MOLECULAR CLONING AND CHARACTERIZATION OF HISTONE GENE CLUSTERS IN TWO SEA STAR SPECIES

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Molecular cloning and characterization of histone gene clusters in two sea star species

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Abstract

The organization and DNA sequences of histone genes from Solaster stimpsoni and Pycnopodia helianthoides have been investigated. The sizes of major histone gene cluster elements were first determined by genomic blots. Partial genomic libraries of Pycnopodia and Solaster were constructed and screened three time with histone gene sequences from Pisaster ochraceus or Dermasteria imbricata. A recombinant bacteriophage containing a 5.4 kb histone gene cluster element was isolated from the the Pycnopodia genomic library. Two recombinant phage carrying either a 6.2 kb or a 7.5 kb histone gene cluster element from Solaster genomic library were identified. The 5.4, 6.2 and 7.5 kb histone gene elements have been characterized.

Genomic blots indicate that Pycnopodia contains a single major histone gene cluster, whereas Solaster contains at least three different sizes of histone gene clusters. The histone genes isolated from either Pycnopodia or Solaster are organized in tandem repeats. Restriction enzyme mapping and Southern hybridization reveal that the arrangement and transcriptional polarity of core histone genes within each gene cluster element are identical (5'-H2B-H2A-H4-H3-3'), and are also the same as those from three other sea stars. The results suggest that there is a remarkable stability in histone gene organization in sea stars.

DNA sequence analysis of H4 and H3 genes reveals a high degree of sequence homology in the coding regions between the

two species. The flanking regions, however, have diverged to the point where sequence identity has been erased. The sequences were compared to those from other sea stars and organisms. Analysis of nucleotide substitutions between sea star H3 genes indicates that saturation of nucleotide substitutions occurred except between Pisaster and Pycnopodia. The pattern of nucleotide substitutions between H3 genes was also observed. The ratio of transversions to transitions appears to be related to the divergence time.

The potential TATA, CAAT, Cap blocks and dyad symmetry sequences are found in the flanking regions of H4 and H3 in both species when compared to the regions from other organisms. These conserved sequences may be important for gene regulation or expression.

Acknowledgements

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TABLE OF CONTENTS

Approval....................ii
Abstract....................iii
Acknowledgements....................v
List of Tables....................viii
List of Figures....................ix
Introduction....................1
Materials and Methods....................5
1. DNA isolation....................5
2. Construction of partial genomic libraries....................6
3. Screen of histone gene clusters....................6
4. Preparation of DNA probes....................8
5. Restriction digestion and gel electrophoresis 8
6. Subclones 9
7. Southern blots and hybridization condition....................9
8. DNA deletion....................10
9. DNA sequencing....................11
Results....................13
Identification and isolation of a histone gene cluster element from _Pycnopodia_ genome....................13
Identification and isolation of histone gene cluster elements from _Solaster_ gemone....................15
Restriction mapping of histone gene cluster elements of _Solaster_ and _Pycnopodia_....................19
Histone gene arrangement within cluster elements of _Pycnopodia_ and _Solaster_....................22

The transcriptional polarity of histone genes..........23

Analysis of the transcriptional polarity of histone genes within the 7.5 Kb element.........................25

Further investigation of the "insertion portion" within the 7.5 Kb element..26

DNA sequence analysis of H3 and H4 genes................28

Comparison of _Solaster_ and _Pycnopodia_ H3 coding regions..32

Comparison of _Solaster_ and _Pycnopodia_ histone H4 coding regions...32

Distribution of nucleotide substitution.................35

5' flanking regions of H3 genes.........................37

5' flanking regions of H4 genes.........................39

3' flanking regions of histone H3 genes.................40

3' flanking regions of histone H4 genes.................42

Discussion...44

Organization and number of histone gene clusters........44

Restriction mapping analysis............................48

Analysis of histone H3 gene evolution among four sea star species......................................48

Analysis of histone H3 and H4 flanking regions..........54

Phylogeny of Sea stars..................................57

Conclusions and perspectives............................59

References...62

LIST OF TABLES

Table 1: Probes: Origins and Descriptions....................7

Table 2: Pattern of nucleotide substitutions at different degenerate sites between sea star H3 genes........36

Table 3: Pairwise comparison of nucleotide change between sea star H3 genes...................................50

Table 4: Codon usage in four sea star H3 genes.............53

LIST OF FIGURES

Figure 1. Southern blots of Pycnopodia genomic DNA hybridized with a H3 histone gene probe....................14

Figure 2. Southern blots of Solaster genomic DNA indicating the different sizes of histone gene cluster elements..16

Figure 3. Southern blot of Solaster genomic DNA from three individual males................................18

Figure 4. Agarose gel electropherogram of restriction enzyme digested plasmids pSoH7.5 and pSoH6.2...........20

Figure 5. Restriction maps of the tandemly repeated histone gene cluster elements of Solaster and Pycnopodia......................................21

Figure 6. DNA sequences of pPyH PstI/EcoRI (A) and pSoH AccI/EcoRI (B) subclones demonstrating the transcriptional polarity of H2B genes...........24

Figure 7. Southern blots of Solaster genomic DNA hybridized with two different probes isolated from Solaster pSoH7.5 subclone................................27

Figure 8. The directed deletion of the pPyH1.4 HincII/SacI subclone from Pycnopodia histone cluster element...29

Figure 9. The entire DNA sequence of H3 and H4 genes and their flanking regions from Pycnopodia tandemly repeated element................................30

Figure 10. The nucleotide sequence of H4 and H3 genes and

their spacer regions from Solaster 6.2 Kb histone gene cluster element.............................31

Figure 11. Alignment of H3 coding sequences indicating positions of divergence among sea star species and between sea stars and sea urchins...............33

Figure 12. Alignment of H4 coding regions..................34

Figure 13. Comparisons of the consensus sequences in the flanking regions of H3 genes....................38

Figure 14. Comparison of DNA sequences in the flanking regions of Solaster and Pycnopodia H3 genes.............41

Figure 15. The phylogenetic tree of sea stars based on the percentage of nucleotide difference between H3 genes 60

Introduction

Eukaryotic genomes consist of single-copy, moderately repeated and highly repeated DNA sequences (Britten and Kohne, 1968). Multigene families provide a special opportunity to understand gene regulation and evolution. The histone gene family is one of the most interesting multigene families. This is due to the involvement of histones in DNA packaging, and the association of histone gene expression with DNA replication.

The structure, organization and expression of histone genes in human (Stein, et,al., 1984), chicken (Engle and Dodgson, 1981), mouse (Marzluff, 1984), Xenopus (Zernik et,al., 1980), sea urchin (Hentschel et,al., 1981), and yeast (Hereford et al., 1979) have been extensively studied. A common character of histone genes is that they do not contain intervening sequences in gene coding regions. Histone mRNAs are generally not polyadenylated (Stein et al., 1984). Histone gene expression is regulated at several levels. Histone mRNA levels and protein synthesis are often coupled to the cell cycle (Detke et al., 1979). The developmental regulation of histone gene expression also occurs (Zweidler, 1980). The DNA sequences of histone genes from various organisms appear highly conserved at both nucleotide and amino acid levels (Wells, 1986). Some of the functionally important sequences

in the flanking regions of histone genes have also been identified. There is significant homology between different organisms in these regions (Wells, 1986).

The best understood histone genes are sea urchin histone genes. The sea urchin genome contains several hundred copies of histone genes which are organized in tandemly repeated units. The analysis of early histone genes in several sea urchin species has indicated a high stability in the organization of histone genes within histone gene repeat unit (Roberts, et al., 1984). Histone gene organization and trancriptional polarity within histone gene clusters are known in three sea star species, Pisaster ochraceus, Pisaster brevispinus, and Dermasterias imbricata (Howell et al., 1986; Cool et al., 1988). The histone genes in these three sea stars are clustered in a tandemly repeated fashion, which is similar to that of sea urchin early histone genes. However, core histone gene arrangement in the histone gene cluster in sea stars (H2B-H2A-H4-H3) is different from that of sea urchins (H2B-H3-H2A-H1-H4). It is still an open question whether the organization of histone genes within histone gene clusters in sea stars is constant or not. There is more to be learned about histone gene evolution in sea stars.

I chose the sea stars, Pycnopodia helianthoides and Solaster stimpsoni, to determine the stability of histone gene organization because these species are the phylogenetically intermediate species to the three other sea stars studied

earlier (Cool et al., 1988). It has been reported that Solaster stimpsoni was distinct from the Dermasterias line more than 200 million years ago (Spencer and Wright, 1966), whereas Pycnopodia was distinct 20-40 myr ago from Pisaster (Smith, et al., 1982). Pycnopodia and Pisaster are in the order Forcipulatida, while Solaster and Dermasterias are representatives of the order Spinulosida. The two orders have been distinct for 500 myr. Complementary to previous studies of sea star histone genes in our lab (Cool, et al., 1988), We are attempting to understand the stability of histone gene organization, the gene transcriptional polarity, as well as histone gene evolution among sea stars.

In the past two years, I prepared a partial genomic library from Solaster in lambda gtWes. A Pycnopodia genomic library in lambda EMBL4 was a gift from M. Smith. Histone gene cluster elements were isolated from the libraries. Restriction maps of the cluster elements and precise localization of histone genes within histone gene clusters were investigated. Complete nucleotide sequences of H3 and H4 genes as well as their flanking regions were determined from these two species.

An unexpected finding is that the number of major histone gene clusters in Solaster is different from Pycnopodia and other sea stars investigated so far. Solaster contains at least three tandemly repeated histone gene clusters. Cluster elements from two of them have been isolated. They are quite

similar, implying that a recent divergence and amplification may have occurred in Solaster. The structure and organization of histone genes within main histone gene clusters of Solaster and Pycnopodia are compared with those of other sea stars and other organisms. The results show that histone gene organization and transcriptional orientation within Solaster and Pycnopodia gene clusters are the same, and identical to those from other sea stars, suggesting that the arrangement and transcriptional polarity of histone genes within gene clusters among sea stars is very stable. The complete DNA sequences of histone H3 and H4 genes from Solaster and Pycnopodia are extensively analyzed and compared to those from other sea stars and other organisms. The ratio of transition to transversion at different positions of codons and the sequence divergence between H3 genes give a clear picture of molecular evolution of histone genes in sea stars. In addition, the homologous sequences in the flanking regions which may be very important for gene regulation are also identified. The phylogenetic tree based on percentage of nucleotide difference has been constructed to show the evolutionary relationship of sea stars.

Materials and methods

Materials

Pycnopodia helianthoides and Solaster stimpsoni were collected in the Indian Arm of Burrard Inlet in British Columbia and were held in 12°C sea water until used (Fraser et al.,1981).

Methods

1. DNA isolation

Genomic DNA was prepared from fresh sperm or -80°C frozen testes by the method of Blin and Stafford (1976) modified by M. Smith (personal communication). The sperm was dispersed in 0.2 M Tris-HCl, 0.2 M EDTA pH 8.0 (sperm buffer). About 100 ml of sperm buffer per ml of sperm was used. The mixture was adjusted to a final concentration of 100 ug ml protease K (preincubated 2 hours at 37°C), 1% SDS (sodium dodecyl sulfate) and incubated at 37°C overnight. The protease K digested sperm was extracted 3 times with an equal volume of phenol:SEVAG (1:1) saturated with 50 mM Tris-HCl pH 8.0. SEVAG consists of chloroform: isoamyl alcohol at a ratio of 24:1. The DNA mixture were dialyzed against 0.02 M Na_2 EDTA, 0.02 M Tris, pH 8.0 at least 2-3 days. The DNA was treated with RNAse A, then protease K and extracted with phenol:SEVAG

(1:1) as above and with equal volume of SEVAG two times. DNA was suspended in the 0.3 N Na acetate overlaying gently 2 volumes of 95% ethanol and wound out of the aqueous phase several times. High molecular weight DNA was ready to digest with restriction enzyme at this stage.

2. Construction of partial genomic libraries

Total genomic DNA was digested with various restriction enzymes such as EcoRI, HindIII, PstI as described (Maniatis et, al., 1978, 1982). The sizes of histone gene cluster elements were first detected by Southern blots of restriction enzyme digested genomic DNA hybridized with each histone gene probe by the method of Southern (1975). All probes used in this study are shown in Table 1.

To prepare genomic libraries, Pycnopodia genomic DNA was partial digested with Sau3A. Size-selected DNA bands ranging from 15-18 kb was isolated from agarose gel and ligated to BamHI-digested EMBL4 phage (Maniatis, 1982). Solaster genomic DNA , however, was completely digested with EcoRI. Size-selected DNA bands ranging from 5.5- 7.5 kb were isolated to prepare a lambda gtWes partial genomic library by the method recommended by BRL as well as the method of Maniatis (1982). Both libraries contain approximately 2 x 10^5 recombinants after packaging.

3. Screen of histone gene clusters

Table 1

Probes----Origins and Descriptions

Specification	Subclone	Description	Reference
H3	pPoH0.8	A 800 bp EcoRI fragment from the *Pisaster ochraceus* histone gene cluster	Howell et al., 1986.
H4	pPbHE/HcII	A 950 bp EcoRI/ HincII fragment from *Pisaster brevispinus* histone gene cluster	Cool et al., 1988.
H2A	pPbHB/E	A 1.1 kb BamHI/EcoRI fragment from the *Pisaster brevispinus* histone gene cluster	Cool et al., 1988.
H2B	pPbH0.4	A 400 bp BamHI/PstI fragment from the *Pisaster brevispinus* histone gene cluster	Cool et al., 1988.

The EMBL4 and lambda gtWes libraries were plated on E.coli Q359 and VCS257 hosts respectively. The cells were grown in the presence of 0.2% maltose to increase phage infectivity. Both libraries were screened three time with denatured ^{32}P end-labeled histone H4 and H3 probes (Table 1). The positive phage clones selected from the libraries were amplified by large scale preparation (Maniatis, 1982). The purified phage DNA was digested with EcoRI. The histone gene cluster elements from recombinant phage were gel purified and subcloned into plasmid puC19.

