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The human *AQP4* gene: Definition of the locus encoding two water channel polypeptides in brain

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ABSTRACT **The aquaporin family of membrane water transport proteins are expressed in diverse tissues, and in brain the predominant water channel protein is AQP4. Here we report the isolation and characterization of the human *AQP4* cDNAs and genomic DNA. Two cDNAs were isolated corresponding to the two initiating methionines (M1 in a 323-aa polypeptide and M23 in a 301-aa polypeptide) previously identified in rat [Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B. & Agre, P. (1994) *Proc. Natl. Acad. Sci. USA* 91, 13052–13056]. Similar to other aquaporins, the *AQP4* gene is composed of four exons encoding 127, 55, 27, and 92 amino acids separated by introns of 0.8, 0.3, and 5.2 kb. Unlike other aquaporins, an alternative coding initiation sequence (designated exon 0) was located 2.7 kb upstream of exon 1. When spliced together, M1 and the subsequent 10 amino acids are encoded by exon 0; the next 11 amino acids and M23 are encoded by exon 1. Transcription initiation sites have been mapped in the proximal promoters of exons 0 and 1. RNase protection revealed distinct transcripts corresponding to M1 and M23 mRNAs, and AQP4 immunoblots of cerebellum demonstrated reactive polypeptides of 31 and 34 kDa. Using a P1 and a λEMBL subclone, the chromosomal site of the human *AQP4* gene was mapped to chromosome 18 at the junction of q11.2 and q12.1 by fluorescence *in situ* hybridization. These studies may now permit molecular characterization of *AQP4* during human development and in clinical disorders.**

Recognition of the aquaporin family of water transport proteins has provided a molecular explanation for the high water permeability of membranes of certain tissues. Characterization of the organization of aquaporin genes and identification of their sites within the human and mouse genomes have established a primary role for some aquaporins in specific clinical disorders (for review, see ref. 1). Recognition that Colton blood group antigens are polymorphisms on red cell AQP1 (2) led to the recognition that the *AQP1* null phenotype is not clinically severe and the prediction that backup water transport mechanisms exist (3). In contrast, mutations in *AQP2* cause a severe form of nephrogenic diabetes insipidus (4), and mutations in the *MIP* gene (encoding a lens aquaporin) cause congenital cataracts in mice (5).

AQP4 is known to be expressed predominantly in brain (6, 7). Although AQP4 may contribute to the pathophysiology of normal pressure hydrocephalus, pseudotumor cerebrii, or postischemic edema, such linkages have not yet been established. To facilitate such studies, isolation and characterization of human *AQP4* cDNAs and genomic DNAs were pursued. While these studies were underway, another group reported on the organization and localization of the gene encoding MIWC (an alternate name for AQP4; ref. 8). Although several observations were in agreement with ours, significant incompatibilities were noted, including unrelated N-terminal sequences, multiple discrepancies in the coding region, dissimilarities in the genomic organization, and conflicting localizations of the gene to different sites on human chromosome 18. Additional studies were therefore undertaken to reconcile these differences.

MATERIALS AND METHODS

cDNA and Genomic DNA Cloning. A 611-bp *Pst*I fragment from the coding region of rat *AQP4* cDNA (6) was random-labeled with [α-^{32}P]dCTP (Boehringer Mannheim, Amersham); this was used to probe 5×10^5 plaques from a human fetal brain cDNA library (λZAP; Stratagene) and an adult human retina cDNA library (λMaxi; CLONTECH). Purified clones were sequenced by dideoxynucleotide chain termination (United States Biochemical). A human genomic DNA library in λEMBL-3 (9) was screened with the rat *AQP4* cDNA fragment, and positives were restriction-digested and subcloned for Southern analysis and sequencing (10). Intron sizes were estimated by restriction mapping and PCR amplification of genomic DNA. A human P1 genomic DNA library (11, 12) was screened similarly, and presence of *AQP4* in clone ICRF P700M1638 (11) was confirmed by cycle sequencing for exons 1, 2, 3, and 4 (4).

