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Fluorescent *in situ* Hybridization to Ascidian Chromosomes

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ABSTRACT—The draft genome of the ascidian *Ciona intestinalis* has been sequenced. Mapping of the genome sequence to the *Ciona* 14 haploid chromosomes is essential for future studies of the genome-wide control of gene expression in this basal chordate. Here we describe an efficient protocol for fluorescent *in situ* hybridization for mapping genes to the *Ciona* chromosomes. We demonstrate how the locations of two BAC clones can be mapped relative to each other. We also show that this method is efficient for coupling two so-far independent scaffolds into one longer scaffold when two BAC clones represent sequences located at either end of the two scaffolds.

Key words: ascidians, fluorescent *in situ* hybridization, mapping of genes

INTRODUCTION

Ascidians, or sea squirts, are marine invertebrate chordates. Their critical evolutionary position as primitive chordates and the simplicity of their embryogenesis have attracted developmental and evolutionary biologists since the turn of the 20th century (Satoh, 1994; Nishida, 2002). Recently, there has been a surge of interest in the ascidian tadpole as a model system for evolutionary developmental biology (Satoh, 2003). Elucidation of the molecular mechanisms underlying the embryogenesis of ascidians (basal chordates) is essential for understanding the ancestral developmental program of chordates, including vertebrates. In addition, at the end of 2002, the draft genome of *Ciona intestinalis* was sequenced (Dehal *et al*., 2002). The availability of the genomic sequence information together with a large quantity of cDNA information (Satou *et al*., 2002a, 2002b) makes *Ciona* an attractive emerging model for whole-genome analyses of the expression and function of developmental genes (Satoh *et al*., 2003).

Analyses of the *Ciona intestinalis* draft genome show that ~117 Mbp of euchromatic sequences predict 15,852 protein-coding genes (Dehal *et al*., 2002). Recent efforts to precisely annotate developmentally relevant genes (Satou *et al*., 2003; Wada *et al*., 2003; Yagi *et al*., 2003), genes encoding the core components of cell death machinary (Terajima *et al*., 2003), genes for innate immunity (Azumi *et al*., 2003), and genes associated with the endocrine system (Campbell *et al*., submitted) have revealed various features of the ascidian genes during the evolutionary transition from non-chordate to chordate animals. The *C. intestinalis* draft genome consists of ~3,500 scaffolds, although nearly 60 Mbp sequences are covered with 119 scaffolds longer than 190 kb (Dehal *et al*., 2002). The number of haploid chromosomes of *C. intestinalis* is 14 (Colombera and Lazzaretto-Colombera, 1978). Given these circumstances, it is key to map the scaffolds to the chromosomes.

Although efforts are being carried out to establish a linkage map of *C. intestinalis* with the aid of polymorphism between different populations (Kano, 2001), fluorescent *in situ* hybridization (FISH) is one of the most promising ways to map the genome sequences to the chromosomes. We

