Molgulid ascidians share a unique gene complex

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ABSTRACT

Typical chordate features found in ascidian tadpole larvae have been evolutionarily lost several times independently within the Molgulidae family. While tailed molgulids retain a tail with muscle cells, a notochord, and a dorsal neural tube, the notochord and muscle cells have been lost within the tailless species. A locus containing an unusual gene arrangement of the *Bobcat* gene within the first intron of the *Manx* gene has been shown to be essential for the development of chordate features in molgulid tadpole larvae. Sequencing and closer examination of ascidian genomes show that there is a unique gene arrangement of *SSNA1* upstream and adjacent to *Manx* and *Bobcat* within the Molgulidae which is not found in cionid ascidians; however, a similar arrangement of *SSNA1* directly upstream of *Bobcat* was found in *Oikopleura dioica* supporting Appendicularia as a sister group to the Molgulidae. *SSNA1* is expressed in tailed *Molgula oculata* gonads and not in tailless *M. occulta* gonads, suggesting *SSNA1* could have a role in the development of tailed larvae. Expression of these key genes could be affected by one another's close proximity, disturbing normal gene expression and thereby larval development of chordate features. We propose that the rearrangement that took place in the molgulid ancestor may be contributing to the numerous instances of the evolution of taillessness found in the Molgulidae.

INTRODUCTION

Ascidians are a class of tunicates that are filter feeding marine invertebrates and are sessile as adults. Most of the nearly 3,000 species of ascidians have a chordate tadpole larva, with a notochord, muscle cells, a dorsal neural tube, and sensory organs, an otolith and an ocellus (Swalla, '04). Recent phylogenomic data supports a chordate phylogeny that places the tunicates, or urochordates, as the sister group to the vertebrates (Bourlat et al., '06; Delsuc et al., '06; '08). Molgulidae is a monophyletic family of ascidians that has evolved tailless larvae at least three times or more independently (Huber et al., '00). Few other extant ascidian species have evolved tailless larva; only two tailless species are found in the Styelidae (Huber et al., '00). Therefore Molgulidae appear to have a propensity to independently evolve tailless species (Huber et al., '00) and we are interested in the molecular basis of this phenomenon. In previous studies, it has been shown that some tailed larval characters can be rescued in the tailless species *Molgula occulta*, by crossing with its tailed sister species *Molgula oculata*, suggesting loss-of-function mutation(s) as the cause for tailless development (Swalla and Jeffery, '90; Jeffery and Swalla, '92).

Manx encodes a zinc-finger transcription factor vital for the development of chordate features in molgulid larva (Swalla and Jeffery, '96). *Manx* is present in the *Ciona intestinalis* genome, but it becomes increasingly difficult to establish homology outside of the tunicates. It was previously reported that in the molgulids, *Bobcat*, a DEAD-box RNA helicase gene, is located within the first intron of *Manx*, with the two genes sharing two small noncoding exons (Swalla et al., '99). This gene complex was the first to be found in ascidians to show trans-splicing and also to be alternatively spliced to

form multiple protein transcripts. Subsequently, trans-splicing has been reported in both *Ciona* (Sierro et al., '09) and the distantly related *Oikopleura* (Ganot et al., '04) genome, so it is likely common in all tunicate genomes.

The expression of *Bobcat* in *M. oculata* and *M. occulta* was found to be required for neural sensory organ, secondary muscle cell, and tail development (Swalla et al., '99). *DDX5* (formerly *p68*) and differentially spliced *DDX17* are paralogous DEAD-box RNA helicases found in vertebrates that are involved in the processing of mRNA and microRNA, both functions that were found to be essential for development in mice (Fukuda et al., '07). The gene sequence of *Bobcat* in *M. oculata* and *M. occulta* is interesting because it lacks introns in its coding region (Swalla et al., '99) that are found in non-molgulid homologs, suggesting a previous retrotransposition event that inserted the processed RNA within the first intron of the *Manx* gene.

We have significantly expanded the mapping of this gene complex to include a third gene, *SSNA1*, closely 5' upstream of *Manx* and *Bobcat*. *SSNA1* (*Sjogren syndrome nuclear antigen 1*) is a nuclear gene highly conserved within eukaryotes. This protein lacks a nuclear localization signal, and it is thought that its small size of 14 kD would allow it to diffuse through nuclear pores into the cytoplasm, but instead it remains in the nucleus (Ramos-Morales et al., '98). *SSNA1* is localized to the centrosome and to sperm flagella (Pfannenschmid et al., '03) and has been shown to be involved in targeting ATPase *spastin* to microtubules in the centrosome (Errico et al., '04).

