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Characterization of an A/T-rich family of sequences from channel catfish (*Ictalurus punctatus*)

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Abstract

A family of highly repetitive DNA sequences referred to as *Xba* elements was identified and characterized from the channel catfish (*Ictalurus punctatus*) genome. The *Xba* elements represent about 5% to 6% of the total genomic DNA of the channel catfish. The *Xba* elements are distributed specifically in the channel catfish and blue catfish (*I. furcatus*), but not in closely related species such as white catfish (*Ameiurus catus*) and flathead catfish (*Pylodictus olivaris*). These *Xba* elements are arranged as head-to-tail tandem repeats. Seven sequences were sequenced. They are A/T-rich (over 65%). Each sequence contains four copies of the ATTA repeat and eight copies of $(A)_{3-6}$ GT/TG motifs whose function is not known. Each unit of the repeats is 325 bp from the Kansas strain, and 321 pb from the Auburn and Stuttgart strains of channel catfish. The *Xba* elements are conserved in length within a specific strain, and highly conserved in sequence identity. Sequence identity is more conserved among copies isolated from the same strain than from different strains. The sequence length polymorphism among strains may be useful for identification of strains by polymerase chain reaction analysis. Many features of these elements could make them potentially important for development of homologous recombination expression vectors and position-independent expression vectors.

Introduction

Tandem repetitive noncoding DNA sequences make up a large fraction of the genomes of eukaryotes. The sequence complexities of these repetitive sequences can vary from 1 bp to over 2 kb. Copies of the tandem repeats can vary greatly, ranging from a total size of 100 bp to more than 100 mega base pairs (Mb) (Milklos and Gill, 1982). Based on the repeat lengths and array sizes, they have been divided into three classes: microsatellite, minisatellite, and satellite DNAs (Levinson and Gutman, 1987; Izsvak et al., 1997). As their names indicate, microsatellites include repetitive sequences with very simple sequence complexity and short arrays of the repeat. Satellite DNA exhibits more sequence complexity (generally 100 bp or longer) with long arrays of total repeat lengths. Minisatellites show intermediate features between satellites and microsatellites.

The three classes of the repetitive sequences also differ in where they reside in the eukaryotic genomes. Satellite DNA is often concentrated in regions of very low recombination (Charlesworth et al., 1986), such as heterochromatic regions near centromeres and telomeres where meiotic recombination is suppressed. Minisatellite sequences are usually located in the euchromatic portions of chromosomes. Recombination at minisatellite loci appears to be higher than at satellite DNA loci (Stephan, 1989; Stephan and Cho, 1994). Microsatellites are interspersed in various regions in chromosomes including within or near expressed genes (Gong et al., 1997; Z. Liu, unpublished results).

Until the mid 1980s, repetitive sequences were regarded as noncoding sequences and attributed to few other functions than structural importance in telomeres and centromeres. Participation of repetitive sequences in nuclear matrix attachment enforced their roles in ''large-scale regulation'' including concentrating transcriptional factors

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(Mirkovitch et al., 1986), transcriptional activation (Cockerill and Garrard, 1986), facilitation in chromosomal segregation, and DNA replication (Johnson, 1993). The fact that the structural functions served by the repetitive sequences can in turn regulate various other important functions forced redefinition of ''coding capacities'' of repetitive DNA sequences (for review, see Vogt, 1992). DNA codes are therefore redefined to be based not exclusively on the primary DNA sequence itself (or protein-coding capacity), but also on functions offered by specific features of higher order structures. In addition to their structural functions in centromeres and telomeres, tandem repetitive sequences have been speculated to function in organizing the locus-specific loop structures along each chromosome (Vogt, 1990; Markova et al., 1994).

