Identification of Transcripts Expressed Preferentially in Hemocytes of *Ciona intestinalis* that can be Used as Molecular Markers

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Abstract

The immunity provided by ascidian hemocytes represents one prototype of innate immune function in vertebrates. However, there are currently no molecular markers of ascidian hemocytes. We accumulated a large number of ESTs of cDNAs derived from hemocytes of *Ciona intestinalis*, a cosmopolitan species of ascidian. By comparing these ESTs with those derived from other tissues and developmental stages of *Ciona*, we were able to extract 81 transcripts expressed abundantly and preferentially in hemocytes. Among them, the *von Willebrand factor type A (vWA)-like* and *complement 6 (C6)-like* transcripts were found to be expressed almost exclusively in hemocytes, based on RT-PCR analysis and whole mount *in situ* hybridization. We propose that *vWA-like* and *C6-like* can be used as molecular markers for *Ciona* hemocytes.

Key words: ascidian; hemocytes; transcript; molecular marker

Ascidians occupy a unique position in the evolution of deuterostome animals in the sense that a host defense mechanism functioning in ascidians represents a prototype of the innate immunity seen in vertebrates.[1,2] A recent report of the draft genome sequence of *Ciona intestinalis*,[3,4] a cosmopolitan species of ascidians, provided us with the chance to search for and identify the genes responsible for immunity.[2] As expected, genes known to belong to the acquired immunity system, such as the immunoglobulin, T-cell receptor, and RAG genes, were not found; whereas genes known to be involved in innate immunity, such as those for complement and the Toll-like receptors, were detected in the genome. It is particularly interesting that some components resembling the vertebrate's *MHC* genes were detected in a physically linked order in the *Ciona* genome. This favors the idea that ascidians closely precede vertebrates in the evolution of immune function.

Hemocytes are the central players of immunity in ascidians. The cells can phagocytose foreign materials, particularly opsonized ones,[5–7] and can synthesize antimicrobial peptides.[8–11] Using cDNA/EST studies, we recently found that various transcripts related to host defense are expressed in *Ciona* hemocytes.[12,13] However, a lack of molecular probes for hemocytes hampers the rapid progress of ascidian immunology. The number of hemocyte ESTs we reported previously (3,353) is very limited for the purpose of identifying such markers. In the present study, we wanted to see firstly what kind of transcripts are abundantly and preferentially expressed in *Ciona* hemocytes. Then, by using this information, we wanted to identify the molecular markers of *Ciona* hemocytes. To accomplish these two aims, we accumulated a relatively large number of cDNAs/ESTs.

The poly(A)$^+$ RNA fraction was isolated from *Ciona* hemocytes as described previously.[12] Conversion of poly(A)$^+$ RNA to cDNA, construction of a cDNA library and EST sequencing were also performed as described previously.[14] The sequences were registered in the DDBJ DNA database and are also available at our

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Table 1. The number of clusters as classified into functional groups.

Class			number of TCs
(A) Functions that many kinds of cells use			
A I	Transpotation and binding proteins for ions and small molecules		0
A II	RNA processing,polymerizing,splicing and binding proteins,and enzymes		0
A III	Cell replication,histones,cyclins and allied kinases,DNA polymerases,topoisomerases,DNA modification		0
A IV	Cytoskelton and membrane proteins		7
A V	Protein synthesis co-factors,tRNA synthetases,ribosomal proteins		0
A VI	Intermediary synthesis and catabolism enzymes		2
A VII	Stress response,detoxification and cell defense proteins		3
A VIII	Protein degradation and processing,proteases		4
A IX	Transpotation and binding proteins for proteins and other macromolecules		1
		Subtotal	17
(B) Cell-cell communication			
B I	Signaling receptors,including cytokine and hormone receptors,and signaling ligands		3
B II	Intracellular signal transduction pathway molecules including kinases and signal intermediates		5
B III	Extracellular matrix proteins and cell adhesion		5
		Subtotal	13
(C) Transcription factors and other gene regulatory proteins			4
		Subtotal	4
(D) Miscellaneous			
D I	Not enough information to classify		6
D II	Not significant similarities to known proteins		41
		Subtotal	47
		Total	81

Functional classification used here is based on that employed by a series of *Ciona*'s cDNA/EST studies.[12–21] As for the details of each TC, see Table 2.

