Korean J Physiol Pharmacol
Vol 1: 195−200, April, 1997

Cloning of Mouse AQP-CD Gene

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Water transport in highly-permeable membranes is facilitated by some specialized pathways, which are called aquaporins (AQP). AQP1 (AQP-CHIP) is the first recognized aquaporin identified from red cells and renal proximal tubules. Up until now 4 other aquaporin homologs have been reported. Each aquaporin has its unique tissue distribution and regulatory mechanims. To elucidate molecular mechanisms for their transcription regulation and tissue-specific expression isolation of aquaporin genes is required. To clone promoters of the AQP family mouse genomic library was screened by the 1st exon-specific probe of AQP4, and 5 different plaques were positively hybridized. Phage DNAs were purified and characterized by restriction mapping and sequencing. One of them is the mouse AQP-CD gene. The gene was consisted of 4 exons and the exon-intron boundaries of mouse AQP-CD gene were identified at identical positions in other related genes. The 5'-flanking region of AQP-CD gene contains one classic TATA box, a GATA consensus sequence, an E-box and a cyclic AMP-responsive element. The cloning of the mouse AQP-CD gene, of which product is expressed in the collecting duct and is responsible for antidiuresis by vasopressin, will contribute to understand the molecular mechanisms of tissue-specific expression and regulation of AQP-CD gene under various conditions.

Key Words: Aquaporin-CD, Gene, Cloning

INTRODUCTION

Water transport is mediated by two distinct pathways through the plasma membrane, simple diffusion through lipid bilayer and channel-mediated transport (Finkelstein, 1987). The first clue of molecular identity of water channels was provided by the discovery of CHIP28 from red cells and renal proximal tubules (Denker et al, 1988). The cDNA cloning of CHIP28 revealed that the protein belongs to an ancient channel family, the major intrinsic protein (MIP) family (Preston & Agre, 1991) and the following study demonstrated that this protein is the first molecular water channel (Preston et al, 1992). Subsequently, four different water channel homologs have been found in mammalian cells (Knepper, 1994; Jung et al, 1994; Raina et al, 1995), which are called 'aquaporins (AQP)' (Agre et al, 1993). Each aquaporin has its unique distribution and regulatory mechanisms in mammalian tissues (Knepper, 1994). Tissue-specific expression and transcriptional regulation of a gene are determined by the binding of transcriptional factors at cis-acting regulatory DNA sequences on the 5'-flanking regions of a gene. Therefore, cloning of aquaporin genes is required to understand molecular mechanisms of their tissue-specific expression and regulation.

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METHODS

Genomic library screening

Rat AQP4 cDNA probes were previously described (Jung et al, 1994): I) 494 bp *Bam*H I-*Sma* I fragment containing between nucleotide +394 and +888 of the coding sequence; ii) 231 bp *Bam*H I fragment containing between nucleotide +162 and +393. A mouse

129 genomic DNA library in FIXII vectors (Stratagene) was provided by Se Jin Lee, Johns Hopkins University.

The ^{32}P-labeled 231 bp *Bam*H I fragment of rat AQP4 cDNA (Jung et al, 1994) was used to screen 106 plaques with moderate stringency. cDNA probes were gel-purified and labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Co.) using a random priming kit (Boehringer Manheim). Phage DNAs from positive clones were purified and digested by single or combinations of restriction endonucleases and analyzed by Southern blot with ^{32}P-labeled probes obtained. DNA fragments which were hybridized by the 1st exon specific probe of AQP4 were subcloned into pBluescript SK(-) vector (Stratagene, USA) and sequenced by the dideoxy chain termination method using T3 or T7 primers (Sanger et al, 1977). To get the whole sequences of inserts nested deletion constructs were made by exonuclease III endonuclease (Promega). Except where listed, standard methods were employed (Sambrook et al, 1989).

