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Detection of a Single-Locus Gene on Channel Catfish Chromosomes by *In-Situ* Polymerase Chain Reaction

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ABSTRACT. An *in-situ* polymerase chain reaction (ISPCR) procedure was applied to chromosomal localization of the gene, *Ig H*, encoding the immunoglobulin heavy chain of channel catfish (*Ictalurus punctatus*). Metaphase chromosomes were prepared by a replication banding procedure and subjected to ISPCR using biotin-labeled primers. The hybridization signals were detected with an avidin-fluorescein isothiocyanate (FITC)-based method, and chromosome bands revealed by simultaneous or sequential treatment methods. Standard fluorescent *in-situ* hybridization (FISH) was performed on chromosome preparations to compare with the ISPCR procedure. The *Ig H* gene was detected at the telomeric position of a chromosome with a relative length of 3.2 ± 0.2%. The *Ig H*-bearing chromosome detected by the FISH method was identical to that found by ISPCR procedure. Visibility of chromosome bands was reduced by heat and salt treatments and could not be analyzed after thermocycling. Therefore, specific identity of the chromosome bearing the *Ig H* gene remains unknown. Banding of fish chromosomes is difficult and poses a barrier for applying current molecular techniques to physical mapping of teleost genomes. Application of the ISPCR to chromosomal mapping is new for fish species and is only in initial stages of development for higher vertebrates. COMP BIOCHEM PHYSIOL 118B;4:793–796, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ISPCR, FISH, gene mapping, chromosome, replication banding, teleost

INTRODUCTION

General acceptance of *in-situ* hybridization (ISH) for physical mapping of genes has occurred during the past decade, led by the development of nonisotopic labeling techniques and use of fluorescent or enzymatic reporters (1). Fluorescent *in-situ* hybridization (FISH) has become a standard method for chromosomal localization of DNA sequences (20) in higher vertebrates including humans (22) and mice (16). Other than application in cytogenetics and gene mapping, FISH is commonly used for diagnosis of chromosomal abnormalities (2).

However, an inherent limitation to the FISH technique is that multiple copies of a target sequence are typically required for detection (17), resulting in limited application for single-locus sequences. *In-situ* polymerase chain reaction (ISPCR) is a newly developed technology that allows multiplication of target DNA sequences. Although ISPCR is theoretically useful in physical mapping, information on this topic is currently limited (7) and only a few studies have addressed chromosomal mapping (23). This is in part because chromosome morphology is degraded by thermal cycling, and it is difficult to retain PCR products *in situ*.

Identification of chromosome banding patterns is an additional and required step of physical mapping. Several procedures have been proposed to allow simultaneous observation of chromosome bands and *in-situ* hybridization signals, such as banding with 4, 6-diamidino-2-phenylindole (DAPI) (9) and use of alkaline antifading and counterstaining solutions (13). Alternatively, hybridization signals and banding patterns can potentially be displayed in successive steps (11).

Compared with higher vertebrates, little information is available about application of ISH for gene mapping and cytogenetic studies of fish species. Ribosomal RNA genes (18) and other highly repeated DNA sequences (10) have been investigated in a few cases. Greater difficulty can be expected in applying ISPCR to fish species because fish chromosomes are small and numerous, and techniques for identifying individual chromosomes are available only in a few species (8,25). Most fish species do not have well differentiated chromosome bands, and therefore, simultaneous display of hybridization signals and banding patterns can be difficult to achieve.

In this study, an ISPCR method was tested for chromosomal localization of the channel catfish *Ig H* gene, which encodes the immunoglobulin heavy chain constant region

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(24). The objectives of this study were to identify the location of the *Ig H* gene on channel catfish chromosomes and to analyze the identity of the *Ig H*-bearing chromosomes by simultaneous and sequential staining methods.

MATERIALS AND METHODS

Preparation of Metaphase Chromosomes

Leukocytes were isolated from peripheral blood of channel catfish and cultured in L15 medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco) and 5% catfish serum. Phorbol ester (Sigma Chemical Co., St Louis, MO) and calcium ionophore A23187 (Sigma) were added for stimulation of mitotic activity (14). Metaphase chromosomes were prepared based on a replication banding procedure and stained by the fluorochrome plus Giemsa (FPG) method to reveal banding patterns (25).

In-Situ *PCR (ISPCR)*

Chromosomes were prepared on slides with a teflon-coated border (Cel-line, Newfield, NJ). Pretreatments, including RNase A digestion and denaturation of chromosomal DNA, were based on a procedure developed for interphase nuclei (25). Primers were synthesized with biotin labeling at the 3′-ends. The primer sequences were (5′ to 3′): TCCCCAA GGTTTACTTGCTCGCTCC and CGATGGATCTGG ATATGTGGCGCAC and the ISPCR was carried out with an initial denaturation step of 95°C for 4 min, and 10 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 1 min.

