Identification of Thirty-four Transcripts Expressed Specifically in Hemocytes of Ciona intestinalis and Their Expression Profiles throughout the Life Cycle

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Abstract

The innate immunity of ascidian hemocytes is considered to be a prototype of that in vertebrates. In this study, we identified as many transcripts as possible that were expressed specifically in hemocytes of Ciona intestinalis, a ubiquitous species of ascidian. Using a large-scale whole-mount in situ hybridization (WISH) technique and young adult specimens of C. intestinalis, 34 such transcripts were identified. Three of these appeared to encode immunity-related polypeptides, whereas 23 encoded hypothetical and/or new genes. Interestingly, different sets of transcripts appeared to be expressed in different subsets of hemocytes, as revealed by double-colored WISH. The 34 genes were categorized into two major subgroups based on their expression patterns during the C. intestinalis life cycle. Based on the gene expression profiles, we speculate that C. intestinalis hemocytes may exert more pleiotropic effects in immunity than previously believed.

Key words: gene expression; ascidian; hemocytes; immunity; WISH; microarray

1. Introduction

Ascidians hold a unique position in the evolution of the animal kingdom. Ascidians were the first chordates, having developed a notochord and dorsal nervous system. As such, ascidians have attracted the attention of developmental biologists as a model system for studying the body planning principles employed by chordates, including vertebrates and humans.¹,² A draft genome sequence of Ciona intestinalis, a ubiquitous species of ascidian used by researchers world-wide, has been prepared, and 15,852 protein-coding genes have been predicted from the euchromatic region of the genome.³ Extensive cDNA/EST studies also have been carried out with C. intestinalis.⁴ These have focused primarily on the various developmental stages of C. intestinalis, including fertilized eggs,⁵ cleavage-stage embryos,⁶ gastrulae and neurulae, tailbud embryos⁷ and tadpole larvae.⁸ The total number of ESTs has reached 454,861 assembled into 20,616 independent clusters. Gene expression profiles of the above-mentioned embryonic stages have been analyzed by means of whole-mount in situ hybridization (WISH).⁵–⁸

The developmental stage following fertilization covers only a short period of 1–2 days in the life cycle of C. intestinalis. Following metamorphosis on days 3–4, the animal begins to feed and grow. By 2–3 weeks several adult organs have formed, including the neural complex, body-wall muscle, heart, endostyle, pharyngeal gill slits, esophagus, stomach, intestine and gonads.⁹ The animal then matures and survives for another 3–4 months. A cDNA/EST study and gene expression profiling using WISH have been performed on young adult C. intestinalis¹⁰ and several endostyle-specific genes have been characterized.¹¹

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Immunity is an important biological function that is generated and mobilized as the animal reaches adulthood. Hemocytes play a central role in immune function. Although hemocytes originate in the trunk lateral cells of the tailbud embryo in the case of Halocynthia roretzi, another species of ascidian, they are first detected morphologically at the larval stage. During or after metamorphosis, hemocytes proliferate significantly.

Ascidians have only an innate type of immunity, rather than an adaptive-type, to protect themselves against foreign invaders. The innate immunity of ascidians has been examined using mainly Halocynthia and Styela and is considered a prototype of that found in vertebrates. C. intestinalis hemocytes also phagocytose foreign materials. Based on the assumption that C. intestinalis hemocytes serve as a model for studying the innate immunity, we so far reported on the gene expression profiles of the cells. First, we performed a cDNA/EST study of hemocytes' transcripts and identified 62 host defense-related clusters from 3357 ESTs. Out of them, 79 newly identified cDNAs were fully sequenced. We subsequently increased the number of cDNAs (ESTs) to 28355 (56709) and identified among them 2 hemocyte marker genes, von-Willebrand factor A-like and complement 6-like. In parallel, we surveyed the C. intestinalis genome and detected many immune system-related genes.

