Identification and expression of *six* family genes in mouse retina

Kiyoshi Kawakami[a,*], Hiromi Ohto[a], Toshihiro Takizawa[b], Takuma Saito[b]

[a] *Department of Biology, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi-ken, 329-04, Japan*
[b] *Department of Anatomy, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi-ken, 329-04, Japan*

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Abstract **We identified five cDNA clones of the *Six* gene family which are expressed in retina. They are Six2, Six3α and Six3β (which are derived from alternative splicing forms), Six5, and AREC3/Six4. All of these *Six* family genes possess extensive sequence similarity among each other in the *so*-homologous region (*Six* domain and homeodomain) but differ greatly in structure in some other regions. The amino acid sequence similarity of the *so*-homologous region to the previously identified AREC3/Six4 is 70.1% for Six2, 57.3% for Six3α and Six3β, and 70.3% for Six5. The expression of these genes was observed in inner and outer nuclear layer, ganglion cell layer, and pigment epithelium of mouse retina by in situ hybridization. The *so*-homologous region of each *Six* family protein has specific DNA binding activity. Six5 and Six2 bind to the same sequence as does AREC3/Six4, while Six3 does not. These observations suggest that some of the *Six* family genes can regulate the same target genes.**

Key words: *Six* gene family; cDNA cloning; Retina development; Homeobox protein; Gel retardation assay; GST-fusion protein

1. Introduction

The mammalian *Six* family genes were identified by homology to the *Drosophila sine oculis* (*so*) gene product [1–3]. The *so* codes a homeobox protein and is essential for the development of *Drosophila* complex eye [4]. Four members of the mouse *Six* gene family have been characterized. *Six1* and *Six2* are restricted to head and body mesenchyme, limb muscles, and tendons [1]. *Six3* was observed in anterior neural plate and in developing eye [2]. We cloned the cDNA of AREC3/*Six4*, which encodes the transcription factor regulating the Na^+,K^+-ATPase α_1 subunit gene (*Atp1a1*) [3,5]. Binding activity of the factor was observed in restricted cell types [5] and was regulated during the cell cycle [6]. The AREC3 protein was produced in the nucleus and cytoplasm of C2C12 myoblast cells and its production was augmented during muscle differentiation [3]. The production of AREC3 protein was specifically observed not only in developing skeletal muscle but also in ganglion cells, inner plexiform and outer segment of retina (unpublished observation), suggesting an important role in the formation of retina structure. We screened a retina cDNA library to seek possible alternative spliced forms of the AREC3 and other *Six* family genes specific to retina. At least four kinds of newly identified *Six* gene family cDNAs were cloned. The roles of these *Six* family genes in retina formation are discussed.

*Corresponding author. Fax: (81) (285) 44 5476.

Abbreviations: SIX, sine oculis related homeobox; Na^+,K^+-ATPase, sodium- and potassium-dependent adenosine triphosphatase; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; ARE, *Atp1a1* regulatory element.

2. Materials and methods

2.1. Screening of the cDNA library and DNA sequencing

The mouse retina cDNA library from 1-month-old BALB/c mice was kindly supplied by Dr. Ananda Swaroop [7]. A *Sal*I-*Dra*I (−17–2486) fragment of AREC3 cDNA (pSVSPORTM18) [3] was labeled with [^{32}P]dCTP with a Megaprime labeling kit (Amersham). About 1.2×10^6 plaques were screened and we obtained 13 positive clones. They were grouped into 4 based on restriction mapping and partial nucleotide sequencing. The overlapping clones were sequenced and we obtained four species of *Six* family cDNAs. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers: D83147 (Six2), D83144 (Six3α), D83145 (Six3β) and D83146 (Six5).

