BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 53–58 (1997)
ARTICLE NO. RC977769

Molecular Cloning and Expression of a Novel Human Aquaporin from Adipose Tissue with Glycerol Permeability

Hiroshi Kuriyama,* Shoko Kawamoto,* Narihiro Ishida,† Ikko Ohno,* Shiro Mita,† Yuji Matsuzawa,‡ Kenichi Matsubara,* and Kousaku Okubo*,[1]

**Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan; †Discovery Research Division, Santen Pharmaceutical Co., Ltd., Ikoma, Nara 630-01, Japan; and ‡Second Department of Internal Medicine, Osaka University Medical School, Suita, Osaka 565, Japan*

Received October 25, 1997

In a systematic analysis of genes expressed in human adipose tissue, we detected a novel gene that is expressed uniquely in adipose tissue. The sequence showed that it encodes a 342-amino-acid protein containing six putative transmembrane domains, and is a new member of the aquaporin family of water-selective membrane channels. We named this gene aquaporin 9. It features a cyclic-AMP protein kinase phosphorylation consensus site in the NH_3-terminal domain. Expression of the cRNA in *Xenopus* oocytes yielded a 7-fold increase in osmotic water permeability blocked by 0.3 mM $HgCl_2$, and also facilitated the uptake of glycerol. Northern blot analysis demonstrated that the mRNA is abundant in adipose tissue, but not in other tissues. Thus, this gene product may participate in glycerol transport in adipocytes.

We initiated a systematic analysis of active genes in adipose tissue by constructing a 3′-directed cDNA library, in which the mRNA population is faithfully reflected (1, 2). By using this method, genes that are uniquely expressed in this tissue have been identified, and three such novel genes, apM 1-3 (*Adi*pose *M*ost Abundant Gene Transcript 1-3) have been cloned and characterized (2, 3). In this communication we report another adipose tissue specific novel gene, that codes for a new member of the aquaporin family.

The aquaporins are a family of water-selective membrane channels found in animals, plants and microorganisms. Eight kinds of mammalian aquaporin have been identified until the present day (AQP0∼7, AQP6 is originally named hKID.) among which AQP3 and AQP7 have glycerol and urea permeability (4-7). The new aquaporin in adipose tissue shows glycerol permeability. In adipose tissue triglycerides are stored, and hydrolyzed to glycerol and fatty acids in response to a demand for energy (8). These products are released from the adipocytes. However, any proteins associated with glycerol transport in adipocytes are unknown. Thus, this protein may play a key role in this process.

MATERIALS AND METHODS

Materials. As previously reported, abdominal subcutaneous and visceral fat tissues from two female patients (aged 35 and 38 yr, respectively) were used for construction of the cDNA library (2, 3).

Cloning of a full-length cDNA. A full-length cDNA was cloned in the same way as described in previous reports (2, 3). Briefly, we selected GS3340, which proved to be an adipose tissue-specific gene in the expression profile, and used this sequence as a probe. A full-length human adipose tissue cDNA library was constructed at the *EcoR* I /*Xho* I sites of pBluescript (ZAP-cDNA Synthesis Kit; Stratagene) according to the manufacturer's protocol. The vector-primed cDNA was blunted at the 5′ terminus with T_4 DNA polymerase, circularized with T_4 ligase and transformed into *E.coli* DH5. About 40,000 transformants of this library were screened with ^{32}P-labeled GS3340 DNA, and among the 12 positive clones a clone that has the longest cDNA insert was selected and sequenced. The insert of this clone was amplified by Vent polymerase (New England Biolabs), using kinated flanking primers, by means of 30 cycles at 96°C for 30 sec, 60°C for 60 sec, 72°C for 120 sec, followed by 72°C for 10 min. The amplified product was purified using Sephacryl S400 column chromatography, then ligated, sheared by sonication and subcloned into M13 for sequencing.

Functional studies. The coding sequence of AQP9 was amplified by PCR using designed sense primer (5′-GAGAAGACAAGCTTG-ATACAGCCACCATGGTTCAAGCATCC-3′, corresponding to bp −25 to +15 of the engineered *Hind*III site and Xenopus b-globin sequence) and antisense primer (5′-TAAACACTAGCCTAGGTA-AGGGGT-3′ containing a *Bam*HI site and corresponding to bp +1139

[1] Corresponding author. Fax: +81-6-877-1922. E-mail: kousaku@imcb.osaka-u.acjp.

Abbreviations: AQP, aquaporin.

Another aquaporin (AQP8) was recently reported (Biochem. Biophys. Res. Commun. 1997, **237,** 714–718).