The DNA regions that bind to the nuclear scaffold are called matrix attachment regions (MARs) or scaffold attachment regions (SARs). There is evidence that repetitive sequences are involved in organization of MARs/SARs (Vogt, 1990). Particular sequences in the MARs may establish independent domains of gene expression, thereby insulating one stretch of DNA from another to permit localized expression of genes (Stratling and Dolle, 1986; Phi et al., 1990; Eisenberg and Elgin, 1991; Boulikas and Kong, 1993; Dillon and Grossveld, 1993; Targa et al., 1994). Because of their insulating role to form independent domains for gene expression, these sequences are also referred to as border elements. Several types of border elements have been reported to direct position-independent gene expression (Bonifer et al., 1991; Kellum and Schedl, 1991; McKnight et al., 1992; Caldovic and Hackett, 1995). Such border elements capable of directing position-independent gene expression are desired for gene transfer to ensure continuous expression after integration of the transgenes.

Certain repetitive *Alu* sequences have some properties of border elements (Small et al., 1982) and have been shown to be associated with the nuclear matrix (Razin et al., 1979; Matsumoto, 1981; Tsongalis et al., 1992; Jackson and Bartlett, 1996) and thus have the properties of MARs. MARs are A/T-rich ranging from 250 to 300 bp (Gasser et al., 1989; Phi et al., 1990). However, no conserved MAR consensus sequence has been identified (Romig et al., 1994) except some AT-rich ATTA or ATTTA motifs.

The idea that a gene might be located in a structural loop bracketed by two MARs has received considerable support from studies on a number of animal (for review, see Bonifer et al., 1991; Eissenberg and Elgin, 1991) and plant genes (e.g., van der Geest et al., 1994; Avramora et al., 1995). Transgenes bordered by two MAR elements have been shown to exert a shielding effect and directed position-independent transgene expression (van der Geest et al., 1994; Caldovic and Hackett, 1995).

Understanding the structure and organization of repetitive sequences in channel catfish facilitates gene mapping of the catfish genome. In this study, we identified and characterized a family of A/T-rich, highly repetitive sequences termed *Xba* elements from channel catfish, the most significant cultured fish in the United States.

Results and Discussion

Specific presence of highly repetitive satellite elements in channel and blue catfish

When genomic DNA of channel catfish was digested with the restriction endonuclease *Xba*I, a prominent band of about 330 bp was noticed. In addition to this prominent band, bands with sizes roughly in multiples of 330 bp were also noticed, indicating base substitution at the restriction site in some copies of the elements (Figure 1). Identities represented by the sequences of the 330-bp fragments are hereby defined as *Xba* elements owing to the use of restriction enzyme *Xba*I for detection of these elements.

The *Xba* elements were observed only from genomic DNA of channel catfish and blue catfish when digested with *Xba*I, not from genomic DNA of closely related species such as white catfish (*Ameiurus catus*) and flathead catfish (*Pylodictus olivaris*) (Figure 1, lanes 8 and 9). This is consistent with current taxonomic classification, in which channel catfish and blue catfish are in the same genus, but flathead catfish and white catfish are in other genera. The absence of the band in white catfish and flathead catfish may mean that the elements are specific to the channel catfish and blue catfish, or that such sequences exist in the white catfish and flathead catfish, but the *Xba*I restriction site was not conserved. If these sequences were also present in white catfish and flathead catfish and the absence of the prominent band after *Xba*I treatment was due to loss of *Xba*I sites, the existence of homologous sequences could be detected by hybridization analysis using the *Xba* elements as probes.

To demonstrate that the *Xba* elements were present specifically in channel catfish and blue catfish, a genomic Southern blot hybridization was performed using the *Xba* elements as probes. As

