Functional and molecular characterization of the human neutral solute channel aquaporin-9

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Tsunaguchi, Hiroyasu, Stanisława Weremowicz, Cynthia C. Morton, and Matthias A. Hediger. Functional and molecular characterization of the human neutral solute channel aquaporin-9. Am. J. Physiol. 277 (Renal Physiol. 46): F685–F696, 1999.—In metabolically active cells, the coordinated transport of water and solutes is important for maintaining osmotic homeostasis. We recently identified a broad selective-neutral solute channel, AQP9, from rat liver that allows the passage of a wide variety of water and neutral solutes (H. Tsukaguchi, C. Shayakul, U. V. Berger, B. Mackenzie, S. Devidas, W. B. Guggino, A. N. van Hoek, and M. A. Hediger. J. Biol. Chem. 273: 24737–24743, 1998). A human homolog (hAQP9) with 76% amino acid sequence identity to rat AQP9 (rAQP9) was described, but its permeability was found to be restricted to water and urea (K. Ishibashi, M. Kuwahara, Y. Gu, Y. Tanaka, F. Marumo, and S. Sasaki. Biochem. Biophys. Res. Commun. 244: 268–274, 1998). Here we report a reevaluation of the functional characteristics of hAQP9, its tissue distribution, the structure of its gene, and its chromosomal localization. When expressed in Xenopus oocytes, hAQP9 allowed passage of a wide variety of noncharged solutes, including carbohydrates, polyols, purines, and pyrimidines in a phloretin- and mercurial-sensitive manner. These functional characteristics are similar to those of rAQP9. Based on Northern blot analysis, both rat and human AQP9 are abundantly expressed in liver, whereas, in contrast to rAQP9, hAQP9 is also expressed in peripheral leukocytes and in tissues that accumulate leukocytes, such as lung, spleen, and bone marrow. The human AQP9 gene is composed of 6 exons and 5 introns distributed over approximately ~25 kb. The gene organization is strikingly similar to that reported for human AQP3 and AQP7, suggesting their evolution from a common ancestral gene. The promoter region contains putative tonicity- and glucocorticoid-responsive elements, suggesting that AQP9 may be regulated by osmolality and catabolism. Fluorescence in situ hybridization assigned its locus to chromosome 15 q22.1–22.2. Our data show that hAQP9 serves as a promiscuous solute channel expressed in both liver and peripheral leukocytes, where it is ideally suited to transport of metabolites and/or nutrients into and out of these cells.

water channel; urea transport; chromosomal localization; gene organization; osmoregulation

SOLUTES SUCH AS IONS, nutrients, metabolites, and neurotransmitters are known to cross cell membranes via a variety of transporters (1, 21, 47). The liver plays a central role in the production and elimination of biological compounds, and hepatocytes must have specific solute transport mechanisms to allow uptake and exit of large amounts of solutes with minimal osmotic perturbation (11). Urea is mostly produced in the liver as a major end product of nitrogen metabolism and is secreted into the urine via the kidney. We initially hypothesized that a specialized urea transporter might be responsible for urea secretion in hepatocytes. Our laboratory previously isolated three members of the urea transporter (UT) family from rat kidney, named UT1, UT2, and UT3 (41). None of these transcripts were found to be expressed in liver, suggesting that hepatocytes have a structurally distinct urea transporter.

To elucidate the molecular basis of this putative urea transporter in liver, we employed expression cloning with Xenopus oocytes and isolated a 295-amino acid residue protein, aquaporin-9 (AQP9), which belongs to the major intrinsic protein (MIP) family (1, 21, 47). Rat AQP9 (rAQP9) displays moderate homology to mammalian AQP3 (9, 15, 29) and AQP7 (13) (46–48% amino acid sequence identity) as well as to the bacterial glycerol facilitator (GlpF) (37% identity) (Ref. 7a). Surprisingly, the substrate selectivity of rAQP9 was found to be strikingly different from that of the other aquaporins (1, 21, 47). Rat AQP9 allows permeation of a wide variety of structurally unrelated solutes including carbohydrates, polyols, purines, and pyrimidines, as well as water, in a phloretin- and mercury-sensitive manner. The protonated forms of monocarboxylates and ketone bodies (β-hydroxybutyrate) were also found to be permeable, whereas amino acids, cyclic sugars, Na+ , K+ , Cl−, and deprotonated monocarboxylates and ketone bodies were excluded. These data revealed that rAQP9 behaves as a broad selectivity neutral solute channel that represents a new branch class of the MIP protein family.

