimethylated lysine, amongst other substrates, and the presence of a complete FACT complex in *A. pacificum* shows that transcription through a nucleosome array does occur [39].

Table 4. Results of histone gene amplification using cDNA as template.

Accession No.	Gene name	PCR product size (bp)	Gene status	ORF coverage	Intron ^a	Gene [Species hit]
KY659046	H1.a	390	Partial	-	-	H1 [<i>Triticum aestivum</i>]
KY659047	H1.b	403	Partial	-	-	H1 [<i>Pisum sativum</i>]
KY659038	H2A.a	715	Partial	87%	Yes	H2A.X [<i>Alexandrium tamarense</i>]
KY659039	H2A.b	440	Partial	85%	Yes	H2A.Z [<i>Anoplopoma fimbria</i>]
KY659040	H2B.a	448	Full	100%	No	H2B [<i>Dacryopinax primogenitus</i>]
KY659041	H2B.b	427	Partial	99%	No	H2B [<i>Babesia bovis</i>]
KY659042	H3.a	428	Partial	83%	No	H3 [<i>Eimeria maxima</i>]
KY659043	H3.b	526	Full	100%	Yes	H3 [<i>Chlamydomonas reinhardtii</i>]
KY659044	H4.a	439	Full	100%	Yes	H4 [<i>Tauraco erythrophus</i>]
KY659045	H4.b	407	Partial	91%	Yes	H4 [<i>Zea mays</i>]

ORF, open reading frame.

^aIntron; while using DNA as a template.

Amplification and Cloning of Variants of Histone Genes

Our results show that a number of histone sequences are present in the *A. pacificum* transcriptome. Thus, to confirm whether or not these sequences are present in the organism, we amplified, cloned, and sequenced 10 genes (*i.e.*, two for each core and linker histone) from cDNA (Table 4). All these sequences were the same as in the transcriptome data, although there were a small number of base changes in some genes. Using the same primer, we then amplified core histone genes from the DNA template; the results of this approach showed that the genes H2B.a, H2B.b, and H3.a lacked introns, whereas H3.b and both variants of H2 (A and B) contained intron sequences (Fig. 1). Linker histone genes were readily amplified using cDNA as the template; however, we were not able to amplify from DNA.

Fig. 1. Amplification of core histone genes from cDNA and DNA.

The core histone genes of *Alexandrium pacificum* were amplified from cDNA and DNA using the same primers.

Histone Gene Relative Expression under Nutrient-Depleted Cultures

Nine histone genes chosen for RT-qPCR analysis were amplified, cloned, and sequenced, and plasmids were extracted. We monitored the relative expression levels of histone genes under two nutrient-depleted conditions (LP and LN) to determine the functional significance of *A. pacificum* histones. We carried out qPCR experiments on mid G-phase cultures. This species showed a significantly high expression of histone mRNA for histone genes under nitrogen stress, except H2B.a, H2B.b, and H1.a. Under phosphorus-limited cultures, histone genes H2A.a, H3.a, H3.b, and H4.b exhibited near stable expression, whereas H1.a, H2B.a, H2B.b, and H4.a were downregulated when compared with 11-day-old cultured cells. Significantly high expression was observed in the H2A.Z variant during phosphorus stress (Fig. 2).

Protein-Level Histone Expression in *A. pacificum*

To evaluate histone expression at the protein level, we employed a range of different sensitive techniques, including western blotting and MS. Acid-extracted proteins from log-phase condition were resolved using SDS-PAGE and stained with Coomassie blue. Results from this approach showed that *A. pacificum* does not exhibit a typical pattern of histones on a gel as is the case, for example, in yeast (Fig. 2). In contrast, *A. pacificum* exhibited a very prominent and darkly stained band in the vicinity of 15 kDa, confirmed via MALDI-TOF-MS to correspond with a HLP with a molecular mass of 11.47 kDa. The reduced mobility of this protein on the gel is likely the result of the highly basic residues that it contains [40]. Immunoblotting was then performed using commercial antibodies of human origin against H3 and H4

Fig. 2. Expression of *A. pacificum* histone genes in nutrient-depleted media and at the protein level.