4. Preparation of DNA probes

The fragments containing histone genes and part of their flanking regions from _Pisaster ochraceus_, _Pisaster brevispinus_ or _Dermasterias imbricata_ (Table 1) were used as DNA probes. The probes were labeled with alpha-^{32}P deoxyribonucleotide triphosphates by a Klenow polymerase fill-in reaction after exonuclease III digestion (Gou and Wu, 1983). Alternatively, DNA was labeled with ^{32}P by nick-translation (Rigby et al., 1977). The radioactivity of the probes used in our experiments ranged from 4x 10^7 to 3x 10^8 cpm./per ug.

5. Restriction digestion and gel electrophoresis

Digests of genomic DNA or subclones were performed according to the method recommended by Bethesda research labs (BRL) or Pharmacia. Double and triple enzyme digestions were

also performed. Digested DNA samples were electrophoresed on 0.7-1.2% agarose gels for several hours to overnight to separate the fragments (Maniatis, 1982). Lambda phage DNA digested with HindIII or EcoRI, or pBR322 digested with HinfI was used as molecular weight markers. The sizes of DNA fragments were determined by reference to the markers.

6. Subclones

DNA fragments containing each histone gene were recovered from agarose gels by the method described below. Briefly, digested DNA samples were electrophoresed on 0.7-0.8% of low melting agarose gel for several hours to overnight. The target DNA band was excised together with low melting gel. The gel was melted at 70°C. Buffer (TE: 10 mM Tris base pH 8.0, 1 mM Na_2-EDTA pH 8.0) was added to a total volume of 500 ul. The mixture was heated for 3 additional minutes and extracted with an equal volume of 70°C pre-heated hot phenol two times, then with SEVAG one time. DNA was precipitated with 95% ethanol. Alternatively, DNA was electroeluted into dialysis bags as described by Maniatis (1982). Gel purified restriction fragments were ligated into the plasmid puC19 (Messing and Vieira, 1982) or pVZ1 (Henikoff and Eghtedarzadeh, 1987). The ligation mixture was used to transform competent E. coli JM 83 cell, and were selected as ampR: lac^- phenotype.

7. Southern blots and hybridization condition

DNA samples digested with restriction enzymes were electrophoresed on 0.7-1.2% agarose gel. DNA was denatured by soaking the gel in at least two volumes of 0.5 N NaOH, 1.5 N NaCl solution for 30 minutes and neutralized by soaking the gel in two volumes of 1 N NH_4Ac, 0.02 N NaOH solution for 0.5 to 1 hour. The gel was bidirectionally transferred to nitrocellulose membranes (Maniatis, 1982). Filters were baked at 80°C under vacuum for 1-2 hours. DNA filters was pre-hybridized for 2 hours or more at 62°C in 5x SET (5x 0.15 M NaCl, 0.03 M Tris HCl, pH 8.0, 1 mM Na_2EDTA), 5x Denhardt's solution (1% Bovine serum Albumin, 1% polyvinylpyrrolidone, 1% Ficoll (M.W. 400,000)), 0.1% Na pyrophosphate, 2.5 mM phosphate buffer pH 6.8, 0.1% SDS and 50/ug.ml sheared denatured calf thymus DNA. Hybridization was done at 62°C with heterologous probes or at 68°C with homologous probes overnight (Maniatis, 1982). Filters were washed in 3 x SET, 0.3% SDS subsequently to 1 SET, 0.3% SDS or to 0.1 SET, 0.1% SDS. The filters were dried and exposed to Kodak XAR or BB film with intensifying screen at -70°C (Laskey and Mills, 1977).

8. DNA deletion

To determine DNA sequences of large fragments, DNA deletion was performed by the method of Henikoff (1984). Briefly, the large DNA fragments were cloned into plasmid puC19 or pVZ1.

DNA (5 ug) was first digested with two different restriction enzymes whose cleavage sites served as direct deletion or protection sites. DNA was dissolved in 30 ul ExoIII buffer (66 mM Tris HCl pH 8, 0.66 mM $MgCl_2$). Exonuclease III (300 units) was used to remove DNA along 3' end of the 5' protruding restriction site. The digestion was performed at 37°C. The digested mixture was removed into a new tube which contained 7 ul of S1 nuclease mixture at each 30-45 seconds interval. The S1 nuclease mixture consists of 86 ul H_2O, 14 ul 10 x S1 buffer (1.1 ml 3 M KOAc pH 4.6, 5 ml 5 M NaCl, 5 ml glycerol, 30 mg $ZnSO_4$, 3 M NaOAc), and 30 units of S1 nuclease. Reaction was stopped by adding 1 ul of S1 stop solution (0.3 M Tris-OH, 0.05 M EDTA). A chase solution (1 ul of 0.5-1 mM dNTP solution) and 2 units of Klenow DNA polymerase were used to fill the gaps in DNA sequences. The samples (2 ul) were used for gel analysis at this stage. The linear deleted DNA was religated at 12°C overnight. A series of overlapping deletion subclones were picked up through this method.

9. DNA sequencing

The subclones containing target inserts were sequenced according to the dideoxynucleotide method (Sanger et al., 1977). DNA templates were prepared by low-melting gel purification, followed by alkali denaturation (Hattori and Sakaki, 1986). Klenow fragment of E.coli polymerase I (2

units) and alpha-^{32}P dATP (20 uCi) were used for each reaction. The reaction was performed at 42°C to 50°C and stopped by adding 5 ul of formamide dye mix (Maniatis, 1982). DNA sequencing gels were composed of six percent acrylamide, 7 M urea. The sequencing gel was pre-run for 10 to 15 minutes. The samples (1.5 ul) from each reaction were loaded and run at 38 or 60 Watts for about 2 hours after the first loading and 1 hour after the second loading. The sequencing gel was dried in vacuum at 80°C for 20 minutes and exposed to Kodak BB film at room temperature for several hours or overnight depending on the radioactivity of the gel.

DNA sequences were analyzed by using Delaney computer program (Delaney Software Ltd, 1985). The Eyeball Sequence program was employed to prepare sequence figures (E.L. Cabot, 1988).

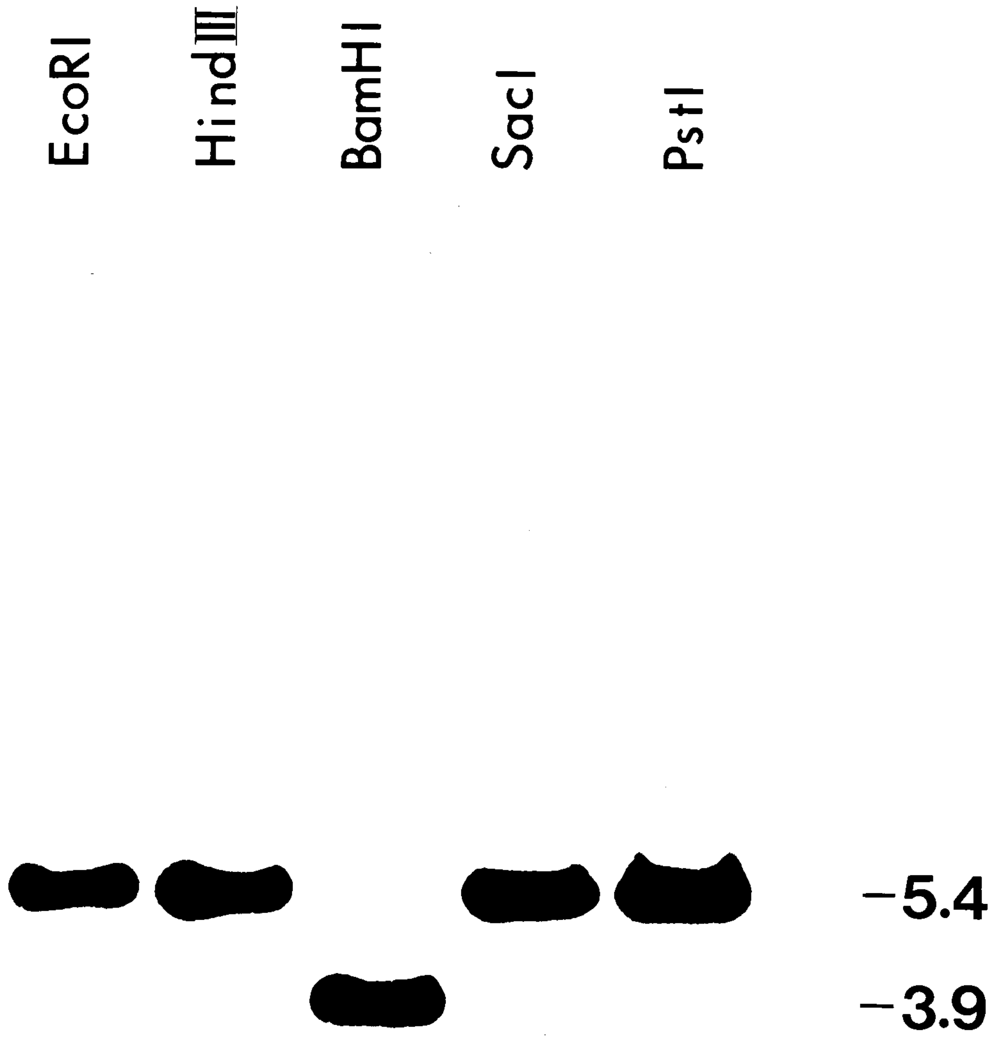
Results

Identification and isolation of a histone gene cluster element from Pycnopodia helianthoides genome

To explore histone genes from Pycnopodia genomes, high molecular weight genomic DNA was prepared from Pycnopodia sperm and digested with various restriction enzymes. Southern transfers of the digests were hybridized with histone H4 and H3 probes (Table 1). The probes contain either histone gene coding region or part of histone gene coding region with a piece of flanking region. Southern blots of Pycnopodia genomic DNA digested with EcoRI, HindIII, PstI and SacI show a 5.4 kb band when hybridized with H4 or H3 probes (Figure 1). BamHI digestions probed with H3 or H4 show a 3.3 kb band. These results suggest that there is one major histone gene cluster present in Pycnopodia genome, organized in 5.4 kb tandem repeats.

To isolate histone genes, Pycnopodia genomic DNA partially digested with Sau3A was cloned in the bacteriophage vector lambda EMBL4. The recombinant phages were screened three time by using end-labeled ^{32}P histone H4 or H3 probes. One positive phage from the genomic library was isolated. The digestion of this clone with EcoRI gives a strong 5.4 kb band, a 2.45 kb and a 1.45 kb bands besides the vector fragments.

Figure 1. Southern blots of *Pycnopodia* genomic DNA hybridized with a H3 histone gene probe. *Pycnopodia* genomic DNA was digested with indicated restriction enzymes and electrophoresed on a 0.8% agarose gel. The filter was hybridized with either the histone H4 or H3 gene sequence from *Pisaster ochraceus*. Both probes give the same pattern of bands. The blot probed with H3 is shown.

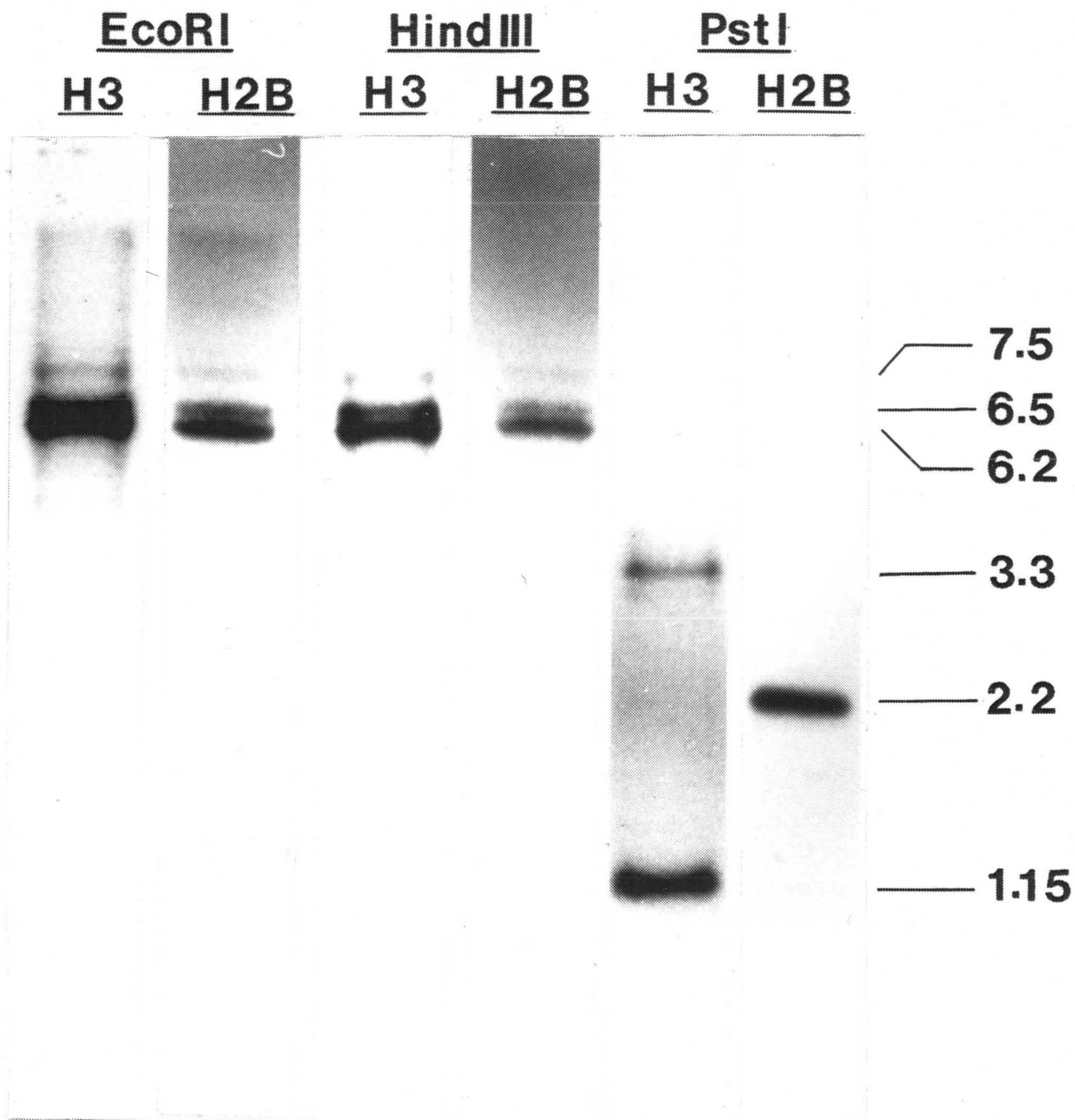

EcoRI
HindIII
BamHI
SacI
PstI
−5.4
−3.9

Southern transfers of the digests probed with H3 show very strong 5.4 kb and a 1.45 kb signals. Transfers probed with H2A show a 5.4 kb and a 2.45 kb band. These results imply that the insert contains two 5.4 kb histone gene cluster elements with partial fragments of repeat cluster element linked to its both ends. The 5.4 kb fragment was isolated and ligated to plasmid puC19, referred to pPyH5.4 subclone.