A long-standing puzzle in transport physiology has been whether water and solutes share a common pathway when they cross cell membranes. The classic concept was that these processes are of distinct biophysical nature. Earlier studies with red blood cells (2) and kidney medulla (20) suggested that there must be a separate transport pathway for water and urea. Consistent with the concept that urea transporters are highly selective carriers for urea, we and others found that the urea transporters UT1, UT2, and UT3 transport urea and urea analogs in a phloretin-sensitive manner but...
are not permeable to water and glycerol (30, 41). In contrast, our functional analysis of rat AQP9 (42) revealed that it forms a common pathway for water and solutes.

A broad selectivity water/neural solute channel may have profound pharmacological and clinical implications. Since the purine and pyrimidine analogs, e.g., 5-fluorouracil, which readily permeate AQP9, are commonly used as chemotherapeutic agents, AQP9 may influence the chemosensitivity and resistance in cancer tissues. We observed significant upregulation of AQP9 mRNA in the liver of streptozotocin-induced diabetic rats, suggesting that AQP9 is particularly important for cells that must adapt to osmotic stress in metabolically active states. Regulation of gene expression by hypertonicity has been extensively studied for several kidney osmolyte transporters and glucose-catalyzing enzymes (6). The observations suggested that the AQP9 gene may be controlled by similar osmoregulatory mechanisms.

In this study, we report the functional characteristics of the human isoform of AQP9 (hAQP9). This analysis is of particular importance, since Ishibashi et al. (14) reported that the permeability of hAQP9 is restricted to urea and water. Furthermore, to study the regulatory mechanisms of AQP9 expression and to search for possible linkage to human diseases, we isolated the hAQP9 gene, including its 5' and 3' flanking regions, and mapped it to the human chromosomes.

**METHODS**

Cloning of hAQP9 cDNA. The cDNA probe (420 bp) was generated by PCR from a human liver cDNA using oligonucleotide primers according to the sequence of rat AQP9 (accession no. AF016406, nucleotides 396–816). A human liver cDNA library was constructed in a 

**ZAP II** phage vector (Stratagene, La Jolla, CA) by using the Superscript Choice System (GIBCO-BRL). Approximately 4 × 10^6 clones were screened with [32P]-labeled PCR probe under high-stringency conditions that included washing with 0.1× SSC and 0.1% SDS, at 65°C. A positive clone was selected and subcloned into PBlluescript II SK(−) (Stratagene).

In vitro translation. cDNA was translated in vitro using a rabbit reticulocyte lysate system in the absence or presence of canine microsomal membrane (Promega, Madison, WI) according to the manufacturer’s protocol. The products were analyzed on a 10% SDS-PAGE gel.

Northern analysis. A multiple tissue Northern blot (Clontech, Palo Alto, CA) was hybridized in 50% formamide with [32P]-labeled full-length hAQP9 cDNA probe at 42°C, and washed with 0.1% SDS and 0.1× SSC, at 65°C. Autoradiography was performed at −80°C for 5 days.

Oocyte expression and radiotracer uptake assay. Human AQP9 cDNA was digested by Not I and BamHI I and blunt-end ligated into the Bgl II site of the high level expression vector pXβ-gal-e1, which contains the β-galactosidase translation region of the Xenopus β-galactosidase cDNA (a gift of Dr. Peter Agre; Ref. 38). AQP9-cDNA was synthesized after linearization with Sma I, using T3 RNA polymerase, and 25 ng of cRNA was injected into collagenase-treated oocytes. After incubation at 18°C for 2–3 days, radiotracer studies were performed as described (42). Briefly, oocytes were incubated for 90 s with Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, and 10 mM HEPES, pH 7.4) containing 1 mM unlabeled compound and 1–2 µCi/ml of radiolabeled compound. Uptake was terminated by adding ice-cold Barth’s solution with 1 mM unlabeled compound, and oocytes were solubilized in 200 µl 10% SDS. The diffusive solute permeability coefficient (P, cm/s) was determined from the relation P = N/A × Δc, where N is radiotracer uptake (in pmol/s), A is the membrane area (0.045 cm²), and Δc is the concentration difference of the solute (in pmol/cm²).