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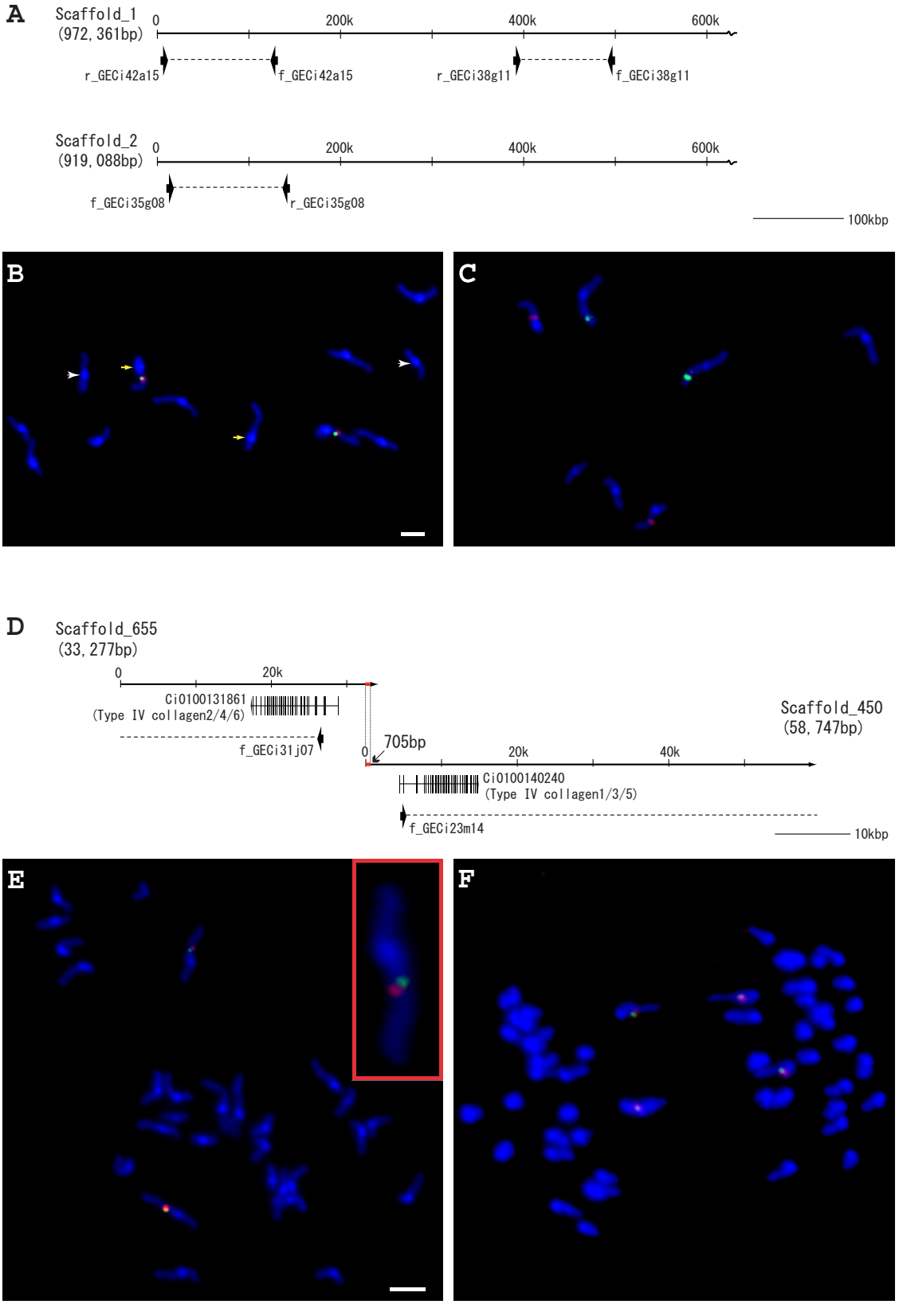
have already made BAC libraries of *C. intestinalis* (Kobayashi *et al.*, 2002). Sequencing of BAC ends permits their assignment to the scaffolds, and the BAC clones can also be used to prepare probes. Here we described a robust and reliable method for mapping genome sequences to *C. intestinalis* chromosomes using FISH to metaphase spreads pre-



pared from embryos. The method can be used with two BAC probes simultaneously (two-color FISH) for comparing chromosomal locations of two scaffolds. We also show that this method is efficient for coupling two so-far independent scaffolds into one longer scaffold when two BAC clones represent sequences located at either end of the two scaffolds.

MATERIALS AND METHODS

Metaphase preparation

Jha *et al*. (1995) and Castro and Holland (2002) demonstrated that embryos of marine invertebrates provide a good source of rapidly dividing cells. We therefore used ascidian embryos to obtain metaphase spreads. The *Ciona intestinalis* were maintained in Maizuru Fisheries Research Station, Field Science Education and Research Center of Kyoto University. Insemination, dechorionation of fertilized eggs and culturing of embryos were carried out as described by Satou *et al*. (2001) with the culturing temperature at 15°C. Embryos at three different developmental stages (32-cell embryos, 64-cell embryos and gastrulae) were used to produce metaphase spreads, although treatment of 32-cell embryos and 64-cell embryos with 0.05% colchicine (Sigma) in seawater caused aggregated and disorganized chromosomes. After treatment with colchicine for 20–30 min, embryos were transferred to a 1.5-ml microfuge tube, to which cold methanol:glacial acetic acid (3:1) fixative was added (chilled on ice). The fixative was changed twice over a period of 1 hr and once after overnight (embryos kept at 4°C). The specimens were then stored in fixative at 4°C.

