

Detection by In Situ Polymerase Chain Reaction of a Channel Catfish Gene Within Cells and Nuclei

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An in situ polymerase chain reaction (ISPCR) procedure was developed for analysis of single-locus genes of the channel catfish (*Ictalurus punctatus*). Intact leukocytes, suspended in phosphate buffered saline (1.0×10^6 cells/milliliter), were distributed on microscope slides and digested with proteinase K (6 μg/ml) for 1 h at 27°C. The PCR was performed on the slides with a thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA). Primers were designed to target the gene *Ig H*, encoding the constant region of the immunoglobulin heavy chain. After amplification with biotin-labeled primers, the intracellular PCR products were detected by color development or fluorescent methods. The cells on treatment slides were strongly positive for the target gene. This was verified by use of an indirect procedure, in which the ISPCR was performed using unlabeled primers, followed by use of a biotin-labeled probe. The ISPCR technique was also applied to the location of the gene in interphase nuclei. Two copies of the target gene were revealed in positive interphase nuclei. For validation of the ISPCR technique, we performed the technique on a mixture of channel catfish leukocytes and fetal donkey dermal cells. More than 90% of the catfish cells exhibited fluorescence associated with the presence of the target gene, whereas no fluorescence was observed on the fetal donkey dermal cells. Liquid-phase PCR was also included as a positive control in each experiment and yielded products of the expected size (303 base pairs) and DNA sequence. Localization of a single-locus gene by ISPCR is new to nonmammalian species and will provide an important tool for gene mapping and transgenic studies in fishes.

Key Words: Genome mapping—*Ictalurus punctatus*—Single locus—FISH.

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In situ hybridization (ISH) involves a hybridization reaction between a labeled nucleotide probe and a complementary strand of target DNA or RNA in tissue sections or intact cells (13). Many refinements have been made in the past decade, changing the original ISH (8) into a technique that provides fast, precise, and sensitive localization of DNA sequences (11). The major technical variations of ISH include probe length and the types of labeling and detection methods. The size of probes can range from 25 to 500 nucleotides for DNA, and from hundreds to thousands of nucleotides for RNA (13). Probes can be labeled with radioisotopes (5), biotin, digoxigenin (6), chemiluminescence (17), or fluorescent markers (22).

Fluorescent in situ hybridization (FISH) is a recent technique for visualizing DNA within cells, interphase nuclei, and on extended chromatin fibers (11). Although the innovation of FISH has enabled mapping of single-locus genes (12), an inherent limitation of this technique is that it typically requires multiple copies of identical DNA or RNA sequences for detection (14). Fewer gene copies require a secondary signal amplification for detection. For this reason, FISH has been employed to study mRNA present in high copy numbers (7) or highly repetitive DNA sequences (10,15). This difficulty has been gradually overcome with introduction of the polymerase chain reaction (PCR) into morphological and diagnostic fields (9). The PCR technique is capable of producing billions of copies of target DNA, although these products do not readily remain associated with the target tissue or chromosome. A combination of ISH with PCR has enabled detection of single copies of DNA or RNA molecules (1). Currently, applications of in situ PCR (ISPCR) are confined mainly to the detection of foreign or mutated genes (3,18).

Direct and indirect methods are two important variations developed for the ISPCR (9). Direct ISPCR uses primers or free nucleotides labeled with biotin, digoxigenin, or protein molecules that allow direct visualization of target sequences after amplification. Of the two,

this method is more straightforward and easier to perform. The indirect method utilizes PCR amplification followed by hybridization with labeled probes. This procedure is more complicated, but the hybridization step provides an additional validation of the specificity of the PCR amplification and greatly reduces the potential for false positive signals (9).

These techniques, although rapidly gaining acceptance in mammalian (medical) research, are little studied and not available for use in fishes. They offer great potential for research, but pose technical barriers to application. We sought to develop these procedures for use in genetic analysis of fish species. In this study, the *Ig H* gene of channel catfish, encoding the immunoglobulin heavy chain constant region, was chosen as a target. The nucleotide sequence of this gene and its mRNA splicing pattern were reported (16). The objectives of this study were to develop an ISPCR procedure for detecting genetic material within intact cells of channel catfish, to expand the use of ISPCR for gene localization in interphase nuclei, and to evaluate the validity and specificity of the ISPCR procedure.