Isolation of genomic clones. Approximately 5 × 10^6 clones from a human lambda phage genomic library (Lambda Fix II, Stratagene) were screened by using the [32P]-labeled full-length hAQP9 cDNA. Two positive clones were isolated, and the inserts were subcloned into the Not I site of PBlluescript SK(−) (λ 1 and λ 2–2). To obtain further 5’ upstream sequence, a human P1 artificial chromosome (PAC) library (Genome Systems, St. Louis, MO) was screened with a 382-bp cDNA probe corresponding to the 5’ end of the hAQP9 cDNA (nucleotides −209 to +173). The isolated PAC DNA (PAC 19-h12) was subjected to restriction enzyme and Southern blot analysis. A 5.5-Kb EcoR I fragment from PAC 19-h12, spanning the translation initiation site as well as the 5’ flanking region, was subcloned into PBlluescript SK(−) (pBS H12). pBS H12 construct was used for sequence analysis to determine the gene promoter sequence and for primer extension analysis. Sequence was determined on both strands and analyzed by the GCG analysis package (version 8.1; Genetic Computer Group, Madison, WI). The size of introns was determined by PCR using two primers placed in consecutive exons.

Primer extension. An antisense oligonucleotide was designed (5’-GCCGTTCCAATTAGGCTGTGGC-3’, nucleotides −144 to −167) and end-labeled with [γ-32P]ATP (6,000 Ci/mmol, New England Nuclear, Boston, MA) by T4 poly-nucleotide kinase. Poly(A)− RNA from human liver (2 µg) was incubated with 0.1 pmol of the primer in a reaction buffer containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 10 mM dithiothreitol, 1 mM of each dNTP, and 0.5 mM spermidine at 58°C for 20 min. The annealed samples were reverse-transcribed by using avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in the same buffer containing 6.25 mM sodium pyrophosphate. After 30-min incubation at 42°C, the samples were analyzed on an 8% acrylamide gel in Tris/borate/EDTA buffer.

Rapid amplification of 5’ cDNA ends. 5’ cDNA ends were amplified by PCR from human liver Marathon-Ready cDNA (Clontech) using two gene-specific antisense primers: AS1, 5’-TAAGCTGCTTCCAAGCCAGCTC-3’ (nucleotides +44 to +66); AS2, 5’-ATGACCCCTCCAAACGCTTCCG-3’ (nucleotides +151 to +173), in combination with adaptor primers 1 and 2 (AP1 and AP2, Clontech). Following the first round of PCR with primer sets AP1 and AS2, the products were subjected to a second round PCR using primer sets AP2 and AS1. PCR products were analyzed by agarose gel electrophoresis, transfered, and hybridized with a hAQP9 cDNA probe corresponding to the 5’ end (nucleotide positions −209 to +173). Hybridization was performed at 42°C in 50% formamide, and filters washed with 0.1% SDS and 0.1× SSC, at 65°C.

Fluorescence in situ hybridization. The human genomic clone λ 2–2 (0.5 µg) was labeled with digoxigenin-11-dUTP, coprecipitated with 10 µg Cot-1 DNA (GIBCO-BRL), and
resuspended in 1× Tris-EDTA buffer at 100 µg/ml. Hybridization of metaphase chromosome preparations from peripheral blood lymphocytes from normal males was performed with the probe at 15 µg/ml in Hybrisol VI as described (34). The probe was detected using the Oncor kit (Oncor, Gaithersburg, MD) according to the manufacturer’s protocol. Twenty-two metaphase chromosomes were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and were assessed for probe localization with a Zeiss Axiophot microscope. Images were captured and photographed using the CytoVision Imaging System (Applied Imaging, Pittsburgh, PA).

Radiation hybrid mapping. The Stanford G3 radiation hybrid panel (Research Genetics, Huntsville, AL) was screened by PCR assay with a set of primers designed on the 3′ untranslated region of hAQP9 cDNA (sense, 5′-TGATATTCTCAGACGAAATG-3′; and antisense, 5′-GAAAGTGCCTTGTGAATTGTTAAGC-3′).
RESULTS

cDNA cloning and tissue distribution of hAQP9. The human AQP9 cDNA we isolated consists of 2,890 nucleotides and its sequence is identical to that previously reported (GenBank accession no. AB008775, Ref. 14) with the exception that our clone has an additional 14 nucleotides at the 5’ end. The sequence encodes a 295-amino acid residue protein with a predicted molecular mass of 31 kDa (Fig. 1A). Human AQP9 cDNA has a potential N-linked glycosylation site at Asn 142, and in vitro translation generated a single band of 28 kDa that increased in size by adding the microsome membrane, suggesting that this site is indeed glycosylated (Fig. 1B). Human AQP9 also has a potential protein kinase C (PKC) phosphorylation site at Ser 11, but there are no protein kinase A (PKA) sites. Hydropathy analysis showed that, in analogy to rat AQP9 (rAQP9), hAQP9 has six putative transmembrane domains (Fig. 1C). The amino acid sequence of hAQP9 is 76% identical to that of rAQP9 (42) and has moderate identity with human AQP3 (47%) (9, 15, 29), human AQP7 (41%) (17), and the bacterial glycerol facilitator GlpF (39%) (7a), whereas it displays only weak identity with AQP1 (28%) (1, 21, 47). High-stringency Northern analysis (Fig. 2) gave a strong signal of 2.9 kb in liver, in analogy to rAQP9 (42). In contrast to rAQP9, hAQP9 is also abundantly expressed in peripheral leukocytes as well as the tissues accumulating leukocytes, such as the lung, spleen, and bone marrow (22, 42). hAQP9 mRNA was not detected in testis and brain, whereas rAQP9 is abundantly expressed in these tissues (42). We did not detect hAQP9 mRNA in cancer cell lines of nonepithelial origin such as leukemia, lymphoma, and melanoma, or of epithelial origin (colon and lung carcinoma).