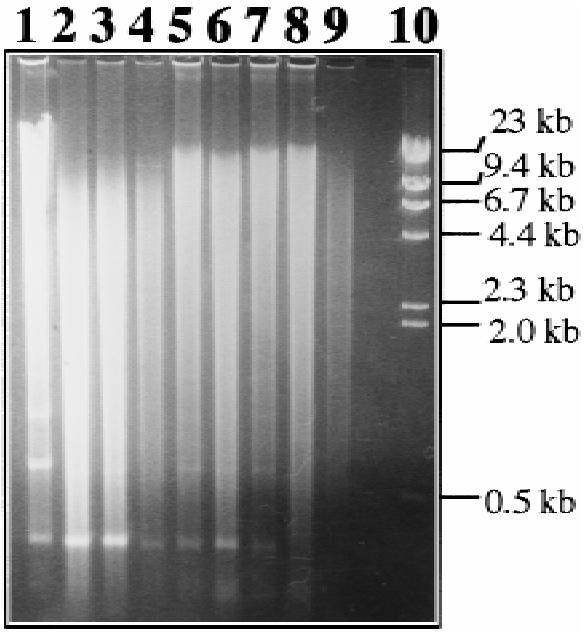

Figure 1. Identification of *Xba* elements from channel catfish and blue catfish. Genomic DNA was digested with restriction endonuclease *Xba*I and subjected to electrophoresis on 1% gel. Note the prominent band at about 330 bp. Genomic DNA was isolated from channel catfish strains Kansas (lane 1), Auburn (lane 2), Stuttgart (lane 3), from blue catfish strains Craft (lane 4), Rio Grande (lane 5), D & B, (lane 6); from white catfish (lane 7); and from flathead (lane 8); control (lane 9); molecular weight markers (lane 10).

shown in Figure 2, the *Xba* element probes hybridized to the DNA from channel catfish (lanes 1–6), but did not hybridize to the DNA from white catfish and flathead catfish (lanes 7 and 8). These results indicate that *Xba* elements exist specifically in the channel catfish and blue catfish. Absence of the *Xba* elements from closely related species of catfish suggests a relatively short history in the evolutionary origin of such sequences. Similar to our results here, studies of the *Alu* family of repetitive sequences in mammals and zebrafish (*Brachydanio rerio*) did not suggest any sequence homology (He et al., 1992).

The Xba *elements exist in the form of tandem repeats*

The repetitive *Xba* elements from the channel catfish and blue catfish exist in tandem repeats. They were detected as multimers such as dimer, trimer, etc., when genomic DNA was partially digested. High molecular weight bands gradually shifted more and more to low molecular weight bands as increasing amount of restriction enzyme was used in the digestion reaction. Southern blot results clearly revealed that the majority of *Xba* elements exist as tandem repeats (Figure 2). The chromosomal origin of these *Xba* elements is not presently known. The large numbers and tandem repeat forms of these elements make them excellent probes for cytologic studies. Further studies using fluorescent in situ hybridization (FISH) would indicate whether they exist in different chromosomes and in what regions on the chromosomes they reside. The tandem repeat arrangement of the *Xba* elements is similar to that identified in zebrafish (He et al., 1992; reviewed by Izsvak et al., 1997).

The Xba *elements are highly repetitive in channel catfish*

The *Xba* elements represent about 5% to 6% of the channel catfish genome. Given that the size of the channel catfish haploid genome is 1×10^9 bp, 5%

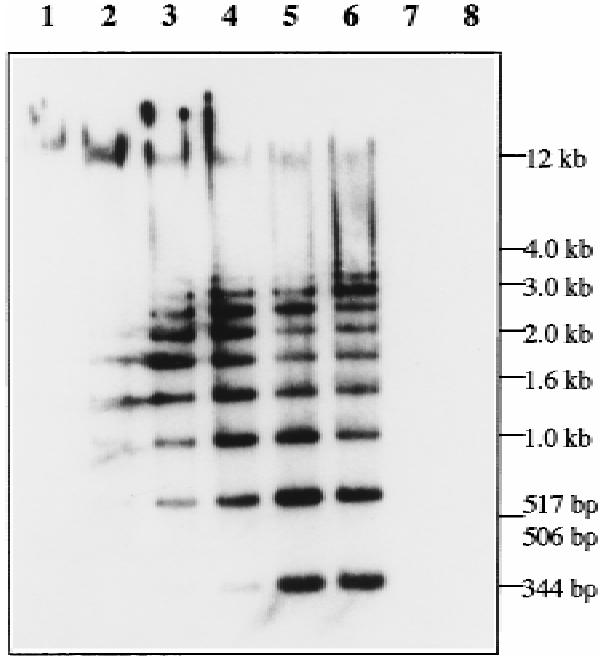

Figure 2. Tandem arrangement of the *Xba* elements in the channel catfish genome. Genomic DNA from channel catfish (Kansas strain) was partially digested with incremental amounts of *Xba*I endonuclease (lanes 1–6): lane 1, 0.2 U; lane 2, 0.4 U; lane 3, 0.8 U; lane 4, 1.6 U; lanes 5 and 6, 3.2 U. Genomic DNA from white catfish (lane 7) and flathead catfish (lane 8) was completely digested with *Xba*I endonuclease. All DNA samples were digested for 1 hour at 37°C and electrophoresed through a 1% agarose gel followed by Southern hybridization with *Xba* repetitive fragments as probes.