2.2. In situ hybridization

A *Hae*III (844–1049) fragment from the *Six2* cDNA, a *Pvu*II (1328–1784) fragment from the *Six5* cDNA, and a *Dde*I (655)-*Hha*I (798) fragment from *Six3α* cDNA were subcloned into the *Sma*I site of pSVSPORT. After linearization, sense and antisense probes were prepared with T7 and SP6 RNA polymerase using a digoxigenin labeling kit (Boehringer).

Eyes from 8-week-old ICR mice were fixed in 4% paraformaldehyde, 8% sucrose in PBS for 30 min at 4°C, and then the cornea and lens were removed. Retina was subsequently fixed for ~4 h overnight at 4°C. After washing with 8% sucrose/PBS followed by PBS, tissue was dehydrated with ethanol and then embedded in paraffin. Sections were cut at 5 μm thickness and mounted on aminopropyltriethoxysilane coated cover slips.

Tissue preparation and in situ hybridization were performed according to Uehara et al. [8] with modifications. The sections were deparaffinized and rehydrated in a graded series of ethanol, rinsed in PBS, and incubated in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA containing proteinase K (5 μg/ml) for 10 min at 37°C. They were washed in PBS containing glycine (2 mg/ml), and post fixed in 4% paraformaldehyde for 10 min. They were then washed in PBS containing glycine (2 mg/ml), rinsed in PBS and treated with 0.2 N HCl for 10 min. They were then washed in PBS, rinsed in 0.1 M triethanolamine, pH 8.0, and acetylated in the same buffer containing 0.25% acetic anhydride by incubation for 10 min at room temperature. Slides were then rinsed in PBS, dehydrated in graded ethanol, and finally air dried.

Hybridization was performed in a mixture of 50% deionized formamide, Denhardt's solution, yeast tRNA(1 mg/ml), 20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, pH 8.0, 300 mM NaCl, 10% dextran sulfate. The hybridization mixture containing digoxigenin-labeled RNA probe (0.65 μg/ml for *Six2* and *Six3α*, 1.0 μg/ml for *Six5*) was applied to tissue sections which were covered by cover slips and incubated in a moist chamber at 50°C for 16 h to allow hybridization. After hybridization, the cover slips were removed gently in 5×SSC, and then the slides were washed in 50% formamide, 2×SSC at 50°C for 1 h, rinsed in 10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA for 10 min at 37°C, treated with 20 μg/ml RNase A in the same buffer for 30 min at 37°C, and washed in the same buffer for 10 min. The slides were then washed in 2×SSC, 0.2×SSC, and 0.1×SSC for 30 min each at 55°C. After rinsing and blocking, the slides were then incubated with anti-digoxigenin antibody, conjugated with alkaline phosphatase (Boehringer) as described.

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A

Six2

MSMLPTFGFTQEQVACVCEVLQQGGNIERLGRFLWSLPACEHLHKNESVLKAKAVVAFHR 60
GNFRELYKILESHQFSPHNHAKLQQLWLKAHYIEAEKLRGRPLGAVGKYRVRRKFPLPRS 120
IWDGEETSYCFKEKSRSVLREWYAHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDR 180
AAEAKERENSENSNSSSHNPLASSLNGSGKSVLGSSEDEKTPSGTPDHSSSSPALLLSPP 240
PPPGLPSLHSLGHPPGPSAVPVPVPGGGGADPLQHHHSLQDSILNPMSANLVDLGS

Six3α

MVFRSPLDLYSSHFLLPNFADSHHCSLLLASSGGGSGASGGGGGAGGGGGGNRAGGGGAG 60
GAGGGSGGGGSRAPPEELSMFQLPTLNFSPEQVASVCETLEETGDIERLGRFLWSLPVAP 120
GACEAINKHESILRARAVVAFHTGNFRDLYHILENHKFTKESHGKLQAMWLEAHYQEAEK 180
LRGRPLGPVDKYRVRKKFPLPRTIWDGEQKTHCFKERTRSLLREWYLQDPYPNPSKKREL 240
AQATGLTPTQVGNWFKNRRQRDRAAAAKNRSVAGTAARPPQAPG