Functional properties of hAQP9. When expressed in oocytes, hAQP9 increased the urea permeability coefficient ($P_u$) from 1.5 ± 0.2 × 10⁻⁶ cm/s (water-injected) to 30.2 ± 1.7 × 10⁻⁶ cm/s (Fig. 3A). The increase in $P_u$ was similar to that observed for the urea transporters (UT2 and UT3) and rAQP9 ($P_u$ = 25–45 × 10⁻⁶ cm/s) (41, 42). Human AQP9 induced permeabilities of a variety of structurally unrelated solutes including polyols (glucitol, mannitol, sorbitol), purines (adenine), pyrimidines (uracil and the chemotherapeutic agent 5-fluorouracil), and urea analogs (thiourea) (data not shown). Also consistent with rAQP9 properties, we found a three- to fourfold higher permeability for the monocarboxylates lactate and β-hydroxybutyrate at reduced pH (pH 5.5) compared with physiological pH (data not shown). As alluded to in our previous study, the pH sensitivity indicates that monocarboxylates permeate AQP9 in their protonated, neutral form. Likewise, the low permeabilities of the purine analogs xanthine and uric acid suggests that these compounds permeate hAQP9 in their protonated form.

When oocytes expressing hAQP9 were transferred from regular Barth’s solution (200 mosmol/kgH₂O) to diluted Barth’s solution (70 mosmol/kgH₂O), we observed osmotic lysis of the oocytes within 2–3 min following exposure to hypotonicity. This indicates that hAQP9 is permeable to water (data not shown), a property that was investigated in detail for rAQP9 (42). Phloretin (0.1 mM) and HgCl₂ (0.3 mM) effectively inhibited the permeability of mannitol, sorbitol, adenine, 5-fluorouracil, and β-hydroxybutyrate (70–90%)

![Fig. 2. Distribution of hAQP9 mRNA in human tissues. Northern blots with 2 µg of poly(A)⁺ RNA of various human tissues were hybridized with a full-length hAQP9 cDNA probe. mRNA sources of cancer cell lines are: promyelocytic leukemia (HL-60, lymphohlastic), HeLa cell (S3), chronic myelogenous leukemia (K-562), lymphohlastic leukemia (MOLT-4, T-lymphohlastic), Burkitt lymphoma (Raji, B-lymphohlastic), colorectal adenocarcinoma (SW480), lung carcinoma (A549), and melanoma (G361).](image-url)
The urea and glycerol permeabilities were inhibited only 20–50% by 0.1 mM phloretin, and glycerol uptake was inhibited only 50% by 0.3 mM HgCl₂. The urea permeability was not significantly affected by 0.3 mM HgCl₂. Overall, these inhibitor sensitivities were very similar to those reported for rAQP9 (42). Thus our data indicate that, despite the report of Sasaki and colleagues (14, 22), the functional properties of human and rat AQP9 are almost identical (42).

Genomic cloning and exon-intron organization. To determine the genomic structure of the human AQP9 gene, we screened a human bacteriophage genomic library using the full-length hAQP9 cDNA as a probe. We isolated two overlapping clones, called λ1 and λ2–2, each containing ~15 kb (Fig. 4). Southern analysis revealed that the two clones span an ~20-kb genomic region that includes the coding region of the last two-thirds of the carboxy terminus of hAQP9, but they...
Table 1. Exon/intron organization of the human AQP9 gene