Identification and isolation of histone gene cluster elements from Solaster stimpsoni genome

Solaster genomic DNA prepared from sperm of one individual was originally digested with EcoRI, HindIII and PstI. Southern blots of Solaster genomic DNA were performed under the same condition as above. Three different sizes of hybridization bands appear in the Southern transfers of EcoRI and HindIII digested Solaster genomic DNA hybridized with heterologous H2A, H2B, H3 and H4 probes respectively from Table 1 (Figure 2). The strongest signal among the three bands is 6.2 kb in size. A second band is approximately 6.5 kb but at least 5 times less abundant than the 6.2 kb band. There is a third band which is 7.5 kb in length. This band is the weakest of the three bands. An additional 8.5 kb signal is seen with a longer exposure. Southern blots of genomic DNA digested with PstI show a 1.15 kb band when probed with H3 and H4; a 2.2 kb band when probed with H2B.

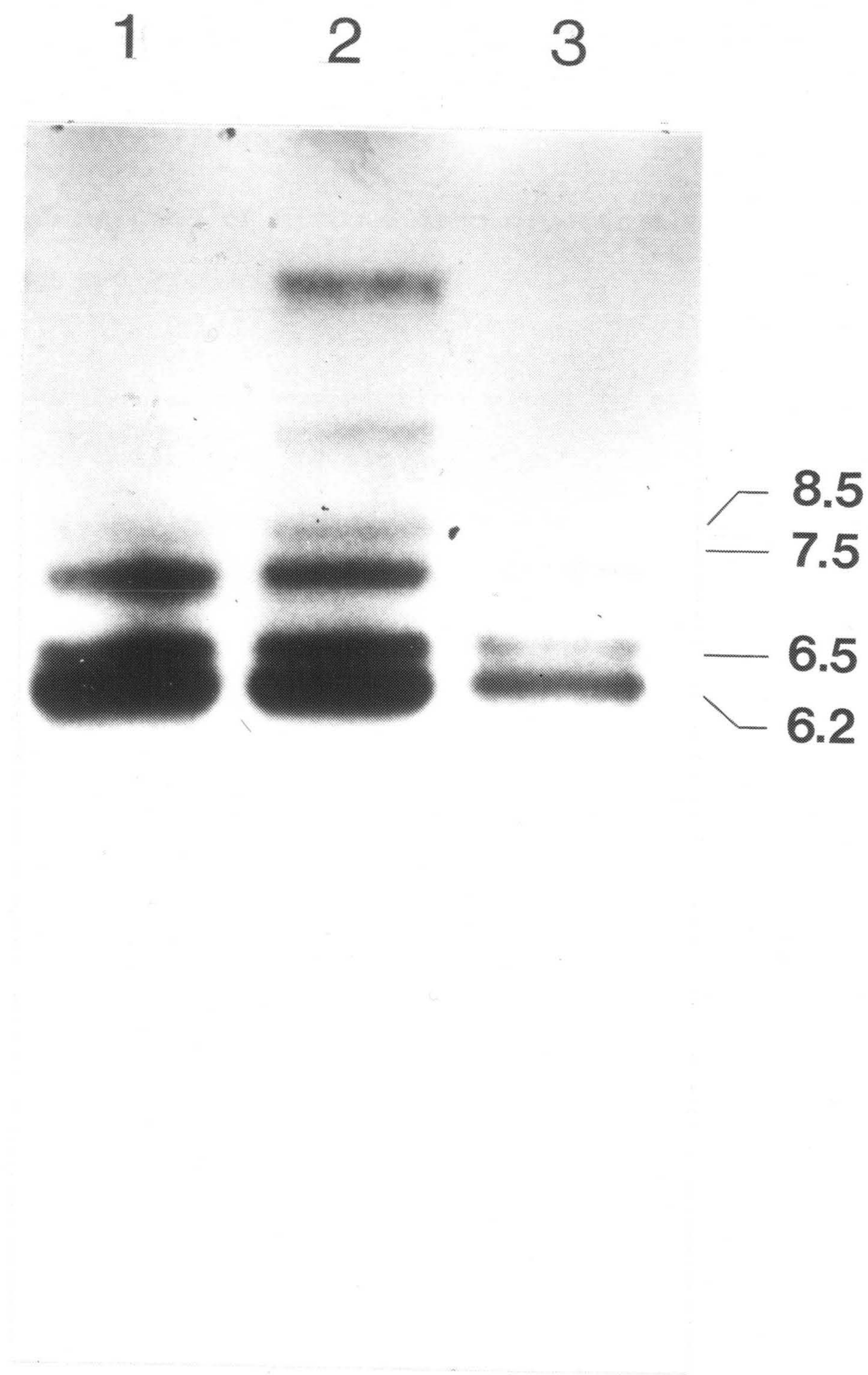
Figure 2. Southern blots of _Solaster_ genomic DNA indicating the different sizes of histone gene cluster elements. Southern blots were done as described in Figure 1. The digests of DNA with HindIII and EcoRI hybridized with either H3, H2B, H4 or H2A show the same pattern of DNA bands. Southern blots probed with H3 and H2B are shown.


EcoRI
HindIII
PstI
H3
H2B
H3
H2B
H3
H2B
7.5
6.5
6.2
3.3
2.2
1.15

To clarify whether this genomic blot pattern represents a common phenomenon in Solaster genome, genomic DNA from two additional Solaster individuals was prepared. Sperm DNA from three individuals cleaved with EcoRI was electrophoresed on agarose gels and hybridized with a H3 and H4 probe isolated from Solaster 6.2 kb histone gene cluster element. All three individuals show the same pattern of Southern blots (Figure 3). The weak 8.5 kb signal seen in Figure 2 shows up more clearly in Figure 3, verifying that there are at least three different sizes of EcoRI repeated histone cluster elements in Solaster genome. The 6.2 kb tandemly repeated element is considered as the major histone gene cluster because it is the most abundant. I have shown above that only one hybridization signal appears on the Southern blot of PstI digested genomic DNA probed with H2B (Figure 2). This suggests that these cluster elements may be organized similarly to each other in some way considering at least three different sizes of histone gene clusters in Solaster.

Solaster genomic DNA has been completely digested with EcoRI. DNA bands ranging from 5.5-7.5 kb were isolated to prepare a lambda gtWes partial genomic library. 1.98×10^5 recombinant phages were contained in this partial genomic library after packaging. The library was screened three time with ^{32}P end-labeled histone H3 and H4 probes (Table 1). Six positive recombinant phages were isolated. Five of the clones carry a 6.2 kb cluster element. The sixth contains a 7.5 kb

Figure 3. Southern blot of Solaster genomic DNA from three individual males. Solaster genomic DNA was cleaved with EcoRI and electrophoresed on a 0.8% agarose gel. The filter was hybridized with an end-labeled 1.15 kb fragment from Solaster pSoH1.15 subclone containing H4 and H3 genes. Note the faint 8.5 kb band which is also seen in Figure 2.


1
2
3
8.5
7.5
6.5
6.2

cluster element. The two different sizes of cluster elements from Solaster have been isolated and subcloned into puC19, and are referred to as pSoH6.2 and pSoH7.5.

Restriction mapping of histone gene cluster elements of Solaster and Pycnopodia

The distribution of restriction sites in the histone gene clusters was determined to provide a detailed restriction map and to localize histone genes within gene cluster. Subclones pPyH5.4, pSoH6.2 and pSoH7.5 were digested with a series of restriction enzymes. To localize certain enzyme cleavage sites, double and triple restriction digestions were also performed.

Comparisons of size patterns of DNA fragments of pSoH6.2 and pSoH7.5 subclones digested with different enzymes indicate that these two subclones are very similar (Figure 4). For instance, PstI cleaved pSoH7.5 (A) and pSoH6.2 (B) give three common fragments at 4.4, 1.15 and 0.75 kb. The difference between these isolates is that pSoH7.5 contains a 3.8 kb fragment, but the pSoH6.2 contains a 2.6 kb fragment. Further analysis of fragment sizes of enzyme cleaved pSoH7.5 and pSoH6.2 subclones reveals that the 7.5 kb cluster element contains an additional 1.2 kb fragment inserted between SphI and AvaI restriction sites of 6.2 kb cluster element (Figure 5).

Figure 4. Agarose gel electropherogram of restriction enzyme digested plasmids pSoH7.5 and pSOH6.2. The pSoH7.5 (A) and pSoH6.2 (B) were digested with EcoRI, HindIII, PstI, PstI/HindIII and PstI/EcoRI restriction enzymes, electrophoresed in 1% agarose gel and stained with ethidium bromide.

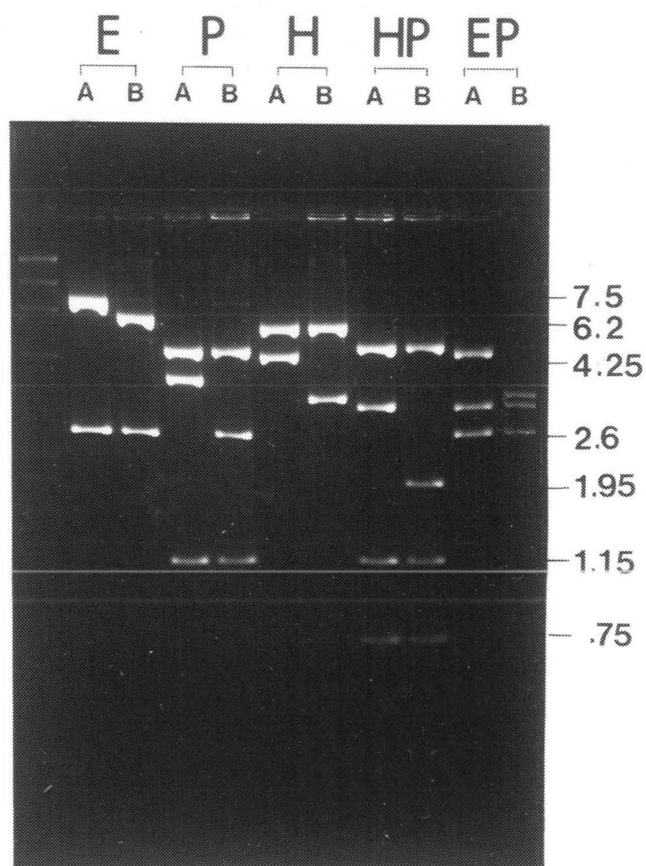
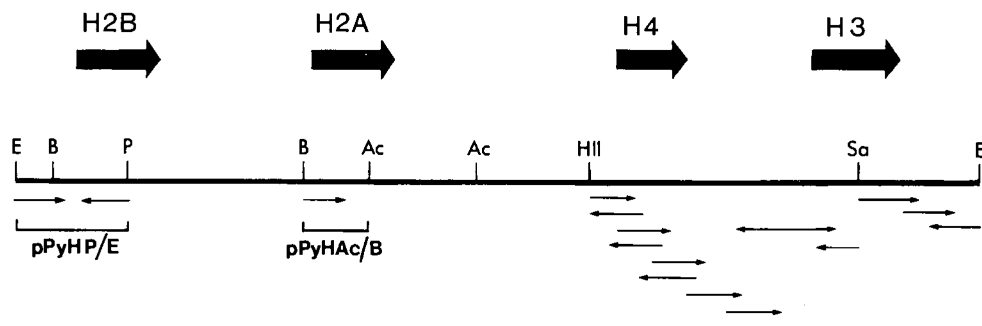
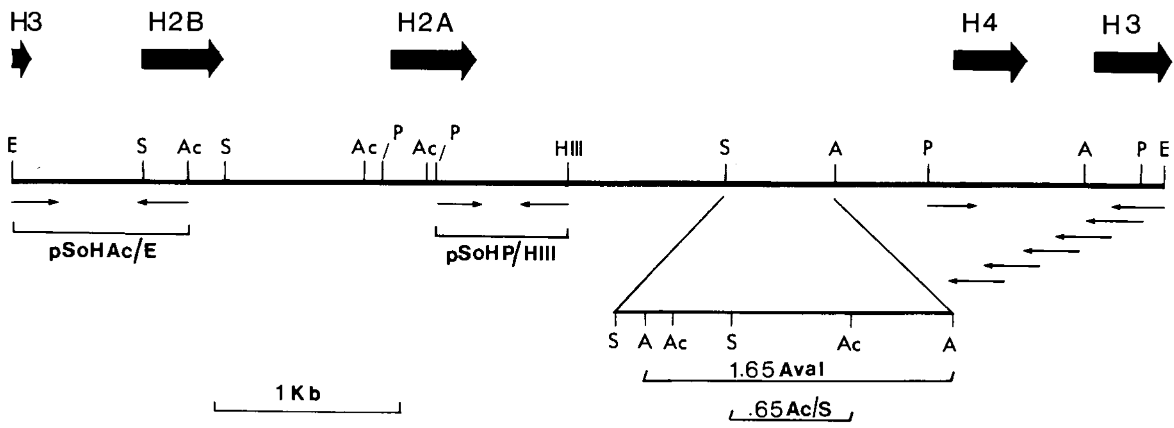

E
P
H
HP
EP
A
B
A
B
A
B
A
B
A
B
7.5
6.2
4.25
2.6
1.95
1.15
.75

Figure 5. Restriction maps of the tandemly repeated histone gene cluster elements of _Solaster_ and _Pycnopodia_. The maps were determined by digesting pPyH5.4, pSoH6.2 and pSoH7.5 with various restriction enzymes. The histone gene coding regions and transcriptional polarity are indicated by the large bars above the maps. The DNA regions sequenced in this study are indicated by the thin arrows. The restriction map of _Solaster_ includes the maps of the 7.5 kb and 6.2 kb cluster elements. Some of the subclones are indicated below the maps. Not all of the HII sites in _Pycnopodia_ are shown. P = PstI, B = BamHI, E = EcoRI, Ac = AccI, HII = HincII, Sa = SacI, S = SphI, HIII = HindIII, A = AvaI.