Six3β

RSPLDLYSSHFLLPNFADSHHCSLLLASSGGGSGASGGGGGAGGGGGGNRAGGGGAGGAG 60
GGSGGGGSRAPPEELSMFQLPTLNFSPEQVASVCETLEETGDIERLGRFLWSLPVAPGAC 120
EAINKHESILRARAVVAFHTGNFRDLYHILENHKFTKESHGKLQAMWLEAHYQEAEKLRG 180
RPLGPVDKYRVRKKFPLPRTIWDGEQKTHCFKERTRSLLREWYLQDPYPNPSKKRELAQA 240
TGLTPTQVGNWFKNRRQRDRAAAAKNRLQHQAIGPSGMRSLAEPGCPTHGSAESPSTAAS 300
PTTSVSSLTERADTGTSILSVTSSDSECDV

Six5

AAADSGSPSGPGSPRETVTEVPTGLRFSPEQVACVCEALLQAGHAGRLSRFLGALPPAER 60
LRGSDPVLRARALVAFQRGEYAELYQLLESRPFPAAHHAFLQDLYLRARYHEAERARGRA 120
LGAVDKYRLRKKFPLPKTIWDGEETVYCFKERSRAALKACYRGNRYPTPDEKRRLATLTG 180
LSLTQVSNWFKNRRQRDRTGTGGGAPCKSESDGNPTTEDESSRSPEDLERGVASMAAEAP 240
AQSSIFLAGATSPATCPASSSILVNGSFLAASSPPAVLLNGSPVIINSLALGENSSLGPL 300
LLTGGSAPQPQPSLQGVSEAKNSLVLDPQTGEVRLDEAQSEAPETKGVHGTTGEEIPGAL 360
PQVVPGPPPASTFPLTPGAVPAVAAPQVVPLSPSSGYPTGLSPTSPRLNLPQVVPTSQVV 420
TLPQAVGPLQLLAAGPGSPVKVAAAAGPTNVHLINSSVGVTALQLPSSTAPGNFLLANPV 480
SGSPIVTGVAVQQGKIILTATFPTSMLVSQVLPPAPSLALPLKQEPAITVPEGALPVGPS 540
PTLPEGHTLGPISTQPLPPASVVTSGTSLPFSPDSSGLLSSFSAPLPEGLMLSPAAVPVW 600
PAGLELSTGVEGLGTQATHTVLRLPDPDPQGLLLGATTGTEVDEGLEAEAKVLTQLQSVP 660
VEEPLEL