RNA Studies. To determine the site of transcription initiation of exon 0, an antisense oligonucleotide primer 5′-AGCCAGAGTGCAGCTCTCAT-3′ was end-labeled with [γ-^{32}P]ATP; 1×10^6 cpm was hybridized to 10 μg of total human brain RNA and extended (10). Transcription initiation for exon 1 was undertaken similarly with the antisense primer 5′-AAGATCATCCAGTTTCACGGAA-3′. The template plasmid for RNase protection assays (6) was constructed with a 200-bp *Eco*RI–*Bam*HI fragment of M1*AQP4* cDNA (corresponding to 70 bp of exon 0 and 130 bp encoding exon 1) inserted into pBluescript II KS(−) and linearized with *Xba*I or *Kpn*I to generate sense or antisense RNAs. A uniformly labeled 311-nt antisense probe was synthesized from the T3 promoter with [α-^{32}P]UTP [800 Ci/mmol (1 Ci = 37 GBq); Amersham]; 10^6 cpm was hybridized at 42°C to 10 μg of total RNA from adult human brain or 60 μg of RNA from lung or salivary gland (CLONTECH). After digestion with RNase A (20 μg/ml) and RNase T1 (200 units/ml), protected fragments (3×10^5 cpm) were analyzed on 10% polyacrylamide sequencing gels next to sequencing standards. Sense RNAs generated from the T7 promoter (5 ng) or yeast tRNA (10 μg) were hybridized and digested similarly.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U63611–U63623).
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Chromosomal Localization. *In situ* hybridizations of R-banded normal human lymphocyte spreads (13) were performed with a 5.8-kb *Bam*HI λEMBL subclone or the P1 clone (ICRF P700M1638). The probes were labeled with biotin-14-dATP (GIBCO/BRL Life Technologies) hybridized to heat-denatured metaphase spreads (14), and visualized with fluorescein isothiocyanate conjugated to avidin-D in three signal amplification rounds (15). 4′,6′-Diamidino-2-phenylindole bands were obtained using standard methods and referred to according to the International System for Human Cytogenetic Nomenclature (16). Chromosomal slides were evaluated by digital imaging microscopy by high-performance charge-coupled device camera (Photometrics, Tucson, AZ).

RESULTS

Isolation and Characterization of Human *AQP4* cDNAs. Nine unique clones were isolated from a human fetal brain cDNA library, and two were isolated from an adult human retina cDNA library by probing with the rat *AQP4* cDNA (6). The longest brain clone contained 40 bp of 5′ untranslated sequence, an open reading frame encoding a 323-aa polypeptide with two potential initiating methionines (corresponding to M1 and M23 from rat), followed by ≈3 kb of 3′ untranslated DNA with a polyadenylylation consensus. Neither of the retina clones extended to an initiation site, but a cDNA was amplified from the retina library with a sense primer designed to the M23 sequence but not with a primer corresponding to M1 (data not shown). The nucleotide sequences of both DNA strands of brain and retina cDNAs were identical from M23 to the translation stop site. The deduced amino acid sequences of these human cDNAs were 94% identical to rat *AQP4* cDNA (6), with 11 of the 19 amino acid differences representing conservative substitutions (Fig. 1).

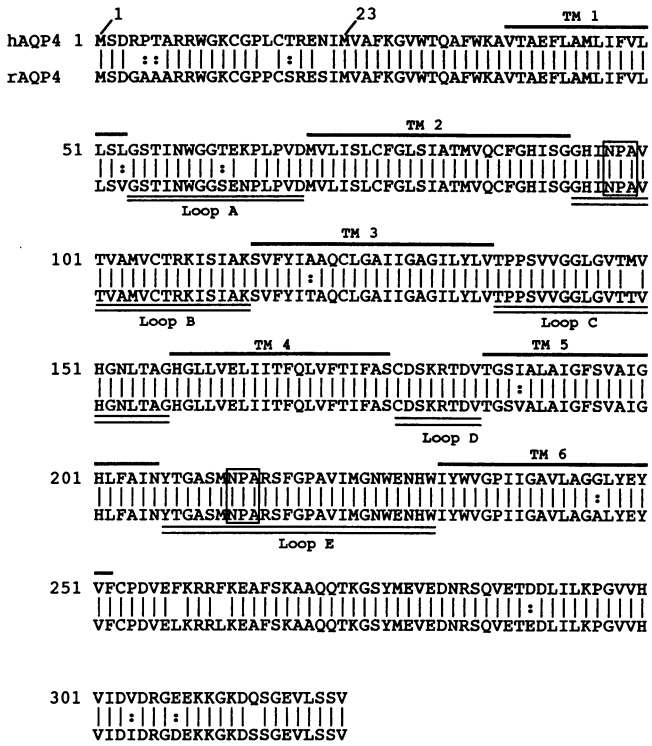

FIG. 1. Deduced amino acid sequences of human and rat *AQP4*. Identical amino acids are connected by straight lines; residues with similar charge, polarity, or structure are joined by dots. Presumed bilayer-spanning domains are labeled TM1–TM6. Connecting loops A–E are double-underlined. Conserved NPA motifs are boxed. The initiating methionines, M1 and M23, are indicated.

