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TECHNICAL NOTE

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Large scale EST analyses in *Ciona intestinalis*

Its application as Northern blot analyses

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Abstract We have conducted large-scale EST analyses of transcripts expressed in the basal chordate *Ciona intestinalis*. The cDNA libraries examined were from fertilized eggs, cleaving embryos, gastrulae/neurulae, tailbud embryos, larvae and whole young adults, and the gonad (ovary and testis), endostyle, neural complex, heart, and blood cells of the adult. Because the libraries were not normalized or amplified, the occurrence of cDNA clones or EST counts in each library may reflect the quantity of transcripts of the corresponding genes. Thus, comparison of the EST counts of a certain gene at the six developmental stages may reflect the temporal expression pattern of the gene, while the comparison of EST counts in different tissues of the adult may reflect the spatial expression pattern of the gene. Here we present evidence that this is the case, and that the EST count can therefore be used like "Northern blot analysis" of *Ciona* genes.

Keywords *Ciona intestinalis* · Gene expression pattern · Large scale EST analyses · Northern blot analyses

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Results and discussion

Large-scale EST analyses of *Ciona intestinalis*

For the last several years, we have conducted large-scale EST analyses of mRNAs expressed in *Ciona intestinalis* embryos and adults (Satou et al. 2002a, 2002b). The first series of the analyses included the five developmental stages of fertilized eggs (Nishikata et al. 2001), cleaving embryos (16–110 cell stages) (Fujiwara et al. 2002), tailbud embryos (Satou et al. 2001), swimming larvae (Kusakabe et al. 2002), and young adults about 2 weeks after metamorphosis (Ogasawara et al. 2002), and testis (Inaba et al. 2002). The second series expanded the first one by adding more data on the previously examined developmental stages, and by adding new data on mRNAs expressed in gastrulae/neurulae, and in the gonad (ovary), endostyle, neural complex, heart, and blood (coelomic) cells of the adult. The number of ESTs so far obtained is summarized in Table 1. A total of 480,753 ESTs, comprising 241,519 ESTs of the 3′ end and 239,234 ESTs of the 5′ end, have been obtained. The 3′ ESTs were used to examine the overlap of the clones in order to

Table 1 ESTs of transcripts expressed in *Ciona intestinalis*

Library	Lib ID	5′ EST	3′ EST	Total
Embryogenesis				
Egg	EG	29,810	29,444	59,254
Cleaving embryo	CL	31,156	26,796	57,952
Gastrula/Neurula	GN	23,066	23,475	46,541
Tailbud embryo	TB	30,282	31,209	61,491
Larva	LV	24,282	24,680	48,962
Young adult	AD	28,547	29,138	57,685
Adult organs/tissues				
Gonad	GD	16,048	16,239	32,287
Testis	TS	4,655	4,717	9,372
Endostyle	ES	2,241	2,497	4,738
Neural complex	NC	10,116	10,029	20,145
Heart	HT	12,904	12,414	25,318
Blood cells	BD	28,412	28,596	57,008
Total		241,519	239,234	480,753

Table 2 EST counts of *Ciona* genes in cDNA libraries derived from whole animals/embryos at six different developmental stages

Gene	EG	CL	GN	TB	LV	AD	cDNA cluster	Reference or note
Ci-pem	33	18	17	12	2	0	01544	Yoshida et al. (1996)
Ci-macho1	3	0	0	3	7	0	09919	Satou et al. (2002c)
Ci-ZicL	0	12	10	1	0	0	03695	[a]Imai et al. (2002b)
Ci-Fkh	0	6	4	7	2	0	05495	Corbo et al. (1997)
Ci-sna	0	2	9	0	0	0	02928	Fujiwara et al. (1998)
Ci-Bra	0	2	2	1	0	0	11351	Corbo et al. (1997)
Ci-FGF9/16/20	0	2	2	5	0	0	00935	[a]Imai et al. (2002a)
Ci-MDF	0	0	2	0	0	0	36253	Meedel et al. (1997)
Ci-fibrinogen-like	0	0	0	6	0	0	00347	Hotta et al. (2000)
Ci-Epi1	0	4	15	117	75	3	00171	[a]Chiba et al. (1998)
Ci-TPM3	0	0	3	36	46	1	00660	Chiba et al. (2003)
Ci-Gsx	0	0	0	2	0	0	13979	Hudson and Lemaire (2001)
Ci-engrailed	0	0	0	0	1	0	13509	Imai et al. (2002b)
Ci-meta6	0	1	0	0	1	37	03531	Nakayama et al. (2002)
unknown	8	0	0	0	0	0	13304	No significant similarity with known proteins
unknown	0	10	8	0	0	0	11523	No significant similarity with known proteins
unknown	0	0	0	7	0	0	13254	No significant similarity with known proteins
unknown	0	0	0	7	18	0	06600	Low similarity with claudin proteins (E $>6e^{-12}$)
unknown	0	0	0	0	0	22	02714	No significant similarity with known proteins

[a] These references describe *Ciona savignyi* orthologues.

categorize the cDNAs into independent (non-duplicated) clusters (groups of clones). Altogether, after examining the overlap of all developmental stages and adult organs, the 241,519 3′ ESTs fell into 17,834 clusters. The reading of the *Ciona intestinalis* draft genome has predicted 15,832 protein-coding genes in the genome (Dehal et al. 2002), suggesting that 85% or more transcripts of this ascidian have been identified by the EST analyses.