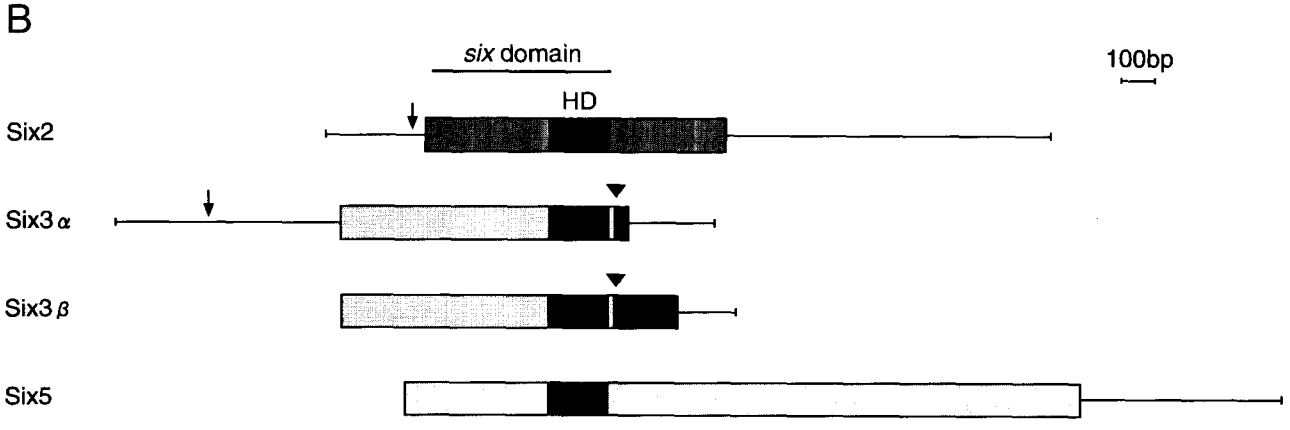

Fig. 1. Amino acid sequence and structural features of cDNA clones of *Six* family genes. (A) Amino acid sequence deduced from the cDNA sequence of the *Six* family cDNAs. The portions used for GST-fusion proteins are underlined. (B) Structural features of five *Six* family cDNAs expressed in retina. The coding regions are shown as boxes. Triangles indicate the position where sequence diversity occurs. Arrows indicate the position of in-frame termination codon upstream of the coding region. HD, homeodomain.

2.3. GST-fusion proteins

For SIXDso, an *Msc*I-*Eco*RI (948–1773) fragment of Six3α cDNA was blunt-ended and ligated into the blunt-ended *Eco*RI site of pGEX-3X. For SIX2so, a *Bss*HII-*Dde*I (281–1007) fragment of Six2 cDNA was blunt-ended and subcloned into the *Sma*I site of pGEX-3X; for SIXGso, a *Sma*I-*Nco*I (44–705) fragment of Six5 cDNA was blunt-ended and subcloned into the blunt-ended *Eco*RI site of pGEX-3X. SMNT has been described by Kawakami et al. [3]. GST-fusion proteins were induced by adding 1 mM IPTG, then purified on a gluthathione-Sepharose column (Pharmacia) following the manufacturer's protocol.

2.4. Gel retardation assays

Gel retardation assays were performed as described [9]. ARE fragment (*Pvu*II-*Mlu*I fragment of *Atp1a1*) [5] was labeled with [^{32}P]dCTP and used as a probe. For competitors, C3WT: 5'-TCGAGCCGGTGTCAGGTTGCTCC and 5'-TCGAGGAG-CAACCTGACACCGGC were annealed and C3MUT: 5'-

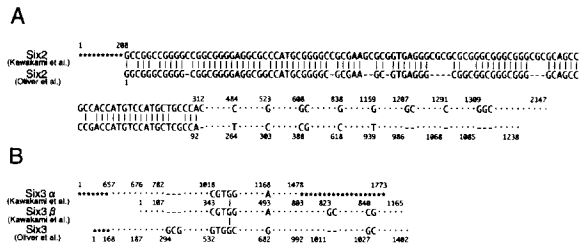

Fig. 2. Summary of the sequence differences between the Six2 cDNAs (A) and among Six3α, Six3β and Six3 cDNAs (B)

TCGAGCCGGTGTGAGGTTGCTCC and 5′-TCGAGGAG-CAACCTCACACCGGC were similarly annealed.