Organization of the Human *AQP4* Genomic Locus. A single copy of the human *AQP4* gene was established by Southern analysis of genomic DNA from two unrelated individuals after digestion with multiple restriction enzymes (data not shown). This confirmed the presence of a single genomic copy of *AQP4* (8), which was also found for all other aquaporins (10, 17–20).

The rat *AQP4* cDNA was used to screen two different human genomic DNA libraries (9). One of the three positive clones contained a 20-kb insert from which restriction fragments were subcloned into pBluescript II KS(−) for Southern analysis and sequencing with oligonucleotides corresponding to the anticipated exon-specific regions of the *AQP4* cDNA (Fig. 2). Four exons were identified with class 0 intron boundaries that coincided exactly with the boundaries of the first four exons in other members of the aquaporin family: the gene-encoding lens homolog *MIP* (17), *AQP1* (18), *AQP2* (19), and *AQP5* (10). The M23 initiating methionine was present in exon 1 (Fig. 3), and the coding sequences in the genomic fragments were identical to the human brain and retina cDNAs. Intron sizes were determined by restriction mapping and PCR using sense and antisense primers designed from the following coding sequences: a 0.8-kb intron between exons 1 and 2; 278 bp between exons 2 and 3; and 5.2 kb between exons 3 and 4 (Fig. 2). Thus splicing of exons 1–4 would correspond to the M23 cDNA from retina (Fig. 2).

The existence of another exon (here designated exon 0) was inferred because the cDNA sequence at the 5′ end of the M1 cDNA was not present in exon 1. Only the first 34 bp in the 5′ DNA flanking exon 1 corresponded to the sequence in the M1 cDNA and was preceded by an intron splice acceptor site. By Southern analysis and PCR amplifications, exon 0 was located 2.7 kb upstream from exon 1. Thus, splicing of exon 0 with exons 1–4 would correspond to the M1 cDNA from brain (Fig. 2). Exon 0 encodes the first 10 and 2/3 amino acids in the M1 transcript followed by a class II splice donor site. The next 11 and 1/3 amino acids preceding M23 are encoded by the 5′ DNA flanking exon 1 (Figs. 2 and 3).

Transcription and Translation of *AQP4*. Primer extension was used to determine the sites of transcription initiation for exons 0 and 1 using human brain RNA as a template. Two potential transcription initiation sites were identified upstream from M1 in exon 0 (Fig. 4*A*). Although two TATA motifs were also identified, only the closer may be functional, because it resides just 28 bp upstream from the second transcription initiation site at nucleotide +42, whereas the farther TATA motif lies 173 bp from the first initiation site (Figs. 3 and 4*A*). Mapping the transcription initiation site of the M23 mRNA was undertaken similarly and revealed a start site 36 bp downstream from the single TATA element (Fig. 4*A*), which differs from a recent report (8).

To confirm that two *AQP4* transcripts exist, RNase protection was performed with total RNA from human brain, submandibular salivary gland, and lung using a ^{32}P-labeled antisense cRNA probe overlapping sequences in exons 0 and 1 (Fig. 4*B*). Two distinct protection fragments corresponding to M1 and M23 mRNAs were seen in all three human tissues, although 6-fold higher concentrations of lung and salivary gland RNA were needed to create signals of intensity equal to that of brain. When brain RNA was present, the 200-nt fragment (corresponding to M1 mRNA) was of higher intensity than the 130-nt fragment (corresponding to M23 mRNA), but the fragments were equivalently protected by RNA from the other two human tissues.

