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Genomes and Developmental Control

Transcriptome dynamics in early embryos of the ascidian, *Ciona intestinalis*

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ABSTRACT

Maternally provided mRNAs and proteins direct early development and activate the zygotic genome. Using microarrays, we examined the dynamics of transcriptomes during the early development of a basal chordate, *Ciona intestinalis*. Microarray analysis of unfertilized eggs, as well as 8-, and 16- and 32-cell embryos revealed that nearly half of the genes encoded in the genome were expressed maternally, and that approximately only one-fourth of these genes were expressed at similar levels among eggs obtained from different individuals. Genes encoding proteins involved in protein phosphorylation were enriched in this latter group. More than 90% of maternal RNAs were not reduced before the 16-cell stage when the zygotic developmental program begins. Additionally we obtained gene expression profiles of individual blastomeres from the 8- and 16-cell embryos. On the basis of these profiles, we concluded that the posterior-most localization, which has been reported for over 20 different transcripts, is the only major localization pattern of maternal transcripts. Our data also showed that maternal factors establish only nine distinct patterns of zygotic gene expression at the 16-cell stage. Therefore, one of the main developmental functions of maternally supplied information is to establish these nine distinct expression patterns in the 16-cell embryo. The dynamics of transcriptomes in early-stage embryos provides a foundation for studying how maternal information starts the zygotic program.

Introduction

The earliest developmental program in animal embryos is carried out with maternally supplied information, which subsequently triggers the zygotic program. This transition has been called the maternal-to-zygotic transition (MZT) (Tadros and Lipshitz, 2009). The MZT often coincides with the mid-blastula transition (MBT) (Newport and Kirschner, 1982; Schier, 2007); at the MBT, cell divisions become asynchronous and cell cycles become longer.

In embryos of a basal chordate, the ascidian *Ciona intestinalis*, cell division becomes asynchronous and the duration of cell cycles becomes longer after the 16-cell stage. In addition, 20 regulatory genes are specifically expressed to start zygotic developmental programs at the 16-cell stage (Bertrand et al., 2003; Hamaguchi et al., 2007; Hudson and Yasuo, 2005; Imai et al., 2004, 2006; Shi and Levine, 2008), while the earliest known zygotic gene expression (*FoxA-a* and *SoxB*1) starts at the 8-cell stage. These previous observations suggest that the MZT begins between the 8- and 16-cell stages in the ascidian embryo. In the present study, we examined the transcriptome dynamics of early *Ciona* embryos around the beginning of the MZT to understand how maternal factors begin zygotic programs. We especially focused on the following three points.

First, since no inbred strains of *C. intestinalis* are available, animals with different genetic backgrounds have been used for experiments in this species. While expression patterns of most of the genes that have been examined are highly reproducible in different animals, several genes are known to show variability in expression patterns. For example, the expression of *Otx* in a6.7 blastomeres at the 32-cell stage is seen in 6% of embryos (Tassy et al., 2006). We also empirically know that the expression of *AP2-like-2* in A5.2 at the 16-cell stage (Imai et al., 2004) is not observed in all embryos (see below). In the present study, to examine whether and how extensively such variation is seen in the maternal mRNA population, we examined the transcriptomes of unfertilized eggs obtained from 11 different adult animals.

Second, localized maternal mRNAs and the spatially controlled destabilization of maternal mRNAs are one of the key mechanisms evoking zygotic programs in a spatially regulated manner (Bashirullah et al., 2001). Because ascidian eggs have historically been thought to be typical “mosaic” eggs, extensive efforts have been made to identify localized mRNAs in early embryos, and over 20 localized maternal mRNAs encoded in the nuclear genome have been identified (Nishida and Sawada, 2001; Sardet et al., 2005;

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Sasakura et al., 2000; Satou and Satoh, 1997; Yamada et al., 2005; Yoshida et al., 1996). These mRNAs are all localized in the posterior pole of the embryo, and therefore they are called posterior end mark (pem) RNAs (or postplasmic RNAs). In an attempt to identify localized RNAs distinct from pem RNAs, a previous study compared the animal half of the 8-cell embryo with the vegetal half, and also the posterior vegetal cells of the 8-cell embryo with the remaining part of the embryos using microarrays (Yamada et al., 2005). However, it remains unclear whether there are maternal mRNAs localized in other subcellular regions or in particular cells of early *Ciona* embryos. For instance, mRNAs localized in the anterior animal cells would not have been identified in this previous microarray study (Yamada et al., 2005). In order to address this ongoing question, here, we analyzed the transcriptomes of individual blastomeres from the 8-cell and 16-cell embryos.

Third, localized and unlocalized maternal gene activity initiates zygotic gene activity. As described earlier, previous comprehensive expression profile assays of regulatory genes identified 20 zygotically activated genes at the 16-cell stage (Bertrand et al., 2003; Hamaguchi et al., 2007; Hudson and Yasuo, 2005; Imai et al., 2004, 2006; Shi and Levine, 2008) (see Fig. S5A). There were only seven variations on the expression profiles of these 20 genes, a much smaller number than the theoretically possible upper limit of 255 ($=2^{(16/2)}-1$: the ascidian embryos are believed to be bilaterally symmetrical). This observation indicates that maternal factors set up a simpler pre-pattern to restrict subsequent patterning decisions to a subset of options. However, it still remains unclear whether these seven identified patterns of gene expression represent the entirety of the early-stage zygotic expression patterns. To understand how maternal factors initiate zygotic programs, in the present study, we tried to obtain the whole repertoire of zygotic genes initially activated by maternal factors.