3. Results and discussion

3.1. Cloning and sequence of Six family gene cDNAs from cDNA library of mouse retina

To identify the *Six* family genes expressed in retina, we screened a retina cDNA library from BALB/c mice using a probe containing the *so*-homologous region of the AREC3 cDNA. The 13 isolated clones were classified into 4 groups. All four cDNAs were distinct from the AREC3 one, but contain the *so*-homologous region and therefore were the *Six* family genes. One of the clones was the full length version of *Six2* (Fig. 1A), which was originally identified by Oliver et al. [1]. The nucleotide sequence has differences at positions 608 and 609 resulting in R to A amino acid change, silent differences at positions 484, 523, 838 and 1159, which might be due to polymorphism. In the 3′ non-coding region, residues at positions 1207, 1208, 1291, 1309, 1310 and 1311 are missing in the sequence reported by Oliver et al. [1]. The nucleotide sequence of the 5′ region (upstream from position 312) of the *Six*2 cDNA has many differences from that reported by Oliver et al. [1] for unknown reasons. These differences are summarized in Fig. 2A. The coding region is from nucleotide position 296 to 1183. Six3α and Six3β are apparently derived from alternatively spliced forms of the same gene (Fig. 1B). They share the *so*-homologous region, but the sequences diverge from position 1478 of Six3α and position 803 of Six3β. Six3α and Six3β share the sequence with the recently identified *Six*3 gene cDNA from E14.5 mouse brain [2]. Sequence differences are noted in several points. The sequence upstream of position 656 of Six3α is quite divergent from that of *Six3*. The GCG sequence at position 294–296 of *Six3* is missing in Six3α and Six3β. The nucleotide sequence at position 532–536 of *Six3* is different in position 1018–1022 of Six3α and 343–347 of Six3β, resulting in the amino acid sequence difference of WP (*Six3*) and VA (Six3α and Six3β). The G residue at position 682 and GC nucleotides at 1027 of *Six3* are different. GC nucleotides (at positions 823 and 824 of Six3β) are missing in between 1011 and 1012 of *Six3*, resulting in the frame shift and amino acid sequence difference between *Six3* and Six3β (Fig. 2B). These difference might be due to alternative splicing, polymorphism, cloning artifacts and/or sequencing error. The existence of the alternatively spliced mRNA in retina was confirmed by RT-PCR for Six3α and Six3β (data not shown). Six5 is the cDNA encoded by the MDAHP gene [10]. The Six5 sequence is identical to that of the exonic region of the mouse gene. It is apparently a partial clone, and the coding region spans up to position 2001.

3.2. Homology of the Six gene family

The isolated cDNAs share homologous sequence only in *Six* and homeodomains. To gain insight into the evolutionary relationship among the *Six* family genes, we analyzed the amino acid sequence of the domains using the Genetyx multiple alignment program. The alignment shows that 84 amino acids were conserved in a total of 178 amino acids (Fig. 3A). The similarity of the sequence to AREC3/Six4 is 70.1% for Six2, 57.3% for Six3α and Six3β, 70.3% for Six5. The phylogenetic relationships are shown in Fig. 3B.

3.3. Expression of cDNAs in mouse retina

To confirm the expression of these cDNAs in retina and to analyze the location of the transcripts, in situ hybridization was performed in mouse retina using antisense and sense probes from the cDNAs. As shown in Fig. 4, *Six2* and *Six3α* show clear expression in the inner and outer nuclear layers, the ganglion cell layer and in the pigment epithelium layer. The expression of *Six2* in retina is contradictory to the report by Oliver et al. [1]. In the case of *Six5*, expression was very weak and showed a pattern similar to those seen with *Six2* and *Six3*. The distribution of the AREC3 mRNA shows a similar pattern (data not shown). Since it is known that *Drosophila so* is essential for eye formation, these homeobox genes might be involved in the determination and maintenance of mouse retina formation. The expression of four kinds of *Six* family genes raises the possibility that all four genes play a role as a functional homologue of *Drosophila so* rather than that only one of the genes is a homologue.