The potential functional significance of the two *AQP4* transcripts was analyzed by immunoblotting brain membranes. Because of proteolysis in postmortem human tissues, rat cerebellum was evaluated with anti-AQP4 (Fig. 4*C*). Reactive bands were observed with electrophoretic mobilities of 31, 34, 59, and 64 kDa. The 31- and 34-kDa bands are in agreement with the predicted sizes of AQP4 protein translated from M1

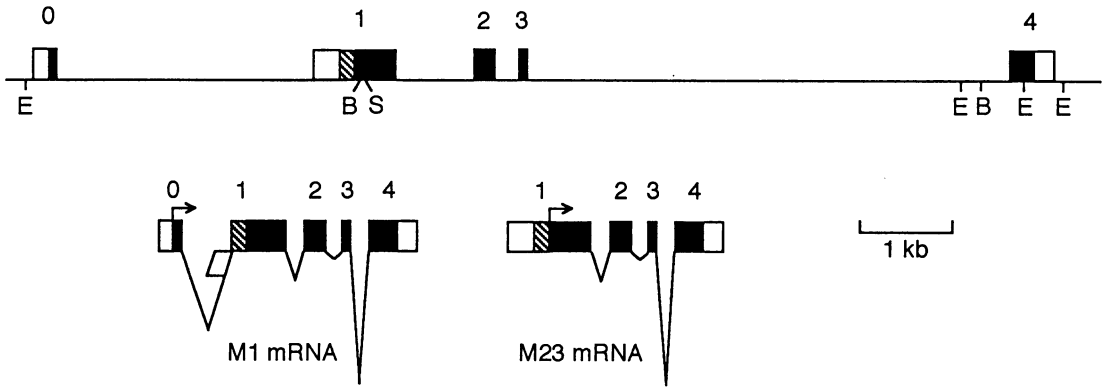

FIG. 2. Exon-intron organization of the human *AQP4* gene. A segment of the 20-kb genomic *AQP4* clone is represented at the top of the figure. Shown are untranslated regions of exons 0 and 1, and to the first polyadenylylation consensus of exon 4 (open rectangles), and coding sequences (filled rectangles). The sequence that encodes residues 12–22 in M1 mRNA but is not translated in M23 mRNA is represented by a hatched rectangle. Restriction sites are also denoted. B, *Bam*HI; S, *Sal*I; E, *Eco*RI). Spliced forms corresponding to M1 mRNA and M23 mRNA are depicted at the bottom of the figure with translation initiation sites (arrows).

and M23 (32.3 and 34.8 kDa), although the abundance of the 31-kDa band was greater than the 34-kDa band. The 59- and 64-kDa bands may represent dimers but do not represent N-glycosylated subunits, because their electrophoretic mobilities were not altered by digestion with Peptide:*N*-glycosidase F (New England Biolabs; data not shown).

Chromosomal Localization of the *AQP4* Gene. Determination of the chromosomal site for *AQP4* was established by fluorescence *in situ* hybridizations. The 5.8-kb *Bam*HI genomic clone containing exon 0 and part of exon 1 yielded fluorescence signals localized at chromosome 18 q11–q12 (data not shown). To establish the chromosomal location with highest

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                                    tacaacatataaccaaacaagacaaattctgaacaattcttcatg   -140 ┐
                                                                                      proximal
gtagaaagaattcaaataaagatttaaggaaatcctcattttccaaaattactgtctcc   -81                    promoter
                                                                                    ┘
aaaaaaaaaaattaagtaaatgtttttgcattcctgaaatgccctgtgtctacagtgat   -22  ┘

                                    caggtacagaaaagctacttc AAGTTCAAATATAACTTAGCGATTGCCACATGGTGCA   +37 ┐
                                                                                                    5' utr
Exon 0                              GAATCTTTCCACCCCTAACACTCCAAAAACCCAATCAGACAAGTGGCCGTAATCTGACT   +96
                                                                                                  ┘
                                    CCCAGCACACAGGGAGCTGCGGGGCAGGCAATGAGAGCTGCACTCTGGCTGGGGAAGGC   +155 ┘

                                    ATG AGT GAC AGA CCC ACA GCA AGG CGG TGG GG gtaagtcctcctttg
                                    (M) (S) (D) (R) (P) (T) (A) (R) (R) (W) (G)  5' splice donor
                                    *1   2   3   4   5   6   7   8   9   10  11