Detection of Hybridization Signals and Chromosome Bands

Hybridization signals were detected by staining with avidin-fluorescein isothiocyanate (FITC) (25). Slides were counterstained with propidium iodide (5 μg/ml) prepared in an alkaline antifading medium (100 mg *p*-phenylenediamine in 100 ml glycerol, pH 11) for simultaneous production of chromosome bands. For sequential detection, after photographing the hybridization signals, slides were rinsed in 2 × SSC buffer to remove the antifading medium. Slides were baked at 65°C overnight and processed by the FPG method for displaying chromosome bands (25).

Fluorescent In-Situ *Hybridization (FISH)*

A standard FISH procedure (17) was performed on chromosome preparations for comparison with the ISPCR technique. Probe DNA was obtained by PCR amplification using biotin-labeled primers. Hybridization was performed at 37°C in a humid box for 36 to 48 hr. The subsequent detection steps were the same as used in the ISPCR.

Chromosome Measurements

Chromosomes were photographed with a microscope-mounted (Microphot-SA, Nikon Inc.) Nikon FX-35DX camera (Nikon Inc., Garden City, NY) with Kodak Ektachrome (400 ASA) color slide film for fluorescent images or with Kodak Technical Pan 2415 film for black-and-white images. The negatives were scanned into a computer with a slide scanner (SprintScan 35, Polaroid scanner model CS-2700, Needham Heights, MA) for analysis. Chromosome length was measured by a "line measurement" function of the Optimas® computer software package (BioScan Inc., Edmonds, WA), and relative length (percent of total complement length: %TCL) was calculated by a standard method (12). Replication banding patterns were analyzed by densitometry (25).

RESULTS

Chromosomal location of the *Ig H* gene was revealed by the ISPCR procedure (Fig. 1a and b) and was consistently found on the telomeric position of a particular chromosome. Fluorescent counterstaining (propidium iodide in alkaline medium) did not reveal chromosome banding patterns, however; nor did sequential treatment by the FPG method performed after the ISPCR. Replication R-bands analyzable by densitometry before ISPCR (Fig. 2a) were not analyzable after thermal cycling (Fig. 2b).

Chromosomal location of the *Ig H* gene was displayed by the standard FISH method (Fig. 1c). Morphology of the *Ig H*-bearing chromosome was identical to that identified by the ISPCR procedure, but without banding information the chromosome remained anonymous. The %TCL of the anonymous chromosome was 3.2 ± 0.2% (N = 10), which was identified to be in the chromosome groups of medium submetacentrics, medium subtelocentrics, small metacentrics, or small submetacentrics of channel catfish (25). Further identification of the chromosome will require knowledge of centromere position and banding.

DISCUSSION

Replication banding has been successfully coupled with ISH (13), and the banding technique is well developed for mammalian species (20). However, replication banding has been reported only in a limited number of fish species including *Rutilus rutilus* and *Scardinus erythrophthalmus* (8) and *Salmo gairdneri* (3). Zhang (25) reported that replication banding of channel catfish chromosomes could only be revealed by harsh treatments such as the FPG technique. Fluorescent stains including acridine orange, propidium iodide, Hoechst 33258, and actinomycin D did not produce analyzable replication bandings in this species. Overall, regardless of tech-

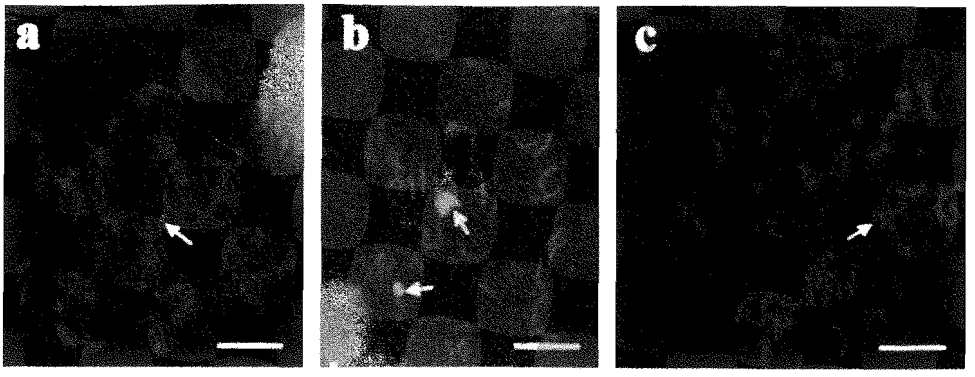

FIG. 1. Chromosomal location of the channel catfish *Ig H* gene by direct *in situ* PCR (a and b). Standard fluorescent *in situ* hybridization procedure was performed for verification (c). Arrowheads indicate location of the target gene. Bars = 10 μ.