In this study, we have tried to identify as many transcripts as possible that are expressed specifically in C. intestinalis hemocytes, using the large amount of EST information and large-scale WISH. We have followed the expression of the identified genes during the entire C. intestinalis life cycle by extracting corresponding transcripts from a microarray dataset, providing a broad view of hemocyte-specific genes in C. intestinalis.

2. Materials and Methods

2.1. Biological materials

Adult C. intestinalis was maintained under constant light to induce oocyte maturation. Eggs and sperm were collected by dissecting gonads and inseminated artificially. The fertilized eggs were cultured at 20°C and larvae hatched ~14 h later. After metamorphosis, juveniles that adhered to trays were cultured with the diatom Chaetoceros gracillis as their food source. They were fed every 2 days and the seawater was changed every 2 days. Adult C. intestinalis survived up to 4 months.

2.2. WISH

Two-week-old young adults were used as the material, since they are of adequate size for WISH and have all types of adult tissue. The organisms were starved for 3 days to evacuate food waste and harvested. Relaxation and fixation of specimens were as described previously.

The cDNA clones were chosen from a cDNA resource of C. intestinalis, and the insert sequence with the T7 promoter was amplified by PCR. Digoxigenin (DIG)-labeled RNA probes were synthesized using T7 RNA polymerase and the cDNA sequences as the templates. Large-scale WISH using InSitu Chips was as described. Preparation and WISH of adult hemocytes were performed as described.

2.3. Double-colored WISH

The procedure for double-colored WISH was the same as for single-colored WISH except that the DIG- and biotin-labeled RNA probes were prepared, mixed and used for hybridization. The biotin signal was detected as green fluorescence using the TSA Biotin System (PerkinElmer, Boston, MA) and streptavidin-FITC. The DIG signal was detected as red fluorescence (HNP/ FastRed TR) with an HNPP Fluorescent Detection Set (Roche Diagnostics, Indianapolis, IN). An Olympus BX51-33-FL2 fluorescence microscope equipped with a Pixera pro 150ES camera was used for the photography.

2.4. Microarray analysis

To prepare total RNA from developing C. intestinalis embryos, the acid guanidine thiocyanate-phenol/ chloroform method was used. For adult RNA preparation, whole bodies were homogenized in a solution containing 4 M guanidine thiocyanate, 1% (v/v) mercaptoethanol, 50 mM Tris–HCl, pH 7.6, 10 mM EDTA and 0.5% (w/v) N-lauroylsarcosinate. The homogenate was ultracentrifuged through a cesium trifluoroacetate gradient. Poly(A) + RNA was purified using an mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Its quality was tested with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Two microgram each of poly(A) + RNA was labeled with either Cy3 or Cy5 using an Agilent Fluorescent Linear Amplification Kit (Agilent Technologies), mixed together and hybridized to the Ciona 22K Custom Oligo DNA Microarray loaded with 21 938 probes. Hybridization and washing protocols were according to the manufacturer’s instructions. The microarrays were scanned with a GenePix4000B DNA Microarray Scanner (Axon Instruments, Foster City, CA). For image analysis, normalization and data extraction, GenePix Pro4.0 Microarray Analysis Software (Axon Instruments) and GeneSpring Software (Silicon Genetics, Redwood City, CA) were used.

A combination of two different RNA preparations was used for hybridization. For the developing embryos, RNA from fertilized eggs served as a reference sample and RNA from post-egg stages were used as test samples. The developing embryos included 2-, 4-, 8-, 16-, 32- and 64-cell embryos, and early gastrulae, late gastrulae, early neurulae, immediate early tailbuds, early tailbuds, middle
tailbuds, late tailbuds, larvae and juveniles. For adults, the RNA combinations were from juveniles and 1.5-month-old adults, from 1.5- to 2.5-month-old adults, and from 2.5- to 4.0-month-old adults. Hybridizations were performed twice, using the same RNA combinations but different dyes. Thus, the average intensity for a given gene was calculated from two independent hybridizations. To draw an expression profile for each gene during the life cycle, the fluorescence intensity obtained from the fertilized eggs served as the reference 1.0, from which relative values could be calculated for each stage of development through adulthood.