                                    ttcgaaatgtaagtagcatataaaatgtactcgaaacttaaaaacttgctctaagggaa
                                    TTAAGTACAAGGTAAATATATTTTATTCTTAA-[250 bp]-GGCTTCTGATGCTGATT
                                    5' utr in M23 mRNA
Exon 1                              TGTTTCTCTTTTCAG T AAG TGT GGA CCT TTG TGT ACC AGA GAG AAC
                                    (3' splice acceptor  (K) (C) (G) (P) (L) (C) (T) (R) (E) (N)
                                     in M1 mRNA)          12  13  14  15  16  17  18  19  20  21

                                    ATC ATG GTG GGC [363 bp] GTC ACC ATG gtagcatctttagctattttc
                                    (I)  M   V   A  [121 aa]  V   T   M   5' splice donor
                                    22  *23  24  25           147 148 149

                                    3' splice acceptor                                  5' splice donor
Exon 2                              tatttttttctttctctag GTT CAT GGA [147 bp] TTA TTT GCA gtaagtttccaagtcca
                                                         V   H   G  [49 aa]  L   F   A
                                                        150 151 152          202 203 204

Exon 3                              ttttcttgtttcctatag ATC AAT TAT [63 bp]  AAC CAT TGG gtaaccctcatattgata
                                                         I   N   Y  [21 aa]  N   H   W
                                                        205 206 207          229 230 231

Exon 4                              cttccatcttttccttag ATA TAT TGG [258 bp] TCT TCA GTA tgactagaagatcgcact
                                                         I   Y   W  [86 aa]  S   S   V   3' utr
                                                        232 233 234          321 322 323
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FIG. 3. Genomic sequences and exon–intron boundaries of the human *AQP4* gene. Shown are nucleotide sequences of the 5′ flanking regions and 5′ and 3′ intron splices (lower case letters) and 5′ untranslated and coding sequences (upper case letters). Deduced amino acids are listed below nucleotide sequences; residues unique to M1 mRNA are enclosed in parentheses. The methionines for M1 and M23 mRNAs conform to the translation initiation consensus (21) and are indicated by an asterisk. The 5′- and 3′-terminal amino acids of each exon are numbered relative to M1 mRNA; numbers corresponding to interior nucleotide and deduced amino acid sequences for exons 2, 3, and 4 are enclosed in brackets. Potential transcription initiation sites of exon 0 and exon 1 identified by primer extension (see Fig. 4) are indicated by arrows, and characteristic tataa motifs are underlined.