EST counts and temporal expression pattern of *Ciona* developmental genes during embryogenesis

Because the libraries were not amplified or normalized, the appearance of cDNA clones (or EST counts) occurs in proportion to their abundance at the corresponding developmental stage. Therefore, if the EST counts of a certain gene are determined at the six different developmental stages, they may represent the temporal expression pattern of the gene. Table 2 summarizes the temporal expression patterns of 14 genes so far reported in *Ciona* embryos as well as five developmentally regulated genes, and their EST counts at the six developmental stages. For example, *Ci-pem* is a *Ciona intestinalis* homolog of *Cs-pem* (*Ciona savignyi-posterior end mark*) (Yoshida et al. 1996). *pem* is so named because the maternal transcripts of the gene are localized at the posterior end of fertilized eggs and early embryos. The transcript is segregated into the posterior-most blastomeres at early gastrulae, and eventually inherited by a few endodermal strand cells, which are likely to be the presumptive germ cells (Fujimura and Takamura 2000). Therefore, it is expected that a large amount of *pem* maternal transcripts would be present in eggs, and that this amount would decrease as development proceeds, but that some transcripts would still remain at the larval stage. As shown in Table 2, the EST count of *Ci-pem* was maximum at the egg stage and decreased gradually during embryogenesis, but a few counts were still evident at the larval stage, and the count finally disappeared in the adult. Thus, the EST count of *Ci-pem* corresponded fairly well with the temporal expression pattern of the gene.

Ci-macho1 is a *Ciona intestinalis* homolog (Satou et al. 2002c) of a muscle-determinant gene (*macho-1*) encoding a Zic-related zinc finger transcription factor of another ascidian, *Halocynthia roretzi* (Nishida and Sawada 2001). *macho-1* is expressed only maternally, whereas *Ci-macho1* is expressed maternally and zygotically, and the zygotic expression begins in cells of the CNS at the early tailbud stage (Satou et al. 2002c). As is evident in Table 2, transcripts (as indicated by EST counts) of *Ci-macho1* were detected at the egg stage, not detected at the cleavage and gastrula stages, and then detected again at the tailbud and larval stages. Another example is the *Ci-sna* or *snail* gene of *Ciona intestinalis* (Fujiwara et al. 1998). This gene has been reported to be expressed zygotically and transiently at the cleavage and early gastrula stages in blastomeres giving rise to larval muscle and mesenchyme cells. The EST counts of *Ci-sna* were detectable in the cleaving embryos and gastrulae/neurulae, indicating a good correlation between Northern blot and EST counts.

Ci-Bra is a *Brachyury* gene, which is essential for the notochord differentiation of the ascidian embryo (Corbo et al. 1997). The gene begins to be expressed at the 64-cell stage and its expression is downregulated by the late tailbud stage. Table 2 indicates that the EST of *Ci-Bra* was obtained at the cleavage, gastrulae/neurulae and tailbud stages only. This is accordance with the known temporal expression pattern of *Ci-Bra*. In addition, the *Ci-fibrinogen-like* gene is one of the *Ci-Bra* targeted genes (Hotta et al. 2000) that are expressed in the differentiating notochord cells in the tailbud embryo (Imai et al. 2002a). It had a detectable EST count only at the tailbud stage

Table 3 EST counts in cDNA libraries derived from the *Ciona* adult organ or tissue

Gene	GD	TS	HT	ES	NC	BD	cDNA cluster	Reference
Ci-Nut	14	0	0	0	0	0	01544	Etani and Nishikata (2002)
Ci-CA8	0	4	0	0	0	0	08548	Chiba et al. (2003)
Ci-MHC2	0	0	25	0	0	0	32003	Chiba et al. (2003)
Ci-TPM3	0	0	45	0	0	0	00660	Chiba et al. (2003)
Orphan bHLH	0	0	0	0	4	0	01932	Satou et al. (2003)
FoxI	0	0	0	0	0	5	13471	Yagi et al. (2003)
β-catenin	36	1	4	2	7	1	00321	Imai et al. (2000)
Cs-HIF	3	0	11	0	1	26	02710	Satou et al. (2003)
Orphan Fox	1	0	2	3	3	10	03488	Yagi et al. (2003)
Ci-HMG1/2	9	8	2	0	2	12	00531	Yamada et al. (2003)

(Table 2). This is another example of a good correlation between the temporal expression pattern of the gene and the EST count. Here we describe in detail 6 of the 14 developmentally regulated genes listed in Table 2. For the remaining eight genes, there was also a good correlation between the temporal expression profile determined by Northern blotting or in situ hybridization and the EST counts.