Southern blot anaysis

100 ng of purified phage DNA and plasmid DNAs was digested with various restriction endonucleases, electrophoresed on a 1% agarose gel, and blotted by a nylon membrane. The membrane was hybridized with the same probe used for the screening for genomic clones and washed in 1 X SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at 55°C.

Primer extension analysis

Total RNA was extracted from rat kidney medulla. A synthetic oligonucleotide corresponding to nucleotides -1 to -24 in the 5'-flanking region of the mouse AQP-CD gene was end-labeled with γ-[^{32}P]ATP, 1 x 10^5 cpm of which was hybridized with 20 μg of total RNA for 55°C for 1h in 250 mM KCl, 10 mM Tris, pH 8.0, 1 mM EDTA. Primer extension was carried out in 75 mM KCl, 10 mM $MgCl_2$, 0.25 mM EDTA, 20 mM Tris-Cl (pH 8.0), 10 mM dithiothreitol, 0.25 mM dNTP, 100 μg/ml with 500 U of Superscript II reverse transcriptase (Life technologies, Inc.). Ths synthesized product was analyzed in denaturing polyacrylamide gel electrophoresis. The sequence ladder was generated by sequencing the 5'-flanking region of mouse AQP-CD gene with the same primer used for primer extension analysis.

RESULTS

Characterization of clone mAQPp1

5 recominants which reacted with the ^{32}P-labeled AQP4 *Bam*H I probe were isolated from a partially *Mbo* I-digested mouse genomic library. The isolates contained inserts of about 15 kb. Restriction map of one genomic clone (designated mAQPp1) was established with digestion with single or combination of *Kpn* I, *Bam*H I, *Sal* I and *Xho* I. To check which

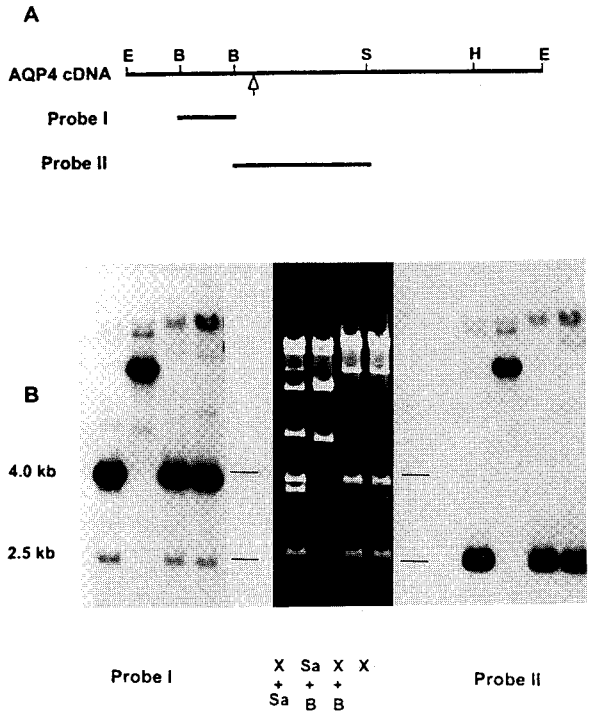

Fig. 1A. Schematic representation of the AQP4 probes used in southern hybridization. Probe I is the *Bam*H I fragment and Probe II is the *Bam*H I/*Sma* I fragment. The arrow indicates the position of the 1st and 2nd exon boundary of the MIP family. Abbreviations: B, *Bam*H I; E, *Eco*R I; H, *Hind* III; S, *Sma* I

