Genomes & Developmental Control

Genome-wide network of regulatory genes for construction of a chordate embryo☆

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Received for publication 7 November 2007; revised 29 December 2007; accepted 9 January 2008

Available online 26 January 2008

Abstract

Animal development is controlled by gene regulation networks that are composed of sequence-specific transcription factors (TF) and cell signaling molecules (ST). Although housekeeping genes have been reported to show clustering in the animal genomes, whether the genes comprising a given regulatory network are physically clustered on a chromosome is uncertain. We examined this question in the present study.

Ascidians are the closest living relatives of vertebrates, and their tadpole-type larva represents the basic body plan of chordates. The Ciona intestinalis genome contains 390 core TF genes and 119 major ST genes. Previous gene disruption assays led to the formulation of a basic chordate embryonic blueprint, based on over 3000 genetic interactions among 79 zygotic regulatory genes. Here, we mapped the regulatory genes, including all 79 regulatory genes, on the 14 pairs of Ciona chromosomes by fluorescent in situ hybridization (FISH). Chromosomal localization of upstream and downstream regulatory genes demonstrates that the components of coherent developmental gene networks are evenly distributed over the 14 chromosomes. Thus, this study provides the first comprehensive evidence that the physical clustering of regulatory genes, or their target genes, is not relevant for the genome-wide control of gene expression during development.

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Keywords: Chordate early embryos; Cell fate determination; Networks of regulatory genes; Chromosomal positioning; Genome-wide control

Introduction

Genes encoding transcription factors (TF) and those encoding cell signal transduction molecules (ST) play pivotal roles in the pattern formation of the embryo and specification and subsequent differentiation of embryonic cells (Davidson, 2001, 2006; Carroll et al., 2001). A big challenge in the era of genome science and systems biology is to explore how the expression of these developmental genes is integratively and/or coordinately controlled. Recent studies have unveiled genetic cascades and/or regulatory networks of developmental genes during the formation of three germ layers of sea urchins (Davidson et al., 2002), mesoderm specification of frog embryos (Loose and Patient, 2004; Koide et al., 2005), dorso-ventral patterning of the Drosophila embryo (reviewed by Stathopoulos and Levine, 2005), vulva formation in Caenorhabditis elegans (Inoue et al., 2005), and early fate determination of the Ciona intestinalis embryo (Imai et al., 2006). These studies show us a wired scope of expressional control of TF genes and ST genes in animal development (Levine and Davidson, 2005; Davidson, 2006).

However, it is largely unknown how the genetic regulatory networks of developmental genes are controlled at the chromosomal level (Cremer and Cremer, 2001). Genes that are expressed in most tissues (housekeeping genes) have been reported to show strong clustering in the human genome (Lercher et al., 2002). Similarly, the housekeeping genes in Caenorhabditis elegans also show more chromosomal clustering than tissue-specific genes (Pauli et al., 2006). One attractive concept is that the genes comprising a given regulatory network are physically clustered on a chromosome, and that the linear
order of the genes influences the genome-wide control of gene expression. Is the idea of the physical clustering of housekeeping genes applicable to genes which compose a certain regulatory network for a specific event of embryonic development?

*C. intestinalis* is a premiere model system for elucidating the genome-wide control of chordate embryogenesis (Satoh et al., 2003; Shi et al., 2005). Ascidians, a member of the urochordates or tunicates, are the closest living relatives of vertebrates (Bourlat et al., 2006; Delsuc et al., 2006), and their tadpole larva shares a common body plan with vertebrates, including a notochord centered in the tail that is flanked dorsally by the nerve cord, laterally by muscle, and ventrally by endoderm (Satoh, 1994, 2003; Nishida, 2002). The ascidian larva is composed of only ∼2600 cells, and embryonic cells are determined to follow specific cell fates during early embryogenesis between the 16-cell and early gastrula (110-cell) stage (Nishida, 1987).

The complete genome sequence assembly of approximately 160 Mb for *C. intestinalis* suggests that the ascidian contains the basic ancestral complement of genes involved in development and cell signaling of chordates including vertebrates (Dehal et al., 2002). Further analyses of developmentally relevant genes in the *Ciona* genome, together with over 670,000 expressed sequence tags (ESTs) and 6700 full-length and sequenced cDNAs, permits the comprehensive identification of known and potential regulatory genes (Satou and Satoh, 2003).