URL, http://ghost.zool.kyoto-u.ac.jp. In the present study, however, we used Release 2.0 ESTs that are available at the TIGR database at http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=c_intestinalis; these ESTs are clustered into tentative consensus sequences (TCs). The hemocyte ESTs collected in this study are seen in the BM7 library (n=56,709, in these, the 5′- and 3′-ESTs represent 28,322 and 28,387, respectively), whereas those in our previous report[12] are in the 9VV library (n=3,353). For the purpose of comparison, we also used *Ciona* ESTs/TCs obtained from other tissues and various developmental stages of *C. intestinalis*.[14–21] The total numbers of *Ciona* ESTs and TCs in the TIGR database are 485,757 and 20,813, respectively.

Firstly, from the 7,940 *Ciona* hemocyte TCs, we extracted the TCs that satisfied both of the following criteria: (1) The TC should contain more than 28 ESTs derived from BM7 and (2) The BM7- and 9VV-derived ESTs in the TC should constitute more than 50% of all the ESTs in the TC. Twenty-eight ESTs constitutes 0.05% of 56,709; therefore, a TC that meets this criterion is considered to represent a transcript that is expressed abundantly in hemocytes. TCs that fulfill the second criterion are considered to represent transcripts that are expressed to a greater degree in hemocytes than in other tissues and developmental stages.

Only 81 of 7,940 TCs fulfilled the above two criteria. We used gene annotations and information about ontology attached to each TC and classified the 81 TCs into several functional groups (Table 1). The functional classifications used here have been previously employed in a series of *Ciona*'s cDNA/EST studies.[12–21] Table 2 describes the details of each TC.

A total of 34 of the annotated TCs belong to classes A (functions that many kinds of cells use), B (cell-cell communication), and C (transcription factors and other gene regulatory proteins). Among these, it is noteworthy that 7 of the TCs encode cytoskeletal proteins (class AIV). These TCs may be related to the high locomotive activity of hemocytes. Two *protein tyrosine phosphatase-like* (TC15785 and TC27235 in class BI) but no *protein tyrosine kinase-like* transcripts were found in the list. Integrin alpha precursor-like (TC25665 in class BIII) is likely a receptor for a complement 3-like molecule in *Ciona*.[22] In addition, the 4 TCs that encode transcription factors include *c-Jun-like* (TC15120), *HMG-box-containing Sox17-like* (TC25149), *Ets-domain containing ESE-3A-like* (TC26559), and *zinc-finger-containing PEM-4-like*

Table 2. Clusters expressed abundantly and preferentially in *Ciona* hemocytes.

