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# Gene expression profile during the life cycle of the urochordate *Ciona intestinalis*

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**Abstract**

Recent whole-genome studies and in-depth expressed sequence tag (EST) analyses have identified most of the developmentally relevant genes in the urochordate, *Ciona intestinalis*. In this study, we made use of a large-scale oligo-DNA microarray to further investigate and identify genes with specific or correlated expression profiles, and we report global gene expression profiles for about 66% of all the *C. intestinalis* genes that are expressed during its life cycle. We succeeded in categorizing the data set into 5 large clusters and 49 sub-clusters based on the expression profile of each gene. This revealed the higher order of gene expression profiles during the developmental and aging stages. Furthermore, a combined analysis of microarray data with the EST database revealed the gene groups that were expressed at a specific stage or in a specific organ of the adult. This study provides insights into the complex structure of ascidian gene expression, identifies co-expressed gene groups and marker genes and makes predictions for the biological roles of many uncharacterized genes. This large-scale oligo-DNA microarray for *C. intestinalis* should facilitate the understanding of global gene expression and gene networks during the development and aging of a basal chordate.


*Keywords:* Ascidian; *Ciona intestinalis*; Gene expression; DNA microarray; Clustering; Development; Aging; Organ-specific gene

## Introduction

Ascidians are basal chordates, sharing a common ancestor with vertebrates. Ascidians are effective experimental model organisms, not only holding a unique position in evolution, but also having advantages for the investigation of developmental mechanisms (Corbo et al., 2001; Satoh, 1994, 2001; Satoh et al., 2003). *Ciona intestinalis* is a ubiquitous cosmopolitan species that spawns all year round and is used by researchers worldwide. Recently, a draft genome of *C. intestinalis* has been published (Dehal et al., 2002), and its ~160 Mbp genome is estimated to contain 15,852 protein-coding genes, similar to the number in other invertebrates, but only half of that found in vertebrates. It reveals that the ascidian has a basic, non-duplicated set of a chordate-type genome. In addition, an in-depth cDNA analysis including the generation of expressed sequence tags (ESTs), the collection and sequencing of a non-redundant set of cDNAs, and the clarification of their expression profiles in *C. intestinalis* was

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carried out (Satoh et al., 2003; Satou et al., 2002a). EST analysis was performed using six cDNA libraries made from different developmental stages, including fertilized eggs, cleaving embryos, gastrulae/neurulae, tailbud embryos, larvae and juveniles. In the analysis, libraries made from testis, ovary, heart, endostyle, neural complex and blood cells of mature adults (Kawashima et al., 2005) were used. Finally, over 450,000 ESTs were compiled and assembled into 20,616 independent clusters. All of the *C. intestinalis* cDNA information can be viewed at the Web site http://ghost.zool.kyoto-u.ac.jp/indexrl.html.

DNA microarray analysis is a very powerful technique for the life sciences, allowing the measurement of the transcriptional levels of thousands of genes simultaneously. Based on the above-mentioned molecular biological characteristics of *C. intestinalis*, we first established a large-scale cDNA microarray, and then developed this to an oligo-DNA microarray analysis. Previously, cDNA microarray or oligo-DNA microarray studies have been performed in embryos of various organisms, including *Caenorhabditis elegans* (Hill et al., 2000), *Drosophila* (Arbeitman et al., 2002; Furlong et al., 2001; White et al., 1999), zebra fish (Lo et al., 2003; Ton et al., 2002), mice (Carter et al., 2003; Miki et al., 2001; Tanaka et al., 2000), *Xenopus* (Baldessari et al., 2005) and sea urchin (Wei et al., 2006). In particular, a panoramic view of the gene expression profiles throughout the life stages has been depicted in *Drosophila* (Arbeitman et al., 2002) and *Xenopus* (Baldessari et al., 2005). This kind of analysis is important for understanding the differential gene expression mechanism differences between embryos and the adult stages.