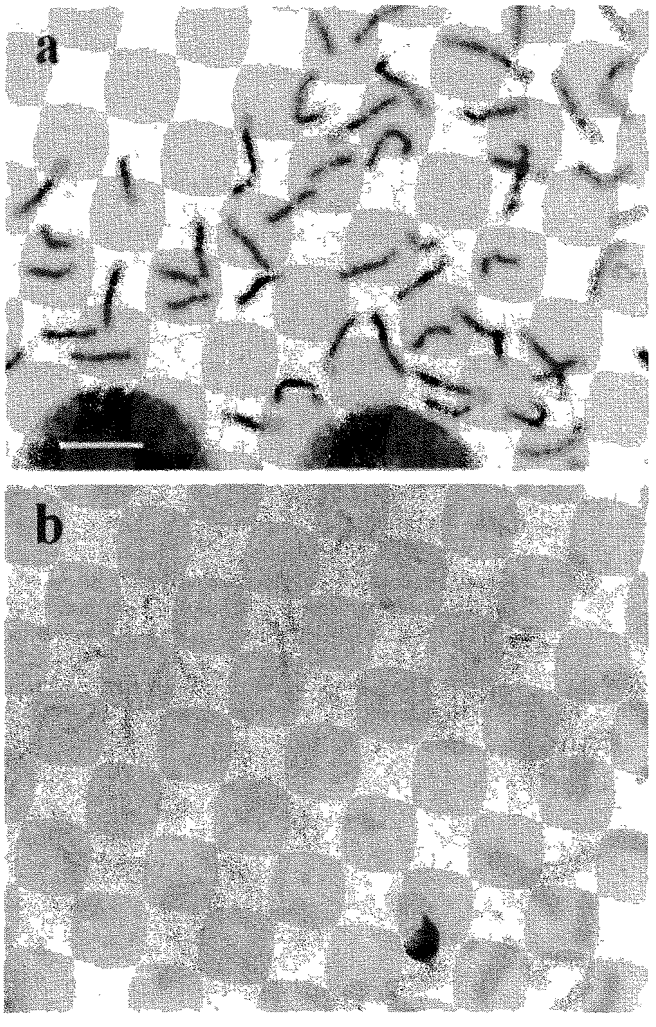

FIG. 2. Replication banding of channel catfish chromosomes prepared by the fluorochrome plus Giemsa staining method: before (a), and after *in situ* PCR amplification (b). The replication R-bands were not analyzable after *in-situ* PCR amplification. Bars = 10 μ.

nique, the banding pattern of fish chromosomes are not as resolvable as those of chromosomes of higher vertebrates such as humans (20).

In this study, the channel catfish *Ig H* gene was mapped to a preliminary chromosome location. However, the identity of this chromosome remains to be definitively established because chromosome banding patterns could not be analyzed after ISPCR or FISH. Although procedures have been developed for simultaneous detection of replication R-bands and hybridization signals in mammalian species, most are not applicable for channel catfish chromosomes because they do not yield banding patterns on chromosomes without ISPCR or FISH treatments. Even when employing sequential detection, the banding pattern was extremely weak and not analyzable after performing hybridization procedures.

These problems could be cumulative effects resulting from repeated exposure to heat and salt (buffer) in the ISPCR or FISH. The contrast of chromosomes was poor after 20 or more cycles of thermocycling, and the chromosomes were difficult to distinguish from background. High concentrations of salt solution reduced nonspecific binding, but also reduced the visibility of chromosomes. Potential solutions for the low visibility of replication banding patterns include densitometric analysis (4) of chromosomes or immunochemical detection methods (19). Other methods, such as restriction enzyme banding (15) or chromosome painting (21) by repetitive DNA probes, could be used as alternatives to replication banding for use with ISPCR.

Application of the ISPCR technique in chromosomal mapping is still in its infancy for fish species and higher vertebrates including humans. To our knowledge, this is the first report using ISPCR for physical mapping of a single-locus gene in a fish species. Although this technique is readily available for use in higher vertebrates, it is hindered in use with fishes. Less than 10% of fish species have been studied cytogenetically, and only a handful of species have yielded reliable banding patterns of any kind (5,6). It is ironic that the success of newer molecular procedures in fish is hampered by a lack of success with cytogenetic techniques that are routine for higher vertebrates. The use of the ISPCR technique in fishes thus awaits improvement of the

procedure and supportive studies on the methodology of chromosome identification.

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