Class	Cluster ID	Hemocytes BM7	Hemocytes 9VV	Total EST	Hemocytes /Total	Tentative annotation	ORF	aa number	Domain
AIV	TC14917	200	6	217	95%	weakly similar to gelsolin {Danio rerio}, partial (41%)	+	737	GEL(6)
AIV	TC24287	161	16	294	60%	weakly similar to nonmuscle myosin heavy chain {Gallus gallus}, partial (80%)	+	1613	MYSc-IQ-coiled coil(2)
AIV	TC15032	111	4	161	71%	weakly similar to probable fimbrin [imported] - Arabidopsis thaliana, partial (34%)	+	634	EFh(2)-CH(4)
AIV	TC15214	73	0	109	67%	weakly similar to KIAA0320 {Homo sapiens}, partial (20%)	+	786	ILWEQ
AIV	TC15217	64	0	112	57%	weakly similar to Fascin 2 (Retinal fascin). [Bovine], partial (41%)	+	487	
AIV	TC25869	34	1	59	59%	similar to C. elegans TBA-9 protein (corresponding sequence F40F4.5) {Caenorhabditis elegans}, complete	+	447	
AIV	TC24881	34	2	58	62%	weakly similar to gelsolin {Danio rerio}, partial (63%)	+	708	GEL(6)
AVI	TC15414	77	2	97	81%	similar to L-lactate dehydrogenase {Styela plicata}, partial (92%)	+	341	
AVI	TC14620	57	5	68	91%	ATP synthase alpha-subunit {Ciona intestinalis}, partial (33%)	+	63or64	
AVII	TC14643	161	0	170	95%	weakly similar to complement component C6 {Branchiostoma belcheri}, partial (18%)	+	565	TSP1-ZnF NFX-TSP1-LDLa-MACPF-EGF
AVII	TC25302	148	6	175	88%	weakly similar to complement component C6 {Branchiostoma belcheri}, partial (11%)	+	566	MYSc-IQ-coiled coil-TSP1-ZnF NFX-TSP1-LDLa-MACPF-EGF
AVII	TC26782	31	0	57	54%	weakly similar to flavin-containing monooxygenase {Gorilla gorilla}, partial (25%)	+	357	TM
AVIII	TC14696	192	10	252	80%	transglutiminase {Ciona intestinalis}, complete (100%)	+	766	TGc
AVIII	TC24838	81	6	144	60%	weakly similar to 72 kDa type IV collagenase precursor (EC			
AVIII	TC16352	31	4	52	67%		+	666	CLECT-Tryp SPc
AVIII	TC14698	30	4	39	87%	transglutiminase {Ciona intestinalis}, partial (27%)	+	212	
AIX	TC26244	39	3	72	58%	weakly similar to Sly1 protein - rat, partial (19%)	+	388	
BI	TC15785	34	3	54	69%	similar to vascular endothelial protein tyrosine phosphatase {Mus musculus}, partial (7%)	+	529	TM-PTPc
BI	TC27235	33	4	41	90%	weakly similar to amPTPR4c {Branchiostoma belcheri}, partial (38%)	+	546	PTPc(2)
BI	TC14267	32	3	44	80%		+	513	PTPc(2)
BII	TC14430	186	9	279	70%	weakly similar to coronin {Hemicentrotus pulcherrimus}, partial (42%)	+	463	WD40(4)-coiled coil
BII	TC14655	106	5	216	51%	homologue to calmodulin {Metridium senile}, complete (100%)	+	149	EFh(4)
BII	TC25991	54	6	102	59%	weakly similar to putative growth regulator 14-3-3 {Echinococcus granulosus}, partial (93%)	+	263	14 3 3
BII	TC26092	42	0	77	55%	weakly similar to Ras GTPase-activating-like protein IQGAP1 (P195). [Human], partial (42%)	+	928	IQ(4)-coiled coil-Ras GAP-coiled coil(2)
BII	TC27312	37	0	43	86%	weakly similar to Raichu404X {Homo sapiens}, partial (24%)			
BIII	TC24677	409	29	467	94%	weakly similar to Vwa1 protein {Boltenia villosa}, partial (29%)	+	1326	CCP(15)-vwa
BIII	TC25663	90	6	112	86%		+	813	Int alpha(3)-TM
BIII	TC26682	48	2	57	88%	weakly similar to Ci-META1 {Ciona intestinalis}, partial (13%)	+	659	TSP1-EGF-EGF CA-PTI-EGF CA(3)-PTI-EGF CA-ZnF NFX- EGF CA(5)-PTI-EGF CA(2)-TM
BIII	TC25600	41	2	71	61%	weakly similar to integrin beta-3 subunit - African clawed frog, partial (36%)	+	840	PSI-INB-VWA-EGF-TM
BIII	TC25665	29	0	29	100%	weakly similar to integrin alpha Hr1 precursor {Halocynthia roretzi}, partial (13%)	+	1250	VWA-Int alpha(4)-TM
C	TC15120	81	6	140	62%	similar to c-Jun protein {Xenopus laevis}, partial (30%)	+	381	BRLZ
C	TC25149	54	4	75	77%	weakly similar to HMG-box transcription factor Sox17 {Danio rerio}, partial (21%)	+	796	HMG
C	TC26559	44	2	58	79%	weakly similar to transcription factor ESE-3A {Homo sapiens}, partial (35%)	+	552	ETS
C	TC15716	38	2	77	52%	similar to PEM-4 {Ciona savignyi}, partial (20%)	+	599	ZnF C2H2-ZnF NFX-ZnF C2H2(2)
DI	TC15029	108	3	167	66%	Not2 {Ciona intestinalis}, complete (100%)	+	412	SERPIN
DI	TC26106	70	2	84	86%	weakly similar to hypothetical protein CL25084 {Homo sapiens}, partial (27%)	+	453	coiled coil
DI	TC24275	65	20	163	52%	homologue to Ci-META4 {Ciona intestinalis}, partial (97%)	+	103	TM
DI	TC15881	34	2	68	53%	weakly similar to FLJ00024 protein {Homo sapiens}, partial (68%)	+	967	