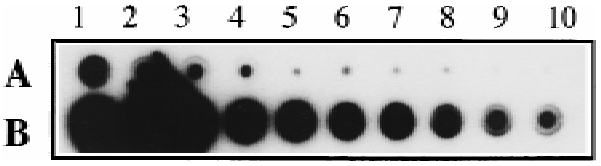

Figure 3. Estimation of copy numbers for *Xba* elements. Known amounts of isolated fragments (A) and genomic DNA (B) were loaded to a nylon membrane followed by dot blot analysis: (A) lanes 1–10, 2× serial dilutions of isolated fragments with 1 ng (lane 1), 500 pg (lane 2), 250 pg (lane 3) . . . 2 pg (lane 10); (B) lanes 1–10, 2× serial dilution of genomic DNA with 2 µg (lane 1), 1 µg (lane 2), . . . 16 ng (lane 8), 8 ng (lane 9), and 4 ng (lane 10).

of the 1 billion base pairs equaled 5×10^7 bp. Because the size of the *Xba* elements is known to be about 330 bp, the *Xba* elements exist at approximately 150,000 copies per haploid channel catfish genome. If copy number of a repetitive sequence in the genome exceeds 1 million in a mammalian haploid genome of 3×10^9 bp, the repetitive sequences are generally regarded as highly repetitive. The *Xba* elements are highly repetitive considering the smaller genome of channel catfish (Figure 3).

The Xba *elements are A/T-rich and contain AT-rich MAR-like motifs*

The sequence of the *Xba-1* element (from Kansas strain) is shown in Figure 4. The isolated *Xba* elements are AT-rich (Table 1). All sequenced elements contained many A or T runs and AT or TA tracts interspersed throughout the sequence, with an A/T content of about 65%, similar to the AT-rich satellite sequences of zebrafish and to A/T-richness of MARs (for review, see Gasser et al., 1989). Repetitive elements with similar A/T-richness isolated from mouse or rat can cause bending of the DNA helix axis and binding of nuclear scaffold proteins (e.g., Hibino et al., 1993), thus exhibiting the properties of the MARs. *Xba* elements contained four copies of ATTA-like AT-rich MAR motifs (Boulikas, 1992; Boulikas and Kong, 1993). Two of the four copies are ATTA and ATTTTA; while the other two copies are the complementary sequences: TAAT and TAAAAT. In addition to these MAR-like ATTA motifs, the *Xba* elements also contained eight copies of $(A)_{3-6}GT/TG$ sequences. The functional importance of these motifs is not known at present.

Table 1. Contents of the *Xba* elements from channel catfish (*Ictalurus punctatus*).

Clone	Source	Length (bp)	(A + T) (%)
Xba-1	Kansas	325	65.2
Xba-2	Kansas	325	65.2
Xba-3	Kansas	325	65.2
Xba-4	Auburn	321	65.1
Xba-5	Auburn	321	64.8
Xba-6	Stuttgart	321	65.7
Xba-7	Stuttgart	321	65.7

The Xba *elements are highly conserved but not identical*

Sequence analysis of seven randomly picked clones of the *Xba* elements revealed that the *Xba*