3. Results

3.1. Identification of genes expressed specifically in hemocytes

One of the goals of this study was to identify as many transcripts as possible that were expressed specifically in hemocytes. Since hemocytes emerge in young adults as a significant tissue throughout the body, cDNA/ESTs obtained randomly from young adults were examined first. Our previously reported young-adult gene expression profile found 12 230 (24 460) cDNAs (ESTs) and 976 assembled clusters.10 Since we had not categorized ‘hemocyte’ expression in that report,10 we carefully re-examined the WISH images of the 976 independent sequences, and found 87 sequences likely to have positive hemocyte staining in the pharyngeal gill. Therefore, these 87 sequences were processed for re-evaluation by WISH.

Furthermore, since the previous report,10 the numbers of cDNAs (ESTs) and assembled clusters from the young adult had grown to 28 414 (56 828) and 7179, respectively. These clusters were classified into several groups, based on putative function as determined by homology to known genes. For this study 550 clusters categorized as of unknown function were processed for WISH. Of these, 66 clusters were found that were likely to stain positively in hemocytes.

In the recent cDNA/EST study of hemocytes themselves, 28 355 (56 709) cDNAs (ESTs) and 7940 assembled clusters were found.22 We chose 81 transcripts as candidates for preferential and abundant expression in hemocytes. However, this was based on an in silico comparison of hemocytes’ cDNA/ESTs with those from other tissues and/or developmental stages, rather than experimental data.

Therefore, 213 clusters (87 + 66 + 81 – redundant clusters) were rigorously and repeatedly examined by small-scale WISH to confirm the strict specificity of gene expression in hemocytes. Thirty-four such cDNA clones eventually were obtained. Among them 03435 and 10410 had been demonstrated as hemocytes’ markers in our previous report,22 and the remaining 32 genes were newly identified as hemocytes-specific transcripts.

Figure 1 illustrates examples of WISH images corresponding to eight genes from the whole body (upper rows) and the pharyngeal gill (lower rows). Only hemocytes inside the vessels stained strongly and specifically.

3.2. Content of genes expressed specifically in hemocytes

Table 1 lists 34 genes, along with the identification of clusters, the status of clusters, and the corresponding ESTs. Where a full cDNA insert sequence or an assembled sequence from ESTs was available, attempts were made to predict an open reading frame (ORF), its amino acid sequence and a domain structure in the polypeptide. ORFs were obtained for 25 transcripts, five of which appeared to encode transmembrane proteins.

Table 1 also includes information on the corresponding gene, as predicted with a genome browser and homologies to the known genes. Eleven clusters showed significant homology to genes with a known motif or a known function. Among them 03435, 10410 and 15640 were homologous to complement component 3b/4b receptor-1 (CR-1, annotated as von Willebrand factor type-A-like previously22), complement 6 (C6) and
### Table 1. A list of Ciona’s hemocytes-specific genes.

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10406 ciad01607 10410 Fully sequenced ciad016m22

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bactericidal permeability-increasing protein (BPIP), respectively. These three are perhaps immunity related. Each of the remaining eight genes (03474, 04837, 05210, 07063, 10406, 15607, 16828 and 37166) showed homology to various known genes, respectively. On the other hand, the 12 genes encoded hypothetical proteins and the 11 genes exhibited no homologies to known genes. Thus, the 34 genes selected as above were diverse in both sequence and domain architecture.

3.3. Differential expression of identified genes in subsets of hemocytes

While identifying the above 34 genes by WISH, we noticed that the percentage of positive cells within the hemocyte population varied from gene to gene. Transcripts of some genes were detected in most of the hemocytes and were referred to as major, whereas transcripts of other genes were detected in only a fraction of the hemocytes and were referred to as moderate or minor. The clusters 02671, 07027 and 15584 were examples of major, moderate and minor patterns of expression in hemocytes, respectively.