Its close proximity upstream to *Bobcat* and *Manx* is a unique feature in the Molgulidae and could be affecting *SSNA1*, *Bobcat*, and/or *Manx* expression. Here, we characterize this expanded gene complex in multiple molgulid species, and show that the

Bobcat retrotransposition is likely to have occurred in the molgulid ancestor. We hypothesize that this gene rearrangement contributes to the propensity for molgulid larvae to evolve a tailless phenotype.

MATERIALS AND METHODS

PCR sequencing

Dr. Dan Seufert did initial PCR and sequencing of the molgulids during his postdoctoral studies at Pennsylvania State University in the late 1990's. Table 1 summarizes the primer sequences used for PCR and sequencing different genes from different molgulid species.

Database sequence retrieval

The *SSNA1, Bobcat,* and *Manx* homolog sequences in other ascidians and animals were taken from online databases and identified via blast searches on NCBI (http://www.ncbi.nlm.nih.gov/), Genoscope (http://www.genoscope.cns.fr/spip/), and Ensembl (http://www.ensembl.org). The *SSNA1/Bobcat/Manx* complex was compared in *Molgula oculata, M. occulta, M. arenata, M. manhattensis, M. provisionalis, M. occidentalis, M. bleizi, M. pacifica, M. citrina, Oikopleura dioica*, and *Ciona intestinalis* in order to examine which ascidian species share this unique gene arrangement.

In situ *hybridization*

SSNA1 was PCRed and inserted into transcription vectors from Roche and linearized with *Xho I* and *Hind III*. Sp6 and T7 RNA polymerases were used to create

anti-sense and sense DIG-labeled transcripts, respectively, to be used for hybridization. After hybridization, the samples were the mounted on slides with permount for imaging.

RESULTS

SSNA1/Bobcat/Manx gene complex

Figure 1 shows the expanded 10kb *SSNA1/Bobcat/Manx* complex was found in nine molgulid ascidians and the *SSNA1/Bobcat* complex found in *Oikopleura dioica*. Previously the 3' part of the molgulid complex comprised of *Bobcat* and *Manx* was reported in Swalla et al. '99. The expanded molgulid complex shows a third gene, *SSNA1*, less than 500bp upstream to the trans-spliced 5' UTR of *Bobcat* and the long *Manx* transcript.

We examined the relative arrangements of *SSNA1*, *Manx*, and *Bobcat*'s homologs in other organisms to confirm the uniqueness of the gene complex in the Molgulidae, the locations of which are summarized in tables 2, 3, and 4. In humans, two homologs of *Bobcat*, *DDX5* and *DDX17*, can be found on chromosomes 17 and 22, respectively, and both genes contain introns that are not found in molgulid *Bobcat*. *Manx* could not be reliably detected in the human genome, but the homologous *SSNA1* gene is on chromosome 9, far apart from the *Bobcat* homologs. All the human genes can be said with certainty to be unlinked to each other in the genome. In the JGI genome of the related tunicate *Ciona intestinalis*, *Manx* and *Bobcat* homologs were found to be at separate contigs instead of within a gene complex (Maglott et al., '11), suggesting that these genes are also not linked in *Ciona*. A similar arrangement of *SSNA1* and *Bobcat* homologs is found in Genoscope's *Oikopleura dioica* genome, shown in Figure 1. A *Manx* homolog could not be found in *O. dioica*, but there have been many gene losses in this species (Seo et al., '01).

The genomic arrangement of *Bobcat* in chordates strongly suggests that it was retrotransposed into the *Manx* locus in molgulid ascidians (Figure 2). Both vertebrate and other tunicate (*Ciona intestinalis* and *Oikopleura dioica*) homologs have 13 exons that are spliced together to produce a mature mRNA (Figure 2). In contrast, *Bobcat* in *Molgula oculata*, *M. occulta*, *M. arenata, M. manhattensis, M. provisionalis, M. occidentalis, M. bleizi, M. pacifica,* and *M. citrina* contain only a single exon, as shown in red in Figure 2. The 5' UTR is trans-spliced onto a single exon containing the entire *Bobcat* coding region. In spite of these dramatically different genomic organization, the coding region of *p68* is highly conserved (Swalla et al., '99).