Pycnopodia
H2B
H2A
H4
H3
E B P B Ac Ac HII Sa E
pPyHP/E
pPyHAc/B


Solaster stimpsoni
H3
H2B
H2A
H4
H3
E S Ac S Ac P Ac P HIII S A P A P E
pSoHAc/E
pSoHP/HIII
S A Ac S Ac A
1.65 AvaI
.65 Ac/S
1 Kb

The restriction maps of pPyH5.4, pSoH6.2 and pSoH7.5 are shown in Figure 5. The restriction map of Solaster includes the maps of pSoH6.2 and pSoH7.5.

Histone gene arrangement within cluster elements of Pycnopodia and Solaster

To determine the location and order of histone genes within each gene cluster element, pSoH6.2 and pPyH5.4 were digested with appropriate enzymes and Southern transferred. pSoH6.2 was chosen because it is the most abundant cluster in the Solaster genome. Blots were hybridized with ^{32}P end-labeled histone gene H4, H3, H2A and H2B probes (Table 1). The H4 probe used in this experiment contains a complete Pisaster H4 coding sequence and 600 bp of its 5' and 3' flanking region. The H3 probe contains 400 bp Pisaster H3 coding region and 400 bp of its 5' flanking region. Both the H2A and H2B probes contain complete gene coding regions and partial flanking regions. The hybridization of the DNA filters with H3 and H4 probes were performed at much higher stringency than that with H2A and H2B probes since that H3 and H4 genes are more conservative than H2A and H2B genes (Wells, 1986). The sequence arrangement of histone genes within gene clusters for both Pycnopodia and Solaster was determined according to the Southern blots. Figure 5 shows the arrangement of histone genes within a tandem repeat unit in both species. The dark

bars above each restriction map indicate the location of each histone gene. The precise gene position was determined from DNA sequences. From Figure 5, we find that the gene arrangement within Solaster and Pycnopodia histone gene clusters is identical, and is the same as seen in three sea star species (Cool et al., 1988).

The transcriptional polarity of histone genes

The transcriptional orientation and position of each histone gene within cluster element was determined from DNA sequence data. To do this, the fragments containing each histone gene were isolated from pPyH5.4 and pSoH6.2. For example, subclone pPyH5.4 was double-digested with HincII and SstI. A 1.4 kb fragment with HincII and SstI cohesive ends was gel purified and subcloned into plasmid puC19, referred to as pPyH1.4 HII/S. The subclones pPyH0.65 SacI/EcoRI, pPyH0.5 EcoRI/PstI and pPyH0.35 BamHI/AccI from pPyH5.4 were prepared in a similar manner. From pSoH6.2, three subclones pSoH1.0 EcoRI/AccI, pSoH0.75 PstI/HindIII and pSo1.15 PstI were prepared. Each subclone was sequenced from both ends with M13 forward and reverse primers. Figure 6 shows the section of DNA sequences from pSoH EcoRI/AccI and pPyH EcoRI/PstI indicating the transcriptional direction of Solaster and Pycnopodia H2B genes. The DNA sequences for determining the transcriptional orientation of histone H4 and H3 genes of

Figure 6. DNA sequences of pPyH PstI/EcoRI (A) and pSoH AccI/EcoRI (B) subclones demonstrating the transcriptional polarity of H2B genes. The arrows indicate the direction of gene transcription.

(A)

```
5'——►3'
  T   D   K   K   R   R   R   R   R   K   E   S   Y   G   I   Y   I   Y   K   V
AACAGACAAGAAGAGGCGACGTCGCCGCAAGGAGAGCTACGGTATTTACATCTACAAGGT
TTGTCTGTTCTTCTCCGCTGCAGCGGCGTTCCTCTCGATGCCATAAATGTAGATGTTCCA 60

  M   K   Q   V   H       D   T   G   I   S   S   K   A   M   S   I   M   N   S
CATGAAGCAGGTCCATCCGACACGGGTATTTCCAGCAAGGCCATGTCCATCATGAACAGC
GTACTTCGTCCAGGTAGGCTGTGCCCATAAAGGTCGTTCCGGTACAGGTAGTACTTGTCG 120

  F   V   N   D   I   F   E   R   I   A   A   E   S   S   R   L   A   H   Y   N
TTCGTCAACGACATCTTTGAGCGCATTGCCGCCGAGTCTTCTCGATTGGCACACTACAAC
AAGCAGTTGCTGTAGAAACTCGCGTAACGGCGGCTCAGAAGAGCTAACCGTGTGATGTTG 180

  K   K   S   T   I   T   S   R   E   V   Q   T   A
AAGAAATCGACCATCACAAGCCGGGAAGTCCAGACTGCAG
TTCTTTAGCTGGTAGTGTTCGGCCCTTCAGGTCTGACGTC                     220
```

(B)

```
5'——►3'
   G   S   G   E   H   K   Q   E   E   R   R   R   R   K   E   S   Y   G   I
AAGGCTCCGGTGAGCACAAACAAGAAGAGCGACGGCGTCGCAAGGAGAGCTACGGTATCT
TTCCGAGGCCACTCGTGTTTGTTCTTCTCGCTGCCGCAGCGTTCCTCTCGATGCCATAGA 60

Y   I   Y   K   V   M   K   Q   V   H   P   D   T   G   I   S   S   L   A   M
ACATCTACAAAGTCATGAAACAGGTGCACCCCGACACAGGAATCTCCAGCTTGGCAATGA
TGTAGATGTTTCAGTACTTTGTCCACGTGGGGCTGTGTCCTTAGAGGTCGAACCGTTACT 120

S   I   M   N   S   F   V   N   D   V   F   E   R   I   A   G   E   S   S   R
GCATCATGAACAGCTTTGTCAACGACGTGTTTGAGCGCATCGCCGGCGAATCTTCTCGCC
CGTAGTACTTGTCGAAACAGTTGCTGCACAAACTCGCGTAGCGGCCGCTTAGAAGAGCGG 180

L   A   H   Y   N   K   K   S
TTGCCCACTACAACAAGAAGTCGAC
AACGGGTGATGTTGTTCTTCAGCTG                                    205
```

Pycnopodia and Solaster are shown in Figure 9 and 10. Sequences which indicate histone H2A gene orientation are not shown. The arrows in front of the large bars above each restriction map in Figure 5 represent the 5' to 3' transcriptional orientation of histone genes. The results show that the transcriptional direction of histone genes within the gene cluster elements of Pycnopodia and Solaster is the same, and is the same as that in Dermasterias and Pisaster. These results indicate that the organization and transcriptional polarity of histone genes within histone gene clusters are very stable among sea star species.

Analysis of the transcriptional polarity of histone genes within the 7.5 kb element.

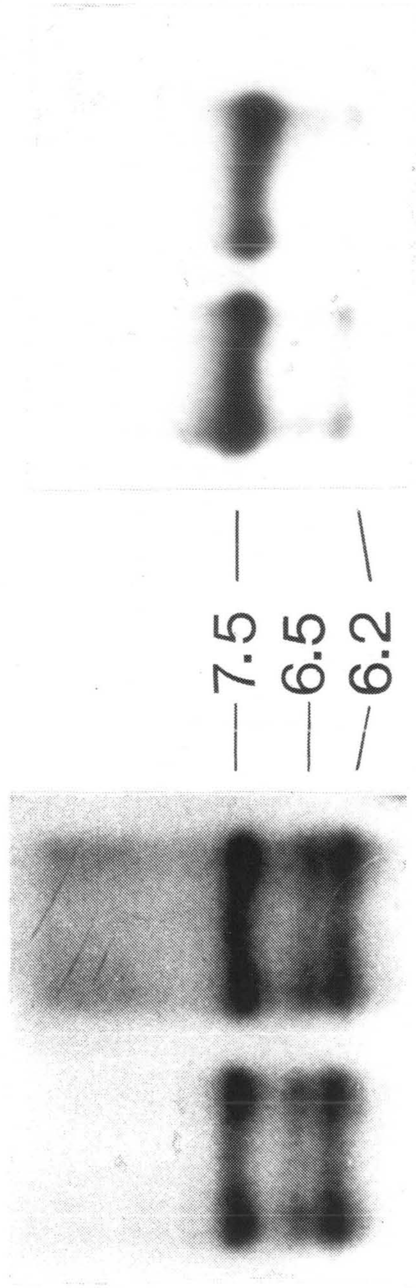
As mentioned above, Southern blots of Solaster genomic DNA digested with EcoRI or HindIII show three bands when probed with core histone DNA sequences (Figure 2). The sizes of the cluster elements are 6.2 kb, 6.5 kb and 7.5 kb respectively. The blots suggest that both the 7.5 kb and 6.2 kb elements contain all core histone genes. The restriction maps of pSoH7.5 and pSoH6.2 are very similar (Figure 5). The fragment size patterns of restriction digested pSoH6.2 and pSoH7.5 were analyzed, and compared with Southern blots of enzyme cleaved pSoH6.2 probed with H2A, H2B, H3 and H4 fragments (Table 1). The analysis reveals that both pSoH7.5 and pSoH6.2 contain a

1.15 kb PstI fragment which hybridizes with H3 and H4 probes, a 0.75 kb PstI fragment which hybridizes with a H2A probe (Figure 4), and a 1.0 kb AccI/EcoRI fragment which hybridizes with a H2B probe (data not shown). To verify the homology of the cluster elements, the DNA sequences of H3 coding region and its 3' flanking region of pSoH7.5 were determined. The sequence data were compared with the same region of pSoH6.2. The result shows that there is not a single base pair change between them. From this together with restriction mapping data, we deduce that the gene order and the transcriptional direction of pSoH7.5 is the same as pSoH6.2 (Figure 5). The size heterogeneity may result from an insertion or deletion between AvaI and SphI restriction enzyme sites within the histone cluster elements.

Further investigation of the "insertion portion" within the 7.5 kb element

To verify our analysis that the insert is located between AccI and SphI restriction sites, a 1.65 kb AvaI fragment and a 0.65 kb AccI/SphI fragment from the "insertion section" of the 7.5 kb cluster element were isolated (Figure 5). The fragments, labeled with ^{32}P, were hybridized with <u>Solaster</u> genomic DNA digested with EcoRI and HindIII. The results are shown in Figure 7. All three histone gene cluster elements hybridize with the 1.65 kb probe (Figure 7A). The strongest

Figure 7. Southern blots of _Solaster_ genomic DNA hybridized with two different probes isolated from _Solaster_ pSoH7.5 subclone. Southern blots were done as described in Figure 1. The 1.65 kb AvaI and 0.65 kb AccI/SphI probes were isolated from the "insertion portion" of the 7.5 kb element shown on restriction map.


EcoRI
HindIII
EcoRI
HindIII
7.5
6.5
6.2
1.65 AvaI Frag
Probe
.65 SphI/AccI Frag

signal is the 7.5 kb band. A very strong 7.5 kb signal appears in the Southern transfer probed with the 0.65 kb fragment (Figure 7B). Very weak 6.2 kb and 6.5 kb bands are seen with a longer exposure. These results confirm the data obtained from enzyme digests that the 1.2 kb insert of the 7.5 kb element lies between the AccI and SphI restriction sites within the histone gene cluster element. The blots also indicate that histone gene arrangement within the 6.5 kb element may be very similar to that of the 6.2 kb and 7.5 kb elements because both the 1.65 and 0.65 kb fragments not only hybridize with the 7.5 and 6.2 kb elements but also hybridize with the 6.5 kb element.

DNA sequence analysis of H3 and H4 genes

The strategy used to determine nucleotide sequences is shown in Figure 5. pPyH1.4 HII/S and pPyH0.65 S/E subclones from pPyH5.4 and pSoH1.15 PstI subclone from pSoH6.2 were subjected to a series of deletions. Figure 8 shows an example of deletion results from pPyH1.4 HII/S subclone. The complete nucleotide sequences of Pycnopodia H4 and H3 genes and their flanking regions have been determined (Figure 9). The amino acid sequences are shown above the gene coding regions. DNA sequences of Solaster H4 and H3 coding region and their flanking regions are indicated in Figure 10. The predicted amino acid sequences are also displayed. The homology blocks

Figure 8. The directed deletion of the pPyH1.4 HincII/SacI subclone from Pycnopodia histone cluster element. The fragments are separated in a 0.8% agarose gel and stained with ethidium bromide. The sizes of the largest and smallest deleted fragments are indicated.