(A) Relative expression levels of *A. pacificum* histone genes under nutritional stress. A change in gene expression was considered significant when expression was increased more than 2-fold (*i.e.*, upregulated) or decreased to less than 0.5-fold (*i.e.*, downregulated). An asterisk (*) on the bar denotes significant difference in gene expression relative to the log phase. (B) Upper panel: Immunodetection of core histones in *A. pacificum* and yeast (*S. cerevisiae*, control). Acid-soluble proteins (ASP) from *A. pacificum* (*A.p*) and *S. cerevisiae* (*S.c*) were prepared for 15% SDS-PAGE analysis and immunoblotting was performed using commercial antibodies against core histones. Both tested antibodies showed a positive reaction against yeast histones, whereas no signal was detected for *A. pacificum*, with the exception of the H3 gene. The identity of this band, however, remains unknown. Lower panel shows ASP from *A. pacificum* (*A.p*) and *S. cerevisiae* (*S.c*). The most prominent band near 15 kDa was excised and subjected to analysis using MALDI-TOF-MS.

histones; this approach successfully detected yeast core histones and failed to detect core histones of *A. pacificum*, whereas antibodies against histone H3 strongly cross-reacted with a protein of unknown identity that showed increased mobility (approx. 11 kDa) compared with the yeast histone H3 (Fig. 2).

A. pacificum Histones Show Sequence Divergence Compared with Other Eukaryotes

The results of this study showed that *A. pacificum* possesses multiple histone gene transcripts with sequences

that are divergent compared with other eukaryotes. Some regions are conserved, however, including the histone fold region. At the same time, the N-termini of most proteins exhibit divergence and additional sequences that elongate the N-terminal region. Because a number of start codons were found in open reading frames (ORFs), we predicted this section of the sequence (Fig. 3) by aligning *A. pacificum* histone protein sequences with those of other species. The results of this comparison showed that the extra start codons before the actual starting position do not belong to a conserved domain and share no sequence similarity with

Fig. 3. Histone protein domains and start codon predictions for *A. pacificum* and human core histones. Because more than one methionine/start codon (triangle) was present at the start of each ORF, one was predicted as the most probable start codon (circle) following alignment of the *A. pacificum* histone sequences with those of other species.

other species. Indeed, the H2A and H4 gene sequences contained two additional start codons in their ORFs that rendered them more elongated than conventional histones, whereas two H3 sequences have just one start codon and lengths that are the same as human H3. One H3 (H3.b) comprised a full-length gene, albeit markedly smaller than conventional histone H3, with the smallest N-terminal tail

in the group. Similarly, the H1 sequences of *A. pacificum* were the most divergent and had very little sequence similarity with other eukaryotes. The molecular characteristics of *A. pacificum* and human histones are shown in Table 5; these data showed that the highest variation in protein length is in H2A compared with the human protein. The GC contents of histone genes in dinoflagellates range

Table 5. Molecular characterization of histone genes in *A. pacificum* and humans.

Gene ID	ORF length	Stop codon	GC%	Peptide residues	Mw (kDa)	PI	Acidic amino acid	Basic amino acid	Most abundant amino acid
H2A.a	489	TGA	62	162	17.35	9.72	18	27	Lys
H2A.b	513	TGA	64	170	18.11	10.05	18	35	Lys
H2B.a	360	TGA	56	119	13.35	10.05	11	25	Lys
H2B.b	423	TGA	56	140	15.76	10.53	11	35	Lys
H3.b	318	TGA	69	105	11.82	8.99	13	16	Ala
H3.xy	411	TGA	64	136	15.34	11.00	11	29	Ala
H4.a	339	TAG	60	112	12.52	10.86	10	26	Arg
H4.b	357	TGA	64	118	13.07	10.86	10	26	Arg
H4.xy	303	TAG	60	100	11.26	10.76	9	22	Arg
Human H2A	393	TAA	63	130	14.11	10.90	9	26	Ala/Leu
Human H2B	381	TAA	60	126	13.94	10.31	10	28	Lys
Human H3	411	TAA	60	136	15.40	11.13	11	31	Ala/Arg
Human H4	312	TGA	61	103	11.37	11.36	7	25	Gly