SSNA1 expression in molgulid gonads

This gene complex arrangement is present in a number of molgulid ascidians so we decided to test whether the expression of *SSNA1* is altered in tailless ascidians, since both *Manx* and *Bobcat* are down regulated in the gonads of the tailless species *M. occulta* (Swalla et al., '99). We performed *in situ* hybridization of *SSNA1* in tailed *M. oculata* and tailless *M. occulta* gonads and in *M.oculata*, *M. occulta*, and hybrid gastrula and neurula stages to detect differential expression. *SSNA1* expression was found only in the ovaries of *M. oculata* and the tailless *M. occulta*, with expression being the highest in *M. oculata* oocytes (Figure 3). Further examination of promoter and enhancer sites within the Molgulid gene complex will be necessary to understand how expression is altered in the different species of molgulid ascidians.

DISCUSSION

Manx was originally isolated as a transcript that was up regulated in the gonads of the tailed species, *M. oculata*, but down regulated in the tailed *M. occulta* gonads (Swalla et al. 1993). Later, it was shown that knockdown of zygotic *manx* in hybrid embryos reverted them to a tailless phenotype, suggesting that it is involved in the elaboration of the tail in ascidian embryos. Genomic analyses showed that *Manx* has a second gene, *Bobcat*, within the first intron, expressed in the gonads of both tailed and tailless molgulid ascidians (Swalla et al. 1999). Here, we show that there is a third gene directly upstream of this complex, *SSNA1*, that is also differentially expressed in the molgulid gonads. This unique gene rearrangment is found in ten molgulid species, suggesting that it is not the close proximity of the genes per se that has facilitated the evolution of taillessness, but that the complex can easily evolve differential expression of the three genes in close proximity.

We show by genome analyses that *Bobcat* is likely to be retrotransposed into the same gene locus in the molgulid genomes. *Molgula occulta* and *Molgula oculata* genomes are highly compact compared to *Ciona intestinalis*, so it is not surprising to find that some genes have lost introns. Similar results have been reported for a pelagic tunicate, *Oikopleura dioica*, which has an even more compact genome (Seo et al., '01). However, the fact that *Bobcat* completely lacks introns, but is flanked by two genes that have the same number of introns as found in *C. intestinalis*, very strongly suggests that it was originally retrotransposed from a *Bobcat* mRNA. Discovery of the *SSNA1/Bobcat* complex in *O. dioica* suggests that the original *Bobcat* gene containing introns must have been lost from the ancestral molgulid genome when it was directly replaced by the intron-

less retrotransposon. We also have not been able to detect a second gene by PCR or southern blot in any of the ten molgulid genomes examined, supporting with the above hypothesis. Complete genome sequencing will be necessary to refute with certainty the existence of the ancestral intron-filled *Bobcat* gene in the extant molgulid genomes.

Phylogenetic analyses by Zeng et al. (2006) showed the Appendicularia were placed as sister group to Stolidobranchia, the same relationship as obtained by Tsagkogeorga et al. (2009). The *SSNA1/Bobcat* gene complex in *O. dioica* shows microsynteny with *SSNA1* and *Bobcat* in the molgulids and is supportive of these phylogenies, placing the appendicularians as a sister group to the stolidobranchs and closer relatives of the molgulids than the cionids. More genomic analysis is needed to support this different phylogeny, but this is the first synteny discovered between *O. dioica* and *M. oculata*, so it is interesting to report.

Genome sequencing and assembly of *Molgula oculata* and *Molgula occulta* will be exciting and useful for further insight into the genomic basis of the loss of a tail in *M. occulta*. Promoter and enhancer analyses of this gene complex will be important in discerning how changes in gene expression evolve. Further studies will also be aimed towards understanding the role that *SSNA1* may play in the development of ascidian eggs and embryos.