MATERIALS AND METHODS

Animals

Channel catfish were obtained from a research stock maintained at LSU and spawned in an indoor recirculating system. Five healthy fish (mean ± SD: 550 ± 58 g) were used for periodic blood sampling in this study.

Isolation of Leukocytes

Leukocytes were isolated from peripheral blood of channel catfish using the density centrifugation method (20). The isolated cells were resuspended in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) at 1.0×10^5, 1.0×10^6, or 1.0×10^7 cells/ml. Ten microliters of the final cell concentrations were placed in each well of two-well slides (Cel-line, Newfield, NJ) coated with a 2% solution of 3′-aminopropyltriethoxysilane (AES) (Sigma Chemical Co., St. Louis, MO). The slides were dried in a laminar hood and fixed for 8 hours in 2% paraformaldehyde (Sigma). After rinsing once with 3-x PBS and twice with 1-x PBS, the slides were treated with 0.3% H_2O_2 overnight at 4°C and dehydrated through a graded series of 70% to 100% ethanol (EtOH) and stored at −80°C until use.

Preparation of Interphase Nuclei

The isolated leukocytes were cultured in L15 medium (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco) and 5% catfish serum. Concanavalin A was added to culture medium for stimulation of mitosis. Cells were harvested after 3 days of culture, and interphase nuclei were prepared with standard procedures used for preparation of catfish chromosomes (20).

Extraction of Genomic DNA

Blood was collected from the caudal vessels of channel catfish into acid-citrate-dextrose solution (Becton-Dickinson vacutainer 4606, Franklin Lakes, NJ). Nuclear DNA was extracted from whole blood using a QIAamp blood kit (Qiagen Inc., Chatsworth, CA). The purity and concentration of DNA was estimated spectrophotometrically using the GeneQuant RNA/DNA calculator (model 80-2104-98, Pharmacia Biotech, Cambridge, England).

Preparation and Analysis of Biotin-Labeled Probe DNA

Probe DNA was synthesized by PCR using primers labeled with biotin-16-dUTP. Primers were synthesized by the LSU Gene Probes and Expression Systems Laboratory and were designed to target the CH4 exon of the *Ig H* gene. The sequences were TCCCCAAGGTTTACTTGCTCGCTCC and CGATGGATCTGGATATGTGGCGCAC (5′ to 3′), which yielded a DNA fragment of 303 base pairs (bp) from nuclear DNA of channel catfish. The PCR reaction conditions were described previously (23), and DNA extracted from fetal donkey dermal (FDD) cells was used as negative control. The PCR products were purified with QIAquick spin PCR purification kit (Qiagen Inc.).

A dot-blot assay procedure (13) with modification for peroxidase-based detection systems was used for verifying biotin incorporation in the DNA probe. Nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) were soaked in 1-x PBS for 5 minutes and blotted dry between filter paper sheets. Five microliters of each of the following DNA solutions were loaded on the membrane: unlabeled probe, labeled unpurified probe, and labeled purified probe. The membranes were held in a laminar hood until completely dried and transferred into 1-x PBS for 1 minute. The membrane was blocked with 1% bovine serum albumin (BSA) and stained by the streptavidin-peroxidase-aminoethylcarbazole (AEC) (color-based) development method (2).

Nucleotide sequences of probe DNA were analyzed with the cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA) and the ABI Prism 310 Genetic Analyzer (Perkin Elmer). Sequences were imported into PC/Gene computer software package (IntellGenetics, Inc., Mountainview, CA), and the final sequence was

determined with complementary information from positive and negative strands and compared with published sequences (16).

In Situ Amplification and Hybridization

The ISPCR procedures were based on methods used for diagnosis of viral genes in human cells (2). Before ISPCR, the whole-cell preparations were digested with proteinase K (6 μg/ml) (Amresco, Solon, OH) at room temperature for 1 hour and heated at 95°C for 1 minute to denature the proteinase. The slides were rinsed with 1-x PBS and distilled water (dH_2O) and dehydrated through a series of 70% to 100% EtOH.

