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Fluorescent *in situ* Hybridisation to Amphioxus Chromosomes

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ABSTRACT—We describe an efficient protocol for mapping genes and other DNA sequences to amphioxus chromosomes using fluorescent in situ hybridisation. We apply this method to identify the number and location of ribosomal DNA gene clusters and telomere sequences in metaphase spreads of *Branchiostoma floridae*. We also describe how the locations of two single copy genes can be mapped relative to each other, and demonstrate this by mapping an amphioxus Pax gene relative to a homologue of the *Notch* gene. These methods have great potential for performing comparative genomics between amphioxus and vertebrates.

Key words: FISH, chromosomes, telomeres, rDNA, gene mapping

INTRODUCTION

There are around twenty species of amphioxus or lancelet comprising the subphylum Cephalochordata, a taxon considered to be the sister group to the vertebrates or craniates (Wada and Satoh, 1994). Amphioxus also retain a basic chordate morphology and embryogenesis, and a genome uncomplicated by extensive gene duplication (Holland and Garcia-Fernandez, 1996). Because of this unique combination of phylogenetic, developmental and genetic characters, amphioxus has emerged as an important model system for studying the evolution of vertebrate development and the origin of vertebrate genomes. In the past ten years, over 200 scientific papers have been published on amphioxus biology, with the majority of these focusing on the analysis of genes and development. In contrast, there have been very few cytogenetic studies on cephalochordate species, despite the fact that mapping genes to chromosomes is a powerful tool in the study of genome organisation and genome evolution.

Howell and Boshung Jr. (1971) described the diploid chromosome number (2n) as 38 for *Branchiostoma floridae;* the same diploid complement was later found for *Branchiostoma lanceolatum* (Colombera, 1974). For *Branchiostoma belcheri*, the chromosome count has been recently demonstrated to be 36 (Saotome and Ojima, 2001). Amphioxus chromosomes are of small size (0.3 to 3 μm) and morphologically very similar to each other (Howell and Boshung Jr., 1971). The majority of the chromosomes are metacentric or submetacentric with the centromere in a subterminal to terminal position in all of the species (Howell and Boshung Jr., 1971). No clear differentiation between chromosomes has been presented, and no individual genes have been mapped to amphioxus chromosomes. This severely limits the capacity to compare amphioxus and vertebrate genomes, thereby holding back our understanding of vertebrate genome evolution. Even repeat DNA regions (such as ribosomal gene clusters) and telomere sequences have not been mapped in amphioxus; rDNA clusters can act as important genomic landmarks, while telomere locations can give clues to fissions or fusions of chromosomes in evolution (Meyne *et al.*, 1992).

Here we described a robust and reliable method for mapping genes and other DNA sequences to *Branchiostoma floridae* chromosomes using fluorescent in situ hybridisation (FISH) to metaphase spreads prepared from embryos. The method can be used with single probes (one colour FISH) or with two or more probes simultaneously for comparing genomic locations (two colour FISH). We demonstrate that the method is sufficiently sensitive to detect single copy genes using cosmid probes, or to detect repeat elements using short DNA probes. We use the method to describe the number and location of 18S rDNA gene clusters, the distribution of telomere sequences, and the location of two single copy developmental genes in relation to each other. These methods have great potential for performing comparative genomics between amphioxus and vertebrates.

MATERIALS AND METHODS

Metaphase preparation

Jha *et al.* (1995), working with polychaetes, previously demonstrated that embryos of marine invertebrates provide a good source of rapidly dividing cells. We therefore used amphioxus embryos to

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obtain metaphase spreads. Adult amphioxus, *Branchiostoma floridae,* were collected in August 2001 from Old Tampa Bay, Florida, USA and in vitro fertilisation performed as described (Holland and Holland, 1993). Embryos at four different developmental stages were used to produce metaphase spreads (7 hr gastrula; 12 hr, 13 hr and 14 hr neurulae). After an initial treatment with 0.025% colchicine in sea water (30 minutes), embryos were transferred with a Nitex filter (20–30 μm mesh) through a series of hypotonic solutions (mix of sea water and 0.075M KCl in ratios 2:1, 1:1, 1:2 and 1:3) for 10 min each. After the final hypotonic wash, embryos were transferred with a Pasteur pipette to a 1.5 ml microfuge tube and cold methanol:glacial acetic acid (3:1) fixative added (chilled on ice). The fixative was changed 2 to 3 times over a period of 1 hour (embryos kept at 4°C) through centrifugation (5 min 1000rpm), and embryos kept in fixative at 4°C before slide making. Specimens could be kept at this stage for up to one year (see below). To prepare slides of metaphase spreads, an excess (x5) of 60% of acetic acid was added to 20–30 embryos and left for a few minutes until the embryos started to burst (monitored with a binocular microscope). This mixture was dropped onto pre- warmed (40°C) acid clean microscope slides (50 μl per slide), left until dry (~ 15 min) and analysed for metaphase index. Slides were kept at –20°C in a dark sealed box.