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TABLES

Table 1. Primers used for sequencing

Species	Primer	DNA sequence	AA sequence	Target Region	References
S. clava	5'LIDF (328)	TTATTGACTTTCTGGAGTCAC			
	3'GDRHV (329)	CCDATICKRTGNACRTARTCYTC		3' of p68	
	U999	TGCCHGGHATTGTHCAYAT	LPAIVHI	Within p68	Seufert et al. 2000 JEZ
	U937 (424)	GCGTGACTGGGTCTTGAATG	RDWVLN region	Upstream of p68 intron	Iggo et al. 1991 MCB:11, p1326
	D1043 (423)	TTATGACAAACTTGACATCGGAC	SDVKFVI region	Downstream of p68 intron	
M. oculata	5-18MR684	CCGATTAGACTAAGTTCGCAG		Sequencing	
	5-18MF712	ATGGTATGGCATTCCAGG		Sequencing	
	M13F1307	CTTTCAGAATGATGTGCGAG		Sequencing	
	NA5MQEE	ATGCARGARGARGARGARAARCA	MQEE	Upstream in NA14	
	NA3IKMYA	ATYTTCATRTANGCTGAYTCNGTYTC	IKMYA	Downstream in NA14	
	NA5TEKLLAKINE	ACIGARAARYTIGCNAARATHAAYGA	TEKLLAKINE	Upstream in NA14	
	P683Mocc	GGTCACTATATCCAGGCATTGTA		Downstream in p68 exon 1	
	NA5SISQV	GYATYTCYCARGTCGAAAGTGG	SISQV	Upstream in NA14	
	NA5VESGK	GTCGAAAGTGGAAAACAAAAAGCAGA	VESGK	Upstream in NA14	
	P68EX1	TCAAGCGGSAACCAACTACC		In p68 exon 1	
M. occulta	NA5VESGK	GTCGAAAGTGGAAAACAAAAAGCAGA	VESGK	Upstream in NA14	
	P68EX1	TCAAGCGGSAACCAACTACC		In p68 exon 1	
M. pacifica	NA5TEKLLAKINE	ACIGARAARYTIGCNAARATHAAYGA	TEKLLAKINE	Upstream in NA14	
	P683Mocc	GGTCACTATATCCAGGCATTGTA		Downstream in p68 exon 1	
M. citrina	NA5VESGK	GTCGAAAGTGGAAAACAAAAAGCAGA	VESGK	Upstream in NA14	

	Prp683 (388)	GATTGG*GTCATAAARATGGCG		In p68	
	P68EX1	TCAAGCGGSAACCAACTACC		In p68 exon 1	

Table 2. Genomic database retrieved sequences of *SSNA1*

***SSNA1* homologs**

Organism	Database	Accession number	Location
B. floridae	NCBI		
C. intestinalis	NCBI		
D. rerio	NCBI		
G. gallus	NCBI		
H. sapiens	NCBI	NP_003722.2	Chromosome 9
N. vectensis	NCBI		
O. dioica	Genoscope Assembly v3	GSOIDT00017550001	Scaffold 9
S. kowalevskii	NCBI		
S. purpuratus	NCBI		

Table 3. Genomic database retrieved sequences of *Bobcat*

***Bobcat* homologs**

Organism	Database	Accession number	Location
B. floridae	NCBI	XP_002599469	BRAFLscaffold_143
C. intestinalis	Ensembl	XP_002130131.1	Chromosome 2q
D. rerio (DDX5)	NCBI	NP_997777.1	Chromosome 3

D. rerio (DDX17)	NCBI	XP_001923830.1	Chromosome 6
G. gallus (DDX5)	NCBI	NP_990158.1	Chromosome 18
G. gallus (DDX17)	NCBI	XP_416260.2	Chromosome 1
H. sapiens (DDX5)	NCBI	NP_004387.1	Chromosome 17
H. sapiens (DDX17)	NCBI	NP_001091974.1	Chromosome 22
N. vectensis	NCBI	XP_001630124.1	NEMVEscaffold_127
O. dioica	Genoscope Assembly v3	GSOIDT00017551001	scaffold_9
S. kowalevskii	NCBI	XP_002731189.1	Skow_1.1 scaffold3004
S. purpuratus	NCBI	XP_780035.1	BCM_Spur_v2.1_Scaffold77863

Table 4. Genomic database retrieved sequences of *Manx*

***Manx* homologs**

Organism	**Database**	**Accession number**	**Location**
B. floridae			Not found
C. intestinalis	Ensembl		NW_001954675.1 scaffold
D. rerio			Not found