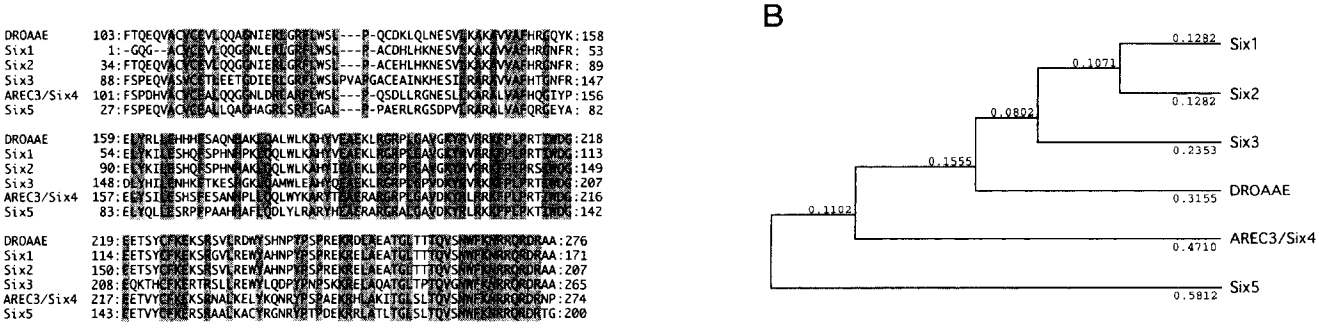

Fig. 3. (A) Alignment of *Six* and homeo domain of *Six* family genes. Conserved amino acids are in gray. (B) Putative phylogenetic relationship of the *Six* family genes.

3.4. Analysis of DNA binding ability and specificity

To ascertain whether the *so*-homologous region can function as a DNA binding domain, as observed in the case of AREC3, we expressed the *so*-homologous region of the *Six* family gene cDNAs as GST-fusion proteins. The purified recombinant protein was tested for DNA binding activity with the use of ARE fragment as a probe in a gel retardation assay. Fig. 5 indicates that Six2, Six3, and Six5 bind to the ARE probe (lanes 1,4,7). Specific competition with the specific oligonucleotide of the AREC3 binding site C3WT but not with the mutation oligonucleotide C3MUT was observed for Six2 and Six5 (lanes 1–3,7–9), and was not observed for Six3 (lanes 4–6). A control experiment which shows specific competition using AREC3 GST-fusion protein and HeLa nuclear extracts is also shown (lanes 10–15). The wild-type competitor interfered with the formation of C3 but not with those of C1 and C2 (lanes 11,14) and the mutant competitor did not (lanes 12,15). These results indicate that AREC3/Six4, Six2 and Six5 can bind to the same DNA element and might compete in binding with each other. Six3 shows DNA binding activity with a sequence unrelated to the C3 consensus sequence. These results are consistent with the observation that Six3 shows the least homology with AREC3 in amino acid sequence. The relationship between the *Six* family genes and other homeobox genes such as *Pax6* [11] and *Chx10* [12], which are involved in the determination of retina will be the crucial step for understanding the mechanism of retina formation.