Table 2. Continued.

Class	Cluster ID	Hemocytes BM7	Hemocytes 9VV	Total EST	Hemocytes /Total	Tentative annotation	ORF	aa number	Domain
DI	TC24361	33	0	50	66%	weakly similar to FLJ00240 protein {Homo sapiens}, partial (59%)	-	1290	LRR RI(6)-coiled coil(2)
DI	TC26489	32	1	51	65%	weakly similar to Hypothetical protein C04D8.1 {Caenorhabditis elegans}, partial (11%)	+	309	Rho GAP
DII	TC24415	299	13	402	78%		+	268	
DII	TC25212	239	8	417	59%		+	123	ZnF NFX
DII	TC15080	106	4	129	85%		+	487	TM-TEP1(3)-EGF-TSP1(2)-EGF
DII	TC14925	102	5	213	50%		+	204	CH
DII	TC15163	100	6	135	79%		+	337	NEBU(3)
DII	TC25608	92	0	141	65%		+	317	TM(4)
DII	TC25643	87	2	155	57%		+	341	TM
DII	TC25166	84	0	95	88%		+	154	TM
DII	TC25165	71	0	77	92%		+	175	TM
DII	TC14720	70	18	130	68%		+	62	TM
DII	TC25213	67	4	92	77%		+	336	coiled coil-TM
DII	TC15215	66	2	125	54%		+	755	CUB(6)
DII	TC15558	62	1	89	71%		+	317	CUB-ZnF NFX
DII	TC25599	49	0	87	56%		+	52	TM
DII	TC25556	49	6	56	98%		-	427	TSP1(5)-ZnF NFX- TSP1(3)
DII	TC26265	47	0	72	65%		+	403	ZP-EGF-TM
DII	TC16155	45	6	59	86%		+	219	TM(4)
DII	TC15992	43	2	65	69%		+	490	JmjC
DII	TC25911	41	0	67	61%		+	146	
DII	TC26361	41	1	55	76%		+	447	SH2
DII	TC15653	40	2	82	51%		+	366	TM(7)
DII	TC25209	40	2	50	84%		+	581	TM(7)
DII	TC26388	40	4	73	60%		+	137	ZnF NFX
DII	TC27213	37	2	45	87%		+	176	TM
DII	TC16675	36	0	44	82%		+	159	
DII	TC26594	35	0	62	56%		+	439	CUB(2)-TM
DII	TC26530	35	0	41	85%		+	158	
DII	TC15312	34	0	41	83%		+	529	TM(7)
DII	TC16853	34	0	40	85%		+	107	TM
DII	TC24905	33	0	47	70%		+	657	ZnMc-ShKT(2)
DII	TC27089	32	2	42	81%		+	647	
DII	TC16049	32	2	36	94%		+	281	LRR TYP(3)
DII	TC16116	32	0	55	58%		+	411	PH
DII	TC25623	31	0	45	69%		+	376	
DII	TC27323	31	3	37	92%		+	560	EGF CA-VWA-EGF CA(3)-VWA
DII	TC24906	30	0	51	59%		+	151	coiled coil
DII	TC25587	30	3	58	57%		+	566	TM(2)
DII	TC16421	30	0	33	91%		+	501	JmjC
DII	TC16408	29	4	49	67%		+	366	TM(5)
DII	TC26927	29	3	49	65%		+	352	
DII	TC26587	28	4	57	56%		+	378	Rho GEF-PH