To prepare slides of metaphase spreads, an excess (x5) of 60% acetic acid was added to a microfuge tube containing 50-100 embryos. One min later, the embryos were pipetted gently and left for about 20 min until the embryonic cells started to burst. Then this mixture was dropped onto prewarmed (48°C) ethanol-cleaned microscope slides (50 µl per slide), and the mixture was blown and expanded with a Pasteur pipette, left until dry (30 min) and then incubated at 65°C for 3 hr in a clean incubator.

DNA isolation and probe generation

The libraries of BAC (bacterial artificial chromosome) clones of *C. intestinalis* were constructed by Kobayashi *et al*. (2002). Five BAC clones, GECi42a15, GECi38g11, GECi35g08, GECi31j07, and GECi23m14, were used in the present study (Fig. 1). To select the clones, *C. intestinalis* genome sequences (www.jgi.doe.gov/ciona4.ciona4/html) and BAC end sequences (http://ghost.zool.kyoto-u.ac.jp/indexr1.html) were extensively referred to. BAC DNA was isolated by alkaline lysis followed by RNase treatment and phenol:chloroform extraction and labeled with biotin or digoxigenin (DIG) respectively using nick translation kits from ROCHE.

Fluorescent *in situ* hybridization (FISH)

The FISH protocol was based on the procedure of Castro and Holland (2002) and modified as follows. Slides were initially incubated in 2×SSC at 37°C for 15 min, followed by dehydration through an ethanol series (5 min each in 70%, 95% and 100% ethanol at –30°C) and left to air dry. To denature the chromosomal DNA, the slides were incubated in 70% formamide in 2×SSC (72°C, 3 min), after which the samples were dehydrated (70% and 95% at –30°C). To denature the DIG and biotin-labeled probes, they were added to hybridization mix (50% formamide, 2×SSC, 10% dextran sulfate, 6 µg sheared *C. intestinalis* sperm DNA, 0.15% SDS) at a final concentration of 0.5 to 2 ng/µl, allowing a total of 30 µl per slide. Denaturation was done at 80°C for 15 min, before chilling on ice. The probe mix was incubated for 15 min at 37°C and applied to the slide, and the slide was covered with parafilm (American National Can). For two-color FISH, both probes were added to the same hybridization mix. Slides were then incubated for 32 hr at 43°C in a water-saturated atmosphere. After hybridization, slides were immersed in 2×SSC (43°C, 5 min) to remove the parafilm, then twice in 20% formamide, 0.1×SSC (43°C, 5 min) and twice in 2×SSC (43°C, 5 min). All washes were static. Slides were then immersed for 30 min and 5 min at 37°C in 4×TNFM (4×SSC, 0.05% Tween20, 5% skim milk (DIFCO); centrifuged for 10 min at 3,800 rpm to remove particles before use). Signals from biotin-labeled probes were amplified and detected with sequential applications of avidin FITC DCS (1:200 in 4×TNFM; Vector Laboratories) for 1 hr, biotinylated anti-avidin D (1:100 in 4×TNFM; Vector Laboratories) for 30 min and then avidin FITC DCS again (1:200 in 4× TNFM) for 1 hr. For detection of DIG-labeled probes, anti-DIG-rhodamine Fab fragments (1:200 in 4×TNFM; ROCHE) was applied for 1 hr and then Texas Red anti-sheep (1:100 in 4×TNFM; Vector Laboratories) for 30 min. Slides were washed in 4×TNFM between applications; 3 times in 4×TNFM (40°C, 5 min) after the first, twice in 4×TNFM (40°C, 5 min) after the second, 3 times in 4×TNFM (43°C, 5 min) after third. All three incubations were carried out at 37°C in the dark in a humidified box. Detection of the two labels in two-color FISH was performed simultaneously. Slides were washed with 4×SSC, 0.05% Tween20 twice for 5 min at room temperature and rinsed with PBS. Vectashield mounting medium with DAPI (Vector Laboratories) was applied to the slides for chromosome counterstaining. Images were collected with a Zeiss epifluorescence microscope equipped with an Axiocam camera. Image processing was done with ADOBE Photoshop 6.0.