B. Autoradiographic results of Southern blot of MAQPp1. Purified MAQPp1 phage DNA was digested with *Xho* I, *Sal* I, *Bam*H I, or their cominations. DNAs were transferred to a nylon membrane and the membrane was hybridized with the probe I or probe II. Abbreviations: B, Bam HI; Sa, *Sal* I; X, *Xho* I

fragments contained exons, southern hybridization was done using AQP4 probes (Fig 1A). The 4 kb fragment by *Xho* I were positively hybridized with Probe I which is supposed to contain the 1st exon only (Moon et al, 1993), whereas the 2.5 kb fragment by *Xho* I was hybridized with the Probe II (Fig. 1B). This result indicated that the 4.0 kb *Xho* I fragment contained the 1st exon and the 2.5 kb fragment contained the remaining part of the gene. The 4 kb fragment contained three *Pst* I sites and the 1.5 kb fragment among the fragments was positively hybridized with the Probe I. To sequence 1.5 kb *pst* fragment, nested deletion constructs were made by exonuclease III enzyme. The sequencing revealed that mAQPp1 is the mouse AQP-CD gene, of which the gene product is only expressed in the kidney collecting duct and is called 'AQP-CD' (Knepper, 1994). The remaining 4 phage clones were also analyzed by the same method as described above. However, they did not show any homology with the aquaporin family.

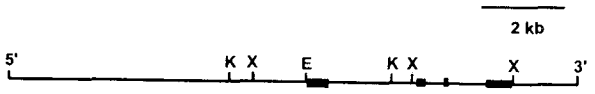

Fig. 2. Genomic structure of mouse AQP-CD gene. The restriction map of mouse AQP-CD gene is shown. The rectangles indicate exon segments. Abbreviations: X, *Xho* I; K, *Kpn* I; E, *Eco*47 III

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mAQP-CD   1 MWEERSIAFS RAVLAEFLAT LLFVFFGLGS  30
Human         L          F
         31 ALQWASAPPS VLQIAVAFGL GISTLVQALG  60
             N T          M       G
         61 HVSGAHINPA VTVACLVGCH VSFLRAAFYV  90
             I
         91 AAQLLGAVAG AAILHEITPV EIRGDLAVNA 120
                       L      A D
        121 LHNNATAGQA VTVELFLTMQ LVLCIFASTD 150
             S ST               L
        151 ERRSDNLGSP ALSIGFSVTL GHLLGIYFTG 180
              GE P T           A       HY
        181 CSMNPARSLA PSVVTGKFDD HWVFWIGPLV 210
                       A
        211 GAVIGSLLYN YLLFPSTKSL QERLAVLKAL 240
              IL       V   PA    S       G
        241 EPDTDWEERE VRRRQSVELH SPQSLPRGSKA 271
                                          T
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Fig 3. The amino acid sequence of mouse AQP-CD and its comparison to human AQP-CD. Putative transmembrane domains are underlined.

Characteristics of mouse AQP-CD gene

Sequencing of the 4- and 2.5 kb- *Xho* I fragments by Exo III-deletion revealed the gene structure of mouse AQP-CD gene. It consists of 4 exons corresponding to amino acids 1-120, 121-175, 176-202, and 203-271 (Fig. 2). Sequence identity of the mouse AQP-CD to the human AQP-CD is 87.5%. The hydropathy profile of mouse AQP-CD predicts six membrane-spanning domains. The conserved amino acid sequence, named NPA box, and tandem repeat in the sequence, which are characteristics of MIP family (Pao et al, 1991; Agre et al, 1993), were also conserved in mouse AQP-CD. One potential N-glycosylation site is present in the sequence of mouse AQP-CD (Asn 124), and one phosphorylation site for protein kinase A (Ser 256) is conserved in the mouse AQP-CD (Fig. 3).

The exon-intron boundaries of mouse AQP-CD genes are identified at identical positions in other related gene, the human AQP-CHIP gene. All splice

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CTGGCTGTCAATGCTgtgagtagcccgagtttc   Exon 1/Intron 1
 L  A  V  N  A
ctccacctctctctccagCTCCACAACAATGCA   Intron 1/Exon 2
                  L  H  N  N  A

GGTCACCTCCTTGGGgtaagtaagtcaaagtcc   Exon 2/Intron 2
 G  H  L  L  G

ctcccactacagATCTATTTCACCGGCTGCTCC   Intron 2/Exon 3
           I  Y  F  T  G  C  S

TTTGATGATCACTGGgtaatggccaaatctctg   Exon 3/Intron 3
 F  D  D  H  W

ccccttcccccagGTCTTCTGGATCGGACCCCTG  Intron 3/Exon 4
           V  F  W  I  G  P  L
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Fig. 4. Exon-intron organization of mouse AQP-CD gene.