Nuclear preparations were processed by the same procedure as described above for whole cells with the omission of proteinase and hydrogen peroxide treatments. However, slides were incubated with DNase-free RNase A (200 μg/ml) (Calbiochem Corp., La Jolla, CA) for 1 hour at 37°C. Slides were treated with 70% deionized formamide (Sigma) and 2-x sodium chloride/sodium citrate (SSC) buffer for 2 minutes at 70°C and placed immediately into 70% EtOH for 5 minutes at −20°C. Slides were dehydrated through a series of 80% to 100% EtOH and dried in a laminar-flow hood.

Biotin-labeled primers were used for direct ISPCR, and unlabeled primers were used for indirect ISPCR. Reaction mixtures were prepared in the same way as for liquid-phase PCR, and 20 μl was applied to each well containing whole cells or nuclei. To prevent evaporation, each well was sealed with a coverglass (22 × 22 mm) attached with Dow Corning® high vacuum grease (Dow Corning Corp., Midland, MI) along its inner edge. The edge of the coverglass was sealed with clear nail polish (Love My Nails, Bari Cosmetics, Ltd., Greenwich, CT). The cell preparations were subjected to PCR amplification on a thermal cycler (PTC-100, MJ Research, Inc., Watertown, MA) as follows: an initial denaturation at 95°C for 2 minutes, followed by 20 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. The nuclear and chromosomal preparations were subjected to 15 cycles of the same parameters as described above.

A hybridization step was performed for indirect ISPCR. This hybridization mixture included the following components: biotin-labeled probe (1.2 ng/ml), 50% deionized formamide, 2-x SSC buffer, 10-x Denhardt's solution, 0.1% sonicated salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS). The hybridization mixture (20 μl) was applied to each well, which was sealed using the method described above. Hybridization was performed on the thermal cycler: slides were heated for 5 minutes at 95°C and cooled gradually to 37°C. Slides were incubated at 37°C overnight.

Detection of Hybridization Signal

Hybridization signals were revealed by a streptavidin-peroxidase-AEC color development method reported previously (2) or by a fluorochrome-based method. For the fluorescent staining, slides were rinsed sequentially with 50% formamide in 2-x SSC (vol/vol) for 2 minutes at room temperature, 2-x SSC twice for 5 minutes at 37°C, and 0.2% Tween 20 in 4-x SSC for 5 minutes at room temperature. Slides were blocked with 1% BSA, 4-x SSC, and 0.05% Triton X-100 (vol/vol) for 15 to 30 minutes at room temperature. Slides were stained for 1 hour at 37°C with avidin-fluorescein isothiocyanate (1:200 in 4-x SSC and 1% BSA) (Boehringer Mannheim Co., Indianapolis, IN). Slides were rinsed twice with 2-x SSC for 10 minutes at 37°C, and 0.2 % Tween 20 in 4-x SSC for 5 minutes at 37°C, and 0.2% Tween 20 in 4-x SSC for 5 minutes at room temperature. Slides were counterstained with propidium iodide (5 μg/ml) prepared in an antifading medium (100 mg p-phenylenediamine in 100 ml glycerol, pH 11).

Internal Control and Data Analysis

Fetal donkey dermal cells were used as an internal negative control in this study. A mixture of FDD cells and channel catfish leukocytes (1:1) was adjusted to 1.0×10^6 cell/ml. The ISPCR procedures performed on cell mixtures were the same as described above.

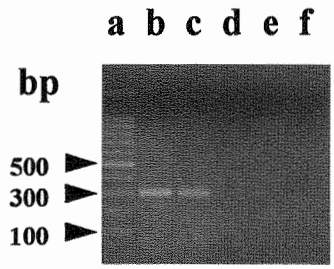
Cell and nuclei preparations were examined under a fluorescence microscope (Microphot-SA, Nikon Inc., Melville, NY) equipped with fluorescence filters for fluorescein isothiocyanate (FITC) (excitation wavelength ~420 to 490 nm) and propidium iodide (excitation wavelength ~330 to 380 nm). Fluorescent images of cells and chromosomes were photographed using Kodak Ektachrome (400 ASA) color slide film (Eastman Kodak Co., Rochester, NY). The slides were scanned using a slide scanner (SprintScan 35, Polaroid model CS-2700, Needham Heights, MA), into a 486 IBM-compatible PC with a 1.2-GB hard drive, 8-MB RAM memory, 2-MB VRAM, and 32-Mhz processor for further analysis. The areas of FDD cells and catfish leukocytes were measured by the area measurement function of the Optimas software, and Student's *t* test was used to evaluate the size difference of these cell types at a significance level of 0.05. The results of the AEC-staining method were recorded and analyzed directly with an image analysis system, in which images were captured with a 24-bit video capture board (Imaging Technology Inc., Bedford, MA) from a light microscope (Microphot-SA, Nikon, Inc.) equipped with a high-resolution color video camera (model A206A, Microimage Video Systems Co., Inc., Boyertown, PA) (19).