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**Table 1**

Chromosomal localization of 373 core transcription factor genes and 111 major cell-signaling molecules genes in *Ciona intestinalis*

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</tr>
<tr>
<td>Total</td>
<td></td>
<td>509 484 65 33 60 42 35 27 29 28 27 25 46 26 7 34</td>
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NR, nuclear receptor.

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Fig. 1. Examples of BAC mapping by two color FISH. (A) With strong DAPI-stained bands (white arrowheads) and previous mapped BAC (GECi37..J17; red arrowheads), new BAC (GECi31..J11; green arrowheads) was mapped onto the long arm of chromosome 1. (B) The broad signals of BAC GECi33..J05 (red arrowheads) containing histone genes were detected onto the short arm of chromosome 2 with GECi38..J22 signals (green arrowheads). On the left interphase nucleus, the signals were observed in two broad territories. Scale bar: 5 μm.
Namely, the Ciona genome contains approximately 670 TF genes, including 390 TF genes with a well-conserved motif. The TF genes with a well-conserved motif, a total of 252, include 45 genes for bHLH family, 26 for bZIP family, 15 for Ets family, 27 for Fox family, 92 for homeobox family, 18 for nuclear receptor family, and 8 genes for T-box family (Imai et al., 2004; Satou et al., 2003a; Takatori et al., 2004; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003). The Ciona genome also contains 138 genes with potential TF activity. In the present study, we have focused on these 390 (252+138) core TF genes (Table 1). In addition, the present study has focused on 119 major ST genes, including 6 for hedgehog signaling, 5 for JAK/STAT genes, 3 for NFκB, 19 for Notch cascade, 42 for RTK/MAPK, 19 for TGF-β, and 25 for Wnt signaling (Table 1).

The cellular and genetic simplicity of the Ciona system mentioned above may provide the first opportunity to superimpose gene networks onto chromosomes. With this aim, we have previously mapped approximately 65% of the genome sequence information onto the 14 pairs of C. intestinalis chromosomes with the aid of two-color fluorescent in situ hybridization (FISH) using 170 bacterial artificial chromosome (BAC) clones (Shoguchi et al., 2006). However, the short arms of chromosomes 2, 7 and 8 were not characterized yet. Using two-color FISH, the present study further mapped the genome sequence of 103 selected BAC clones with the aid of bioinformatics from the newly-assembled C. intestinalis sequences (genome browser ver 2), and determined the position on the chromosomes of most of the core TF and major ST genes. Then, we investigated whether genes comprising given networks are physically clustered on a chromosome or the components of coherent developmental gene networks are evenly distributed over the 14 chromosomes.

**Materials and methods**

**Biological materials**

Handling of gametes and embryos of C. intestinalis was carried out as described previously (Shoguchi et al., 2005). Neurulae and initial tailbud embryos were used in the present study.
Fig. 4. Chromosomal map of 373 core transcription factor genes and 111 major cell signaling molecule genes in Ciona intestinalis. Families of transcription factors are shown by discs with different colors; cell signaling molecules are shown by arrowheads with different colors (bottom-right corner). Centromeric regions are shown by dark blue dashed-lines. Red dashed-lines indicate three rDNA cluster regions, green-dashed lines a histone cluster region. Blue dashed-lines indicate unmapped regions. The left and right vertical lines of each chromosome indicate the 5' to 3' and 3' to 5' alignment, respectively. The telomeric regions on the short arms of chromosomes 12, 13 and 14 are ordered arbitrarily. Clustered genes in other chordate genomes are shown using color code. The enclosed genes in red lines are those analyzed as elements of the regulatory network for chordate body plan construction (Figs. 5A and 6).
Two-color FISH

Metaphase spread was carried out as described in Shoguchi et al. (2005). FISH was carried out as follows (Shoguchi et al., 2006). Briefly, the hybridization mix consisted of 50% formamide, 2× SSC, 10% dextran sulfate, 0.1 μg/μl of sheared C. intestinalis sperm DNA, and 0.15% SDS. An aliquot containing about one-eighth of each labeled probe (derived from 1 μg of BAC DNA) was added to the hybridization mix. A total of 15 μl of this mix was used per approx. 300 mm² (16 mm × 19 mm). Hybridization was performed for about 56 h at 42 °C. Signals from biotin-labeled probes were amplified and detected with sequential applications of avidin FITC DCS (Vector Laboratories), biotinylated anti-avidin D (Vector Laboratories) and then avidin FITC DCS again. For detection of DIG-labeled probes, anti-DIG-rhodamine Fab fragments (ROCHE) were applied and then Texas Red anti-sheep (Vector Laboratories).
Image processing was done with ADOBE Photoshop 6.0. Images were collected with a Zeiss epifluorescence microscope equipped with an Axiocam camera.