Materials and methods

C. intestinalis embryos and cDNA clones

All of the *C. intestinalis* adults we used in the present study were raised in the sea near the Maizuru Fisheries Research Station of Kyoto University under the National Bio-Resource Project for *Ciona*, and were maintained in aquaria. Eggs from batches c and d (Fig. 1A) were pushed out by hand from their gonoducts. For the rest of the animals, eggs and sperm were surgically obtained from gonoducts. Unfertilized eggs were collected after dechorionation. Embryos were dechorionated after insemination, and reared at 18 °C in filtered seawater. We kept a fraction of embryos at each sampling for checking the ratio that they developed into morphologically normal larvae. We used only eggs and embryos, for which at least 60% of the cohort developed into normal larvae (Table S1). cDNA clones were obtained from our EST clone collection (Satou et al., 2005). Blastomeres were isolated with a fine glass needle under a binocular microscope, and isolated blastomeres were immediately stored in RNAlater (Ambion) until RNA extraction.

Microarray

Total RNA was prepared from unfertilized eggs, embryos and isolated blastomeres with an RNeasy Mini Kit (QIAGEN). After checking the quality of the extracted RNA with a Bioanalyzer 2100 (Agilent), 50 ng of RNA was amplified and labeled with Cy3 using a Low Input Quick Amp Labeling Kit (one-color kit; Agilent Technologies). We designed two microarrays, manufactured by Agilent Technologies, which contained 39,652 and 218,674 non-overlapping probes covering 15,243 and 15,255, respectively, out of 15,274 KH gene models (version 2010) (Satou et al., 2008a) (we call them 40k- and 220k-arrays; GEO accession numbers: GPL16870 and GPL16886). We followed the manufacturer's instructions for hybridization and washing. Fluorescence intensity was scanned with a G2505C microarray scanner (Agilent Technologies) and quantified with Agilent Feature Extraction software (version 10). Some probes that were judged beyond analysis by the software were eliminated from the following analysis. The microarray data is deposited in the GEO database under the accession number GSE45575.

Raw signal values were normalized for comparison among different arrays on the basis of an assumption that the majority of genes are not differentially expressed. This assumption is commonly made, and is not likely to be violated in our experiment. For calculations, we first excluded both the 30% of probes exhibiting largest differences between the arrays, and the probes that corresponded to the top and bottom 5% with respect to signal strength. Then, the average signals of the remaining probes were calculated. Next, we adjusted all signal values so that the average signals were equal. After this normalization among arrays, we calculated the average signals for each gene; the 40k- and 220k-arrays contained an average of 2.6 and 14.3 probes per gene, respectively. The sensitivities, and thus signal strengths, differed among the probes for a single gene. Therefore, when we performed statistical tests by using multiple probes for a single gene as technical replicates, we normalized the signal values of these probes as follows. First, we summed up all of the values for each probe across arrays. Second, for every gene, the maximum value among the sums was selected, and the signal values for each probe were adjusted so that the sums of the values were equal to this maximum value. All values were log-transformed for statistical tests. Gene ontology analysis was performed with the Blast2Go program (Conesa et al., 2005). The enrichment analysis with the two-tailed Fisher's exact test is implemented in this software.

In situ hybridization and RT-qPCR

DIG-RNA probes for whole-mount in situ hybridization (WMISH) were synthesized by in vitro transcription with T7 RNA polymerase. A detailed procedure for WMISH has been previously described (Imai et al., 2004).

For reverse transcription and quantitative PCR (RT-qPCR), the same amount of total RNA from five different batches was reverse transcribed by an MMLV reverse transcriptase (Invitrogen). Quantitative PCR was performed using a StepOnePlus PCR machine with the SYBR green method (Invitrogen). The same cDNA pools were used for all reactions. Amplification of a specific product in each reaction was confirmed by determining a dissociation curve. The following primers were used: 5′-AGTTACAGCACCCAGAGAACCAA-3′and 5′-CGTCAATGAGCCGTTTTGTG-3′ for KH.C1.29; 5′-GGAA ATGGACGTTGACCAGAGA-3′ and 5′-GGCGAGAGGAGCGGACTT-3′ for KH.C7.98; 5′-AGTGACGTCGGCGAGGAA-3′ and 5′-CCAAATCGT-TAACCAAGTACTCTGTGT-3′ for KH.C3.605; 5′-TAGGCTTAGCGGAG CCACAA-3′ and 5′-CACACACCGGATCGAATACG-3′ for KH.C4.191; 5′-GGTCCTCCTGGGAATAAAGGTTT-3′ and 5′-TGGGAAGCGTATGAA GTTGCT-3′ for KH.C4.36; 5′-TGCTTACGCCTGGAATGTCA-3′ and 5′-CAAGCACTGTGCCCTGAATG-3′ for KH.C9.710; 5′-TCGGAGCCAGT-GAGCATCT-3′ and 5′-TCGTGCATTCCTCCAACCAT-3′ for KH.C11.314; 5′-AAGAAATGCAATCACATACTGCAATAG-3′ and 5′-TAGCAGACATCG GTTCTACGATTC-3′ for KH.S1081.1; 5′-CTTGTGGGCCTGGGTGTAGA-3′ and 5′-TGACATCCCGCCACACACT-3′ for KH.S1462.1; 5′-ACCA-GAGTTACGGCCATCATTAC-3′ and 5′-ACTCGCTGATGATAAACTTCCAT GT-3′ for KH.S606.2.