Note that linker histones and the H3.a gene are not included in this table because they have incomplete ORFs. Two extra histone variants from the transcriptomes of *A. pacificum*, H3.xy (Algae_064-2_Unigene_BMK.1001) and H4.xy (Algae_064-1_Unigene_BMK.87970/68734), as well as human core histones sequences, were included for sequence analysis.

between 56% and 69%, a larger span than that seen in humans, which are concentrated between 60% and 63%. The highest GC content (69%) was present in the gene H3.b, a typical dinoflagellate histone; indeed, the histones are highly basic proteins that comprise large numbers of

basic amino acids (*i.e.*, Lys+Arg). Data showed that Lys, Ala, and Arg are the most abundant amino acids present in the histones H2AB, H3, and H4 of *A. pacificum*, respectively. Although the highest PI value was seen in the human H4 histone (11.36), the most abundant amino acid encoded by

Fig. 4. Secondary structural predictions for the core histones of *A. pacificum* and humans.

These secondary structures were predicted using Phyr2, although extended histone tails could not be resolved by the software.

this gene is Gly, rather than Lys or Arg (basic amino acids).

Secondary Structure of Histone Proteins in *A. pacificum*: Comparison with Human Histones

To gain insights into the structures of encoded proteins based on their sequences, we made secondary structural predictions. To do this, we predicted the secondary structures of *A. pacificum* and human core histones using the software Phyr2 (Fig. 4). Our results showed that the human H2A structure comprises five helices, and almost the same was predicted for the protein product of the *A. pacificum* H2A.b gene; however, the product of H2A.a has four helices in its secondary structure. Similarly, the proteins encoded by the human H2B and H3 genes both have four helices in their structures, very similar to the situation in *A. pacificum*, with the exception of H3.b. In contrast, the structure of the human H4 gene product comprises three helices, identical to those seen in all H4 variants. The most conserved and normal length protein sequences were observed for H3, whereas H2A contained the most extended sequences. Extended N and C termini were unable to be resolved by the software because no conserved or additional domains were present in it.

Discussion

Conservation and Divergence of Dinoflagellate Histone Proteins

Dinoflagellates are divergent from other eukaryotes because they do not have nucleosomal chromatin organization [41]. Indeed, with the exception of two groups, their basic nuclear proteins do not show typical histone patterns on gels [42]. Exceptions to this are the binucleate dinoflagellates (*e.g.*, *Peridinium balticum*) that possess typical eukaryote and dinoflagellate nuclei [43], and *Perkinsus marinus*, which has a nucleus that resembles a typical eukaryotic cell [44]. This species also possesses both linker and core histone proteins [40], but lacks HLPs, the major nuclear proteins in core dinoflagellates.

One outcome of this study is the revelation that *A. pacificum* expresses all core and linker histones, as well as a number of histone modification enzymes. To resolve potential conflict and determine whether or not the detected histone sequences belong to dinoflagellate species, we present two observations. In the first one, histone sequences obtained from the transcriptomes of dinoflagellates exhibit a level of sequence divergence that makes them unique, and secondly, both axenic and non-axenic dinoflagellate cultures contain

histones [45]. The largest number of histone genes was observed in the transcriptomes of “dinotom” dinoflagellates, so-called because they possess both dinoflagellate and diatom nuclei, as they harbor a tertiary endosymbiont of the latter [46]. Although we observed elongated histone sequences in the transcriptome data, many sequences of normal length were also observed in *A. pacificum*. An elongated histone tail in *A. pacificum* does not mean that additional protein domains are necessarily present, as only a core histone domain was identified. However, some dinoflagellates do possess additional domains, including the TRAM-LAGI-CLN8 and NAD(P) binding domains in H2A [45]. The role of these additional domains remains unclear.