RESULTS

The sizes of the probe and the liquid-phase PCR product were fragments approximately 303 bp, as expected (Fig. 1a). No product was detected from DNA extracted from FDD cells or from the reactions in which no template DNA or polymerase were added (Fig. 1a). Biotin incorporation was detected in the probe DNA synthesized by PCR. The nucleotide sequence of the PCR-amplified *Ig H* gene fragment was compared with the published sequence (16) (Fig. 1b). Complete agreement was found in sequence alignment.

Amplification efficiency was best obtained with cell suspensions adjusted to 1.0×10^6 cells/ml. For the color-development method (Fig. 2), the direct ISPCR procedure was applied first to detect intracellular PCR products. Control cells for the direct method (amplified with unlabeled primers) were not stained with AEC (Fig. 2a). Therefore, only the outlines of cells were visible. Cells retaining the PCR products amplified with biotin-labeled primers were stained intensely in the nucleus and cytoplasm (Fig. 2b). These results were verified by indirect ISPCR. Control cells for the indirect method (amplified with unlabeled primers and hybridized with unlabeled probe) were not stained (Fig. 2c). Cells amplified with unlabeled primers, but hybridized with labeled probe, provided heavy staining with AEC and nondistinct outlines of cells (Fig. 2d).

For the fluorescent method (direct method), control cells maintaining the PCR products amplified by unlabeled primers were not stained by FITC and displayed only propidium iodide counterstaining (Fig. 3a). The



(a)

Primer 1

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[1]  - TAGGCAATCCGGAATTCCCCAAGGTTTACTTGCTCGCTCCACCAGAGAGC -50
[2]  - --------------TTCCCCAAGGTTTACTTGCTCGCTCCACCAGAGAGC -36

[1]  - TCTGGTGAATCAGTGACCCTGACTTGCTATGTTAAAGACTTCTACCCTAA -100
[2]  - TCTGGTGAATCAGTGACCCTGACTTGCTATGTTAAAGACTTCTACCCTAA -86

[1]  - GGAGGTGGCTGTGTCTTGGCTTGTTAACGATAAACAAGTGGAAGAAGTGG -150
[2]  - GGAGGTGGCTGTGTCTTGGCTTGTTAACGATAAACAAGTGGAAGAAGTGG -136

[1]  - TCGGCTATGAGCAGAACACCACTGCAGTTATCGACAGAAACAACCTCTTT -200
[2]  - TCGGCTATGAGCAGAACACCACTGCAGTTATCGACAGAAACAACCTCTTT -186

[1]  - TCAGTGTACAGCCAGCTGATTATCAAAACTGCAGACTGGAACAGTGGCAG -250
[2]  - TCAGTGTACAGCCAGCTGATTATCAAAACTGCAGACTGGAACAGTGGCAG -236

[1]  - TGTGTTCAGCTGCCTGGTTTATCATGAGTCCATCAAGGACTGTGTGCGCC -300
[2]  - TGTGTTCAGCTGCCTGGTTTATCATGAGTCCATCAAGGACTGTGTGCGCC -286

[1]  - ACATATCCAGATCCATCGCTAAAGATTCAAAAACGCCCACCTTAGTGAAT -350
[2]  - ACATATCCAGATCCATCGGGA---------------------------- -307
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Primer 2

(b)

FIG. 1. Analysis of PCR-amplified probe DNA by agarose gel (2%) electrophoresis, and nucleotide sequences of the PCR products determined by DNA sequencing. The size of PCR products and probe DNA were ~303 bp **(a)** [DNA marker (a), channel catfish DNA (b), probe DNA (c), DNA from FDD cells (d), no template DNA (e), and no polymerase (f)]. **b:** The nucleotide sequence of the PCR-amplified fragment (**b**:2) agreed completely with the published sequence of the *Ig H* gene (16) (**b:**1).