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-402 CTGCAGGAACTGGTGCTGGTGGTCATTGTGGGGGCTGGGGCA
                                          spl
-360 GCCTGAGGCAGCTCCATGGGGTAACTGAGGAAAAAACGAGGA
-318 AAACAGAGACGTCAATCCTTATCTGGAGTCCATTAATGAGGA
          CRE
-276 GAACATTAGTCAGCTGTGAAAGCTAAGATGGGGTGATAGGCC
           E box                    GATA
-234 TTCGGGTGGGCAGGAGCAGGGATGGGGGCAAGTCCGCCATGG
-192 GGGAGCACAGGGTTGGCAGGAACGCCTCCTCCACCCCACGTG
-150 CCCAGGTCTACGATAGGAAGGCCCTATAAGTGCCCCACAGTC
-108 TAGCCTCTCCGGAGGCCCAGAGGAAGAGAGAAGAGAAAGAGA
 -66 GAGGGAGGGAGGAAGAGCCACCCCCGTGGCCCAGACCACTGG
 -24 CCAGCGCTCAGAAGGCCGAGCAGCATGTGGGAACTCCGGTCC
                          M  W  E  E  R  S
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Fig. 5. 5'-flanking sequence of the mouse AQP-CD gene. The first ATG codon is double underlined. CRE, E-box, GATA and SP1 consensus sites are underlined.

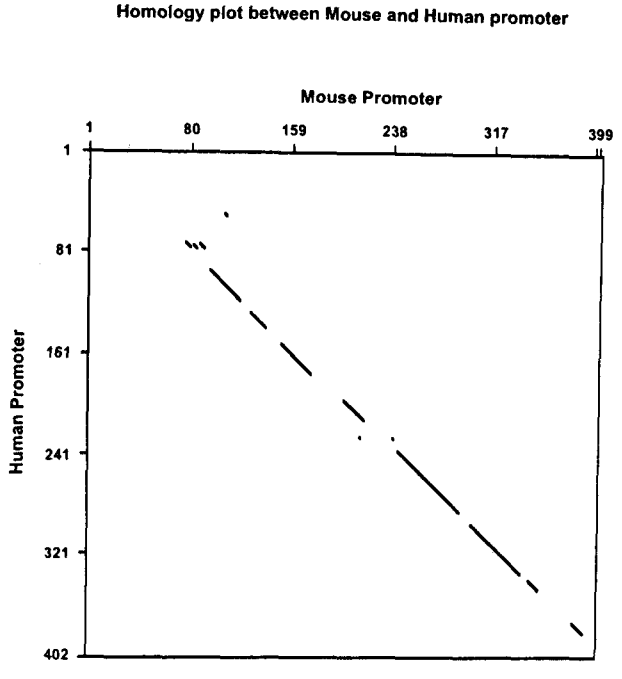

Fig. 6. Homology plot between the mouse and human AQP-CD promoter. The sequences between the TATAA sequence and -402 bp from the translation initiation site are highly conserved between two species.

sites conform to the GT-AG rule and the exon-intron boundaries are class 0 (Fig. 4).