See the main text as for the numbers and the percentages of ESTs that constitute each TC cluster. Briefly, the sources of ESTs are from hemocytes (BM7 and 9VV) or others and "Total" represents the sum of hemocytes- and others-derived ESTs. Contribution of hemocytes' ESTs to the total ESTs in each TC is indicated as the percentage. Tentative annotation represents the gene to which each TC cluster showed the highest homology by the BLAST program[24] and the percentage of identical amino acid residues between the two genes is shown in parenthesis. TC16352, TC14267 and TC25663 are classified as AVIII, BI and BIII, respectively, based on their domain architecture, although they did not show any significant homology to known genes. The number of amino acid residues in the polypeptide encoded by the open reading frame (ORF) is presented. Minus of ORF means the lack of initiating methionine codon in the encoded polypeptide. The feature and number of domains identified by the SMART program[25] is also shown.

(TC15716). These transcription factors may play a role in the development, differentiation, and immune function of hemocytes. Non-annotated, 47 TCs belonged to class D (miscellaneous).

Secondly, we selected two genes from the TCs in Table 2, *von Willebrand factor type A (vWA)-like* (TC24677) and *complement 6 (C6)-like* (TC14643), to see whether they could be used as molecular markers of *Ciona* hemocytes. We chose these two TCs because each has a large number of hemocyte-derived ESTs (438 for *vWA* and 161 for *C6*) and each represents a high proportion of the hemocyte contribution to the total ESTs (94% for *vWA* and 95% for *C6*). In addition, as shown in Fig. 1A, domain analysis of the encoded polypeptides revealed that *vWA-like* and *C6-like* each possess characteristic features, the vWA and membrane-attack complex/perforin (MAC/PF) domains, which are unique to mammalian blood coagulation protein and complement,