<table>
<thead>
<tr>
<th>No.</th>
<th>Size, bp</th>
<th>Position</th>
<th>5' Exon boundary</th>
<th>Intron Sequence and Boundaries</th>
<th>3' Exon boundary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>398</td>
<td>-286 to 111</td>
<td>ATCTGTGATT</td>
<td>gt aagattaatttcctta</td>
<td>ttggtaattttcttcctc</td>
</tr>
<tr>
<td>2</td>
<td>127</td>
<td>112 to 238</td>
<td>GGTGCTCTGT</td>
<td>gt aagcgattagaa</td>
<td>attccag GTGCTTGGGA</td>
</tr>
<tr>
<td>3</td>
<td>138</td>
<td>239 to 376</td>
<td>ATTACATATG</td>
<td>gtagttaaggctctgag</td>
<td>ttcctcaatctccc</td>
</tr>
<tr>
<td>4</td>
<td>119</td>
<td>377 to 495</td>
<td>GCAGATCACA</td>
<td>gt aagatgtagtag</td>
<td>atctcag ATGAGCTTAATG</td>
</tr>
<tr>
<td>5</td>
<td>218</td>
<td>496 to 713</td>
<td>GAAGTTTGCA</td>
<td>atctcag AGCTGGGCC</td>
<td>tcaactttctctccc</td>
</tr>
<tr>
<td>6</td>
<td>1,958</td>
<td>714 to 267</td>
<td></td>
<td></td>
<td>atttccag AGCTGGAAAC</td>
</tr>
</tbody>
</table>

Positions of exons are indicated on the left. First base (A) of the translation start site (ATG) is numbered nucleotide 1. Exon sequences are shown in upper case, and intron sequences are in lower case. Splice consensus sequences are shown in bold. First exon was defined by primer extension and 5’-RACE analysis. AQP, aquaporin.

Table 2. Comparison of splicing site between human AQP9 and AQP3 genes

<table>
<thead>
<tr>
<th>Boundaries</th>
<th>Human AQP9</th>
<th>Human AQP3</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1–2</td>
<td>ATC TGG ATT</td>
<td>ATG CTC TTT GCC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ile Leu Ile Val Leu Gly</td>
<td>Ile Leu Val Met Phe Gly</td>
<td></td>
</tr>
<tr>
<td>Exon 2–3</td>
<td>GGT GTC TCT GGT GGT CAC</td>
<td>CAG GTC TCT GCC GCC CAC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Gly Val Ser Gly His</td>
<td>Gln Val Ser Gly Ala His</td>
<td></td>
</tr>
<tr>
<td>Exon 3–4</td>
<td>TAC TAT GAT GGA CTT ATG</td>
<td>TAT TAT GAT GCA ATC TGG</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Tyr Tyr Asp Gly Leu Met</td>
<td>Tyr Tyr Asp Ala Ile Trp</td>
<td></td>
</tr>
<tr>
<td>Exon 4–5</td>
<td>GCA GAT CAA GTG GTG GCC</td>
<td>TTT GAC CGA TTC ATA GCC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ala Asp Gln Val Val Ala</td>
<td>Phe Asp Gln Phe Ile Gly</td>
<td></td>
</tr>
<tr>
<td>Exon 5–6</td>
<td>GAA GTC TTC AGA GCT GGA</td>
<td>GCA GTC TTC ACC GCC</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Gln Val Phe Arg Ala Gly</td>
<td>Ala Val Phe Thr Thr Gly</td>
<td></td>
</tr>
</tbody>
</table>

Phase class refers to the positions of the introns relative to the coding sequence (Ref. 35); class 0, intron located between the codon; class 1, intron sequence between the first and second nucleotide of a codon; class 2, intron sequence between the second and third nucleotide of a codon. cDNA sequence and gene structure of human AQP3 was referred to GenBank AB001325 and Ref. 12. The gene structure of human AQP7 was recently reported to be similar to that of human AQP3 (6 exons were interrupted at identical sites with the same phase classes of introns, Ref. 17).
tively, within the 5’ end of the cloned AQP9 cDNA. To confirm the transcription initiation sites, we performed 5’-RACE and subsequent Southern analysis. For 5’-RACE analysis, we used human liver Marathon-Ready cDNA (Clontech), which has 5’ adaptors attached containing primer sites AP1 and AP2 (Fig. 5B). The 5’ ends of hAQP9 cDNA were then amplified using 5’ AP1 or AP2 primers and 3’ AQP9-specific primers (AS1 or AS2, see Fig. 5B) and analyzed by Southern blotting. Whereas in the cloned hAQP9 cDNA the distance between the AS1 primer site and its 5’ end is ~300 bp, the two 5’-RACE products that were generated gave a strong band at ~350 bp (5’ sequence of TS1 product) and a weaker band ~270 bp (5’ sequence of TS2 product), again consistent with the existence of two transcriptional start sites, TS1 and TS2. We conclude that nucleotide ~286 relative to the AUG translation start site is the major transcriptional start site and therefore corresponds to the beginning of exon 1.