G. gallus			Not found
H. sapiens			Not found
N. vectensis			Not found
O. dioica			Not found
S. kowalevskii			Not found
S. purpuratus			Not found

Figure Legends

Fig. 1 ***SSNA1/Bobcat/Manx* gene complex found in molgulid ascidians and *Oikopleura dioica*.** *SSNA1*, shown in orange, is upstream and contains two exons. In molgulids, the first exon of both *Bobcat* and the long minor *Manx* transcript share a 5' UTR, which is indicated as an open green box; minor *Manx* also shares its second exon in its 5' UTR with the 3' UTR of the *Bobcat* transcript. In *O. dioica*, *SSNA1* also contains two exons, and is just 35 base pairs upstream of *Bobcat* containing 13 exons. Noncoding UTRs are depicted as open boxes, coding exons as solid boxes. *SSNA1* is orange, *Manx* is red, *Bobcat* is blue, *Bobcat* and *Manx* shared exons are purple.

Fig. 2 **Gene structure of *Bobcat* and its homologs.** Shown are the gene structures of *Bobcat* and its homologs in human and *Ciona intestinalis*. The homologs in human, *C. intestinalis*, and *S. clava* are seen with 13 coding exons with introns separating them, contrasting with the long single coding exon found in *Bobcat* in *M. oculata*. Predicted retro-transposition would be responsible for the lack of introns in *Bobcat* in *M. oculata*. Noncoding UTRS are depicted as open boxes, coding exons as solid boxes.

Fig. 3 ***SSNA1* expression in molgulid gonads.** Expression of *SSNA1* was imaged via in situ hybridization. Tailed *M. oculata* developing oocytes show significant *SSNA1* transcripts present while the tail-less *M. occulta* shows little or no transcripts.

FIGURES

Fig. 1

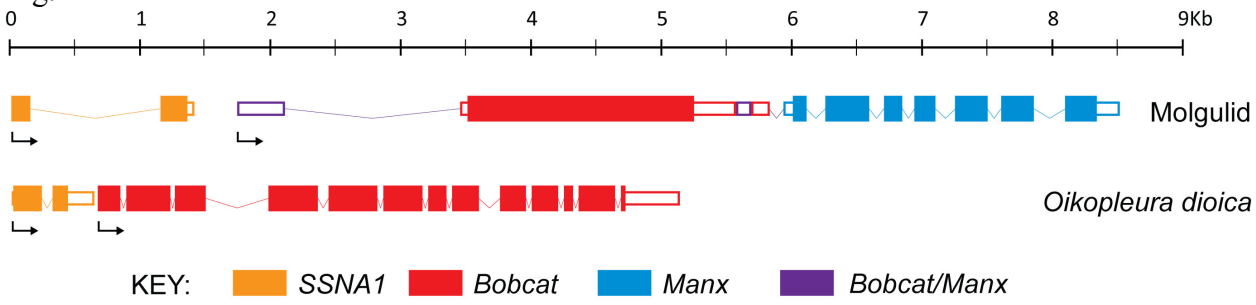

Fig. 2

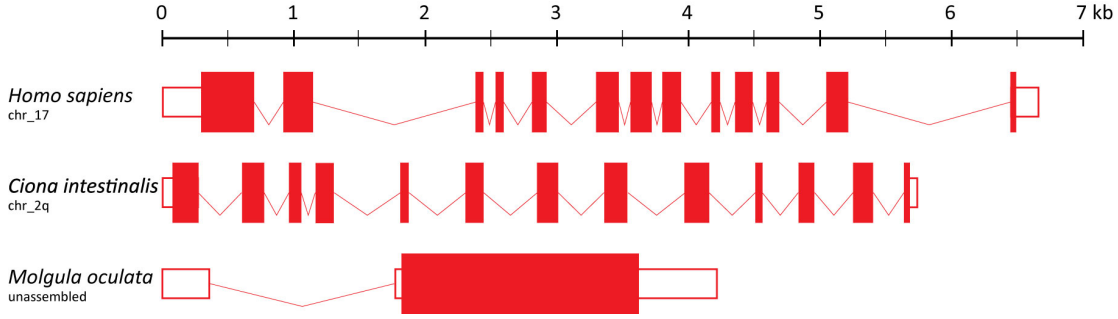

Fig. 3