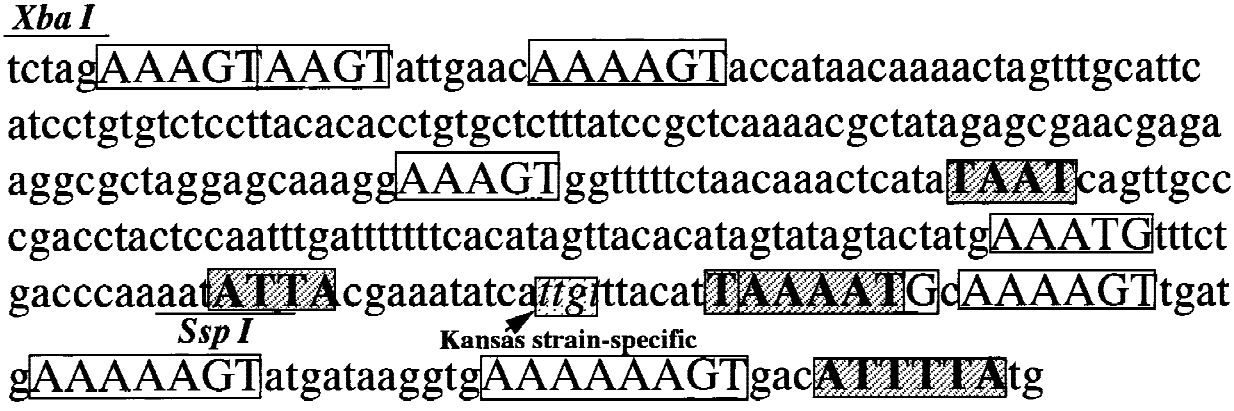

Figure 4. The *Xba* elements contain ATTA MAR-like motifs. Some MAR-like (ATTA) and $(A)_{3-6}GT$ motifs are indicated by uppercase letters. Four *ATTA* motifs are boldface and highlighted with shaded boxes. Eight copies of $(A)_{3-6}GT/TG$ motifs are highlighted by boxes. The 4-bp region specific to the Kansas strain (*arrowhead*) is italicized and highlighted by the dot-filled box. Restriction sites for *Xba*I and *Ssp*I are indicated.

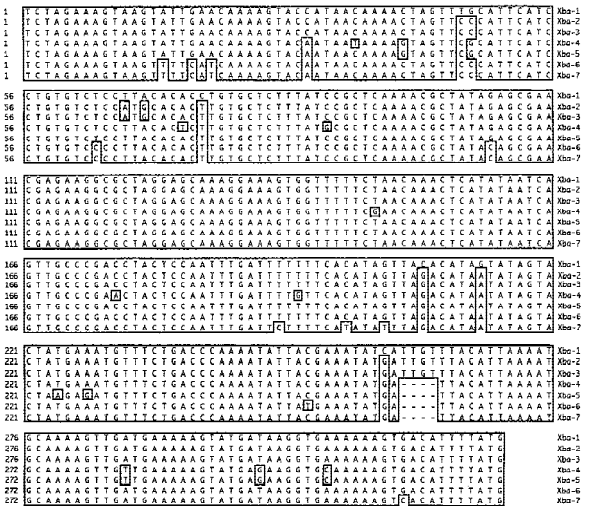

Figure 5. Alignment of seven sequenced *Xba* elements. *Xba-1*, *Xba-2*, and *Xba-3* were isolated from the Kansas strain. *Xba-4* and *Xba-5* were isolated from the Auburn strain of channel catfish. *Xba-6* and *Xba-7* were isolated from the Stuttgart strain of channel catfish.

elements are highly conserved in sequence identity (Figure 5). *Xba-1* differed from *Xba-2* and *Xba-3* (all three were isolated from Kansas strain), with 8 base substitutions. Similar results were obtained with comparisons between *Xba-4* and *Xba-5* (both were isolated from the Auburn strain) and between the *Xba-6* and *Xba-7* (both were isolated from the Stuttgart strain). About 97% of sequences were conserved among clones sequenced from a specific strain of channel catfish. Sequence analysis of *Xba* elements from different strains of the channel catfish indicated a higher level of sequence variation (Table 1). Approximately twice as many base substitutions were observed among clones isolated from different strains.