In ascidians, global analysis of gene expression profiles in embryogenesis has been carried out using large-scale cDNA microarrays or oligo-DNA microarrays (Azumi et al., 2003a; Ishibashi et al., 2003, 2005; Yamada et al., 2005). In the present study, we applied the DNA microarray analysis to delineate transcriptional profiles for 65.7% (10,415) of the *C. intestinalis* genes (15,852) throughout its life cycle, beginning at fertilization, and containing 13 embryonic stages, the larval and juvenile stages, and 4 adult stages. We revealed basic and characteristic patterns of gene expression in *C. intestinalis*, and co-expressed and functionally related gene clusters. The present analysis provides an expansive view of gene expression in *C. intestinalis*, which allows for predictions of gene function.

## Materials and methods

### *Ascidian eggs, embryos, juveniles and adults*

*C. intestinalis* were cultivated at the Maizuru Fisheries Research Station of Kyoto University, Maizuru, Kyoto, Japan. Adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were washed and maintained in seawater at room temperature (18–20 °C). Larvae hatched after about 17 h of development. After metamorphosis, juveniles that adhered to trays were cultured with the diatom *Chaetoceros gracillis* as their food source. Two weeks after metamorphosis, juveniles adhered to trays were suspended in Maizuru Bay. These juveniles were grown to adulthood on the trays and survived for up to several months.

### *Microarray design*

For hybridization, we used the custom-made oligo-DNA microarray, *C. intestinalis* Oligoarray ver. 1, which was manufactured by Agilent Technologies (Santa Clara, CA). It is loaded with 21,939 probes consisting of 21,617 independent 60-mer oligonucleotides derived from 22,445 cDNA/EST sequences, selected using the sequence information from over 450,000 *C. intestinalis* ESTs and 4062 cDNAs (Satou et al., 2002b). More details of the array are described by Yamada et al. (2005).

### *RNA preparation, labeling, hybridization and scanning*

To prepare total RNA from *C. intestinalis* eggs, embryos and juveniles, the acid guanidine thiocyanate-phenol/chloroform (AGPC) method (Chomczynski and Sacchi, 1987) was used. For total RNA preparation from *C. intestinalis* adults, tunics were removed and the inner whole bodies of three individuals per stage were homogenized and RNA was isolated by ultracentrifugation (Azumi et al., 2005). Poly $(A)^+$RNA was purified using an mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). The RNA quality was verified by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies). Two micrograms of poly $(A)^+$RNA was labeled with either Cy3 or Cy5 using an Agilent Fluorescent Linear Amplification Kit (Agilent Technologies), mixed and hybridized with the *C. intestinalis* Oligoarray ver. 1. Hybridization and washing protocols were according to the manufacturer's instructions. The microarrays were scanned with a GenePix 4000B DNA Microarray Scanner (Axon Instruments, Foster City, CA). The resulting fluorescence intensity for each spot was quantified using GenePix Pro4.0 microarray analysis software (Axon Instruments) (Azumi et al., 2003a,b).

### *Statistical data analysis*

Each gene probe was characterized using corresponding cDNA/EST sequences. Annotation information of these sequences was obtained from the non-redundant amino acid (nr-aa) database of NCBI released on December 2005 and June 2006, using the BLAST X algorithm. A filtering analysis of the array data set was carried out, whereby it was divided into three groups based on the expression level. We also performed a combination analysis of microarray data and EST counts of each clone obtained from the database http://ghost.zool.kyoto-u.ac.jp/index.html. We used cluster analysis in order to reveal the similarities and higher order structure present in the data set. Hierarchical and *k*-means clustering were carried out using GeneSpring software (Agilent Technologies). The measure used in both cases was Pearson correlation. The details of data filtering, differential expression analysis and clustering analysis are described in Supplementary methods in Appendix A. The microarray data in this article have been deposited in the National center for Biotechnology Information Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo [accession nos. GSE6308 (series)].

## Results

### *Microarray experiments using C. intestinalis Oligoarray ver. 1*

We attempted to obtain global gene expression profiles during the life cycle of *C. intestinalis* using the Oligoarray ver. 1 for this organism. We chose oligo-DNA microarrays for this analysis rather than cDNA microarrays, by reason of their strict specificity and the feasibility for a larger number of genes. We collected 15 test samples for the developmental stages, comprising fertilized eggs (FE), 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, and 64-cell embryos, and early gastrulae (EG), late gastrulae (LG), early neurulae (EN), embryos at the initial