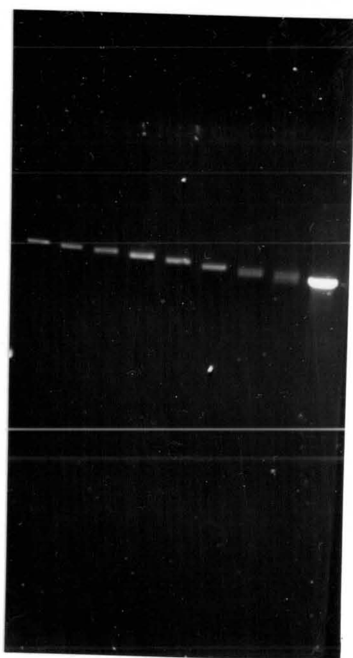

—4.1
—2.9

Figure 9. The entire DNA sequence of H3 and H4 genes and their flanking regions from Pycnopodia tandemly repeated element. The single letter amino acid is presented above the DNA coding regions. The potential Cap (I), TATA (II), CCAAT (III) blocks and other consensus sequences are underlined.

```
                        (IV)      (III)            (II)
CAGGTCAACAAGTCACGCATGGTTTCCCCGCTCACGTCCGCAACGCCGGCCTGAAATAGGCCATCTTTGG 70
             (I)                                            (H4)  -
TTGGTATCAAGTCATTCGCTCTCGTCAGCTAGTAAACAATCTCCTTGGTGNTACTACGTTCTGCTCTGCA 140

      M  S  G  R  G  K  G  G  K  G  L  G  K  G  G  A  K  R  H  R  K  V
CAACTATGTCTGGTCGCGGTAAAGGTGGAAAGGGGCTAGGCAAAGGGGGTGCCAAGCGCCATCGCAAAGT 210

 L  R  D  N  I  Q  G  I  T  K  P  A  I  R  R  L  A  R  R  G  G  V  K
TTTGCGGGACAACATCCAGGGTATCACCAAGCCAGCCATCCGTCGTCTGGCCCGCCGTGGGGAGTCAAG 280

 R  I  S  G  L  I  Y  E  E  T  R  G  V  L  K  V  F  L  E  N  V  I  R
AGAATCTCCGGTCTCATCTACGAGGAGACCCGCGGTGTTCTCAAGGTCTTCCTGGAGAATGTCATCCGGG 350

D  A  V  T  Y  C  E  H  A  K  R  K  T  V  T  A  M  D  V  V  Y  A  L  K
ATGCTGTAACGTACTGCGAGCACGCCAAGAGAAAGACGGTCACCGCCATGGATGTCGTGTACGCGCTCAA 420

 R  Q  G  R  T  L  Y  G  F  G  G  *
GAGACAAGGCCGTACTTTGTACGGATTCGGAGGTTAGGCAGTGCCGTGCAAGAATCCCACATCGAAAACA 490
   -stem loop-                 -AAGAGA-
ACGGCTCTTTTCAGAGCCACCAAAAGTTCAAAAAAGAGAGAAACAGCTTGTCCTTTTGTTTCCTAAAAT 560

CCCCCATTCCTTCGTTAGGTATTATTTACAGTTTATTTCACTCGAAAAGTAGTTCTAAAATACCTCTGGG 630

GCACTAGCATATAACATGCATATAATTATAGTCCTGAAATGTTTTTCTGATATTTTATAAATCAATTTCC 700

TCTAATAGTGGGGATGGATGGGTCTTTATTTCATGATCCACGTATTTTGAATCCCACCAAGTTGTTAAAG 770
                                                   (IX)
TTTGAAAGCTGTTTGCAAATTCCCCCCGGTTTTTCTAAAACCTCCCTCAACACTTTTCTTCTCGCGATA 840
                     (VIII)                                        (VII)
GGTCACACAATGGTACATGTAAAATGTTATTCAGGATGCTTAACCTTGTTCTTGAAAAACCTGTTTCGTC 910
   (VI)
CGTTTGTGCTTAGTTTCGTTTTGTTTTGGCCGGTGGTGCTTGAGCAGCCCCGTGAAATTCGGCCCCCAT 980
    (V)                            (IV)                          (III)
GATGACCACTCAAGCGGTTTGGGCGGATAGCCACTGGCCAATCGCGTTTCCGCCTTTGTCATGGGTGAC 1050
                       (II)                               (I)
CCTTCAACGGACTTCCCATTCCTTTAAGAACTGCTCGGGAATTGGACCAGAACACACAACTTTGGAAAAC 1120

 (H3) -         M  A  R  T  K  Q  T  A  R  K  S  T  G  G  K  A  P  R  K
AGTGATCAAAGATAATGGCCCGTACCAAGCAGACAGCACGCAAGAGCACTGGGGGGAAAGCCCCGCGAAA 1190

 Q  L  A  T  K  A  A  R  K  S  A  P  A  T  G  G  V  K  K  P  H  R  Y
GCAGTTGGCGACCAAGGCTGCCCGAAAGAGTGCCCCGGCCACCGGCGGTGTGAAGAAGCCCCATCGGTAC 1260

 R  P  G  T  V  A  L  R  E  I  R  R  Y  Q  K  S  T  E  L  L  I  R  K
AGACCGGGAACTGTGGCACTGCGGGAGATTCGCCGTTACCAGAAAAGCACAGAGCTGCTCATCCGAAAAC 1330

L  P  F  Q  R  L  V  R  E  I  A  Q  D  F  K  T  E  L  R  F  Q  S  S  A
TCCCTTTCCAGAGACTTGTCCGTGAAATAGCGCAAGACTTCAAGACCGAACTCCGATTCCAGAGCTCAGC 1400
```

```
 V  M  A  L  Q  E  A  S  E  A  Y  L  V  G  L  F  E  D  T  N  L  C  A
GGTGATGGCGCTTCAAGAAGCAAGCGAGGCATACCTTGTGGGACTCTTCGAAGACACCAATCTATGTGCC 1470

 I  H  A  K  R  V  T  I  M  P  K  D  I  Q  L  A  R  R  I  R  G  E  R
ATCCACGCCAAGCGGGTGACCATCATGCCCAAAGACATCCAACTCGCCCGCCGTATTCGCGGTGAACGAG 1540

A  *                                     -stem  loop-              -AAGAGA-
CCTGAGAAATGAAAACTCGTTTCTACTAATCAAAACGGCTCTTTTCAGAGCCACCACTTCTTGGAAGAGA 1610

TTATAATCATGCCCAATGTCATCGTTTTCTTTACAAAATAAAAAATTAATGAATTATAGCTATACGCCGC 1680

AATATTAGGTCTCCTCGTTAAATAAGTAAGATAAGTCAGCCTCAATAGATAACACTTTGTTTCTTTTGTC 1750

GAATTATGAAGTTCGACTGCTCTATTATATATAACTGTAAACTGTTACATAACAGTTTCTGTCTCTTTAT 1820

GTCGTTTCTTTCTTTCTGCGGGTGACTTTCAGCCCGTTGGGCCAACCATTCGTTCTATGCGATGTTTTTG 1890

GTCTCTATGTTTTATACATTCTCGAAAACCATCTGCACACTGAATGTATTCTTACTAAAGCTTAAATACA 1960

CAAATACATCGGAACAAGGAGCTTGTGTCACTTTCATTTTTATTCAACATTCTCTATTATTGCTTGNNTG 2030

AATTCATTGATTTGAGTTTCTATTTCGTTTATTATTTTGATAAAATAATAATAGTAACTTTTCGTGGAGG 2100

GGAAACTATTGGGATCGCCTGTGGAGTCGGGTTCAGCAATAATTAGACAATCATCGGACCGCTTCACTGC 2170

TCACACAGCGAGGCGCGAATTTGAACAATGCCCACTGGACACCGGATCCTCTGGTCGCAGTATAAAGCTA 2240

AACGTCATCCGGTGTTACATCCATCAGCCTGATTTAGACCACTATTCAGTACGCTTGGACAGGAATACTC 2310

TACTCATCATCACGCCTCCGAAGCA                                              2335
```

Figure 10. The nucleotide sequence of H4 and H3 genes and their spacer regions from Solaster 6.2 kb histone gene cluster element. The predicted amino acid sequences are shown above the coding regions. The homology boxes in gene flanking regions are underlined.

```
                                                      (IV)            (III)
CTGCAGGGGATTTGTTGCTAGAGGGCGCCCTGACTTTCGTTTGCCCCGCGTGCTCCTATCGCGTCCGGAT 70
          (II)                 (I)
AGGTGTGATATATAATACACTGCCTCGCCCATTCACTCGATCTAGTCTGAGCAGAGGAGAAGAAGAAAAA 140

     (H4) -      M  S  G  R  G  K  G  G  K  G  L  G  K  G  G  A  K  R
AGACAAGAAAAATAAACATGTCTGGCCGTGGCAAAGGAGGAAAAGGACTGGGCAAAGGGGGTGCCAAGCG 210

 H  R  K  V  L  R  D  N  I  Q  G  I  T  K  P  A  I  R  R  L  A  R  R
TCACCGCAAGGTTCTTCGCGACAATATCCAGGGCATCACTAAACCAGCCATTCGTCGGCTGGCCCGCCGT 280

 G  G  V  K  R  I  S  G  L  I  Y  E  E  T  R  G  V  L  K  V  F  L  E
GGAGGAGTAAAGCGCATCTCCGGTCTCATCTACGAAGAAACCCGCGGAGTGCTCAAGGTGTTCCTGGAGA 350

N  V  I  R  D  A  V  T  Y  C  E  H  A  K  R  K  T  V  T  S  M  D  V  V
ATGTCATCCGCGATGCAGTCACCTACTGCGAGCACGCCAAGAGGAAAACCGTTACTTCGATGGATGTGGT 420

 Y  A  L  K  R  Q  G  R  T  L  Y  G  F  G  G  *
GTACGCGCTGAAGAGACAGGGGCGCACGCTGTACGGTTTCGGCGGATAGATGGCTGGTCCAATTTTCAAA 490
    -stem  loop-                      -AAGAGA-
ACAAACGGCTCTTTTCAGAGCCACCACGATTTCCCAAAAAGAGATGATTCCAGGTTTTAATTTGTGTGTA 560
                                                          (IX)
CTCACTGTGTATTTATTTGAGTTTGGATCTTCCTCCCTGTGTCCATTAAGTTTTCTCTTTTCGTTTATCG 630
                                                        (VIII)
CCCGTAATTATTCATGGGATGTCTTTATTTAAGAAACGGCTCTATTTGTGTTTATGTTGGTAGCTTGGGG 700
                                                   (VII)
AAATTCATTTGCTTGCAAAGTATCATGTGGGTAAAATTTAGCGTGATGTTTTGGAGGGTAAATAAGTTGC 770
                                       (IV)       (III)2
GATCAGTGAAGCTCCCGCCATGTGTCCGAATTACCCAATAGGAAGATGCCCATTGTTAGACTTCAGTCCA 840
 (III)1          (II)                        (I)
AGGATGCTTTTCTCTAGTCTTAAGAGACGGTCTGTTCGTTAATTTGGCATTCACTTCTGATCTACTCTCC 910

                 (H3) -      M  A  R  T  K  Q  T  A  R  K  S  T  G  G  K
AATCTGACCAATCTACTCTCAAATCATGGCACGCACCAAGCAGACGGCAAGAAAGAGCACCGGTGGAAAA 980

 A  P  R  K  Q  L  A  T  K  A  A  R  K  S  A  P  A  T  G  G  V  K  K
GCCCCGAGGAAGCAGTTGGCCACCAAGGCGGCACGCAAGAGCGCACCGGCGACTGGCGGTGTCAAGAAAC 1050

P  H  R  Y  R  P  G  T  V  A  L  R  E  I  R  R  Y  Q  K  S  T  E  L  L
CCCATCGGTACAGACCGGGAACTGTAGCCCTTCGCGAGATTCGTCGCTACCAGAAGAGCACCGAACTGCT 1120

  I  R  K  L  P  F  Q  R  L  V  R  E  I  A  Q  D  F  K  T  E  L  R  F
GATCCGCAAGCTGCCGTTCCAGAGACTTGTGCGTGAAATTGCACAGGACTTCAAAACAGAACTGCGCTTC 1190

 Q  S  S  A  V  M  A  L  Q  E  A  S  E  A  Y  L  V  G  L  F  E  D  T
CAGAGTTCCGCCGTCATGGCACTGCAGGAGGCGAGCGAAGCCTACCTCGTTGGCCTCTTTGAAGACACCA 1260

N  L  C  A  I  H  A  K  R  V  T  I  M  P  K  D  I  Q  L  A  R  R  I  R
ACCTTTGCGCCATCCATGCCAAGAGGGTCACCATCATGCCCAAGGACATCCAGTTGGCTCGCCGAATTCG 1330
```

```
 G  E  R  A  *
                                                                       -stem  loop-
CGGTGAACGTGCTTGAGTCACAGCTAGCTGCGTGCACAAAACAAACCGAACGGCCCTTTTCAG-GCCACT 1400
           -AAGAGA-
ACACTTTTAAAAGAGAGATTAATCCATCGCGTTTTCTGTACAGTTTTTGACGTTAAGTTCGGTTACTATA 1470

TGTAAATACGAATTTTAACGGGCCAGGGTTCCGAAAAACGGATTAATTAATGAAACCATTAAGATTAGTC 1540

TCTATCCGTTTTGTTTCTTTGTCTGTCTCTGATTTTTGTCGTTCCATACCCCTTTAA              1595
```

which may be important for gene regulation are underlined in both Figures.

The comparison of Solaster and Pycnopodia H3 coding regions

Complete H3 coding regions of Pycnopodia and Solaster are compared in Figure 11 with the H3 coding regions of two other sea stars as well as to the sea urchin Strongylocentrotus purpuratus. There are sixty-eight nucleotide substitutions found in the 411 bp H3 coding regions between Pycnopodia and Solaster. Most of these changes occur at the third positions of the codons. Only four occur in the first position of the codons. All the substitutions are silent. The homology of the nucleotide sequences between these two genes is 82.7%.