Identified genes were examined to determine whether they were uniformly or differentially expressed in hemocytes. Double-colored WISH was performed with the above mentioned 02671, 07027 and 15584. Row A in Fig. 2 depicts an entire young adult individual in which signals from 02671 and 15584 were from red (HNP/FastRed TR) and green (FITC) fluorescences, respectively. The red fluorescence was detected only within a minor population of hemocytes, with no overlap. A similar result was obtained with the major transcript 02671 and the moderate transcript 07027. Row B shows the pharyngeal gill. The green fluorescence from 02671 and the red fluorescence from 07027 did not overlap. The yellow fluorescence in Row B likely resulted from endogenous fluorescence since it also was observed in the controls without hybridization. Thus, the expression of each of these three genes in hemocytes appeared to be distinct.

An analysis similar as above was extended to the immunity-related genes. The probes used corresponded to CR-1-like (03435), C6-like (10410) and BPIP-like (15640), respectively. In Fig. 3, the left two columns represent the pharyngeal gill, whereas the right two columns represent isolated hemocytes. In Row A, green fluorescence of CR-1-like and red fluorescence of C6-like were detected in distinct hemocytes and did not overlap. Similarly, overlap was neither observed between BPIP-like and C6-like (Row B) nor between BPIP-like and CR-1-like (Row C). The result indicates that there are at least three different subsets within the hemocyte population and that each subset expresses a distinct set of genes.

3.4. Expression profiles of hemocyte-specific genes in the life cycle of C. intestinalis

Since hemocytes are first detected morphologically at the larval stage, emerge during and after metamorphosis and persist in adult C. intestinalis, the expression patterns of hemocyte-specific genes throughout the life cycle were examined. Data were extracted from microarray analyses.
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performed at each stage of development, including adulthood. The expression patterns of hemocyte-specific genes could be categorized into two major groups, as shown in Figure 4. The expression of gene 15640, *BPIP-like*, was low or undetectable during early stages of development, became detectable at the late-tailbud stage and reached its maximum level at the juvenile/young-adult stage (Fig. 4A). This appeared to parallel the morphological appearance of the hemocytes. The expression of 15640 gradually declined as the adult aged, probably because the percentage of positive hemocytes among the total body cell number decreased with age. The 17 more genes could be categorized as the type of 15640 and they were as follows; 03435 (*CR-1-like*), 10410 (*C6-like*), 02671, 03257, 03268, 03538, 04837, 06033, 06837, 06854, 10250, 10406, 15571, 15584, 15627, 15746 and 16828. Each of them showed somewhat variable but essentially similar patterns with that of 15640.

A second pattern of expression was seen with the gene 05210, *05210*. Its expression gradually decreased during development, re-emerging at the larva stage, although the peak was less evident. Genes 07063, 09947, 10247, 15607, 15691 and 37166 also followed this pattern of expression. Thus, the seven genes were categorized into this group.

The two genes, 02662 and 03474, showed distinct patterns, as compared with the two major categories, and the reason for this is not clear. For remaining genes, either the corresponding probes were not loaded on the microarray chips (5 genes) or the fluorescence intensities were too low to measure (2 genes).

3.5. **Identification of genes expressed predominantly in hemocytes**

The survey of the 213 WISH clusters revealed genes that were expressed in hemocytes but were not strictly specific for hemocytes. These were classified tentatively into two groups: type-1 and type-2. The expression of type-1 genes was detected in hemocytes as well as in one other young adult tissue (Fig. 5A and B). The genes 02764 and 07623 were expressed in the endostyle and intestine, respectively, as well as in hemocytes. The expression of type-2 genes was detected in multiple organs including hemocytes. The type-2 gene 02021 was expressed in the endostyle and neural complex, as well as in hemocytes (panel C). Nineteen type-1 and 35 type-2 genes were found. Table 2 illustrates in which tissues in addition to hemocytes each of the type-1 and type-2 genes were expressed. These results suggest that, as in other animals, there are multiple machineries in *C. intestinalis* that direct gene expression in different sets of tissues including hemocytes.