Overall, based on the molecular characterization of core histones, it is clear that H3 genes are the most conserved in *A. pacificum*, whereas the lowest degree of conservation is seen in H2A. This might present one explanation as to why human origin antibodies can readily detect dinoflagellate H3 proteins after immunoblotting.

Histone modification enables the performance of several functions, including transcriptional initiation and chromatin dynamics, that are linked to epigenetic regulation [47]. *A. pacificum*, for example, expresses a number of proteins that act as chromatin mark readers, writers, and erasers.

This species possesses a large number of acetyltransferases, deacetylases, methylases, demethylases, and histone chaperones; their presence corroborates a role for histones in the regulation of gene expression.

Expression Control and Management of Histone Proteins

It is generally accepted that canonical histone proteins are encoded by replication-dependent (RD) genes, the only known eukaryotic mRNAs that contain a conserved stem-loop at the 3' end instead of a polyA tail [3, 48]. These RD mRNAs are expressed in high concentrations during the S-phase of the cell cycle (expression increases 35-fold during this phase), decreasing rapidly towards the end of this phase [3, 49]. The synthesis of variants known as replication-independent (RI) histones or polyadenylated mRNAs persists outside of the S-phase [48]. Our recent study (unpublished data) showed that expression of histone genes remain stable during the G and S phases of the cell cycle. Although we hypothesized the presence of a distinct mechanism in dinoflagellates to control the expression of histones, no data are presently available. Expression analysis of histone mRNAs has so far only been completed using transcriptome data, which are limited owing to the absence of a polyA tail

in RD histones [48].

To date, some RD histone genes have been identified that remain active and encode mRNAs during the G phase, even when cells are no longer in a dividing state [3]. For example, H2A.X exhibits characteristics of both RD and RI histone genes and produces adenylated and non-adenylated mRNAs. At the same time, H2A.X mRNAs and proteins are synthesized throughout the cell cycle [50]. Our results showed that variants of histone genes are expressed in *A. pacificum* (Table 4). H2A. An X-type variant plays a significant role in DNA damage repair in other eukaryotes. In the present study, H2A.a maintained a steady level of transcription in phosphorus-limited culture during the G phase while histone H2A.b expression was significantly upregulated. The H2A.Z variant has been previously proposed to regulate transcription in many eukaryotes in a number of ways, especially during phosphate stress [51] and other stress conditions [52]. Under the limited availability of phosphorus, dinoflagellates stop their cell division as an adaptive mechanism [53]. Similarly, in nitrogen-depleted culture, dinoflagellate cells slow down or stop their cell division. Nitrogen availability is very important for dinoflagellate growth, especially for toxin production. In reduced cell division, higher expression of histone genes indicates their role during stress management in cells, possibly by controlling transcription.

Histone hairpin-binding protein or stem-loop-binding protein (SLBP) specifically binds to RD histone's conserved stem loop and helps in mRNA transport, stability, and translation regulation [54]. Five unigenes encoding SLBP were detected in the *A. pacificum* transcriptome; however, none of the histone gene sequences contain conserved motif in 3' UTR that can compose a conserved stem loop. There could be two possibilities for this. First, most of the histone sequences are not represented completely in the transcriptome; and second, deep-sequencing techniques are based on the polyA tail section, so there is little information about the transcripts that lack a polyA tail, such as RD histones [55]. Instead of a conserved stem loop sequence, conserved 3' UTR sequence motifs (Brd-box (AGCUUUA) (in H2B.a) and K-box (cUGUGAUa) (in H3-Algae_064-2_Unigene_BMK.91286)) were detected. These conserved motifs in 3' UTR negatively regulate the transcription of notch target genes, and any mutation in it can lead to overexpression of protein as reported previously in *Drosophila* [56]; however, the function of these motifs in *A. pacificum* is unclear.