Comparisons of Solaster and Pycnopodia histone H4 coding region

The alignment of nucleotide sequences of histone H4 genes from Pycnopodia and Solaster is shown in Figure 12. Data from the comparison of amino acid sequences indicate that there is one amino acid substitution which occurs at codon 84 from Ala in Pycnopodia to Ser in Solaster. This amino acid change is a neutral substitution. At the DNA level, however, there are fifty nucleotide changes between these two genes. The

Figure 11. Alignment of H3 coding sequences indicating positions of divergence among sea star species and between sea stars and sea urchins. The dots(.) indicate sequence identity. Sequences of H3 from Solaster stimpsoni(SoH3) and Pycnopodia (PyH3) are extracted from Figure 9 and Figure 10. PoH3 and DiH3 sequences represent sea star Pisaster ochraceus and Dermasterias imbricata H3 genes (Cool, et al., 1988). SUSPH3 is the early sea urchin H3 gene (I. Sures et al., 1978)

```
      M   A   R   T   K   Q   T   A   R   K   S   T   G   G   K
SoH3 ATG GCA CGC ACC AAG CAG ACG GCA AGA AAG AGC ACC GGT GGA AAA
PyH3 ... ..C ..T ... ... ... ..A ... C.C ... ... ..T ..G ..G ...
DeH3 ... ..T ... ... ... ... ..A ... ... ... ... ... ..C ..G ...
PoH3 ... ..T ... ..T ... ... ..A ... C.. ... ..T ..T ..G ..T ...
SUSp ... ... ... ... ... ... ..C ..T C.C ..A TCT ..A ..A ..G ..G

      A   P   R   K   Q   L   A   T   K   A   A   R   K   S   A
SoH3 GCC CCG AGG AAG CAG TTG GCC ACC AAG GCG GCA CGC AAG AGC GCA
PyH3 ... ... C.A ... ... ... ..G ... ... ..T ..C ..A ... ..T ..C
DeH3 ... ..A ... ... ... ... ... ... ..A ... ... ..A ..A ..T ...
PoH3 ... ..A C.A ... ... ... ..G ... ... ..T ..C ..A ... ..T ..C
SUSp ..T ..C C.C ... ... C.. ..A ... ..A ..T ..C A.A ... ..T ..C

      P   A   T   G   G   V   K   K   P   H   R   Y   R   P   G
SoH3 CCG GCG ACT GGC GGT GTC AAG AAA CCC CAT CGG TAC AGA CCG GGA
PyH3 ... ..C ..C ... ... ..G ... ..G ... ... ... ... ... ... ...
DeH3 ... ..T ... ... ..C ... ... ... ... ..C ... ... C.. ... ...
PoH3 ... ..T ..C ... ..C ..G ... ... ... ..C ..T ... ... ..C ...
SUSp ..C ..C ... ..A ..A ... ... ..G ..T ... ..A ... ..G ..T ..C

      T   V   A   L   R   E   I   R   R   Y   Q   K   S   T   E
SoH3 ACT GTA GCC CTT CGC GAG ATT CGT CGC TAC CAG AAG AGC ACC GAA
PyH3 ... ..G ..A ..G ..G ... ... ..C ..T ... ... ..A ... ..A ..G
DeH3 ..C ... ..T ..C ..T ... ..C ... ... ... ... ... ... ... ..G
PoH3 ... ..G ..A ..G ..G ... ... ..C ..T ... ... ..A ..T ..A ..G
SUSp ..A ..C ... ..G A.A ... ... ..C ... ... ... ... ... ..T ..G

      L   L   I   R   K   L   P   F   Q   R   L   V   R   E   I
SoH3 CTG CTG ATC CGC AAG CTG CCG TTC CAG AGA CTT GTG CGT GAA ATT
PyH3 ... ..C ... ..A ..A ..C ..T ... ... ... ... ..C ... ... ..A
DeH3 ... ..T ... A.A ... ... ..C ... ... ... ..C ... ... ... ...
PoH3 ... ..T ... ..A ..A ..C ... ... ... ... ..G ..C ... ... ..A
SUSp ..T ..C ... ..A ..A ... ..A ... ... C.T ..A ... ... ..G ...

      A   Q   D   F   K   T   E   L   R   F   Q   S   S   A   V
SoH3 GCA CAG GAC TTC AAA ACA GAA CTG CGC TTC CAG AGT TCC GCC GTC
PyH3 ..G ..A ... ... ..G ..C ... ..C ..A ... ... ..C ..A ..G ..G
DeH3 ... ..A ... ... ..G ..T ... ... ..A ... ..A ... ..A ... ...
PoH3 ..G ..A ... ... ..G ..C ... ..C ..A ... ... ..C ..G ..G ..G
SUSp ... ... ... ... ..G ... ..G ..A ..T ... ... ... ... ..T ..G

      M   A   L   Q   E   A   S   E   A   Y   L   V   G   L   F
SoH3 ATG GCA CTG CAG GAG GCG AGC GAA GCC TAC CTC GTT GGC CTC TTT
PyH3 ... ..G ..T ..A ..A ..A ... ..G ..A ... ..T ..G ..A ... ..C
DeH3 ... ... T.. ... ... ..A ... ... ..T ... ..G ... ... ..G ..C
PoH3 ... ..G ..T ..A ..A ..A ... ..G ... ..T ..T ..G ..A ... ..C
SUSp ... ..C ..T ..A ..A ..C ... ..G ..A ... ..A ... ... .NN NNN
```

```
       E   D   T   N   L   C   A   I   H   A   K   R   V   T   I
SoH3 GAA GAC ACC AAC CTT TGC GCC ATC CAT GCC AAG AGG GTC ACC ATC
PyH3 ... ... ... ..T ..A ..T ... ... ..C ... ... C.. ..G ... ...
DeH3 ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
PoH3 ... ... ... ..T ..C ... ... ... ..C ..T ... C.. ..G ... ...
SUSp NG. ... ... ... ..G ..T ... ... ..C ... ... ... ..T ... ...

       M   P   K   D   I   Q   L   A   R   R   I   R   G   E   R
SoH3 ATG CCC AAG GAC ATC CAG TTG GCT CGC CGA ATT CGC GGT GAA CGT
PyH3 ... ... ..A ... ... ..A C.C ..C ... ..T ... ... ... ... ..A
DeH3 ... ... ... ... ... ... ... ..C ... ... ..C ..G ... ... ..G
PoH3 ... ... ..A ... ... ..A C.C ..C ... ... ... ..T ... ... ..A
SUSp ... ... ..A ... ... ... C.C ..C ..T ... ..C ... ..A ... ..C

       A   *
SoH3 GCT TGA
PyH3 ..C ...
DeH3 ... ...
PoH3 ..C ...
SUSp ..C .AG
```

Figure 12. Alignment of H4 coding regions. The predicted amino acids are shown at the top of the alignment. There is an amino acid change from Alanine (A) in Pycnopodia (PyH4) and sea urchin Strongylocentrotus purpuratus H4 (SUSP) to Serine (S) in Solaster (SoH4). The single amino acid difference is marked with *. S. purpuratus H4 gene is from Grunstein et al.(1981).

```
      M   S   G   R   G   K   G   G   K   G   L   G   K   G   G
PyH4 ATG TCT GGT CGC GGT AAA GGT GGA AAG GGG CTA GGC AAA GGG GGT
SoH4 ... ... ..C ..T ..C ... ..A ... ..A ..A ..G ... ... ... ...
SUSp ... ..A ... ..A ..A ... ..A ... ... ..A ..C ..A ..G ..T ...

      A   K   R   H   R   K   V   L   R   D   N   I   Q   G   I
PyH4 GCC AAG CGC CAT CGC AAA GTT TTG CGG GAC AAC ATC CAG GGT ATC
SoH4 ... ... ..T ..C ... ..G ... C.T ..C ... ..T ... ... ..C ...
SUSp ... ..A ..T ... ... ..G ... C.A ..A ..T ... ... ..A ..C ...

      T   K   P   A   I   R   R   L   A   R   R   G   G   V   K
PyH4 ACC AAG CCA GCC ATC CGT CGT CTG GCC CGC CGT GGG GGA GTC AAG
SoH4 ..T ..A ... ... ..T ... ..G ... ... ... ... ..A ... ..A ...
SUSp ... ... ..T ..A ... ... ..A ..N ..T A.A A.G ..A ..T ... ...

      R   I   S   G   L   I   Y   E   E   T   R   G   V   L   K
PyH4 AGA ATC TCC GGT CTC ATC TAC GAG GAG ACC CGC GGT GTT CTC AAG
SoH4 C.C ... ... ... ... ... ... ..A ..A ... ... ..A ..G ... ...
SUSp ..G ... ..T ... ... ... ... ..A ... ..A ... ... ..A ..G ...

      V   F   L   E   N   V   I   R   D   A   V   T   Y   C   E
PyH4 GTC TTC CTG GAG AAT GTC ATC CGG GAT GCT GTA ACG TAC TGC GAG
SoH4 ..G ... ... ... ... ... ... ..C ... ..A ..C ..C ... ... ...
SUSp ... ... ... ... ... ... ... ..T ... ..A ..C ..C ... ... ...

      H   A   K   R   K   T   V   T   A*  M   D   V   V   Y   A
PyH4 CAC GCC AAG AGA AAG ACG GTC ACC GCC ATG GAT GTC GTG TAC GCG
SoH4 ... ... ... ..G ..A ..C ..T ..T T.G ... ... ..G ... ... ...
SUSp ... ..T ... C.. ... ..T ... ..A ... ... ..C ..G ... ..T ..A

      L   K   R   Q   G   R   T   L   Y   G   F   G   G   *
PyH4 CTC AAG AGA CAA GGC CGT ACT TTG TAC GGA TTC GGA GGT TAG
SoH4 ..G ... ... ..G ..G ..C ..G C.. ... ..T ... ..C ..A ...
SUSp ..A ... ..G ..G ..T ... ..A ... ... ..C ... ..C ..C ..A
```

nucleotide homology of H4 genes between these two species is 83%. This value is similar to that from the comparison of H3 genes.

Distribution of nucleotide substitution

Observation of the pattern of nucleotide substitution between Pycnopodia and Solaster H3 genes reveals that among 68 nucleotide changes, there are an equal number of transversions and transitions. Among 50 nucleotide changes found in H4 genes between Pycnopodia and Solaster, 23 of the changes are transversions.

Analyses of transition and transversion patterns at different positions of codons between Solaster and Pycnopodia H3 genes indicate that the distribution of nucleotide change pattern is remarkably non-random (Table 2). There are no transversions in non-degenerate or two-fold degenerate positions. Transversions are found only at four-fold degenerate sites. The number of transversions at four-fold degenerate sites between Solaster and Pycnopodia H3 genes is 34, while the number of transitions is 14 (Table 2). This result indicates that the higher ratio of transversions to transitions between Solaster and Pycnopodia are due to the increase of transversions at four-fold degenerate sites. The same is also true for H4 genes. These suggest that nucleotide substitutions are highly constrained in H3 and H4 coding

Table 2. Pattern of nucleotide substitutions at different degenerate sites between sea star H3 genes. The number of transitions and tranversions (in parentheses) is indicated in each pairwise comparison. The nucleotide changes at four-fold degenerate sites are shown in the upper right corner, the substitutions at two-fold degenerate sites are shown in the lower left corner.

Table 2. Pattern of nucleotide substitutions at different degenerate sites between sea star H3 genes

Four-fold degenerate / Two-fold degenerate	Solaster	Pycnopodia	Pisaster	Dermasterias
Solaster	____	14.5 (34.0)	19.5 (33.0)	13.0 (13.0)
Pycnopodia	22.5 (0)	____	9.0 (9.0)	19.5 (27.0)
Pisaster	23.5 (0)	7.0 (0)	____	17.5 (28.0)
Dermasterias	15.5 (0)	25.5 (0)	24.5 (0)	____

regions.

5' flanking regions of H3 genes

About 350 nucleotides 5' of the H3 genes in *Pycnopodia* and *Solaster* were sequenced. In contrast to their gene coding regions, there is a great divergence of DNA sequences in the flanking regions between *Pycnopodia* and *Solaster*. However, some homology blocks which may be functionally important have been found in these regions.

Nucleotide sequences of H3 5' flanking regions of *Solaster* and *Pycnopodia* are shown in Figure 9 and 10. There is about 51% sequence identity between *Solaster* and *Pycnopodia* H3 5' flanking regions. The homology blocks which may be important for gene regulation are underlined in both Figures. These blocks are compared with those found in other sea stars and in sea urchin (Figure 13A). The blocks are numbered with Roman numerals. A consensus sequence (I) similar to the cap site described by Busslinger et al.(1980) is found in a short distance upstream from both in *Pycnopodia* and *Solaster* H3 start codon ATG. In *Pycnopodia*, the potential cap sequence is CAACTT. *Solaster* potential cap sequence, however, is CATTCA. The TATA homology box (Breathnach and Chambon, 1981) is also found in both species at approximately 20 bp to the 5' region of the cap site, and is referred as TTAAGAGA (II). The TAAGA sequence shows no difference among five H3 genes. Sequences

Figure 13. A): Comparisons of the consensus sequences in the 5' flanking regions of H3 genes. Numbers between sequence indicate the number of intervening nucleotides. The potential Cap site (I), TATA box (II), GATCC (III) and CAAT sequence (IV) and other homology blocks of sea stars are compared to those of sea urchin (Susp 17/2) (C. C. Hentschel, et al., 1981). **B): Comparisons of consensus blocks in the 5' flanking regions of histone H4.** The sequences of 5' regions from Pycnopodia and Solaster are compared with those from sea urchin (Susp17/2) and Drosophila (Drom500)(C. C. Hentschel et,al., 1981). The potential cap site (I), TATA box (II), histone special sequence (III) and GC rich sequences (IV) are shown.

(A)

```
                    (IX)            (VIII)          (VII)            (VI)
SoH3  (-323)  CTCTTTT--61--TATGTTG--56--TGTTTTG------------49----
PyH3  (-316)  CACTTTT--34--AATGTTA--32--TGTTTCG--05--TTGTGC--63--
PiH3  (- ??)  CACTTTT--72--AATGTTA--37--TGTTTCT--??--TTCTAC--63--
DeH3  (-650)  CACTTTT--63--AATGTTA--53--TGTTTCT-289--TTCTAC--38--
SUSp  -----------------------------------------------------------

              (V)                (IV)             (III)            (II)
SoH3  -------------------ACCCAATA--04--GATGC-23-GATGC--12--TTAAG
PyH3  TGACCACTCAAGC--21--GGCCAATC--23-------GACCC------21--TTAAG
PiH3  TGACCACTCAAGC--31--ATCCAATT--22-------GATCC------08--GTAAG
DeH3  TGACCACTCAAGC--21--ATCCAATC--33-------GATCC------13--TTAAG
SUSp  ------------------ GACCAATC--25-------GATCC------09--TATAA

                   (I)
SoH3  AGA--20--CATTCA--39--ATCATG
PyH3  AAC--25--CAACTT--19--ATAATG
PiH3  AGG--16--CAACTT--19--ACAATG
DeH3  AGA--25--TCACTT--31--ACCATG
SUSp  ATA--22--CATTCA--46--ACTATG
```

(B)

```
                       (IV)          (III)          (II)            (I)
SoH4  ....(-117)-GCCCCGC--11--CGTCC--12--TATATAA--14--CATTCA--
PyH4  ....(-124)-TCCCCGC--03--CGTCC--12--TGAAATA--24--CATTCG--
SUSp  ...................... AGTCC--09--TAACAAT--21--CATTCG--
DROm  ...............................TATAAAT--21--TAGTTC--

SoH4  --49--AACATG
PyH4  --54--ACTATG
SUSp  --57--ATCATG
DROm  --44--AAAATG
```

homologous to the histone gene special motif GATCC (Hentschel, 1981) appear twice in *Solaster* but once in *Pycnopodia* (III). Another sequence block ACCCAATA (IV) common to many RNA polymerase II transcribed genes, the CCAAT box, is located at further upstream of the histone gene special motif. Two other homology boxes TTCTACGA (VI) and TGATGACCACTCAAGCG (V) pointed out by Cool et al.(1988) are found in *Pycnopodia* but not found in *Solaster*. Three homology blocks are seen 200 bp upstream of the start codon ATG, and are referred as (VII), (VIII) and (IX) boxes (Figure 13A). These three boxes are also found in *Pisaster* and *Dermasterias* (Cool, et al., 1988). Similar boxes have been shown in H4 3' flanking regions. It should be pointed out that the 3' flanking region of histone H4 is also the 5' flanking region of H3 (Figure 5).