Analysis of known repetitive sequences indicates that there are relationships between the repeat lengths, interrepeat variability, and allelic heterozygosity due to copy number variation (Stephan and Cho, 1994). As the recombination rate relative to the mutation rate increases, both interrepeat variability and repeat length decrease. In other words, the recombination rates of microsatellite DNAs are greater than those of minisatellite DNAs, which are greater than those of satellite DNAs. In contrast, the interrepeat variations and repeat lengths of satellite DNA are greater than those of minisatellite DNAs, which are greater than those of microsatellite DNAs. The repeat length of the isolated *Xba* elements resembles that of satellite DNA. However, satellite DNA should show a higher level of interrepeat variability, but the isolated *Xba* elements are highly conserved in interrepeat sequence identity. Thus, the isolated *Xba* elements show some features of satellite DNA, but share some of minisatellite DNA.

Potential use of the Xba *elements as markers to identify the Kansas strain of channel catfish*

The channel catfish strain represents an important concept for aquaculture. A strain is defined as a breeding population having a similar history and possessing unique characteristics (Dunham and Smitherman, 1984). Each strain has a distinct history and exhibits at least one distinguishable trait. Strains represent genetic resources that originated from different geologic areas. However, usually there are no noticeable differences in appearance. This makes it difficult to identify strains. Identification of strains is not only needed to study catfish genetics, but is also important for making crosses and for quality control of crossbreeds. For example, the Kansas strain has shown a faster growth rate than other strains as well as several other superior traits. Commercial channel catfish fingerling suppliers should have ways to identify strains and their crossbreeds. Without a notable marker in morphology, this task relies on discovery of fixed molecular markers (R. Winn, personal communication).

The length polymorphisms of the *Xba* elements can potentially be useful for identification of the Kansas strain after extensive population genetic analysis is performed. Results shown in Figure 6 indicate that a 4-bp difference can be readily detected by polymerase chain reaction (PCR) analysis.

Identification of *Xba* repeats in the channel catfish genome may benefit mapping of integrated transgenes, as well as mapping of normal cellular genes. If *Xba* elements exist in centromeres as *Alu* elements do in zebrafish (Ekker et al., 1996; R. Phillips, personal communication), they should be useful for marking chromosomes of channel and blue catfish.

Experimental Procedures

DNA isolation

Genomic DNA was isolated from blood cells of various strains using the sodium dodecyl sulfate (SDS) lysis and proteinase K digestion (Strauss, 1989). A 0.5 ml sample of blood was obtained from

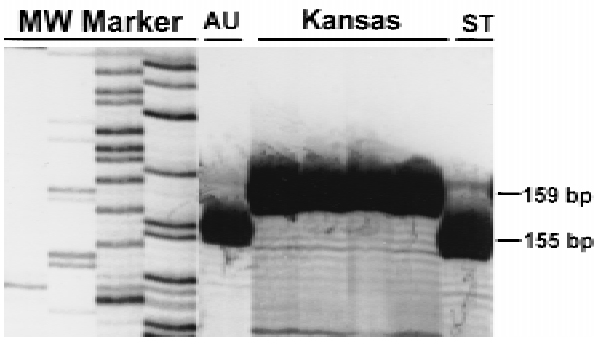

Figure 6. PCR analysis of length polymorphism of the *Xba* elements from three strains of channel catfish. Labeled PCR products were analyzed on a sequencing gel. Sequence ladder was used for molecular weight markers. AU indicates PCR products amplified from the Auburn strain; Kansas, PCR products amplified from the Kansas strain; ST, PCR products amplified from Stuttgart strain. Sizes of the products are indicated at the right margin.

each fish. Blood cells were collected by brief centrifugation. Blood cells were first dispersed by adding 0.5 ml distilled water and pipeting up and down several times until cells were completely dispersed. The cells were then digested in the lysis buffer (Strauss, 1989) overnight. DNA was then extracted once with phenol, once with phenol plus chloroform, and then once with chloroform. DNA was precipitated by adding one-half volume of 7.5 M ammonium acetate and two volumes of ethanol. DNA was collected by a brief spin (2 minutes) at 3000 rpm in a benchtop centrifuge (Beckman CL-6). DNA was then washed with 70% ethanol three times, air-dried, and resuspended in Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 7.4) buffer.