**Naming of C. intestinalis genes**

Names of genes for transcription factors and cell signaling molecules are described in Satou and Satoh (2005), in which the genes were annotated by using gene models of *C. intestinalis* Ghost Database (Satou et al., 2005) (http://ghost.zool.kyoto-u.ac.jp/index1.html) and cDNA sequences, their orthology being examined molecular phylogenetic analyses (Hino et al., 2003; Satou et al., 2003a, 2003b; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003; Takatori et al., 2004).

**Bioinformatics**

Previous chromosomal mapping of 170 BAC clones was carried out based on the *C. intestinalis* ver. 1 assembly (Shoguchi et al., 2006). In the present study, we first mapped the genes according to the *C. intestinalis* ver. 1 assembly and construction of joined-scaffolds by using BAC end sequences and cosmid end sequences (Shoguchi et al., 2006; http://genome.jgi-psf.org/Cioin2/Cioin2.home.html). BACs corresponding to the terminals of joined-scaffolds were selected. Then, we examined the previous mapping again. The mapping of developmental genes in several regions of chromosomes may be a halfway due to incomplete genome sequence assembly but additional sequence data do not appear to change most of the present mapping. It was confirmed by comparing with the recently released *C. intestinalis* ver. 2 assembly (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html).

**Results and discussion**

**Chromosomal mapping of the C. intestinalis genome information**

Previous karyotype analyses as well as a chromosomal mapping study of 170 BAC clones showed that the short arms of chromosomes 4, 5, and 6 exhibit high polymorphism with rDNA clusters (Shoguchi et al., 2005, 2006). The short arm of chromosome 2 also exhibited high polymorphism in its length, and no BAC clones were mapped on this arm in the previous studies. We speculate that the short arm of chromosome 2 contains some other clustering genes such as those encoding histones. In the present mapping of an additional 103 BAC clones (for example, Fig. 1A), BACs GECi46_c05 (hereinafter, GECi designation was omitted in the clone ID), 13_f05, and 18_c15 were selected because the former contains 5S rDNA and histone genes. The three BACs were found on the short arm of chromosome 2, indicating the presence of a cluster of SS rDNA genes and histone genes on this arm (Fig. 1B, Supplementary Tables S1 and S2).

The previous studies also failed to map BAC clones on the short arms of chromosomes 7 and 8, and found only one BAC (20_b17) on that of chromosome 13, although considerable numbers of BAC clones were mapped on all the other arms (Shoguchi et al., 2006). Of the 103 BACs examined in the present study, BACs 20..e05 and 36..j08, BACs 38..g14, 47..f07 and 42..h16, and BACs 23..i17 and 46..e02 were mapped on the short arm of chromosomes 7, 8, and 13, respectively (Fig. 2, Supplementary Table S2). Thus, all arms of the 14 pairs of *C. intestinalis* chromosomes have now been characterized with considerable genome sequence information (Fig. 2). Only Scaffold_437 (ver.1) was mapped using the best similarity to *C. intestinalis* ver. 2 assembly by the BLASTN algorithm (Altschul et al., 1990). Two ver. 1 Scaffolds, 24 and 104, are also found on different chromosomes (Supplementary Table S3). In total, approximately 82% of the genome sequence information have been mapped on all arms of the *Ciona* chromosomes (Fig. 2; Supplementary Table S3).

**Distribution of core TF and major ST genes in the Ciona genome**

The previous and present large-scale mappings have revealed the chromosomal localization of 373 of the 390 core TF genes (96%) and 111 of the 119 major ST genes (93%) (Table 1; Figs. 3 and 4; Supplementary Table S4). These analyses suggest several characteristic features of chromosomal localization of the regulatory genes, as summarized below.