5' flanking regions of H4 genes

About 150 nucleotides in the 5' flanking regions of *Pycnopodia* and *Solaster* H4 genes have been determined. The sequences from these two species can be found in Figure 9 and 10. From these two Figures it can be seen that the 5'-flanking regions of *Pycnopodia* and *Solaster* H4 genes are significantly diverged. There is only 44.4% nucleotide identity between the 5' regions of *Solaster* and *Pycnopodia* H4 genes. There is a 40 base pair purine-rich sequence (90% AG) immediately flanking *Solaster* H4 start codon ATG (Figure 10).

The 5' region of H4 gene in Pycnopodia, however, does not have such a purine-rich sequence.

A summary of the homologous sequences in the 5' flanking regions are shown in Figure 13B. The consensus boxes from sea urchin and Drosophila are also shown in this Figure. The potentially important sequences are numbered. The potential cap site CATTCA (I) in Solaster is found 20 bp upstream of the purine-rich region. The potential cap box in Pycnopodia H4 5' region is CATTCA at approximately the same position as in Solaster. The potential TATA box, TATATAAT in Solaster, is found about thirteen base pairs upstream from the cap site (II). The Pycnopodia TATA homologous motif TGAAATA is found 24 bp upstream from the cap site. There is a homologous motif CGTCC (III) present 12 bp upstream from the TATA boxes in both Pycnopodia and Solaster. A GC rich sequence GCCCCGC (IV) is found 100 base pair upstream ATG in both species. Similar sequence has been supposed to replace the CCAAT sequence in other H4 genes (Clerc, et al., 1983; Sugarman, et al., 1983).

3' flanking regions of histone H3 genes

The sequences of the 3' flanking region of Pycnopodia and Solaster H3 genes are aligned in Figure 14A. There is only 52% of sequence identity between these two regions. However, a 25 bp conserved sequence located 28 and 32 bp downstream from the stop codon TGA is seen in both Pycnopodia and

Figure 14. A): Comparison of DNA sequences in the 3' flanking regions of _Solaster_ and _Pycnopodia_ H3 genes. The arrows indicate the potential dyad symmetry sequences. The homology blocks are indicated and underlined. B): Comparison of the consensus blocks in the 3' flanking regions of _Solaster_ and _Pycnopodia_ H4 genes. Only part of the 3' flanking region are shown.

(A)

```
                 -Stem loop-                        -AAGAGA-
SoH3-3' TGA--32--AACGGCCCTTTTCAG-GCCACTAC--8--AAGAGAGATTAATCCA
PyH3-3' TGA--28--AACGGCTCTTTTCAGAGCCACCAC--7--AAGAGATTATAATCAT

         A-T rich region........
SoH3-3' TCGCGTTTTCTGTACAGTTTTTGACGTTAAGTTCGGTTACTATATGTAAATACG
PyH3-3' GCCCAATGTCATCGTTTTCTTTACAAAATAAAAAATTAATGAATTATAGCTATA

SoH3-3' AATTTTAACGGGCCAGGGTTCCGAAAAACGGATTAATTAATGAAACCATTAAGA
PyH3-3' CGCCGCAATATTAGGTCTCCTCGTTAAATAAGTAAGATAAGTCAGCCTCAATAG

SoH3-3' TTAGTCTCTATCCGTTTTGTTTCTTTGTCTGTCTCTGATTTTTGTCGTTCCATA
PyH3-3' ATAACACTTTGTTTCTTTTGTCGAATTATGAAGTTCGACTGCTCTATTATATAT

SoH3-3' CCCCTTTAA
PyH3-3' AACTGTAAA
```

(B)

```
                  -stem loop-                        -AAAGA-
PyH4-3' TAG--31--CAACGGCTCTTTTCAGAGCCACCA--10--AAAGAGA (+72)

SoH4-3' TAG--23--AAACGGCTCTTTTCAGAGCCACCA--11--AAAGAGA (+65)
```

Solaster. This sequence is the dyad symmetry motif which forms a stem loop structure and is important for the maturation of pre-mRNA. A second homologous sequence 5'-AAAAGAGA-3' is 7-8 base pair further downstream. This sequence is also necessary for the splicing process (Busslinger,et al., 1979). In the 3' region 100 bp from the stop codon, the DNA sequences are highly AT-rich. This kind of phenomenon has been found in the 3' flanking region in most histone genes (Perry, 1985). In the extensive search of sequence homology in the downstream 3' regions in both species, several consensus sequences are found. They are 5'-CGTTTTCTTTTACA-3', 5'-AATTAATGAA-3', 5'-TTTGTTTCTTT-3' boxes, which are underlined in both sequences in Figure 14A.

3' flanking regions of histone H4 genes

The nucleotide sequences of H4 3' flanking regions of Pycnopodia and Solaster are also shown in Figure 9 and 10. There is only about 50% sequence identity between these two sequences. The conserved sequence elements are shown in Figure 14B. Both the dyad symmetry motif and purine-rich boxes seen in Pisaster and Dermasterias are also present in Pycnopodia and Solaster H4 3' region. A search for DNA sequence homology indicates that there are three conserved boxes shared between Pycnopodia and Solaster. They are AATGTTA, CACTTTT and TGTTTCG blocks, which are also found in

other species of sea stars in H4 3' regions (Cool, et al., 1988). These three blocks are indicated in the 5' flanking regions of H3 genes in this thesis (Figure 13A).

Discussion

Organization and number of histone gene clusters

I have demonstrated that histone gene order and transcriptional polarity in _Pycnopodia_ and _Solaster_ major tandem repeats are the same as seen in other sea stars. The histone genes are arranged in the order H2B H2A H4 H3 within each cluster element. The four core histones are transcribed from the same strand. The transcription proceeds in the direction 5'-H2B-H2A-H4-H3-3'. H1 gene has not been localized in my experiments. These results together with the data from Cool et al. (1988) and Raff et al. (1984) suggest that as in sea urchins, the organization and the transcriptional polarity of histone genes within cluster elements are very stable in sea star species. These results support the idea that the gene rearrangement within histone gene cluster may have occurred in the common ancestor of sea stars and sea urchins (Raff et al., 1984).

It has been shown that there is one major type of histone tandem repeat in _Pycnopodia_ genome, which is similar to that in _Pisaster_ and _Dermasterias_. The _Solaster_ genome, however, is organized into at least three different lengths of tandemly repeated clusters. Two of them have been isolated. The 6.2 kb tandem element is the most abundant in the _Solaster_ genome.

Weaker bands showed up when Solaster genomic blots were subjected to longer exposure. These may result from the heterogeneity of restriction sites dispersed in histone gene elements or represent the ends of tandemly repeated clusters. This feature has been discovered in some multigene families (Cohn, et al., 1979; Cool, et al., 1988).

I have pointed out above that according to the analysis of restriction digestion and DNA sequences, gene arrangement and transcriptional orientation in both Solaster 6.2 kb and 7.5 kb tandemly repeated clusters are the same. It is predicted that the arrangement of histone genes within the 6.5 kb cluster element is the same as that in the 6.2 kb or 7.5 kb cluster elements. Presumably it contains an extra insert in the spacer region of the 6.2 kb cluster element or a deletion occurred in the insertion portion of the 7.5 kb cluster element. This prediction has been supported by the Southern blot pattern of PstI digested Solaster genomic blot probed with H2B (Figure 2) and by the results of the hybridization of the 1.65 kb AvaI or 0.65 kb AccI/SphI fragments isolated from pSoH7.5 subclone with Solaster genomic DNA (Figure 7).

These results bring up several interesting questions. Firstly, how and when different histone gene clusters were formed in Solaster genome and why this kind of phenomenon occurred in Solaster but not in Pycnopodia and other sea star species investigated. The presence of more than one type of tandemly repeated histone gene cluster in sea urchins has been

reported (Roberts et al., 1984). The gene arrangement and the protein subtypes in different gene clusters are not the same. The histone genes expressed in the early blastula stage are clustered in tandem repeats. Those expressed in the post-blastula stage are dispersed in the genome. An exceptional situation is seen in the sea urchin *Lytechinus pictus*, where two different histone gene clusters are expressed in early development (Cohn and Kedes, 1979). The gene arrangement within these two repeat units is the same, but DNA sequences in the spacer regions show heterogeneity. Two distinct types of histone gene clusters are also found in *Xenopus* (Perry et al., 1985). However, the arrangement of histone genes between different tandemly repeated clusters in *Xenopus* varies. DNA sequences of histone gene coding regions from different clusters are not the same, though there is no amino acid change between them. As I have described above, my results are different from the situation in *Xenopus* and sea urchins, because it has been found that the gene organization and DNA sequences of histone H3 coding regions and part of its 3' flanking regions in both *Solaster* cluster elements are identical. It is possible that an insertion or deletion took place in the *Solaster* histone gene cluster during evolution, followed by a series of amplification events. We suppose that the amplification events happened recently because of the identity of DNA sequences of H3 genes and their 3' flanking regions between these two different cluster elements.

Alternatively, gene conversion may maintain nucleotide homology between different histone gene clusters. This is consistent with a duplication event that occurred a long time ago.

It is interesting to ask how the different types of histone cluster elements were fixed in _Solaster_. _Solaster_ has a different developmental pattern from that of _Pycnopodia_, _Pisaster_ and _Dermasterias_ (Dan, 1968). The _Solaster_ egg contains an unusually large amount of yolk, which leads to the development of a non-feeding larva. _Pycnopodia_, _Pisaster_ and _Dermasterias_, however, produce eggs that are ten times smaller than _Solaster_ eggs and development is via feeding larva. It is possible that the presence of multiple tandemly repeated histone gene clusters in the _Solaster_ genome may relate to selection or adaptation. It is known that during oogenesis and maturation, large amounts of histone mRNAs in sea urchin are accumulated for subsequent use during early embryogenesis (Maxson et al., 1982). A very low level of maternal histone mRNA is observed in _Pisaster_ eggs (Banfield et al., 1988). We do not know whether _Solaster_ eggs store much more maternal mRNA than _Pisaster_ or not. The question whether the change in the number of major histone gene clusters in _Solaster_ affects gene expression or the developmental pathway remains to be answered. Some basic data, such as the amount of maternal mRNA in _Solaster_ egg, the copy number of histone genes in _Solaster_ genome would be helpful to understand this question.

Restriction mapping analysis

The restriction maps of histone gene cluster elements among five sea stars (two from this study and three from Cool et al. (1988)) are compared. The analysis reveals that though the size and restriction enzyme sites of histone gene cluster elements in sea stars diverge dramatically, similarity between the restriction maps still can be found. Firstly, the size of histone gene cluster in Solaster is 6.2 kb, which is like that in Dermasterias. Both Pycnopodia and Pisaster histone gene cluster elements are around 5.3 kb, which is also comparable. In addition, several restriction enzyme sites are common in both Pycnopodia and Pisaster histone gene clusters. For example, restriction sites SacI, PstI, BamHI and HII are found in both histone gene cluster elements. Gene localization from our results has confirmed that these restriction sites are at the same positions between Pycnopodia and Pisaster histone gene tandem repeats. There is no restriction site similarity between Solaster and Dermasterias histone gene cluster elements. The restriction mapping supports that Pycnopodia and Pisaster are more closely related species.

Analysis of histone H3 gene evolution among four sea star species

To understand the evolution of histone genes among sea stars, I compared H3 coding regions from _Solaster_ and _Pycnopodia_ with those of two other sea stars (Cool, et al., 1988). The alignment of H3 nucleotide sequences between four sea stars (Figure 11) reveals that all nucleotide substitutions are synonymous substitutions. Pairwise comparisons of nucleotide changes are shown in Table 3. These data give a clear picture that _Pycnopodia_ and _Pisaster_ are the most closely related species among the sea stars compared. _Solaster_ and _Dermasterias_ have a relatively closer relationship when compared with _Pisaster_ or _Pycnopodia_. These results agree well to the DNA hybridization data (Smith, et al., 1982) and evidence from the fossil records (Spencer and Wright, 1966). The failure to find any amino acid substitutions among four species of sea stars suggests the functional importance of histone H3.

Comparisons of nucleotide substitutions at different degenerate sites shown in Table 2 indicate the non-random distribution of nucleotide changes between H3 genes. No transversions are involved at two-fold degenerate sites between H3 coding regions compared. This suggests selection pressure on the amino acid level, because all transversional changes at two-fold degenerate sites are non-synonymous. An equal number of transitions and transversions are seen at four-fold degenerate sites in the comparisons between _Pycnopodia_ and _Pisaster_ or _Solaster_ and _Dermasterias_ (Table

Table 3. Pairwise comparison of nucleotide change between sea star H3 genes. Values in the lower left are the number of nucleotide substitutions between sequences. Values in the upper right are the percentage of nucleotide difference.

Table 3 **Pairwise comparison of nucleotide change between sea star histone H3 genes**

	Solaster	Pycnopodia	Pisaster	Dermasterias
Solaster	___	0.173	0.185	0.099
Pycnopodia	71	___	0.061	0.175
Pisaster	76	25	___	0.170
Dermasterias	41	72	70	___

2). The ratio of transversions to transitions rises with greater divergence time. The transversions are about twice as numerous as the transitions at four-fold degenerate sites between Pisaster and Solaster or Pycnopodia and Dermasterias. These results suggest that the increase of transversions in H3 genes among sea stars is due to the rise of transversions at four-fold degenerate positions, which obviously relates to the divergence time of species.

The transition rate of mtDNA in human is about 10 times higher than the transversion rate (Wilson, 1987, Brown, 1982). In nuclear DNA, transitions greatly outnumber transversions among the closely related species. As divergence time becomes longer, the ratio of transitions to transversions decreases (Brown, 1982). This may be caused by multiple substitutions at the same position when divergence time is long. Our results support this suggestion. A recent report proposed that the relative proportion of transitions to transversions might possibly result from mutator genes, favoring either transitions or tranversions during DNA replication (Jukes, 1987). Evidence supporting this idea is that it has been found that transversions are more common than transitions in a special region of mitochondrial DNA sequences between Drosophila virilis and Drosophila yakuba (Clary and Wolstenholme, 1987). However, what factor may play a major role in affecting the ratio of transitions to transversions remains unclear.