DNA isolation and probe generation

The amphioxus 18S rDNA probe was derived from a 500 bp recombinant PCR fragment amplified using primers JM8 (5' GCCACTAGCATATGCTTGTCTC 3') and JM9 (5' AGACTTGCCTCCAATGGATCC 3') (Holland *et al*., 1991). The whole plasmid was purified by a QIAGEN miniprep kit and labelled with digoxigenin (DIG) using the DIG-Nick translation mix from ROCHE. The telomere probe used was a commercial DIG-labelled probe complementary to telomere repeats (All Human telomeres Appligene Oncor). To isolate a cosmid clone of *AmphiPax1*, we designed PCR primers from a published cDNA sequence (Hetzer-Egger, Schorpp and Boehm, 2000) and used these to amplify a 600-bp genomic fragment. This was cloned and used to screen an amphioxus cosmid library (MPMGc117 from the Resource Centre and Primary Database www.rzpd.de) under high stringency conditions (65°C in the buffer of Church and Gilbert, 1984, and washed at 65°C in 2X SSC, 0.1% SDS). We found clone MPMGc117 A0413 to contain *AmphiPax1* and confirmed this by DNA sequencing. The cosmid containing *AmphiNotch* has been previously described with coordinates MPMGc117 E1080 (Holland *et al*., 2001). Cosmid DNA was isolated by alkaline lysis followed by phenol:clorophorm extraction. The *AmphiPax1* and *AmphiNotch* cosmids were labelled with biotin or DIG respectively using the nick translation kits from ROCHE.

Fluorescent in situ hybridisation (FISH)

The protocol for fluorescent in situ hybridisation was modified from Schwarzacher and Heslop-Harrison (2000) and Ferrier and Akam (1996) as follows. Slides were initially incubated in 2X SSC at 37°C for 15 min, followed by dehydration through an ethanol series (5 min each in 70%, 95% and 100% ethanol) and left to air dry. To denature the chromosomal DNA, slides were incubated in 70% formamide in 2X SSC (72°C, 3 min), after which slides were dehydrated as before (the 70% and 95% at –20°C). To denature the DIG and biotin-labelled probes, these were added to hybridisation mix (50% formamide, 2X SSC, 10% dextran sulphate, 1 μg sheared salmon sperm DNA, 0.15% SDS) at a final concentration of 2.5 to 7 ng/μl, allowing a total of 30 μl per slide. Denaturation took place at 80°C for 15 min, before chilling on ice, after which the probe mix was applied to the slide, covered with a coverslip and sealed with rubber cement (Fixogum, Marabuwerke GmbH). For two colour fish, both probes were added to the same hybridisation mix. Slides were then incubated overnight at 37°C to 40°C in a water-saturated atmosphere. After hybridisation, slides were immersed in 2X SSC (43°C/44°C, 5 min) to remove the coverslip, then twice in 20% formamide, 0.1X SSC (43°C/44°C, 5 min each) and twice in 2X SSC (43°C/44°C, 5 min each). All washes were static. Slides were then immersed for 30 min and 5 min 37°C in 4xTNFM (4X SSC, 0.05% Tween20, 5% Marvel non-fat milk powder; centrifuged 10 min at 3000 rpm to remove particles before use). Signals from biotin-labelled probes were amplified and detected with sequential applications of avidin FITC DCS (1:500 in 4XTNFM; Vector Laboratories) for 1 hr, biotinylated anti-avidin D (1:250 in 4xTNFM; Vector Laboratories) for 30 min and then avidin FITC DCS again (1:500 4xTNFM; Vector Laboratories) for 30 min. For detection of DIG-labelled probes, anti-DIG-rhodamine Fab fragments (1:200 4xTNFM; ROCHE) was applied for 1 hr and then Texas Red anti-sheep (1:100 4xTNFM; Vector Laboratories) for 30 min. Slides were washed in 4xTNFM between applications (3x after first, 2x after second and third; all washes were for 5 minutes at 43°C/44°C). All three incubations were carried out at 37°C in the dark in a humidified box. Detection for the two labels in two colour FISH was performed simultaneously. Slides were washed with 4XSSC, 0.05% Tween20 twice for 5 min at room temperature and rinsed with PBS. Vectashield antifadent agent containing 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 5.5.