Analysis of the 5’ flanking region. We analyzed ~1 kb upstream of the AUG translation initiation codon (Fig. 6) by sequencing a pBS H12 plasmid. The 5’ flanking region did not contain a typical TATA box sequence (TATAWA, Ref. 4) nor a CCAAT box (7) at the transcriptional start site. The human AQP5 gene is reported to lack a TATA box (24), whereas most other human

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**Fig. 5. Mapping of the transcription initiation site.** A: primer extension. An antisense oligonucleotide, ASPE (nucleotide positions -144 to -167 of the human AQP9 cDNA), was end-labeled and annealed to 2 µg of the human liver poly(A)^+ RNA. The annealed mixture was further extended by avian myeloblastosis virus reverse transcriptase, and the reaction products (PE) were analyzed on an 8% polyacrylamide gel. Sequencing products of a plasmid pBS H12, generated from the antisense primer, were run on the gel as a size marker. TS1 and TS2 indicate the positions of the extension products that represent the major and minor transcriptional start sites. Asterisks indicate the nucleotide position of the initiation sites (the numbers at either end of the sequences represent the nucleotide positions relative to the translation start site). B: diagram of 5’-RACE cDNA products. Coding and 5’-untranslated regions of the hAQP9 cDNA are shown by the solid box and the bold line, respectively. Positions of the two transcriptional start sites (TS1 and TS2) determined by primer extension analysis are indicated. Human AQP9 cDNA-specific primers are AS1 and AS2, and two overlapping adaptor primers are AP1 and AP2. A 295-bp PCR product is expected from the 5’ end of the cloned cDNA (accession no. AF016495) when primer set AP2-AS1 is used. C: Southern blot analysis of 5’-RACE products. The PCR products from the primer pair AP1-AS2 were further amplified with the nested sets of primers AS1-AP2. The resulting PCR products (AS1-AP2) were electrophoresed, transferred to nitrocellulose filters, and probed with ^32P-labeled hAQP9 cDNA (nucleotide -209 to +173). The sizes of the upper and lower bands are consistent with the positions of the TS1 and TS2 sites shown in A and B.
The aquaporin genes (AQP0, AQP1, AQP2, AQP3, and AQP4) contain a TATA box element (12, 28, 32, 37, 44). Thus AQP9 provides the second example of a "TATA-less" promoter in the aquaporin gene family. The gene promoter also did not contain GC-rich elements (G+C content was 35% at positions from 2900 to 2400) nor Sp-1 binding sites, although these motifs were reported to contribute to transcription regulation in TATA-less gene promoters (8, 39).

When searching for regulatory cis elements, we identified several putative transcription factor recognition sites. The 5′ flanking sequence contained AP-1 (activator protein-1, TGANTMA, position -2580; Ref. 25) and GATA-1 (WGATAMS, position -2602; Ref. 43) sites. Potential binding sites were also found for the transcription enhancer factor TEF-1 (TGGAATGT, position -849), the sis-inducible factor SIF (CCCGTTC, position -371), and the histone H4 transcriptional factor H4TF (GATTTC, position -238). The sequence TGTCCT at position -723 corresponds to a binding motif for the glucocorticoid receptor (GR) (19). We furthermore found two TonE tonicity response enhancer consensus motifs (TGGAANNYYNY; Refs. 6, 40) at position -847 and -886.

Transcription factor recognition sites may be involved in tissue-specific expression. We identified a CCAAT/enhancer-binding protein (C/EBP) site (TKNNGYAAK, position -766; Ref. 18) and a hepatocyte nuclear factor (HNF-1) site (ATTAATCATTACC, in reversed orientation at position -2273, distal to TS1; Ref. 36), both of which are known to be involved in liver-specific expression. There are also putative gamma-interferon inducible binding protein 1B (AAGTGCA, position -763, Ref. 43) and nuclear factor NF-κB (GGGNN-YYY, position -238, Ref. 26) sites that may be associated with lymphocyte-specific expression.
Localization of hAQP9 on the human chromosomes. We analyzed the chromosomal localization by fluorescence in situ hybridization (FISH), using a 15-kb genomic probe (Fig. 7). The map position was determined by visual inspection of the fluorescent hybridization signals on DAPI-stained metaphase chromosomes. In all of the 22 metaphase preparations, a hybridization signal was found on the long arm of chromosome 15 in bands q22.1–22.2. In 21 metaphase spreads, both copies of chromosome 15 were labeled and, in one metaphase spread, the signal was detected on a single chromosome 15. We next performed a radiation hybrid mapping, using the Stanford G3 radiation panel. PCR amplification with primers specific to the 3′-untranslated region of hAQP9 cDNA indicated that it is most likely linked to the polymorphic locus D15S117 (marker AFM098yg1) with a centiray distance (cR10000) of 10.95 (lod score = 13.05). The physical position of D15S117 correlated with the cytogenetic localization of the band q22.1-q22.2 (3), confirming that the hAQP9 gene exists at this locus. This locus is 10 centimorgans proximal to the centromeric marker of Bardet-Biedl syndrome (chromosome 15q22.3-q23) (5), an autosomal recessive disorder that is characterized by retinal degeneration, polydactyly, obesity, and hypogenitalism (OMIM 209900).