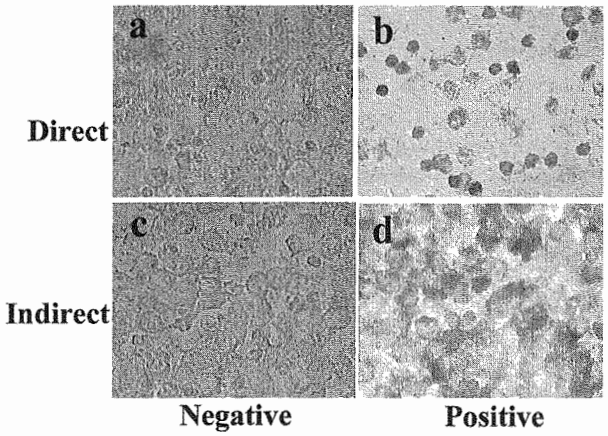

FIG. 2. ISPCR amplification of DNA from catfish leukocytes with unlabeled primers as a negative control **(a)** or with biotin-labeled primers **(b).** Intracellular PCR products were detected directly by the streptavidin-peroxidase-AEC color development method (original magnification ×1,000). An indirect ISPCR procedure was included for comparison, in which cells were amplified using unlabeled primers, and intracellular PCR products were hybridized with biotin-labeled probe **(d)** or unlabeled probe as control **(c)** (original magnification ×1,000). No intracellular PCR products were detected within cells from the control treatments **(a, c);** only the outlines of these cell were seen.

cells with the products amplified by biotin-labeled primers were stained brightly by avidin-FITC, and little counterstaining was observed (Fig. 3b). Direct (Fig. 4a) and indirect procedures (Fig. 4b) were used to detect the intranuclear presence of the *Ig H* gene. Two copies of the target gene were found in positive nuclei for each method.

The in situ amplification of the channel catfish *Ig H* gene was conducted on channel catfish leukocytes (Fig. 5a), FDD cells (Fig. 5d), and a mixture of channel catfish

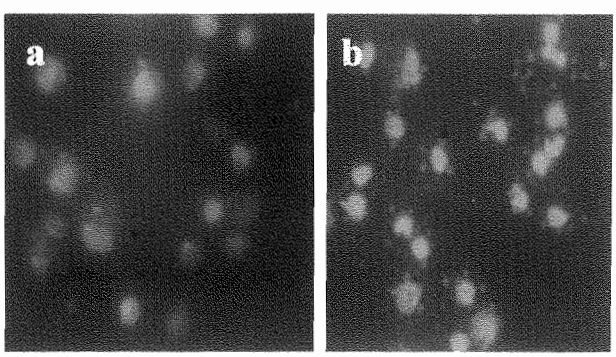

FIG. 3. ISPCR amplification of DNA from catfish leukocytes with unlabeled primers as a negative control **(a)** or with biotin-labeled primers **(b)** (original magnification ×1,000). Intracellular PCR products (yellow fluorescence) were detected by the avidin-FITC-based fluorescence method, and cells were counterstained with propidium iodide (red fluorescence).

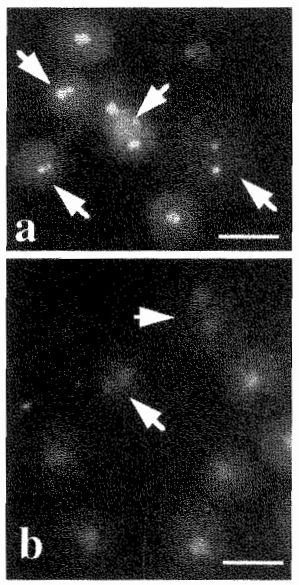

FIG. 4. Localization on interphase nuclei of the channel catfish gene *Ig H* encoding the immunoglobulin heavy chain constant region by direct **(a)** and indirect **(b)** ISPCR methods. Positive nuclei were found to have two copies of the target gene (arrowheads) (original magnification ×1,000).

leukocytes and FDD cells (Fig. 5b and c). The channel catfish leukocytes were significantly smaller than the FDD cells ($p = 0.001$) enabling reliable identification of cell type. The yellow-green fluorescence (FITC) was found on approximately 90% of the catfish cells. This fluorescence did not appear on FDD cells.