First, it has been shown that *Hox* genes of *C. intestinalis* are not located within a single gene complex, and the *Hox7*, 8 and 9 genes are undetectable probably due to degradation of the genes during retrograde evolution of the ascidians (Dehal et al., 2002), although there are short stretches of linearity for certain *Hox* genes (Ikuta et al., 2004). The present study revealed that *Hox1*, 2, 3, 4, 5, 6, and 10 are on the long arm of chromosome 1, while *Hox12* and 13 are in the middle of the long arm of chromosome 7 (Fig. 4). *Hox1* is present near the centromere, *Hox2, 3* and 4 are clustered near the telomeric region, while *Hox10*, 5 and 6 are present at the end of the telomeric region (Fig. 4). Therefore, it is now evident that the *Hox* genes of *C. intestinalis* were split into chromosomes 1 and 7. The genome of a larvacean, *Oikopleura dioica*, also appears to have lost *Hox3, 5, 6, 7*, and 8, and remaining *Hox* genes are scattered in the genome showing a rather severe breakdown of the *Hox* cluster (Seo et al., 2004). Nevertheless, they still retain colinearity in their expression pattern in the larva. It is likely that the complete set and strict linearity of *Hox* cluster genes are not indispensable for embryonic development of tunicates (Satoh et al., 2006).

Second, *Para-Hox* genes are also found on different chromosomes, *gxs* on the long arm of chromosome 2 and *CdX* and *IPF1* on chromosome 14 (Fig. 4). Similarly, partial *NK* gene clusters seen in the amphioxus and human genomes (Luke et al., 2003) are dispersed in *C. intestinalis* (Fig. 4). Moreover, *Fox* transcription factor genes that are clustered in both the
amphioxus and human genomes (Wotton and Shimeld, 2006), are not clustered in Ciona (Fig. 4). Therefore, the Ciona genome has lost many of the gene arrangements that are conserved among metazoans, which may be why the Ciona genome has less synteny with vertebrate genomes (Dehal et al., 2002).

Third, genes that arose by duplication of a single ancestor appear to be clustered in the genome. For example, Foxlc, Foxlb and Foxla are localized in the middle of the long arm of chromosome 8, and EphrinAa, EphrinAb, EphrinAc, and EphrinAd are clustered near the centromere on the long arm of chromosome 3 (Fig. 4). On the other hand, genes arising from five lineage-specific duplication events, including E(spl)/hairy, p53/p73, Evx, LAG1-like and STAT, have dispersed between chromosomes 1 and 3 (Fig. 4).

This genome-wide mapping of the Ciona regulatory genes onto the chromosomes also reveals that the 373 core TF genes and 111 major ST genes appear to be distributed rather evenly over the 14 chromosomes (Fig. 4). This was the case for each gene family (Table 1; Fig. 3). For example, the Ciona genome contains 92 homeobox genes, and 91 of them were mapped in the present study (Table 1). Eighteen of them are present on chromosome 1, three on chromosome 2 and 12 on chromosome 3. The remaining 58 genes were mapped on chromosomes 4 through 14, suggesting no special localization or clustering of homeobox genes in the Ciona genome (Fig. 3). A similar scenario is seen for the 111 major ST genes, which include coordinated expressed signaling components in common signal transduction pathways. For example, 18 Notch ST genes are distributed over seven chromosomes, each of them containing one to five genes (Table 1; Fig. 3). Although four of the five JAK/STAT genes were mapped on chromosome 3, they do not necessarily map near one another (Fig. 4). These observations reveal that regulatory genes are widely distributed throughout the genome (Table 1). The chromosome 1, which is the longest of all chromosomes and is composed of about 14-Mb euchromatin, contains 51 core TF and 14 major ST genes. However, these numbers are roughly proportionate to the fraction of the genome contained on this chromosome (Figs. 3 and 4).

