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Application of Ascidian DNA Microarray Analysis for Risk Assessment of Marine Chemical Pollutants

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Abstract—Ascidians (Phylum Chordata) are sessile, filter feeding marine animals. We have developed a large-scale oligo DNA microarray of the ascidian *Ciona intestinalis*, and have obtained the expression patterns for 10,415 genes of *C. intestinalis* expressed during its life cycle. These were categorized into 49 clusters, which were then grouped into 5 super-clusters. Using these DNA arrays, we identified genes that were up- or down-regulated in ascidians following exposure to organotin compounds, which are recognized marine contaminants. Organotin compounds induced up- or down-regulation of many ascidian genes, most of which belonged to a specific gene cluster or super-cluster, such as the "juvenile-specific gene cluster" or "embryonic and adult gene super-cluster " in our classification system. This result suggested that organotin compounds might affect embryogenesis and metamorphosis in ascidians. To verify this possibility, we investigated the inhibitory effects of organotin compounds on embryogenesis and metamorphosis in ascidians and found that low concentrations of organotin compounds completely inhibited both phenomena. These studies show that DNA microarray analysis, using our new classification method for ascidian genes, is a valuable tool for predicting the biological effects of chemicals on ascidians.

Keywords: ascidians, DNA microarray, gene expression, organotin compounds, embryogenesis, metamorphosis, risk assessment

INTRODUCTION

Ascidians are commonly known as "sea squirts" and are distributed worldwide. They are sessile marine animals that mostly live in shallow water and survive by filtering plankton and nutrients from seawater. Ascidians have unique biological properties. They are chordates and share common ancestors with vertebrates.

Ascidian larvae resemble tadpoles and have notochords in their tails; however, after metamorphosis, they loose their notochords and become effectively "invertebrates". *Ciona intestinalis* is a cosmopolitan species that spawns all year round and is used by researchers worldwide, especially in the field of developmental biology. Rapid developments have resulted in a recent increase in the availability of molecular information about ascidians. For example, the draft genome of *C. intestinalis* has been determined (Dehal *et al.*, 2002) and many EST clones have been sequenced and registered in the NCBI database (Satou *et al.*, 2002; Satoh *et al.*, 2003). Research techniques for whole mount *in situ* hybridization and the construction of transgenic mutants have been greatly improved (Sasakura *et al.*, 2005). Based on this molecular biological background, we have succeeded in producing a large-scale oligo DNA microarray for this ascidian, and have been analyzed global gene expressions at various stages of development and under various conditions (Ishibashi *et al.*, 2005, Azumi *et al.*, 2007). Using this oligo DNA microarray, we obtained expression profiles for 10,415 genes of *C. intestinalis* during its life cycle. Based on their expression patterns, these genes were categorized into 49 clusters, which were then grouped into 5 super-clusters; an embryonic gene cluster (A), an embryonic and adult gene cluster (B), an adult gene cluster (C), a stably-expressed gene cluster (D) and a maternal gene cluster (E) (Azumi *et al.*, 2007). The 49 clusters contained genes whose expression occurred in the same period, such as during embryogenesis, metamorphosis and during development of adult tissues. Furthermore, some clusters contained functionally related genes, which we classified as a "juvenile-specific gene cluster", a "testis-specific gene cluster" and an "immune-related gene cluster".

In this study, we have used the DNA microarrays to detect genes that were up- or down-regulated in ascidians after exposure to marine chemical pollutants. Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) are marine chemical pollutants that have been widely used in marine anti-fouling paints. Because of their toxicity, the use of organotins has been restricted for nearly thirty years. However, they are still detected in marine sediments and in animals collected from coastal zones in Asian countries (Hong *et al.*, 2002; Shim *et al.*, 2002). We have found that many genes were up- or down-regulated in ascidians after exposure to organotin compounds and we were able to predict the biological functions affected by these organotin compounds using DNA microarray data and our new classification method for ascidian genes.