No other genes of aquaporins and transporters were found in the vicinity of 15q22.1–22.2.

DISCUSSION

An important goal of this study was the reevaluation of the functional characteristics of human AQP9. Ishibashi et al. (14) reported that the permeability of human AQP9 is restricted to urea and water, a finding which would be in contrast to the broad permselectivity of its rat homolog (42). In addition, the same group recently reported that rat AQP9L (22), of which amino acid sequence is 99% identical to rat AQP9 we reported (42), is permeable only to urea and glycerol as well as to water. The discrepancy between our findings and those of Sasaki and colleagues (14, 22) is not clear. Our data establish that the permselectivity of human AQP9 is very similar to that of rat AQP9.

Northern analysis showed that human AQP9 mRNA is expressed in leukocytes and in tissues that accumulate white blood cells, including spleen, bone marrow, and lung. Notably, in rat, AQP9 mRNA is not expressed in peripheral leukocytes. The distinct pattern of tissue distribution in rat and human may be due to different requirements of metabolites among the two species. The
AQP9 expression may also be developmentally regulated and may change according to the metabolic state of tissues. We assume that cell types of leukocytes expressing hAQP9 are neutrophils and/or B-lymphocytes, because 1) no AQP9 mRNA was detected in thymus (~100% of the cells are T-lymphocytes) and in T-lymphoblastic cell lines (MOLT-4), 2) moderate AQP9 expression was detected in spleen and lymph node (most of the cells are B-lymphocytes), and 3) the most abundant expression was found in peripheral leukocytes, of which cells are usually a mixture of ~80% neutrophil, 15% T-lymphocytes, and 5% B-lymphocytes.

As a first step toward understanding the regulatory mechanisms of hAQP9, we isolated and characterized its promoter. Interestingly, there are no typical TATA or CCAAT box elements in the hAQP9 promoter. The A-T rich region found at position ~320 to ~340 may serve as a TATA box. Alternatively, the Inr initiator element was reported to participate in the transcription of genes which do not have TATA or CAAT boxes (27, 45). Inr elements typically consist of an A (adenine) at the transcriptional start site and flanking polypyrimidine clusters, i.e., _3YYCAYYYY_Y5 (27, 45). The putative hAQP9 transcriptional start sites (Figs. 5 and 6) have an adenine at the initiation position, but the flanking sequence does not have typical polypurine motifs as reported previously. These findings were not unexpected for AQP, since the AQP5 gene also lacks TATA box and Inr elements (24).

With regard to tissue-specific expression of AQP9, Northern analysis (Fig. 2) showed that hAQP9 is expressed mainly in liver and leukocytes. The 5' flanking region of the hAQP9 gene contains several motifs that may serve as recognition sites for tissue-specific factors such as the hepatocyte-specific regulatory factors HNF-1 and C/EBP (18, 36) and the lymphocyte-specific transactivating factors IKB-1 and NF-kB (26, 43). Since we observed that AQP9 mRNA is upregulated in the liver of streptozotocin-treated diabetic rats (see footnote 1), we were further interested in whether AQP9 expression is controlled by osmoregulation.
volume changes. Numerous studies with kidney medul-
lar cells (6) suggested that regulation of osmolyte
transport by external hypertonicity is important for
these cells to adapt to osmotic stress (6). Recently, an
osmotic response element has been identified in several
gene promoters responsible for the osmoregulation of
the sodium-dependent
aptinmyo-inositol transporter (SMIT),
the betaine-y-amino-n-butyric acid transporter (BGT),
and the aldose reductase. We found two toxicity re-
response enhancer (TonE) consensus motifs in the hAQP9
promoter, which reside in close proximity, only 18 bp
apart, 600 bp upstream of the transcription initiation
site (Fig. 6). A glucocorticoid-responsive element motif
at position –723 may also affect AQP9 gene expression.
AQP9 may be regulated by the catabolic hormones such
as glucagon and glucocorticoid, because these hor-
mones promote the breakdown and oxidation of stored
body fuels, thereby increasing the requirement for
transport of metabolites.