RESULTS AND DISCUSSION

Chromosome preparation

We examined metaphase spreads of three different developmental stages (32-cell, 64-cell, and gastrula stages), and found that the 32-cell and 64-cell stages yielded a good score of metaphase chromosomes, but the treatment with colchicine resulted in disorganized chromosomes (data not shown). Although gastrula stage embryos contained large numbers of interphase nuclei in contrast with the 32-cell and

Fig. 1. FISH to chromosomes of *Ciona intestinalis.* (A) Three BAC clones, GECi42a15, GECi38g11 and GECi35g08, corresponding to sequences of Scaffolds 1 and 2, were analyzed. Each BAC clone is about 120 kb in length, and the 5' and 3' ends are indicated by arrows. Probes were made from the BAC clones. (B) FISH with GECi42a15 (red) and GECi38g11 (green) BAC probes. Both signals are seen on the same pair of chromosomes, suggesting that the two scaffolds are mapped to a single chromosome. Bar, 2 µm for (B) and (C). (C) FISH with GECi38g11 (red) and GECi35g08 (green) BAC probes. Signals are seen on two different pairs of chromosomes, suggesting that the two scaffolds are mapped to independent chromosomes. (D) A BAC clone, GECi31j07, located at the 3' end of Scaffold 655, which contains a gene encoding Type IV collagen 2/4/6, and a BAC clone, GECi23m14, located at the 5' end of Scaffold 450, which contains a gene encoding Type IV collagen 1/3/5. The 694-bp sequence of the 3' end of Scaffold 655 is identical to that of the 5' end of Scaffold 450 (95%). (E,F) FISH with GECi31j07 and GECi23m14 BAC probes. GECi31j07 (red) labeled with DIG and GECi23m14 (green) labeled with biotin. Bar, 2 µm. Inset in E shows a stretched chromosome with GECi23m14 (green) and GECi31j07 (red) signals at higher magnification. (E) FISH to metaphase chromosomes treated with colchicine and (F) metaphase chromosomes not treated with colchicine.

64-cell embryos, treatment of these stages with colchicine yielded stretched metaphase chromosomes, applicable to mapping by FISH. We found that the FISH probes hybridized with equal efficiency to metaphase spreads and to interphase nuclei (data not shown).

The scoring of chromosomal number per metaphase plate confirmed the previous counts of 28 chromosomes per diploid genome of *Ciona intestinalis* (Colombera and Lazzaretto-Colombera, 1978; Fig. 1E). Although differentiation among the *Ciona* 14 chromosomes (haploid set) is extremely difficult due to their small and similar size, in many cases we obtained metaphase plates with stretched chromosomes by gentle colchicine treatment, and strong DAPI staining in those chromosomes was likely to represent the centromere (Fig. 1B, white arrowheads). If this is the case, several chromosomes look metacentromeric (Fig. 1B, white arrowheads), while others look telocentromeric (Fig. 1B, yellow arrows). In some other animal species, DAPI staining is reported to show characteristic banding patterns, which assist the identification of individual chromosomes. However, no banding pattern was observed in the *Ciona* chromosomes after DAPI staining. Despite the inability to identify each chromosome, we found that genome sequences could be mapped relative to each other, by using combinations of probes in two-color FISH experiments, as described below.