RESULTS AND DISCUSSION

Applicability of the protocol

From the four different developmental stages used to produce metaphase spreads, we found that gastrulae produced the highest metaphase index, presumably reflecting highest mitotic activity. Nevertheless, all developmental stages tested yielded a good score of metaphase chromosomes, in the order of 50 to 150 metaphase spreads per microscope slide prepared from 50 μl aliquots of cells. In addition, all stages yielded large numbers of well spread interphase nuclei. We found that the FISH probes hybridised with equal efficiency to metaphase spreads and to interphase nuclei (Fig. 1a); these give complementary information on gene or sequence localisation.

We also tested whether it was possible to produce chromosome spreads from the fixed embryos after prolonged storage at 4°C. We detected little difference in the quality of metaphase spreads between freshly prepared slides and from material that had been fixed and stored for one year of storage prior to slide preparation. In both cases, slides gave excellent signals after probe hybridisation using the protocol described in this paper. This information is particularly relevant due to the seasonality of amphioxus spawning.

Furthermore, since descriptions of embryonic development are highly congruent between three different amphioxus species (*B. floridae, B. lanceolatum* and *B. belcheri*), we anticipate that the protocol described here will be broadly applicable to these species.

Chromosome number

The scoring of chromosomal number per metaphase plate confirmed previous counts of 38 chromosomes per diploid genome (Howell and Boshung Jr., 1971). As noted

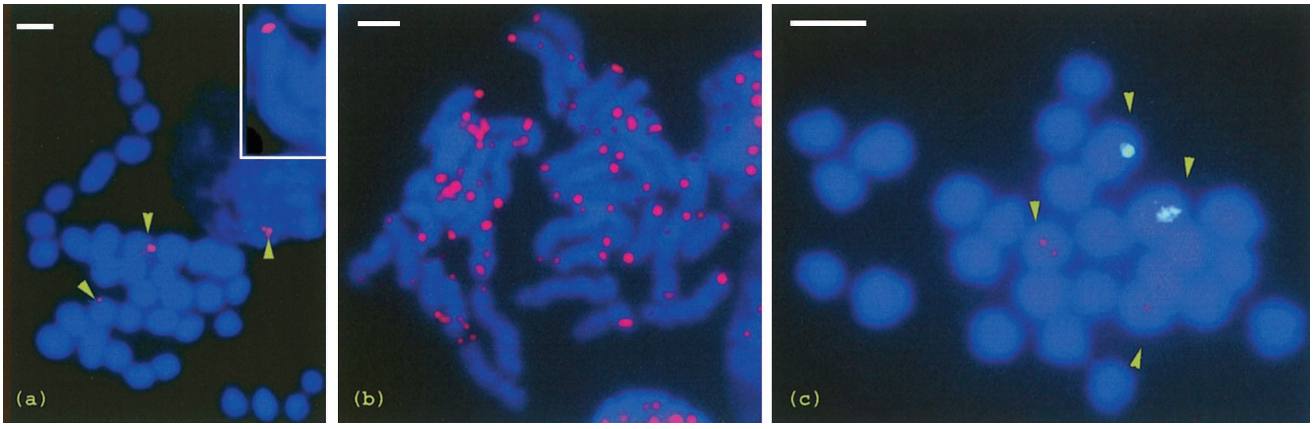

Fig. 1. FISH to chromosomes of *Branchiostoma floridae*. **(a)** FISH with 18S rDNA probe to a metaphase spread and, on the right side of main panel, an interphase nucleus. The boxed insert shows a stretched metaphase chromosome with the subterminal position of the rDNA gene cluster in amphioxus. **(b)** FISH with all-human telomere $(TTAGGG)_n$ probe. **(c)** FISH with *AmphiPax1* (green) and *AmphiNotch* (red) cosmid probes. Bars, 2 μm.

by others, however, differentiating between the 19 amphioxus chromosomes (haploid set) is extremely difficult due to their small and similar size. In some cases we obtained metaphase plates with stretched chromosomes but no clear difference was observed between chromosomes (Fig. 1b). Howell and Boshung Jr. (1971) have proposed the existence of two to four pairs of chromosomes with the centromere in a median to submedian position. In our observations, we did not detect this differentiation. In some other animal species, DAPI staining is reported to show characteristic banding patterns, which assist the identification of individual chromosomes. No banding pattern was observed in amphioxus chromosomes after DAPI staining. Despite the inability to distinguish chromosomes, we find that genes can still be mapped relative to each other, by using combinations of probes in two colour FISH experiments.

Not every chromosome spread contained exactly 38 chromosomes, although this was by far the most frequent number observed. We detected a proportion of spreads with fewer than 38 chromosomes; these can be accounted for by cell breakage and chromosome loss during specimen preparation. Saotome and Ojima (2001) have recently described the appearance of cells with higher ploidy than 2n after preparing metaphase spreads from adult testis of *B. belcheri*. We also detected a significant proportion of spreads with higher numbers than 38 chromosomes. In both cases, spreads are still useful for gene mapping experiments using FISH (Fig. 1).