To clarify whether there is a preferential codon usage in H3 genes in sea stars, I analyzed codon usage of histone H3 genes among four sea stars (Table 4). It is shown that the amino acids specified by two codons including Phe, Tyr, Gln and Asp have strong preference for codons ending with C or G. Amino acids specified by four codons such as Val and Pro show the same preference. There are some codons which are not used in sea star H3 genes. They are GAU (Asp), UCU (Ser), UUA (Leu). The average value of codons ending with G+C is 64% among sea star species. There is an average 22% of codons ending with A in both histone H3 and H4 gene among species compared. Similar values of codon usage have been observed among sea urchin early histone genes (Wells, et al., 1986). High constraints of codon usage of four histone H3 genes from mouse have been reported (Taylor, 1986). The percentage of G and C at the third position of codons in mouse is more than 70%. This observation is even higher than what we have seen in sea stars. It may be possible that the high degree of conservation in histone genes is due partly to the strict pattern of codon usage.

It has been reported that functional rather than phylogenetic relationship specifies codon usage in the H3 genes of higher eukaryotes (Wells, 1986). A comparison of codon usage of sea star H3 with those from other organisms reveals that the pattern of codon usage is similar to the sea urchin early histone genes and the histone H3.1-like gene in

Table 4. Codon usage in four sea star H3 genes

TTT	Phe	1	TCT	Ser	0	TAT	Tyr	1	TGT	Cys	1
TTC	Phe	15	TCC	Ser	1	TAC	Tyr	11	TGC	Cys	3
TTA	Leu	0	TCA	Ser	2	TAA	Term	0	TGA	Term	4
TTG	Leu	7	TCG	Ser	1	TAG	Term	0	TGG	Trp	0
CTT	Leu	11	CCT	Pro	1	CAT	His	3	CGT	Arg	14
CTC	Leu	14	CCC	Pro	10	CAC	His	5	CGC	Arg	18
CTA	Leu	1	CCA	Pro	2	CAA	Gln	8	CGA	Arg	17
CTG	Leu	15	CCG	Pro	11	CAG	Gln	24	CGG	Arg	9
ATT	Ile	8	ACT	Thr	9	AAT	Asn	2	AGT	Ser	7
ATC	Ile	18	ACC	Thr	24	AAC	Asn	2	AGC	Ser	13
ATA	Ile	2	ACA	Thr	6	AAA	Lys	16	AGA	Arg	10
ATG	Met	12	ACG	Thr	1	AAG	Lys	36	AGG	Arg	4
GTT	Val	2	GCT	Ala	12	GAT	Asp	0	GGT	Gly	8
GTC	Val	8	GCC	Ala	29	GAC	Asp	12	GGC	Gly	9
GTA	Val	2	GCA	Ala	19	GAA	Glu	21	GGA	Gly	7
GTG	Val	12	GCG	Ala	12	GAG	Glu	11	GGG	Gly	4

vertebrates.

Analysis of histone H3 and H4 flanking regions

It has been shown that nucleotide substitutions between H3 or H4 coding regions compared are highly constrained, but the flanking regions have diverged dramatically. I analyzed the DNA sequences of H3 and H4 flanking regions of Solaster and Pycnopodia in order to understand the potentially important sequences for gene regulation as well as to know the actual divergence of DNA sequences among sea star species. The conserved sequences were identified according to the sequence identity and their location compared to the those of other sea stars as well as to other organisms.

As I have pointed out, the 5' flanking regions of histone H3 genes between Solaster and Pycnopodia have diverged to the point where little sequence identity can be found. However, some similar blocks still can be detected. Comparing the H3 5'-flanking regions of Solaster and Pycnopodia to those from other sea stars and sea urchins (Figure 13A), I have shown that the potential cap site in Solaster, CATTCA, is the same as those in sea urchins (Hentschel et al., 1981), while the Pycnopodia potential cap site CAACTT is identical to that of other sea stars (Cool, et al., 1988). In addition, the conserved boxes TTCTAC (V) and TGACCACTCAAGCG (VI) which appear in H3 5' regions of Pycnopodia are 95% homologous to

those in Pisaster and Dermasterias (Figure 13A). These two boxes are not found in Solaster. The difference in the 5' region of Solaster H3 gene compared to other sea stars implies that different transcriptional factors may be involved in Solaster histone expression.

The three homology blocks CTCTTTT (IX), AATGTTA (VIII) and TGTTTCG (VII) (Figure 13A) detected in 5' flanking regions of Solaster and Pycnopodia H3 genes are also found in that of Pisaster and Dermasterias. These blocks in Pisaster and Dermasterias were shown in the 3' flanking regions of H4 genes (Cool et al.,1988). However, I place them in the 5' flanking regions of H3 genes due to the fact that they are very close to H3 genes of Solaster and Pycnopodia. The sequence identity between species for these three boxes is higher than 90%. The spacer region between each block among five species are about the same. It seems unlikely that this is a coincidence considering they are present among five highly divergent species. Rather it suggests that these sequences may be very important for histone gene expression or regulation in sea stars.

The 5'-flanking regions of H4 genes of Solaster and Pycnopodia are also highly diverged. A major difference is that Solaster H4 5' region contains a 40 base pair purine-rich sequences (90% AG) immediately flanking the start codon (Figure 10). A similar sequence is not found in the 5'-flanking region of Pycnopodia H4 (Figure 9). A comparison

of the H4 5' flanking regions with those from sea urchin and Drosophila shows that the cap site, TATA box and histone special motif CATCC are conserved between sea stars and sea urchins. As in sea urchin and Drosophila, the 5' flanking regions of Solaster and Pycnopodia H4 gene do not contain a CAAT-like box. Instead, there is a G-C rich sequence at almost the same position of CAAT box. The GC rich sequence has been suggested to play the function of CAAT box in sea urchins (Hentschel et al., 1981).

The 3'-flanking sequences of all four histone genes investigated contain a dyad symmetry motif GGCTCTTTTCAGAGCC. Such a symmetry motif has been demonstrated in histone genes of various organisms. There is one base pair deletion in this region in Solaster H3 3'-flanking region (Figure 14A). This deletion should not affect the formation of the stem loop structure of the pre-mRNA. The purine-rich sequence AAAGAGA is also common to sea star histone H3 and H4 3' regions. These two sequences have been implicated in transcript processing (Busslinger, 1979). Three other homology blocks downstream of the AAAGAGA sequence of Solaster and Pycnopodia H3 genes (Figure 14A) do not appear in Dermasterias and Pisaster, indicating that these sequences may be not important or they are species-specific.

It is interesting to ask how the conserved motifs are maintained since the flanking regions have greatly diverged among species or between organisms. Some kind of correction

mechanisms may have been involved in maintaining the homology.

Phylogeny of Sea stars

It has long been a question whether _Solaster_ and _Dermasterias_ belong to the same order or not. Spencer and Wright (1966) suggested that _Solaster_ and _Dermasterias_ are both in order Spinulosida, but Perrier (1875) and Blake (1981) put _Solaster_ in Spinulosida, and _Dermasterias_ in order Valvatida. DNA sequence analysis between H3 genes from our results shows that the number of nucleotide substitutions between _Solaster_ and _Dermasterias_ is much less than that between _Solaster_ or _Dermasterias_ and _Pisaster_ or _Pycnopodia_ (Table 3). The ratio of transversions to transitions between the genes of the former two species is much lower (Table 2). Sequence comparisons of histone H3 and H4 flanking regions among sea stars show that the histone H3 5' flanking region in _Solaster_ is highly diverged from _Dermasterias_. The homology blocks identified from _Solaster_ are highly distinct from _Dermasterias_, _Pycnopodia_ and _Pisaster_. This suggests that functional constraints are present in the regions. It may be possible that different transcriptional factors are involved in gene expression so that the conservative blocks in the flanking regions of histone gene H3 and H4 between _Solaster_ and other sea stars investigated are significantly different.

To understand the phylogeny of sea stars, I estimated

nucleotide divergence based on the data of nucleotide substitutions of histone H3 genes among four sea stars. Two methods of analyses, LWL (Li, et al., 1984) computer program and Perler method (Perler, 1980), were applied to estimate nucleotide divergence among sea star H3 genes. The results indicate that saturation has occurred in histone H3 genes between most comparisons except between Pisaster and Pycnopodia (data not shown).

The rate of synonymous substitution in histone H4 and H3 genes from comparisons between mammals is higher than those obtained from the comparisons between mammals and chicken due to saturation or other reasons (Li, et al., 1984). Perler et al.(1982) reported that the accumulation of synonymous substitutions in the C peptide of chicken preproinsulin genes is not linear with time, possibly breaking at around 85 myr. This evidence indicates that the estimation of nucleotide divergence in our case may not be reliable since saturation has been observed between most of sequences compared. Unfortunately, there is still no perfect method available for estimating nucleotide divergence, especially for the sequences diverged over a long time. In addition, gene conversion in histone genes may take place, which will cause a serious underestimation of nucleotide divergence.

The 34% nucleotide divergence between Pycnopodia and Pisaster H3 indicates a divergence time of approximately 30-40 myr, assuming the rate of nucleotide substitution is constant

at 1-1.2% per myr per site (Ochman, 1987). This estimate of divergence time between Pycnopodia and Pisaster is in good agreement to the data from DNA reassociation (Smith, et al., 1982).

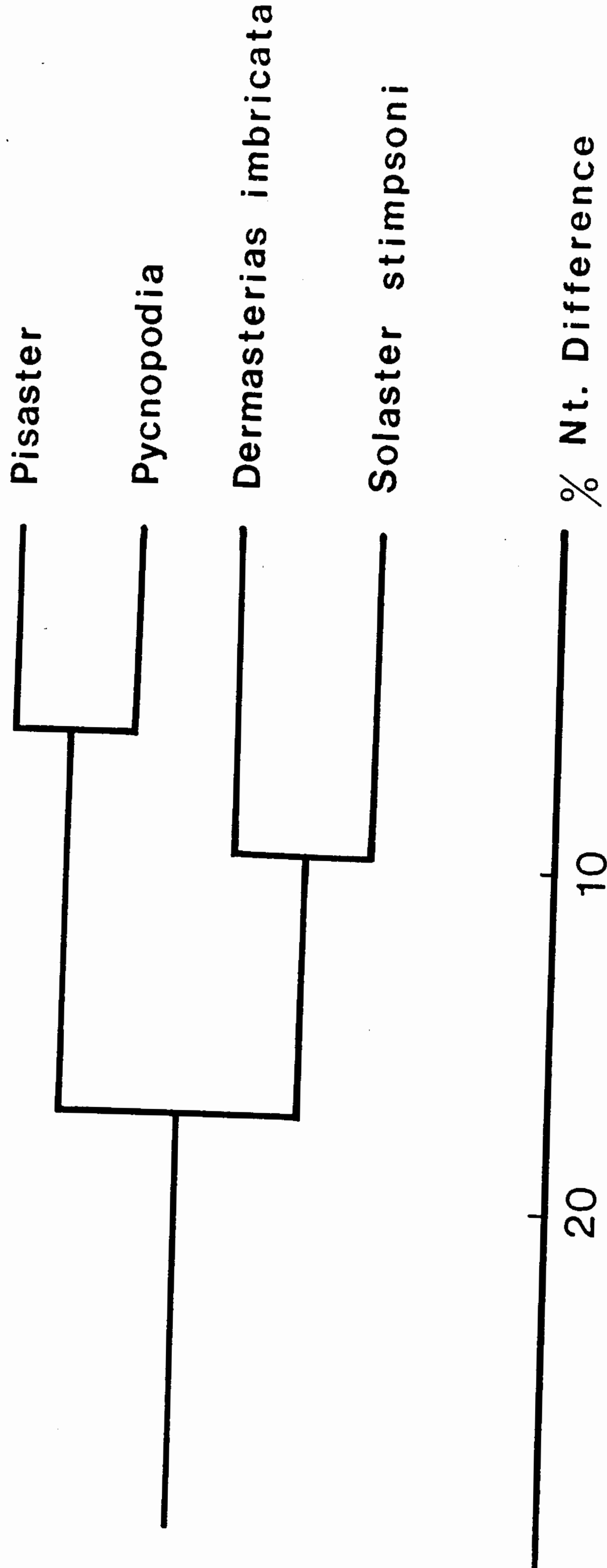
A relative phylogeny of sea star species based on the percentage of DNA sequence difference is shown in Figure 15. This result agrees with the phylogenetic tree reported by Spencer and Wright (1966).

Conclusions and perspectives

This study has extended our understanding of sea star histone gene evolution at two levels: the gene organization and nucleotide sequences. It allows me to make the following conclusions.

1. The class of genes described here appears to code for sea star early histones. The Solaster and Pycnopodia histone genes are clustered in tandem repeats. Comparisons of histone gene organization indicate that there is a remarkable stability in the arrangement and transcriptional polarity of histone genes within gene clusters in sea stars.
2. The number of major histone gene clusters in the Solaster genome differs from that of other sea stars examined to date. Individual Solaster contains at least three different sizes of EcoRI or HindIII histone gene cluster elements. The 7.5 kb and 6.2 kb elements are organized in the same fashion. Both

Figure 15. The phylogenetic tree of sea stars based on the percentage of nucleotide difference between H3 genes compared. Data used for this tree are from Table III.


Pisaster
Pycnopodia
Dermasterias imbricata
Solaster stimpsoni
% Nt. Difference
10
20

these two elements contain histone H4, H3, H2A and H2B genes.
3. The sequences of H3 and H4 genes show extensive conservation in coding regions. Nucleotide substitutions saturate between most of sequences compared, except between *Pycnopodia* and *Pisaster*. The ratio of transversions to transitions increases when compared sequences are from highly diverged species. No amino acid change among sea star H3 genes suggests functional importance in the coding region.
4. The common sequences in the H3 and H4 5' regions of *Solaster* and *Pycnopodia* identified may be of importance for the regulated expression of these genes. The identity of sequences at the 3' flanking region of H3 and H4 genes may represent either a recognition site for pre-RNA process or an initial target site for degradation.

Histone genes in sea star appear to be a typical example of Echinoderm histone gene organization. There is more to be learned about the organization of major and variant histone genes in sea star genome. It will be of interest to see whether the gene-specific sequences in the promoter regions have a functional role in the control of sea star H3 gene transcription.

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