In *A. pacificum*, histone expression at the protein level is

very little; however, the presence of high expression of histone mRNA is not surprising. Cells have a strict regulatory mechanism at the translation and post translational levels because of lethal effects of unbound histones in the cell. Positively charged histone proteins can attach nonspecifically to negatively charged DNA and other cellular bodies and hamper their function [57]. Histone chaperones (HIRA family) control histone gene expression and repress the expression of histone genes not only in G phase but also in S phase, and overexpression of HIRA proteins leads to repression of histone genes in humans [58]. The FACT complex in yeast also prevents the accumulation of transcription evicted histones [59]. SLBPs control the translation of histone mRNA, and DNA checkpoint proteins degrade the unbound/excess histone protein [60]. Eight unigenes for DNA checkpoint proteins were identified in *A. pacificum* from the transcriptome data. Although histones are quite stable proteins, with a half-life of 4–5 months [61], unbound histone proteins are rapidly degraded with the half-life of 30–40 min in yeast [62]. Thus, it is equally possible that either *A. pacificum* strictly inhibits the excess translation of histone mRNA or rapidly degrades the unbound histone proteins owing to their limited role in DNA packaging.

We were unable to detect histone proteins even when using a range of different sensitive techniques, including immunoblotting and MS analysis. Immunogens used to generate antibodies against H3 and H4 shared 88% sequence similarity; still, no positive reaction was observed in this report. Our species H3 and H4 sequences are highly conserved with other eukaryotes, so a negative reaction strongly indicates very little expression of histone proteins in dinoflagellates. In addition, our results showed that antibodies against the histone H3 protein cross-react with a protein of unknown identity; we refer to this as the *A. pacificum* histone H3 because one variant in *A. pacificum* (H3.b) has a molecular mass of 11.89 kDa (Table 5). The greatest degree of similarity that was observed between different variants of this gene supports our hypothesis that this reduced-sized band was correctly identified. We were unable to detect the H3 protein using MS because less-abundant proteins are often masked by more abundant ones in samples [63]. In any case, histone expression at the protein level is very limited in dinoflagellates. In dinoflagellates, only 0.2% of the genome is transcriptionally active. Hence, even if very low amounts of histone proteins are present in dinoflagellates, they can still regulate transcription.

A. pacificum Major Basic Nuclear Proteins

Although histone proteins are responsible for DNA compaction in other eukaryotes, this appears not to be the case for dinoflagellates. Previous studies have identified HLPs in dinoflagellates, and a novel DNA-binding protein called DVNP has recently been identified in this protist. Core dinoflagellate species even contain both HLPs and DVNP (e.g., *Alexandrium* and species of *Lingulodinium*). *A. monilatum* possesses 38 unigenes for DVNPs, the highest number amongst dinoflagellate species studied to date [45]. DVNP unigenes were also detected in the *A. pacificum* transcriptome. The results of this study show that HLP is present in *A. pacificum* as a major nuclear protein. Interestingly, in *A. pacificum* only two variants of HLPs are present; however, the transcript abundance of HLP is nearly 300 times more than the histone transcripts (HLP: RPKM 11,795; and histones: RPKM 5–37). Of note, genes for histones, HLPs, and DVNPs are present in *A. pacificum* but expression at the protein level is only detectable for HLPs to date. DVNP and histone protein expression levels need more precise techniques to measure their low levels.

The presence of different histone and histone-modifying gene sequences, conservation of the core histone domain, and reasonably high transcript level of histones during stress all strongly suggest that histones are not only present in dinoflagellates, but also have functional significance. Advanced techniques for protein extraction and detection are required to measure the low histone levels seen in dinoflagellates. Although these are not the major nuclear proteins in *A. pacificum*, the functional significance of histones cannot be underestimated, and further work will be required to investigate their importance in dinoflagellate biology.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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