DISCUSSION

The success of the ISPCR on whole-cell preparations was affected by microscope slide preparation, control of proteinase digestion, in situ amplification conditions, and posthybridization treatment. The use of coated slides prevented cells from detaching during treatments. In a pre-

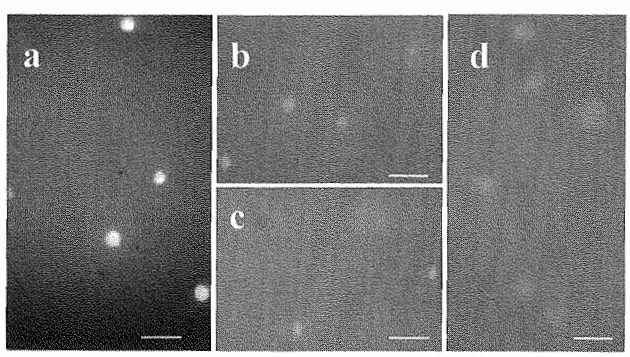

FIG. 5. ISPCR was performed on channel catfish leukocytes **(a),** FDD cells **(d),** and mixtures of channel catfish leukocytes and FDD cells **(b, c)** (original magnification ×600). Intracellular PCR products were detected by the avidin-FITC-based fluorescence method (yellow fluorescence), and cells were counterstained by propidium iodide (red fluorescence). Yellow fluorescence appeared only within the catfish leukocytes, which were significantly smaller in area ($p < 0.001$) than the FDD cells.

viously described method (2), slides were rinsed with water immediately after coating with AES. In our study, better results were obtained when 100% acetone was used to clean the freshly coated slides.

Digestion of cell preparations with proteinase was perhaps the most important step of the entire procedure. A criteria for timing of digestion, the appearance of "peppery dots" on cell membranes, established in previous methods (2), was only visible under 1000-x magnification for catfish leukocytes. Therefore, an improved approach needs to be developed for monitoring the digestion process in these cells.

The indirect method is a widely accepted procedure because the hybridization step is a confirmation of the specificity of the in situ amplification. However, excessive hybridization resulting in heavy staining by AEC interfered with the visualization of target cells (Fig. 2d). The direct method yielded more distinct cell morphology, but has been found to produce false positive signals in other studies (9).

In this study, two different signal detection methods were compared: color development and fluorescent staining. In each case, the buffering system affected the efficiency of detection. Use of the 1-x PBS enhanced color development of the peroxidase-AEC and reduced background noise. Use of the 2-x SSC increased the brightness of the fluorescent staining. Slides treated by the color development method can be stored and analyzed repeatedly, whereas fluorescent treatments have limited viewing time and shorter storage life.

The goal of using the ISPCR procedure with nuclear preparations was to increase the sensitivity of target amplification and retention of PCR products. The ISPCR performed on interphase nuclei included procedures similar to those used in whole-cell preparations but with several different steps. Instead of proteinase digestion, slides were treated with RNase A to increase the sensitivity of target binding. Predenaturation was another critical step in the protocol of interphase nuclei, but it was not necessary for cell preparation. Washing stringency after hybridization was reduced to retain PCR products in association with the location of the *Ig H* gene. High annealing temperature (65°C) improved specificity, as demonstrated by the simultaneous incubation of channel catfish cells (approximately 90% positive) and mammalian cells (100% negative).

The FISH technique, although applied successfully to map multiple-copy genes in higher vertebrates and a few fishes (4,15), is less powerful in localization of single-locus genes because of low abundance of the target. With the ISPCR techniques developed in our laboratory, we have been able to detect a single-locus gene on catfish chromosomes (22). Our results demonstrate the potential of ISPCR techniques for study of fish genomes. The ISPCR techniques developed in this study can be applied for the identification of genetic material foreign to the genome of channel catfish, which is especially useful in gene transfer studies (21). The integration of transferred genes in the genome of host fish can be investigated in embryonic through adult stages. Besides identifying physical location, the activity of these genes can be detected by reverse transcription-ISPCR, which would be an important tool for studying the expression of transferred genes in transgenic fishes. □

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