Southern hybridization analysis

Genomic DNA was digested with *Xba*I endonuclease and electrophoresed on a 0.8% agarose gel (Southern, 1975), then transferred to a piece of Zetabind nylon membrane (Wattman, Wis.) by capillary transfer with 0.4 M NaOH overnight (Reed and Mann, 1985). Membranes were dried at 80°C under vacuum and prehybridized in 50% formamide, 5X SSPE (Sambrook et al., 1989), 0.1% SDS, 5X Denhardt's, and 100 µg/ml sonicated and denatured calf thymus DNA overnight. Hybridizations were conducted overnight at 42°C in the same solution with probes added (>10^9 cpm/µg DNA). The probes used were fragments isolated from genomic DNA digested by *Xba*I and were prepared using the random primer method (Sambrook et al., 1989) with a labeling kit from Boehringer Mannheim (Indianapolis, Ind.). The Zetabind membranes were washed first in 500 ml of 2× SSC for 10 minutes, followed by three washes in 0.2× SSC with SDS at 0.2% at 65°C for 15 minutes each. The membranes were then wrapped by Saran wrap and exposed to Kodak BioMax MS film for 30 minutes and overnight (to observe any signals from white catfish and flathead catfish).

Dot blot hybridization

Known amounts of genomic DNA and isolated *Xba* fragments of the repetitive sequences were first serially diluted. Genomic DNA and isolated fragments of *Xba* elements were denatured by mixing with an equal volume of 1 M sodium hydroxide and kept at room temperature for 5 minutes. An equal volume of 2 M ammonium acetate was added and vortexed to neutralize the samples. The samples were then loaded onto a nylon membrane by using a dot blot apparatus (BRL, Bethesda, Md.). One milliliter of 5× SSC was then added to each sample spot. The filter was removed and air dried before baking at 80°C for 2 hours prior to hybridization as described above for Southern blot analysis. The hybridization signals were quantified by using the GS-525 Molecular Imaging System (Bio-Rad, New York, N.Y.).

Cloning the Xba *repetitive DNA fragments*

Each sample of genomic DNA was digested with restriction endonuclease *Xba*I and subjected to electrophoresis on 1 % agarose gel. DNA fragments of *Xba* elements were recovered by using NA45 DEAE membranes (Sambrook et al., 1989). The purified fragments were ligated into the *Xba*I site of pBluescript SK^+ (Stratagene, La Jolla, Calif.). Ligations and bacterial transformations were performed with standard procedures (Sambrook et al., 1989). Mini-scale plasmid preparations were done to screen for the clones with proper inserts. Clones with expected sizes were picked for sequencing analysis.

DNA sequencing and sequence analysis

All cDNA clones were sequenced by the Sanger's dideoxy termination method (Sanger et al., 1977) using the cycle sequencing kit (Applied Biosystems, Inc., Calif.). Minipreparation plasmid DNA (1 µl, about 200–500 ng) was used for each reaction. The profiles for cycling were: 94°C for 1 minute, 72°C for 1 minute, 55°C for 1 minute, for 30 cycles.

An initial 2 minutes of extra denaturation at 94°C was always used. Both the universal and the reverse sequencing primers were used for sequencing both strands of the cloned inserts. DNA sequences were read and analyzed by using the DNAStar software package (DNAStar Inc, Madison, Wis.) or using the DNAsis software package (Hitachi, distributed by National Biosciences, Inc., Plymouth, Minn.).

PCR amplification

PCR reactions of 50 µl were carried out in 50 mM KCl, 10 mM Tris (pH 9.0 at 25°C), 0.1% Triton X-100, 0.25 mM each of deoxynucleotide triphosphate (dNTPs), 1.5 mM $MgCl_2$, 20 µM each of the upper and lower PCR primers, about 200 ng of genomic DNA, and 2.5 units of *Taq* DNA polymerase. The temperature profiles used to amplify the cDNAs were 94°C for 30 seconds, 45°C for 1 minute, and 72°C for 2 minutes, for 35 cycles. Upper primer of *Xba* element reads AGTGGTTTTTCTAACAAACTC, and lower primer of *Xba* element reads CTTTTTCATCAACTTTTGC. PCR reactions were labeled by adding 1 µl ^{33}P-dATP in the PCR reactions. PCR products were analyzed on a sequencing gel using a sequencing reaction ladder as markers.

Acknowledgments

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