Table 2 also includes five examples of EST counts of developmentally regulated genes whose function is still unknown. For example, the ID 13304 gene encodes a protein with no sequence similarity to known proteins. Its EST count was detectable only in eggs, suggesting that the gene is expressed only maternally. The ID 11523 gene also encodes a protein with no significant similarity with known proteins. Because its EST count was detectable at the cleavage stage only, it is highly likely that this gene is expressed zygotically and transiently in cleaving embryos. Another example is the ID 02714 gene, whose EST count was detectable only in young adults, suggesting that this gene is not expressed during embryogenesis.

EST counts and spatial expression of *Ciona* developmental genes in organs and/or tissues of the adult

In addition to analyzing transcripts expressed at the six developmental stages, we carried out large-scale EST analyses of transcripts expressed in organs and/or tissues of *Ciona* adults (Table 1). Analysis of the EST counts of each gene suggests its spatial expression pattern. For example, Etani and Nishikata (2002) reported that *Ci-Nut* is expressed specifically in the larval neural tube. As shown in Table 3, the EST count of *Ci-Nut* was detectable in the adult gonad, indicating that the gene is also expressed in the gonad and thus may play role(s) in gametogenesis. *Ci-CA8* encodes a type of cytoplasmic actin. The fact that its EST count was only detectable in the testis suggests that it is specifically expressed there, and thus that it may function in sperm formation. Both *Ci-MHC* (*myosin heavy chain*) *2* and *Ci-TPM* (*tropomyosin*) *3* showed high EST count scores in the heart library. At present we have not examined ESTs derived from the body-wall-muscle, and therefore the possibility remains that *Ci-MHC2* and *Ci-TPM3* are also expressed there. However, it is likely that the two genes are strongly expressed in the heart. The EST count of a gene encoding an orphan bHLH protein was detectable in the neural complex, while that of *Ci-FoxI* was detectable in the blood cells (Table 3). Thus, *Ci-orphan bHLH* may be specific to the neural complex, and *Ci-FoxI* specific to blood cells.

On the other hand, the EST counts of *β-catenin*, *Ci-HIF*, *orphan Fox*, and *Ci-HMG1/2* were detectable in various libraries, although the testis library showed no EST counts for *Ci-HIF* or *orphan Fox*, and the endostyle library lacked detectable EST counts for *Ci-HIF* and *Ci-HMG1/2*. These genes are expressed more ubiquitously in order to play roles in various tissues and organs of *Ciona* adults.

It is sometimes difficult to prepare samples of certain isolated tissues or organs without contamination by other tissues. Therefore, the possibility remains that a given library (and therefore the EST counts) contains some transcripts originating from unrelated tissues. Nevertheless, on the whole, the present large-scale EST analysis of mRNAs expressed in various organs and tissues of the *Ciona* adult strongly suggests that the EST counts reflect the spatial expression pattern of a given gene. Further EST analyses of mRNAs expressed in the epidermis, body-wall muscle, pharyngeal basket, and digestive system are now underway to clarify the expression patterns of *Ciona intestinalis* genes.

Conclusion

Altogether, very large-scale EST analyses of *Ciona intestinalis* provide information about the profiles of gene expression. In many cases, these analyses can be used in place of Northern blot analyses. Even if it does not strictly coincide with the result of Northern blot analyses, the profile of the EST counts clearly suggests the temporal and spatial expression patterns of a given gene. In addition, the information about EST counts may be used in various ways. For example, if a gene shows detectable EST counts only at the tailbud stage, it may be sufficient to examine its spatial expression pattern only at this stage by in situ hybridization, without performing in situ hybridization in embryos at other developmental stages.

The draft genome of *Ciona intestinalis* has been sequenced (Dehal et al. 2002). During a series of studies

to identify developmentally regulated genes in the *Ciona intestinalis* genome (e.g., Satou et al. 2003; Wada et al. 2003), we have concluded that the strategy of sequencing the genome is more precise than Southern blot analyses for determining the number of copies of a given gene in the *Ciona* genome. For example, Southern blot analysis of *Ci-Tbx6* yielded only one major band, suggesting that *Ci-Tbx6* is a single-copy gene (Mitani et al. unpublished). In contrast, the *Ciona intestinalis* genome survey demonstrated the presence of four copies of the *Ci-Tbx6* gene (Dehal et al. 2002). This means that Southern blot analysis is not required any longer in this ascidian. In addition, demonstrated by the present study, Northern blot analysis is not generally required any longer to analyze gene expression patterns in *Ciona intestinalis*.

The final goal of our research on *Ciona intestinalis* is first to obtain a complete set of information on the temporal and spatial expression of every developmentally regulated gene and then to discover the molecular pathways that govern the complex network of the expression of these genes. Ultimately, when large-scale in situ hybridization can be performed to cover nearly all the *Ciona intestinalis* genes, *Ciona* may provide an experimental system for exploring the genetic network that governs the formation of the basal chordate body plan.

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