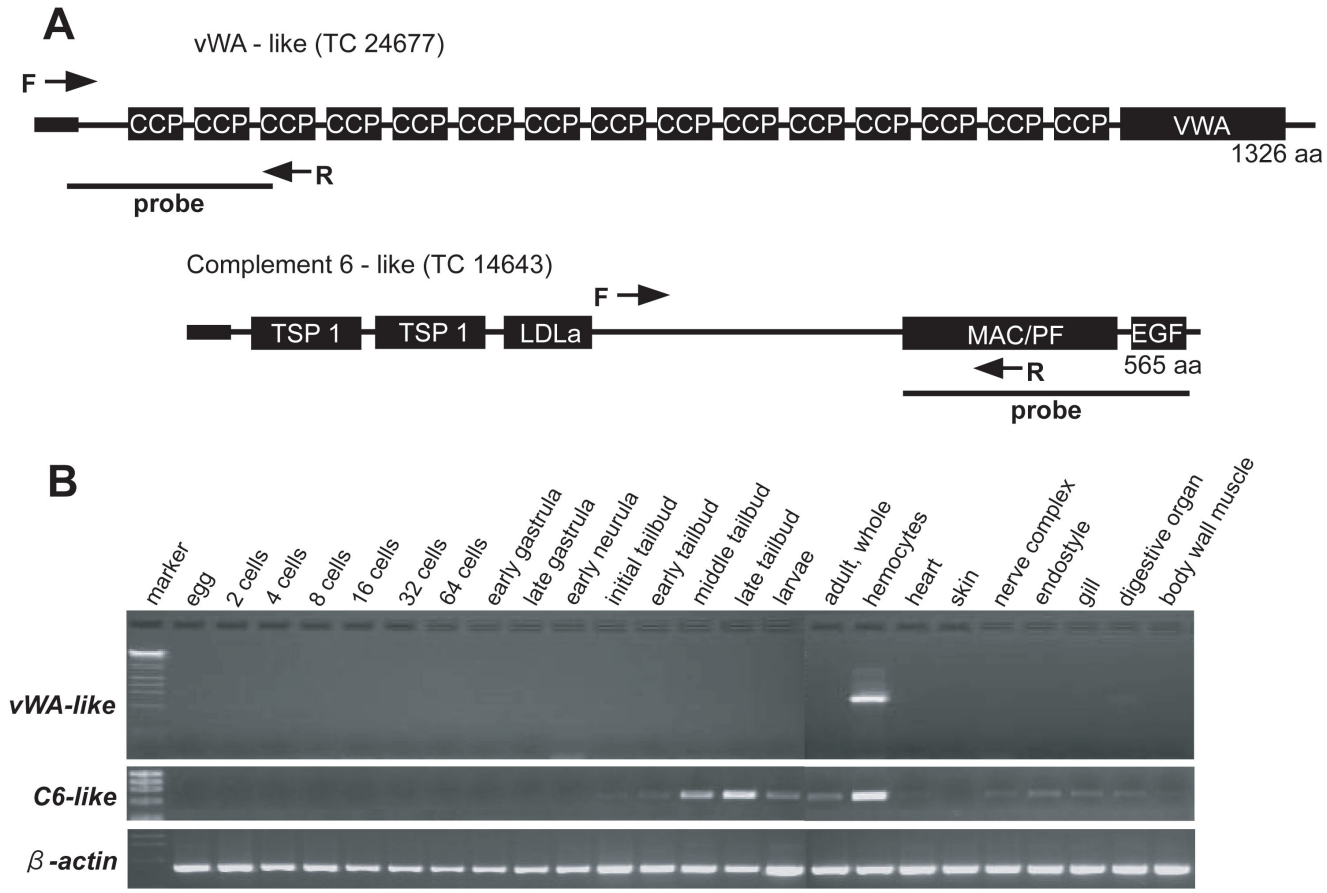

Figure 1. Expression of *vWA-like* and *C6-like* transcripts. (A) Domain analysis of the vWA-like and C6-like polypeptides, which are encoded by TC24677 and TC14643, respectively. Numbers represent amino acid residues. The locations of forward (F) and reverse (R) primers and hybridization probes are indicated. The primers were used in RT-PCR, whereas the probes were used in WISH. CCP, complement control protein modules; vWA, von Willebrand factor type A domain; TSP1, thrombospondin type1; LDLa, low density lipoprotein receptor domain class A; MAC/PF, membrane-attack complex/perforin; EGF, epidermal growth factor receptor. (B) RT-PCR analysis of *vWA-like* and *C6-like* transcripts. RNA was prepared from *C. intestinalis* at various stages of development and from various tissues of 3-month-old adult individuals. These included fertilized eggs, 2-cell embryos, 4-cell embryos, 8-cell embryos, 16-cell embryos, 32-cell embryos, 64-cell embryos, early gastrula, late gastrula, early neurula, immediate early tailbud, early tailbud, mid tailbud, late tailbud, larvae, and 3-month-old whole individuals, hemocytes, heart, skin, nerve complex, endostyle, gills, digestive organ, and body wall muscles. RNA was converted to cDNA by SuperScript II reverse transcriptase (Invitrogen) and a fixed amount of cDNA was used for RT-PCR analysis. The forward and reverse primers were as follows: for *vWA-like*, 5′-ACGGTCTAAAATGTTGGGTGTGCGAC-3′ and 5′-TCCAATCAAAGAAAATCCGGGCGCG-3′, for *C6-like*, 5′-GTTACACTGTGAGGAGTGGTGAAC-3′ and 5′-CCGCCTACTACGTTTGAAATAACT-3′, and for *beta-actin*, 5′-GTGCTTTCATTGTACGCTTCTGGTC-3′ and 5′-CGGCGATTCCAGGGAACATAG-3′. One cycle of PCR was carried out for 60 sec at 94°C, for 30 sec at 60°C, and for 60 sec at 72°C serially, and this cycle was repeated 35 times, using each set of primers and ExTaq (Takara). The amplified products from *vWA-like*, *C6-like*, and *beta-actin* cDNAs were run through agarose gels together with a DNA molecular weight marker.