Two-color FISH mapping of two BAC clones relative to each other on the chromosomes

The main aim of the present study was to test the power of FISH for mapping two BAC clones relative to each other onto the *C. intestinalis* chromosomes. First, we performed one-color FISH with a certain BAC probe and analyzed at least 20 metaphase spreads to determine the efficiency of FISH. In every case, we found that the probe hybridized to two different chromosomes per diploid genome, as predicted (data not shown). We then examined two-color FISH with two BAC probes.

In the first experiment, we analyzed three BAC clones, GECi42a15, GECi38g11, and GECi35g08. As shown in Fig. 1A, extensive analyses of the genome sequences and BAC end sequences revealed that GECi42a15 and GECi38g11 correspond to sequences situated in Scaffold 1 (*C. intestinalis* v1.0), and GECi35g08 to sequences in Scaffold 2. If our FISH protocol worked well, a signal of GECi42a15 should be seen on the same chromosome as the signal of GECi38g11. As shown in Fig. 1B, red signals of GECi42a15 and green signals of GECi38g11 were evident on a pair of the same chromosomes, revealing the accuracy of the present two-color FISH protocol. The results also suggest that Scaffold 1 is mapped to the long arm of a telocentromeric chromosome (Fig. 1B).

We next examined the relative location of Scaffolds 1 and 2 on the chromosomes. BAC clone GECi38g11 is present in Scaffold 1, and GECi35g08 in Scaffold 2 (Fig. 1A). If both scaffolds were located on a single chromosome, two signals would be seen in a pair of the same chromosomes. If the two scaffolds were located on different chromosomes, two signals would be seen in two pairs of chromosomes. As shown in Fig. 1C, red signals of GECi38g11 were seen in a pair of the same, telocentromeric chromosomes, while green signals of GECi35g08 were seen in another, different pair of chromosomes. GECi35g08 is located at a region near the telomere of one arm of a metacentromeric chromosome (Fig. 1C). This suggests that Scaffolds 1 and 2 are mapped to two independent chromosomes. In other experiments, we confirmed with two-color FISH that Scaffold 1 and Scaffold 13 are located on the same chromosome (data not shown). Therefore, the present two-color FISH method is useful for the mapping of genome sequences relative to each other.

Two-color FISH method is efficient for coupling two different scaffolds into one longer scaffold

Finally, we examined whether the two-color FISH method is useful for coupling different scaffolds into one longer scaffold. Such an example is shown in Fig. 1D–F. As shown in Fig. 1D, a BAC clone, GECi31j07, corresponds to sequences located at the 3' end of Scaffold 655, which contains a gene encoding Type IV collagen 2/4/6 (Sasakura *et al.*, 2003). Another BAC clone, GECi23m14, corresponds to sequences located at the 5' end of Scaffold 450, which contains a gene encoding Type IV collagen 1/3/5 (Sasakura *et al.*, 2003). Assembly version 4 of the *Ciona* draft genome showed that Scaffold 655 and Scaffold 450 were not contiguous. However, our recent analyses of the scaffold ends demonstrated that a 694-bp sequence of the 3' end of Scaffold 655 is identical to that of the 5' end of Scaffold 450 (unpublished data), suggesting that the two scaffolds are contiguous. If this is the case, signals of the probe from GECi31j07 and those from GECi23m14 should be seen in a pair of the same chromosomes. As shown in Fig. 1E and F, red signals of GECi31j07 and green signals of GECi23m14 were evident on a pair of the same chromosomes. In addition, PCR reactions using two specific primers complementary to each collagen gene and *C. intestinalis* sperm DNA as template yielded a product band of about 13 kbp (data not shown). We therefore conclude that Scaffold 655 and Scaffold 450 are contiguous for making a longer Scaffold.

As mentioned above, the present two-color FISH method is useful for mapping *Ciona intestinalis* genome sequences to the chromosomes. By scaling up the FISH analyses, we would be able to map numerous BAC clones. If such mapping classifies the scaffolds into 14 independent pairs of chromosome, we may be able to obtain the physical map of the *C. intestinalis* genome sequences. This is an indispensable step for genome-wide analyses of the regulatory mechanisms responsible for the expression and function of genes of this basal chordate.

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