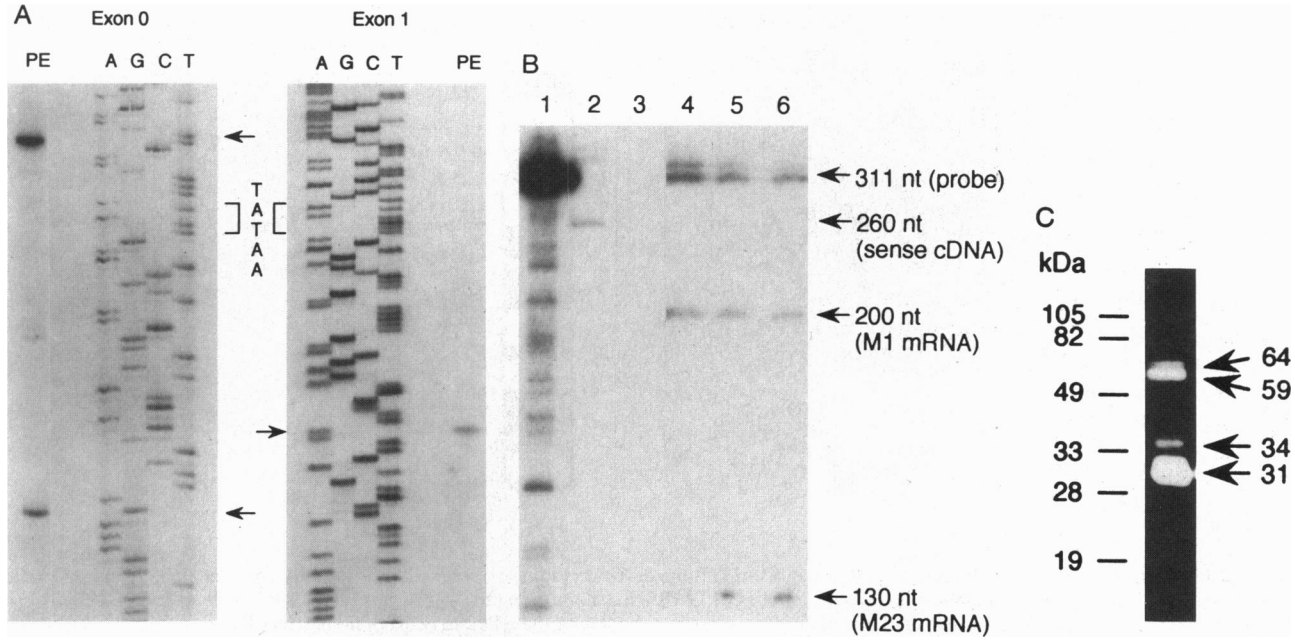

FIG. 4. M1 and M23 mRNAs transcribed from the *AQP4* gene. (*A*) Transcription initiation sites of exons 0 and 1 were mapped by primer extension. An antisense oligonucleotide was ^{32}P-end-labeled, hybridized to total RNA from human brain, and subjected to primer extension. After RNase A digestion, the products (PE) were analyzed on a 10% polyacrylamide, 8 M urea gel alongside an adjacent sequencing ladder from a 6-kb *Bam*HI genomic fragment generated with the same primer (antisense sequence shown). (*B*) Identification of M1 and M23 transcripts by RNase protection. A 311-nt antisense RNA probe containing a 200-nt sequence that overlaps the exon 0–exon 1 cDNA junction was ^{32}P-labeled and hybridized to sense *AQP4* cDNA (a control) or total RNA from human tissues before digestion with RNase. Fragments protected from RNase were analyzed on a 10% polyacrylamide gel. Lane 1, undigested 311-nt antisense probe; lane 2, 260-nt fragment protected by sense *AQP4* cDNA; lane 3, no fragment protected by tRNA; lanes 4, 5, and 6, 200-nt fragment (corresponding to M1 mRNA) and 130-nt fragment (corresponding to M23 mRNA) protected by hybridizations with total RNA from brain, submandibular salivary gland, or lung, respectively. (*C*) Anti-AQP4 immunoblots of adult rat cerebellum. Total membrane protein from rat cerebellum (12 μg) was solubilized in the presence of protease inhibitors at 37°C in 1% SDS, electrophoresed into a 12% polyacrylamide gel, immunoblotted with affinity-purified antibody specific for the C terminus of rat AQP4, and visualized (ECL, Amersham) as described (22).

resolution, hybridizations were undertaken by probing with the P1 clone (ICRF P700M1638) containing the *AQP4* gene and approximately 100 kb of adjacent genomic sequence. Clear fluorescent hybridizations were identified in 15 of the 20 metaphases examined, and all signals were localized to the long arm of chromosome 18 at the precise boundary of q11.2–12.1 (Fig. 5).

DISCUSSION

The studies reported here define the organization, transcription initiation sites, and chromosomal location of the *AQP4* gene. Identification of exon 0 and two distinct mRNAs provides a molecular genetic explanation for the functionally active M1 and M23 isoforms demonstrated in rat (6). Although alternative splicing of exons encoding N-terminal domains is common in other families of transport proteins such as the anion exchangers (25–27), this mechanism for generating diversity is unique to *AQP4* in the aquaporin gene family and is due to sequences in exon 0, a DNA structure without homologs in other aquaporin genes.

It remains to be established if the M23 and M1 mRNAs encode 31- and 34-kDa polypeptide subunits of a single heterotetramer, or if they are segregated into homotetramers comprised exclusively of either 31- or 34-kDa subunits. It is also unknown if the 31- and 34-kDa subunits are expressed at distinct tissue sites during development. With use of antipeptide antibodies corresponding to the C terminus of AQP4, immunoblots have been reported to show reactive bands with mobilities of 31 and 52 kDa in brain and in kidney (22) but only a 30-kDa form in several other tissues (27). These problems may be clarified with an antipeptide antibody specific for the N terminus of the M1 polypeptide. If distinct M1 or M23 tissue distributions or developmental expression patterns exist, analysis of the exon 0 and exon 1 promoters may identify the transcriptional controls.