The sequencing of the 5'-flanking region of mAQP-CD gene revealed the existence of the several consensus sequences (Fig. 5). The region contained the sequence TATAA located at nucleotides -126 to -122 with respect to the translation initiation site. No other potential ATG translation initiation signals were identified and no CAATT sequence was present. SP1 sequence (KRGGCKRRK) was found at -372. A search for the 5'-flanking region for canonical consensus sequences (Fiasst & Meyer, 1992) revealed the presence of a GATA motif (A/TGATAR) at -243, one cAMP-responsible element (GACGTCA) at -311, and one E-box (CANNTG) at -266. The site of transcription initiation was determined by primer extension. Primer extension assay revealed that the site of transcription initiation was found at -94 proximal from the site of translation initiation (data not shown).

Fig 6. represents the homology plot between the human and mouse AQP-CD promoter. Although the 5'-untranslated regions of AQP-CD gene in both species are quite different, sequences between TATA box and -402 from the translation initiation site revealed a strong homology between two species.

DISCUSSION

The water permeability of collecting duct is significantly increased by vasopressin, so that the water in collecting ducts is reabsorbed and urine becomes concentrated to conserve water from body. This is accomplished by insertion of water channels following stimulation by vasopressin (Verkman, 1989). The increase of intracellular cyclic AMP (cAMP) concentration by vasopressin via V2 receptor triggers this event (Handler, 1988). The water channel which is responsible for this process was cloned (Fushimi et al, 1993) and named as 'Aquaporin-CD' (AQP-CD). AQP-CD is only expressed in the collecting ducts in the kidney and its mutation results in autosomal recessive nephrogenic diabetes insipidus (Deen et al, 1994). Recent studies showed that dehydration increases expression of AQP-CD proteins, indicating that vasopressin regulates transcription of AQP-CD gene (Nielsen et al, 1993). To elucidate molecular mechanisms of its tissue-specific expression and

transcriptional regulation, studies using 5'-flanking region of AQP-CD gene is required.

In this study the mouse AQP-CD gene was cloned and characterized. It spans about 5 kb and consists of four exons like the human AQP-CHIP gene (Moon et al, 1994). The sites of exon-intron boundary was exactly same with that of the human gene (Uchida et al, 1994). The 5'-flanking region of the mouse AQP-CD gene has a typical TATA box but lacks a CCAAT box. There are other consensus sequences for cis-elements, a GATA motif, an E-box, a cAMP-responsive element (CRE), and a sp1 site.

The mRNA abundance of the rat AQP-CD has been demonstrated to be increased by dehydration (Fushimi et al, 1993). This study showed that the 5'-flanking region of the mouse AQP-CD gene has a CRE. Therefore, the increase in transcription rate of the AQP-CD gene by dehydration can be explained by increase of cAMP level via vasopressin V2 receptor (Terada et al, 1993). Binding of CREB activated by protein kinase A to CRE may enhance transcriptional rate of the gene. Recently, it has been reported that hypertonicity itself enhanced transcription rate of the sodium-dependent transporters gene of osmolytes by activation of unique transcriptional factors (Takenaka et al, 1994 & 1995) or the aldose reductase gene (Ferraris et al, 1994). Therefore, we cannot exclude the possibility of the presence of tonicity-responsible elements in the 5'-flanking region of AQP-CD gene. GATA factors and the basic helix-loop-helix transcriptional factor family that recognizes E-box may be involved in cell-specific expression, as described other tissues (Yamamoto et al, 1990; Murre et al, 1989). Further studies are necessary to elucidate the roles of these cis-elements in the transcriptional regulation of AQP-CD gene.

As shown in the homology plot between human and mouse promoter, the sequences of 5'-franking region between human and mouse are highly conserved and the positions of some binding sequences are well conserved in the human and mouse, suggesting that this region is important for transcriptional regulation of AQP-CD gene.

Studies using the 5'-flanking region of the mouse AQP-CD gene, of which product is expressed in the collecting duct and is responsible for antidiuresis by vasopressin, will contribute to elucidate the molecular mechanisms of the tissue-specific expression of AQP-CD gene and its transcriptional regulation under various conditions.

ACKNOWLEDGEMENTS

This study was supported by a grant from Ministry of Education (1995).

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