MATERIALS AND METHODS

C. intestinalis were cultivated in Maizuru Bay, Kyoto, Japan. Several *Ciona* adults were exposed to different concentrations of TBT or TPT for 24 h. mRNAs were prepared from three organotin-exposed and three non-exposed individuals without tunics. DNA microarray analysis using *Ciona* oligo DNA chip version1 were performed according to the method previously reported (Azumi *et al.*, 2007). The effects of organotin compounds on embryogenesis and morphogenesis in ascidians were measured. Several different concentrations of TBT, TPT or 0.01% ethanol (control) were added to the wells containing 100–200 fertilized

eggs or larvae of *C. intestinalis* and the numbers of hatched larvae (around 20 h later) or attached juveniles (around 2 days later) were counted under a microscope.

RESULTS AND DISCUSSION

DNA microarray analysis

To investigate the biological effects of organotin compounds, we used a *Ciona* oligo DNA microarray to analyze differentially expressed genes in *Ciona* adults treated with organotin compounds. Throughout the whole body, 152 genes were up-regulated and 199 genes were down-regulated in *Ciona* adults exposed to 50 nM TPT for 24 h. We also found a similar number of up- or down-regulated genes in ascidians exposed to 100 nM TBT for 24 h. The biological effects of organotin compounds could not be predicted based on the list of up- or down-regulated genes due to difficulties in identifying the genes by sequence homology. More than half of the *Ciona* gene sequences did not coincide with any sequence by BLAST search, or there were many orthologs for functionally unknown genes of other animals, including humans.

Estimation of biological effects of organotin compounds

We previously classified around 10,000 genes of *Ciona* into 49 clusters, on the basis of their expression patterns during the life cycle. We then grouped them into five super-clusters; an embryonic gene super-cluster (A), an embryonic and adult gene super-cluster (B), an adult gene super-cluster (C), a stably-expressed gene super-cluster (D), and a maternal gene super-cluster (E). Each cluster contained between several dozen and several hundred genes, all of which showed expression and function during the same life stage, e.g. during embryogenesis, metamorphosis or during the development of adult tissues. We applied this new classification of *Ciona* genes to the genes that were up- or down-regulated in ascidians after exposure to organotin compounds. Interestingly, we found that most genes that were up- or down-regulated by either TBT or TPT were categorized into the "embryonic and adult gene cluster (B)" or the "juvenile-specific gene cluster". We also found that organotin compounds down-regulated some genes in the "immune-related gene cluster". Based on these results, we predicted that organotin compounds would cause disruption to embryogenesis and metamorphosis in ascidians, and would also have inhibitory effects on the immune system in this animal. To confirm our predictions, we observed the inhibitory effects of organotin compounds on embryogenesis and metamorphosis of ascidians.

Effects of organotin compounds on embryogenesis and metamorphosis in ascidians

50 nM TBT or TPT strongly inhibited both embryogenesis and metamorphosis. Our prediction of the abnormal effects caused by organotin compounds on ascidian embryogenesis and metamorphosis was confirmed by bioassays using ascidian embryos and larvae. Studies to confirm other predictions, such as the

inhibitory effects of organotins on the immune system of ascidians, have yet to be performed, though it has been reported that organotin compounds inhibited the phagocytotic activity of ascidian hemocytes *in vitro* (Cooper *et al.*, 1995).

New method for risk assessments of marine chemical pollutants

We have established the following unique method to assess the risks to ascidians from chemical pollutants: First, *Ciona* adults are treated with the chemicals, and RNA is prepared; second, array analysis is performed using *Ciona* oligo DNA microarrays to detect up- or down-regulated genes; and third, the up- or down-regulated genes are categorized based on our new classification method for *Ciona* genes. This allows a prediction of the biological effects of the chemicals on ascidians to be made. Finally, bioassays for the chemicals are performed using ascidian embryos and larvae. This method is suitable for assessing the risks posed by chemical pollutants to marine animals.

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