3.6. **Construction of a data base of C. intestinalis hemocyte transcripts**

The details of this report have been presented as a *Ciona intestinalis* Blood Data Base (CiBlooDB)
4. Discussion

In this study, we have identified as many _C. intestinalis_ hemocyte-specific transcripts as possible. Large-scale WISH of young adult specimens, a technology developed in our laboratory, has proved to be near-ideal for this purpose. With this method InSitu Chips can be used to screen 96 independent sequences at one time. Thus, the screened 1526 (976 + 550) sequences correspond to 16 rounds of hybridization of 96 column-based InSitu Chips. The 1526 sequences correspond to 9.6% of the predicted genes in the genome and 21% of the clusters expressed in young adults. Since the 160 rounds of hybridization that would be needed to cover the entire genome would be impractical, we incorporated into the WISH the 81 candidate sequences that were selected from hemocyte ESTs based on an _in silico_ subtraction. This enabled us to identify 34 hemocyte-specific transcripts, compared with the 2 hemocyte-marker genes we reported previously.

The identification of 34 specific genes is likely to shed some light on the nature of hemocytes themselves. For example, in homology searches with the identified genes, clusters 03435, 10410 and 15640 were found to be homologous to _CR-1_, _C6_ and _BPIP_, respectively. Therefore, these three genes possibly are associated with the immunity-related function of hemocytes. In contrast, 12 clusters have homologies with hypothetical proteins and 11 clusters show no homology to any known gene. Twelve of the 34 clusters likely encode polypeptides of <100 amino acid residues. One interesting possibility is that some of the 34 genes encode low-molecular weight anti-microbial polypeptides. However it must be noted that sequence comparisons alone do not necessarily predict whether a polypeptide has anti-microbial activity. The sequences of anti-microbial peptides reported thus far vary from species to species and from substance to substance. Nevertheless, the identification of significant numbers of hypothetical and/or new genes suggests that _C. intestinalis_ hemocytes either function in the immune response or mobilize genes that are not known among the innate immunity genes of mice and humans.

The expression patterns of hemocyte-specific genes have a second implication. Different sets of some clusters (02671, 07027 and 15584 in Fig. 2, and _CR-1-like_, _C6-like_ and _BPIP-like_ in Fig. 3) are expressed in subsets of hemocytes that appear to be different. This suggests that the _C. intestinalis_ hemocytes can be classified into several, at least three subgroups based on gene expression. The hemocytes of _Halocynthia roretzy_ are categorized into five subgroups, L1 through L5, based on morphology. Furthermore, most of the 34 _C. intestinalis_ genes display one of two different expression patterns in the animal’s life cycle. The genes from one group show a clear peak of expression at the larval/young-adult stage, at the time when hemocytes emerge as a significant proportion of the body cells. The genes from a second group are expressed not only in hemocytes of young adults but also in eggs. Although the significance of one of these genes as a
possible maternal gene is not known, these observations imply the existence of multiple mechanisms for hemocyte-specific gene expression. The identification of hemocyte-dominant genes further strengthens this hypothesis. Thus, from the aspect of gene expression, *C. intestinalis* hemocytes may exert more pleiotropic effects than previously believed.

The promoter regions that are responsible for neuron-specific expression of *C. intestinalis* genes, such as G-protein alpha subunit, arrestin and vesicular acetylcholine transporter, recently have been reported. This study used transgenic *C. intestinalis* embryos in which heterologous green fluorescent protein genes were under the control of the 5’ flanking regions of *C. intestinalis* genes. Thus, in the case of these genes, the regulatory elements for tissue-specific expression appear to be compacted into a relatively small region of 3000–4000 bp. If this proves to be the case for the hemocyte-specific genes as well, the identification of the 34 transcripts in this study will provide a valuable opportunity for analyzing their promoters, by using them in transgenic animals and visualizing hemocytes under live conditions.

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