The results from the genomic analysis of AQP9
provide information on how MIP family members with
diverse permselectivities may have emerged. Based on
functional analysis, we propose that the MIP family
can be divided into three classes: class I, which includes
water-selective channels such as AQP1, AQP2, AQP4,
and AQP5; class II, which has limited permselectivity
for certain neutral solutes, e.g., AQP3 and AQP7; and
class III which has broad permselectivity, e.g., AQP9
from rat and human. Class II and class III channels are
also known as aquaglyceroporins (1). The division into
three distinct classes is consistent with the structures
of the human AQP genes: in general, class II and III
genes have six exons, whereas class I genes have four
exons. Recently, the human AQP8 gene has been shown
to have six exons but to belong to the class I water-
selective channels (23), raising the possibility that it
may represent another phylogenic branch. Identifica-
tion of other AQP members will help to define more
detailed characteristics of such a subclass. Further-
more, genes within a given class often cluster on
specific chromosomes. For example, the class I genes
AQP2 and AQP5 cluster on a single band of chromo-
some 12 (12q13) (47), and the class II genes AQP3 and
AQP7 cluster within a single band on chromosome 9
(9p13) (16, 17). As shown in Fig. 8, the class III AQP9
gene is located on chromosome 15 (15q22.1–22.2), and
it is conceivable that additional AQP9-like genes clus-
ter in this region. Given the close homology (~40% amino acid sequence identity) of class II and III but not class I genes with the water-impermeable bacterial
glycerol facilitator GlpF (7a), we propose that class I
and class II/III genes evolved from a common ancestral
gene ("primitive pore") and that the II/III prototypes
further evolved to form subtypes with specialized perm-
selectivities (Fig. 8).

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REFERENCES
1. Agre, P., M. Bonhivers, and M. J. Borgnia. The aquaporins,
blueprints for cellular plumbing systems. J. Biol. Chem. 273:
Mokady, A. Banks, and D. C. Ward. Integration of the cyto-
getic, genetic, and physical maps of the human genome by FISH
4. Brethnach, R., and P. Chambron. Organization and expres-
sion of eucaryotic split genes coding for proteins. Annu. Rev.
Thomson, A. T. Moore, M. J. ay, M. Warburg, A. Schinzel,
N. Tommerup, K. Tornqvist, T. Rosenberg, M. Patton, D. C.
Mansfield, and A. F. Wright. Linkage mapping in 29 Bardet-
Biedl syndrome families confirms loci in chromosomal regions
expression by hypertonicity. Annu. Rev. Physiol. 59: 437–455,
1997.
Sharp. Human CCAAT-binding proteins have heterologous sub-
proteins of plant and animal cells. Trends Biochem. Sc. 9:
9. Dusing, M. R., and D. A. Wiginton. Sp1 is essential for both
enhancer-mediated and basal activation of the TATA-less human
adenosine deaminase promoter. Nucleic Acids Res. 22: 669–677,
1994.
Cloning and expression of AQP3, a water channel from the
metabolic disposal, and product formation in the liver. In: The
Liver: Biology and Pathobiology (3rd ed.), edited by I. M. Arias,
J. L. Boyer, N. Fausto, W. B. Jakoby, D. Schachter, and D. A.
13. Inase, N., K. Fushimi, K. Ishibashi, S. Uchida, M. Ichio-
ka, S. Sasaki, and F. Marumo. Isolation of human aquaporin 3
14. Ishibashi, K., M. Kuwahara, Y. Gu, Y. Kageyama, A. Toh-
saka, F. Sugaki, F. Marumo, and S. Sasaki. Cloning and
functional expression of a new water channel abundantly
expressed in the testis permeable to water, glycerol, and urea.
15. Ishibashi, K., M. Kuwahara, Y. Gu, Y. Tanaka, F. Marumo,
aquaporin (AQP9) abundantly expressed in the peripheral leuko-
cytes permeable to water and urea, but not to glycerol. Biochem.
16. Ishibashi, K., S. Sasaki, K. Fushimi, S. Uchida, M. Kuwa-
hara, H. Saito, T. Furukawa, K. Nakajima, Y. Yamaguchi, T.
Gojobori, and F. Marumo. Molecular cloning and expres-
sion of a member of the aquaporin family with permeability to
glycerol and urea in addition to water expressed at the basolateral
Ikeuchi, F. Marumo, and S. Sasaki. Molecular characteriza-