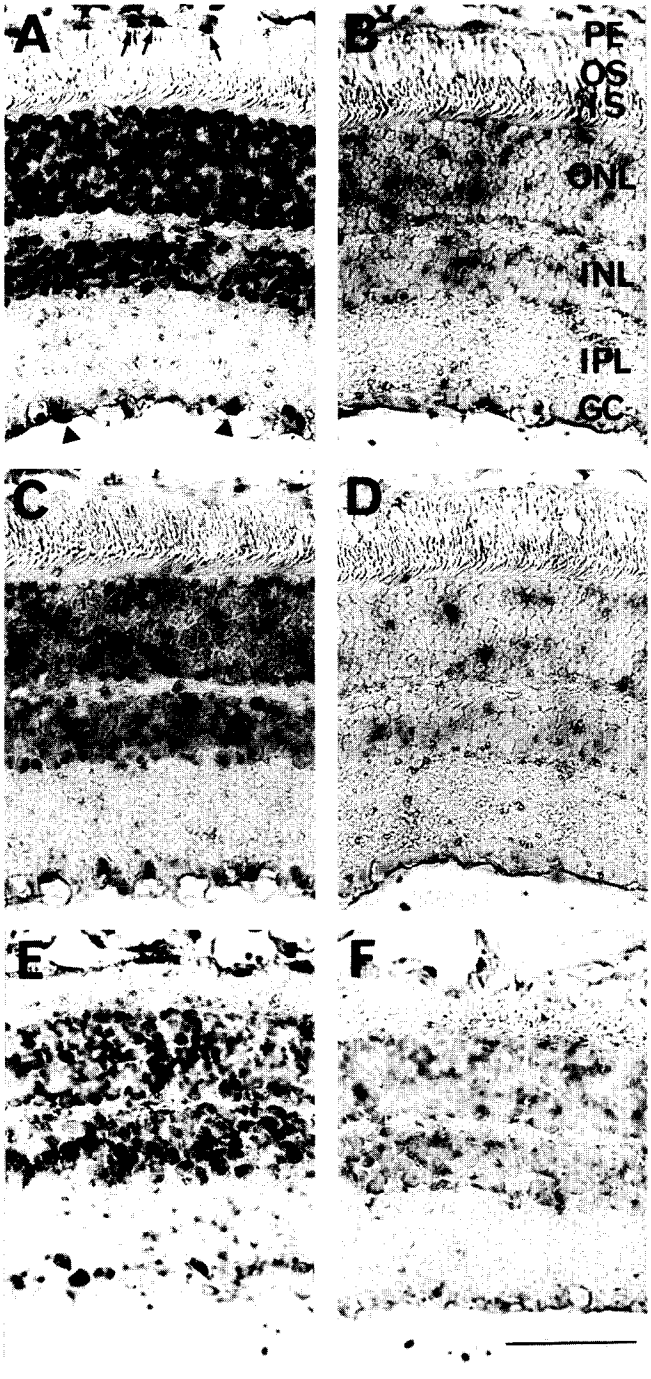

Fig. 4. In situ hybridization analysis of *Six* family gene. Antisense probes for *Six2* (A) *Six5* (C) *Six3* (E), and sense probes for *Six2* (B) *Six5* (D) *Six3* (F) were used. PE, pigment epithelium layer; OS, outer segment; IS, inner segment; INL, inner nuclear layer; ONL, outer nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer. Arrows indicate the positions of signal in PE and triangles indicate those in GC. Bar = 50 μm.

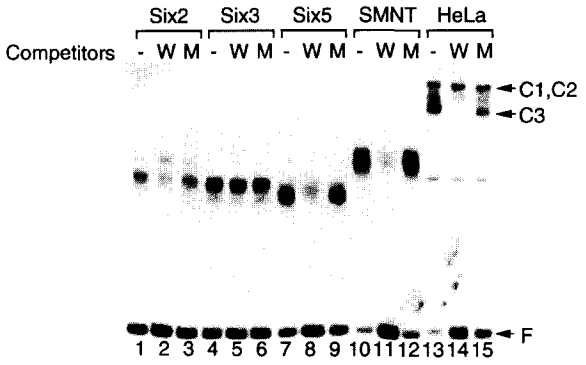

Fig. 5. Gel retardation analysis of *Six* family genes. DNA binding domain and binding specificity of *Six* family genes. GST-fusion proteins of Six2 (lanes 1–3), Six3 (lanes 4–6), Six5 (lanes 7–9), AREC3 (lanes 10–12) and 10 μg protein of HeLa cell nuclear extract (lanes 13–15) were added. The positions of free probe (F) and the retarded complexes of HeLa cells are indicated. 5 fmol of the labeled probe was used. 100 fmol of the specific competitor oligonucleotide C3WT (lanes 2,5,8,11,14) and the mutant competitor C3MUT (lanes 3,6,9,12,15) were added.

4. Conclusion

Four of the *Six* family genes which are expressed in retina were identified by molecular cloning and in situ hybridization analyses. They are AREC3/Six4, Six2, Six3α and Six3β, which are alternatively spliced forms of *Six3*, and the newly identified Six5. The *so*-homologous region functions as the specific DNA binding domain and at least AREC3, Six2 and Six5 share the specific binding sequence. These results strongly suggest the complex regulatory network of the *Six* family genes during retina formation.

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