Ribosomal DNA and telomeres

To examine the distribution of ribosomal DNA (rDNA) clusters and telomere sequences, a minimum of 20 metaphase plates were analysed for each probe. Performing FISH with an amphioxus 18S rDNA probe yielded two labelled chromosomes per standard metaphase spread, with each chromosome showing two adjacent signals marking the two sister chromatids (Fig. 1a). Although difficult to visualize in the majority of metaphase plates, we observed that the rDNA signal resides at the tip of a chromosome (Fig. 1a insert). In line with these findings, we detected two clear signals in each interphase nucleus (right hand signals in Fig. 1a). These results indicate that there is a single ribosomal DNA gene cluster per haploid genome in amphioxus. We suggest this represents the primitive condition for chordates, and that the multiple rDNA clusters of other organisms including vertebrates are a derived condition (Pascoe *et al*., 1996).

We detected the telomeric repeat $(TTAGGG)_n$ at the tip of every chromosome in amphioxus, although in some spreads signal was not always seen on both chromatids and not always in the same focal plane (Fig. 1b). In occasional metaphase spreads we detected interstitial signals, but these were not consistent. We interpret the latter as background hybridisation due to the weakness of the labelling. It is worth noting that we do not know the DNA sequence of the endogenous telomere repeat in amphioxus; our probe was synthesized from a repeat cap typical of vertebrate telomeres (Meyne *et al*., 1989). Although this sequence is also present in many other taxa (e.g. Annelida, Vitturi *et al*., 2000; Gastropoda, Colomba *et al*., 2002), there are variations to this repeat in insects $(TTAGG)_n$ and some other animals (Okazaki *et al*., 1993). All other probes used in the present study were based on endogenous amphioxus sequences.

We have found no evidence for chromosome rearrangements in *B. floridae*, as might be suggested by interstitial telomere sequences. It will be interesting to compare the pattern of telomere hybridisation between *Branchiostoma* species to address the issue of genome conservation within this group. Particularly interesting will be the case of *Branchiostoma belcheri* which has a diploid set of two fewer chromosomes than *B. floridae* or *B. lanceolatum*. It is possible that a simple chromosome fusion, or a centric fission of a biarmed chromosome, is the underlying reason for this

karyotype difference. We suggest that single copy probes could be used to map orthologous genes in the two species, and thereby identify equivalent chromosomes. This would reveal whether two distinct chromosome pairs in *B. floridae* relate simply to one chromosome pair in *B. belcheri*. The fusion scenario would also predict interstitial hybridisation with the TTAGGG repeat in a chromosome pair of *B. belcheri*.

Single copy genes and two-colour FISH

To test the power of FISH for studying genome evolution in chordates we undertook the task of mapping two single copy genes in amphioxus. *AmphiPax1* is a paired class gene, homologous to the of *PAX1* and *PAX9* genes of humans. *PAX1* maps to human 14q13 and *PAX9* maps to 20p11; these chromosomal bands contain other paralogous genes and form paralogy regions thought to have arisen by chromosome or genome duplication (Wang *et al*., 2000; Castro, Luke and Holland unpublished results). *AmphiNotch* encodes a single-pass transmembrane receptor; this gene has four homologues in human mapping to 9q34.3, 1p13-p14, 19p13.2-p13.1 and 6p21.3 respectively. Since none of the human *NOTCH1-4* genes co-localises with either *PAX1* or *PAX9*, we predicted that the homologues would also be unlinked in amphioxus.

We performed two-colour FISH and analysed 20 metaphase spreads to determine the relative positions of *AmphiPax1* and *AmphiNotch*. In every case, we found these probes to hybridise to four different chromosomes per diploid genome, as predicted (Fig.1c).

Using the protocol described, we detect no significant difference in the efficiency of FISH with most single copy gene probes, as compared to the repetitive DNA probes used (18S rDNA and telomeres). It should be noted, however, that we used short probes for the repeat sequences, and much longer cosmid probes for the single copy regions. (the cosmids in the amphioxus library used range from ~36 to ~43 kb). We suggest that the greater probe complexity in these long probes compensates for the single copy nature of the target sequences. In some FISH protocols, genomic DNA is used to suppress spurious hybridisation by interspersed repetitive sequences when large genomic clones are used as probes. We did not include this step in our protocol, as we found that the majority of probes hybridised cleanly without its inclusion. Only 5% to 10% of cosmid probes we have used did not give clean results (data not shown); this problem may be related to repetitive elements, although we did not test this. Instead, we find the simplest way to overcome this problem is to use genome walking to isolate an overlapping cosmid clone.

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