respectively.

Expression of the *vWA-like* and *C6-like* transcripts was examined with RT-PCR analysis, using RNAs that were prepared from various tissues of 3-month-old adult individuals and from various stages of development. As shown in Fig. 1B, the *vWA-like* transcript was detected only in hemocytes, whereas the *C6-like* transcript was detected mainly, but not exclusively, in hemocytes. Expression of *C6-like* was detected to a lesser degree in tailbud and larva but not in the other tissues or developmental stages. The *beta-actin* transcript was used as a control and was detected throughout development.

We next performed whole mount *in situ* hybridization (WISH) of the *vWA-like* and *C6-like* transcripts, using 3-week-old specimens (Fig. 2). The antisense probes of both *vWA-like* (panels A and B) and *C6-like* (panels E and F) revealed similar multiple dot staining. The blood vessels in the field appeared as transverse and longitudinal bars but the vessels themselves were not stained. To observe more closely what kind of cells were stained, the stained specimens were viewed at a higher magnification. The floating, round cells inside the vessels were positive

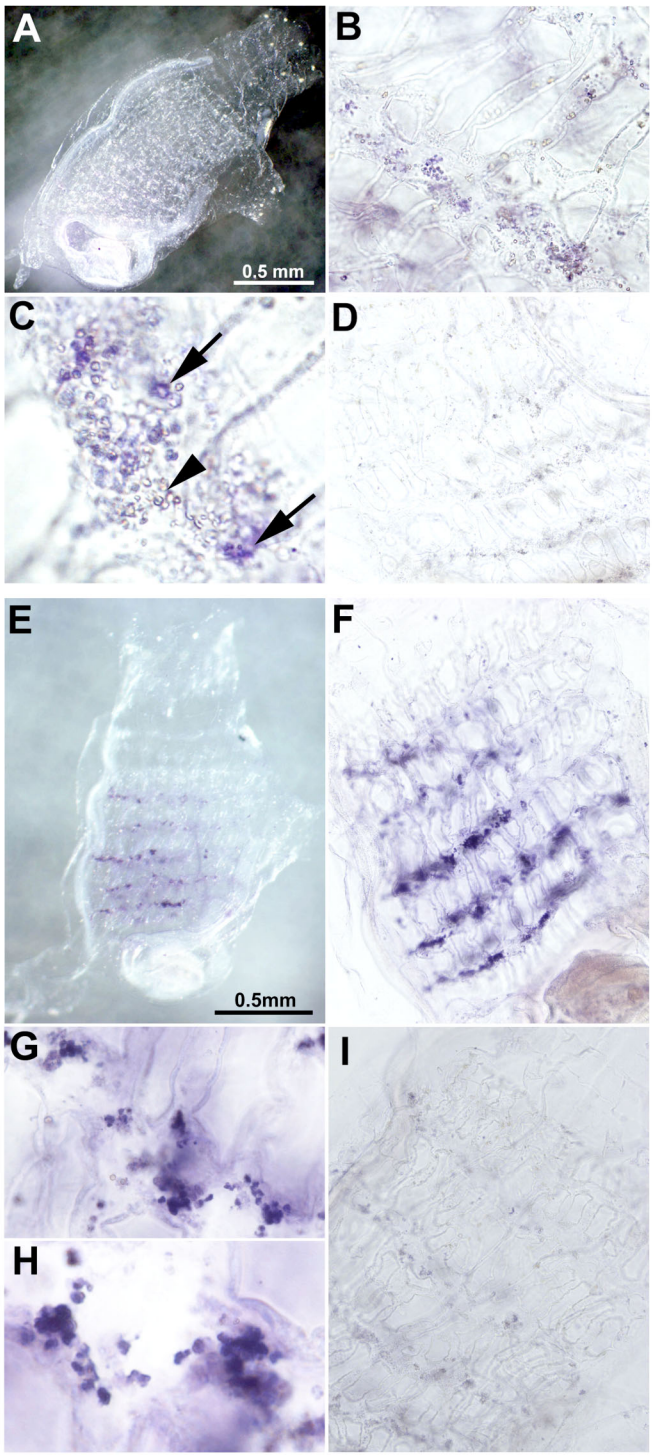

Figure 2. WISH of *vWA-like* and *C6-like* transcripts. Three-week-old adult specimens were collected after 5 days of starvation, relaxed with L-menthol, fixed in a solution containing 4% (w/v) paraformaldehyde, 0.5 M NaCl, and 0.1 M MOPS at 4°C for 12 hr, and stored in 80% ethanol at −25°C until use. For WISH, the methods were based on a published protocol.[26] To prepare the RNA probes, the cDNAs inserted into the pBluescript SKII or p-GEM-T vectors were linearized with an appropriate restriction enzyme and used as a template. The reaction was done in a digoxigenin RNA labeling mixture (Roche), using T7 and T3 (or SP6) RNA polymerases to make antisense and sense RNA probes, respectively. The cDNA sequences were derived from the clone ciad046b20 in the case of *C6-like* and the RT-PCR product in the case of *vWA-like*. The RNA probes used were *vWA-like* antisense (A, B and C), *vWA-like* sense (D), *C6-like* antisense (E, F, G and H), and *C6-like* sense (I). The scale bars in A and E are 0.5 mm. The magnifications of the objective lenses used for the microscopic observation were as follows: ×10 for D, F and I; ×20 for B and G; ×40 for C and H. In panel C, the cells indicated by the arrows and arrowheads represent the positively stained and unstained cells, respectively.

for either *vWA-like* (panel C) or *C6-like* (panels G and H). It must be noted, however, that not all of the floating cells were stained by the *vWA-like* probe, and that some cells were certainly not stained (see the stained cells indicated by arrows and the unstained cells indicated by arrowheads in panel C). Probably, *Ciona* hemocytes are not a uniform population but rather consist of functionally heterogeneous cell types, as in the case of *Halocynthia roretzi*.[23] In contrast, the *C6-like* probe stained most of the cells inside the vessels (panels G and H). As controls, the sense probes of *vWA-like* (panel D) and *C6-like* (panel I) did not produce any positive staining. Thus, based on the results of Figs. 1 and 2 together, *vWA-like* and *C6-like* were considered to be useful as markers of hemocytes.

It must be noted that not all of the TCs listed in the tables were good candidates as molecular markers of *Ciona* hemocytes. For example, RT-PCR analysis revealed that each of TC14917 and TC25665, in which the contribution of hemocyte ESTs was more than 95%, was more or less detected in various tissues. On the other hand, another *C6-like* transcript, TC25302, was detected mainly in hemocytes by RT-PCR as well as WISH analysis (data not shown). Therefore, the number of ESTs accumulated in the present study appears not to be sufficient to extract conclusively the marker genes specific to hemocytes. These situations, however, do not reduce the significance of the TCs listed in the tables. As described above, these TCs that represent abundantly and preferentially expressed transcripts should reflect characteristic features associated with gene expression in *Ciona* hemocytes.

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