The deduced amino acid sequences in our study conflict with sequences from a recent publication (8). These discrepancies seem unlikely to represent simple polymorphisms, because we found identical nucleotide coding sequences in the cDNA and genomic DNA clones isolated from three different libraries prepared from different humans. Moreover, other investigators reported multiple rat–human differences (8) that were not identified in our study (Fig. 1) or in a recent independent report (29). This is a cause for concern, because these investigators previously reported a coding sequence for rat *Aqp4* (7) that was found to encode a nonfunctional protein (6). Of possibly greater significance is the N-terminal amino acid sequence previously reported for a human *AQP4* cDNA (referred to as hMIWC2; ref. 8), which appears to represent a splicing error (i.e., not conforming to gt–ag splicing rules) into the 5′ untranslated sequence of exon 0. DNA corresponding to the deduced N-terminal sequence of hMIWC2 was not present in the 3 genomic or 11 *AQP4* cDNA clones that we isolated, nor were we able to amplify sequence corresponding to the N terminus of hMIWC2 from the retina or fetal brain cDNA libraries by PCR (data not shown). In addition, we observed a different splicing error at the 5′ end of one clone (data not shown), a finding that suggests that cloning artifacts may be common in this region of *AQP4* and underscoring the hazards of interpreting possible alternatively spliced mRNAs by analysis of cDNA sequences alone. Until clarified, we suggest that investigators should not design antipeptide antibodies corresponding to the N-terminal sequence of hMIWC2 (8).

The chromosomal site of the *AQP4* gene was localized by hybridization with a P1 clone to human chromosome 18 at the

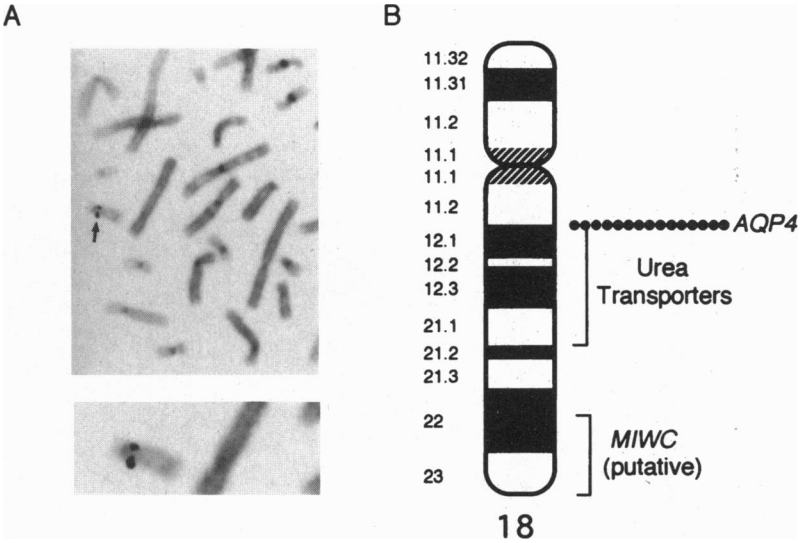

FIG. 5. Chromosomal site of the human *AQP4* gene. Examination of 20 metaphase chromosomal spreads from normal R-banded human lymphocytes after *in situ* hybridization with fluorescently labeled P1 DNA clone containing the *AQP4* gene (ICRF P700M1638); signals were visible in 15 spreads, and all were localized to the long arm of chromosome 18 precisely at the q11.2–q12.1 boundary. (*A*) Representative negative print of metaphase revealing signals over chromosome 18 (*Upper*, arrow) and higher magnification (*Lower*). (*B*) Ideogram of human chromosome 18 showing site of *AQP4* signals. Chromosomal sites reported for hybridization with probes for urea transporters (23, 24) and *MIWC* (another name for *AQP4*; ref. 8) are indicated.

junction of q11.2 and q12.1. This is consistent with the chromosomal site of mouse *Aqp4* gene determined by interspecies backcross matings (N. Jenkins and P.A., unpublished data) but conflicts with the telomeric site on chromosome 18 proposed by others on the basis of fluorescence hybridization with a much smaller probe (8). Search of the human genome data base failed to reveal a human phenotype at human chromosome 18 q11.2–q12.1 that is consistent with the predominant site of *AQP4* expression in brain. Of possible interest are recent reports that localized both members of the human urea transporter family to this region of human chromosome 18 (Fig. 5*B*) (23, 24). Like the aquaporins, urea transport proteins are probably involved in osmoregulation, so their close chromosomal proximity to the *AQP4* gene may represent a cluster of functionally related genes.

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