Chromosomal localization of 76 zygotic regulatory genes for construction of a chordate embryo

Previous studies have shown that in Ciona embryos, 65 TF genes and 26 ST genes are zygotically expressed between the 16-cell and early gastrula stage, at the time when naive blastomers are determined to follow specific cell fates (Imai et al., 2004). Systematic gene disruption assays with 76 of the 91 zygotic genes have produced more than 3000 combinations of gene expression profiles, which allow computational visualiza-
expressed at the 32- to 110-cell stages (including Eph1, SoxB1, COE, sFRP3/4-b, Hex, and others; Fig. 6, right column). These 249-paired interactions include 14 on the same chromosome and 22 auto-regulatory interactions; the remaining 213 represent inter-chromosomal interactions (Figs. 5 and 6; Supplementary Table S5). The low ratio of the same chromosome interactions to non-autoregulatory interactions indicates upstream–downstream genes are dispersed (Table 2), while one of upstream gene, ZicL, is involved in three same chromosome interactions (Fig. 5A). There are several examples of fixed interactions from one chromosome to another. For example, an upstream gene (FGF9/16/20) on chromosome 2 regulates downstream genes on chromosomes 1 and 5, while FoxA-a on chromosome 11 regulates target genes on chromosome 1. However, in general, the interactions are distributed over the genome and no special tendency was found for chromosome-level interactions (Fig. 6). Therefore, we concluded that the clustering of developmental control genes is not essential for the networks establishing the blueprint of the chordate body plan.

Recent studies have elucidated gene regulatory networks (GRNs) that control the embryogenesis of sea urchins (Davidson et al., 2002), frogs (Loose and Patient, 2004; Koide et al., 2005), Drosophila (Stathopoulos and Levine, 2005), Caenorhabditis elegans (Inoue et al., 2005), and C. intestinalis (Imai et al., 2006). The comparative analysis of GRNs provides insights into shared features, such as feed forward loops and auto-regulation (Levine and Davidson, 2005; Davidson, 2006). The present study provides the first attempt to integrate GRNs with genome organization. The 373 core TF genes and 111 ST genes examined in this study are generally dispersed among all 14 chromosomes (Figs. 3 and 4). We therefore conclude that chromosomal clustering is not an essential feature of the genetic cascades controlling the formation of the chordate body plan. Although the present study does not support the idea, it is still possible that the clustering of genes is responsible for spatially and temporally coordinated expression of structural genes associated with the final phase of cell differentiation (Boutanaev et al., 2002; Lercher et al., 2002; Roy et al., 2002; Pauli et al., 2006). Namely, the conclusion obtained from the study of early embryos is not necessarily applicable to genes that are expressed coordinately in the final phase of differentiation in chordate embryos. Future studies will address the possibility of linked “gene batteries” underlying the terminal differentiation of key tissues such as the tail muscles.

It has been suggested that tunicate genomes have lost a considerable number of genes during their evolution, with little genetic synteny when compared to vertebrates (Dehal et al., 2002). Nevertheless, the tunicate genome is sufficiently well organized to produce a tadpole-like larva representing an approximation of the ancestral chordate body plan. The C. intestinalis genome shows mosaic DNA methylation patterns (Simmen et al., 1999). Although CpG methylation appears to function to suppress spurious transcriptional initiation within infrequently transcribed genes (housekeeping genes), it is likely that CpG methylation is not necessarily involved in the developmental control of gene expression (Suzuki et al., 2007). The present study suggests that the key to understanding the genome-wide control of development is not in the two-dimensional structure of the genome. We propose the gene regulation network at early development is hidden in the three- and four-dimensional structure of the genome (Cremer and Cremer, 2001). We expect that Ciona will provide crucial insights into these complex processes due to its remarkable cellular and genetic simplicity.

Acknowledgments

We thank Yutaka Satou, Kaoru S. Imai, and Takeshi Kawashima for providing unpublished data concerning gene annotations and gene regulatory networks and useful suggestions. We appreciate Mike Levine for critical reading of the manuscript. Kazuko Hirayama and Maizuru Fisheries Research Station of Kyoto University are acknowledged for their help in culturing of Ciona intestinalis, which was supported by the National Bioresource Project (NBRP), MEXT, Japan. This research was a part of Core Research for Evolutional Science and Technology (CREST) project by the Japan Science and Technology Agency, Japan. This research was also supported by a Grant-in-Aid from MEXT, Japan to NS (17018018).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.009.

References

Davidson, B., Shi, W., Beh, J., Christiaen, L., Levine, M., 2006. FGF signaling

Table 2

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Note that the intrachromosomal rate is lower than 1/14 (7.1%).
delineates the cardiac progenitor field in the simple chordate, *Ciona intestinalis*. Genes Dev. 20, 2728–2738.