0006-291X/97 $25.00

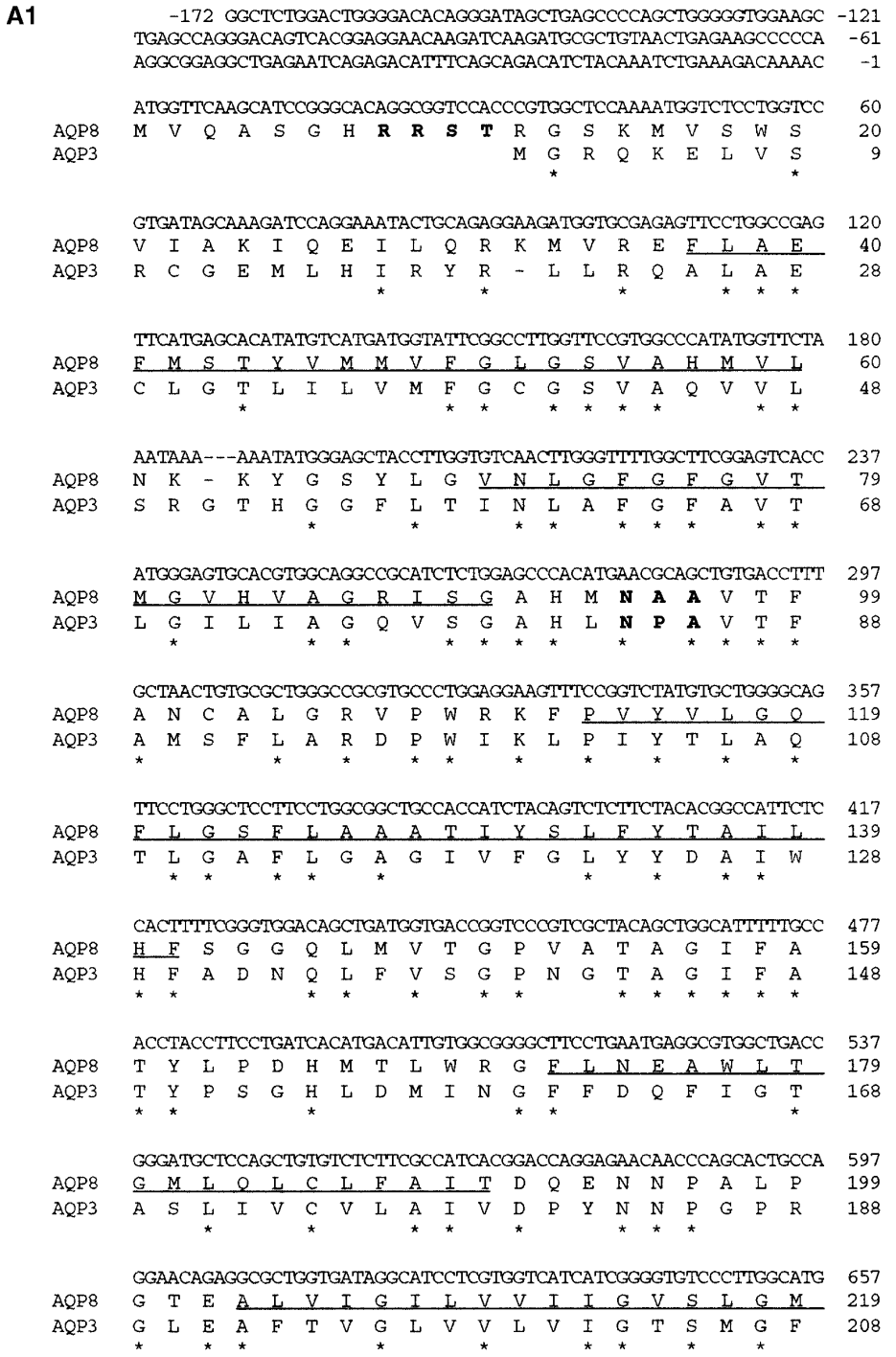

FIGURE 1.

to +1162 of AQP9). The PCR product was subcloned into pSP64-poly(A) at the *Hind* III and *Bam* HI sites. pSP.AQP9 contained the *Xenopus* β-globin 5′-untranslated region located 10 bp upstream of the AQP9 ATG initiation codon. cRNA encoding AQP9 was transcribed/capped using an *in vitro* transcription kit (Stratagene). Oocytes were prepared as described by Taylor et al. (9). Mature female *Xenopus laevis* were anesthetized, then the oocytes (Stage V-VI) were removed and placed in Ca^{2+}-free Barth's buffer. The oocytes were then defolliculated by gentle agitation for 1 h in Ca^{2+}-free Barth's buffer containing 2 mg/ml of type II collagenase. After the oocytes were washed sufficiently with Barth's buffer, they were completely denuded. The oocytes were incubated overnight at 18°C in Barth's buffer and injected with RNA or water the following day. Fifty nanoliters of water or cRNA (0.2 mg/ml) in water were microinjected into the oocytes using a Drummond microinjection system with sterile glass micropipettes. Oocytes were maintained

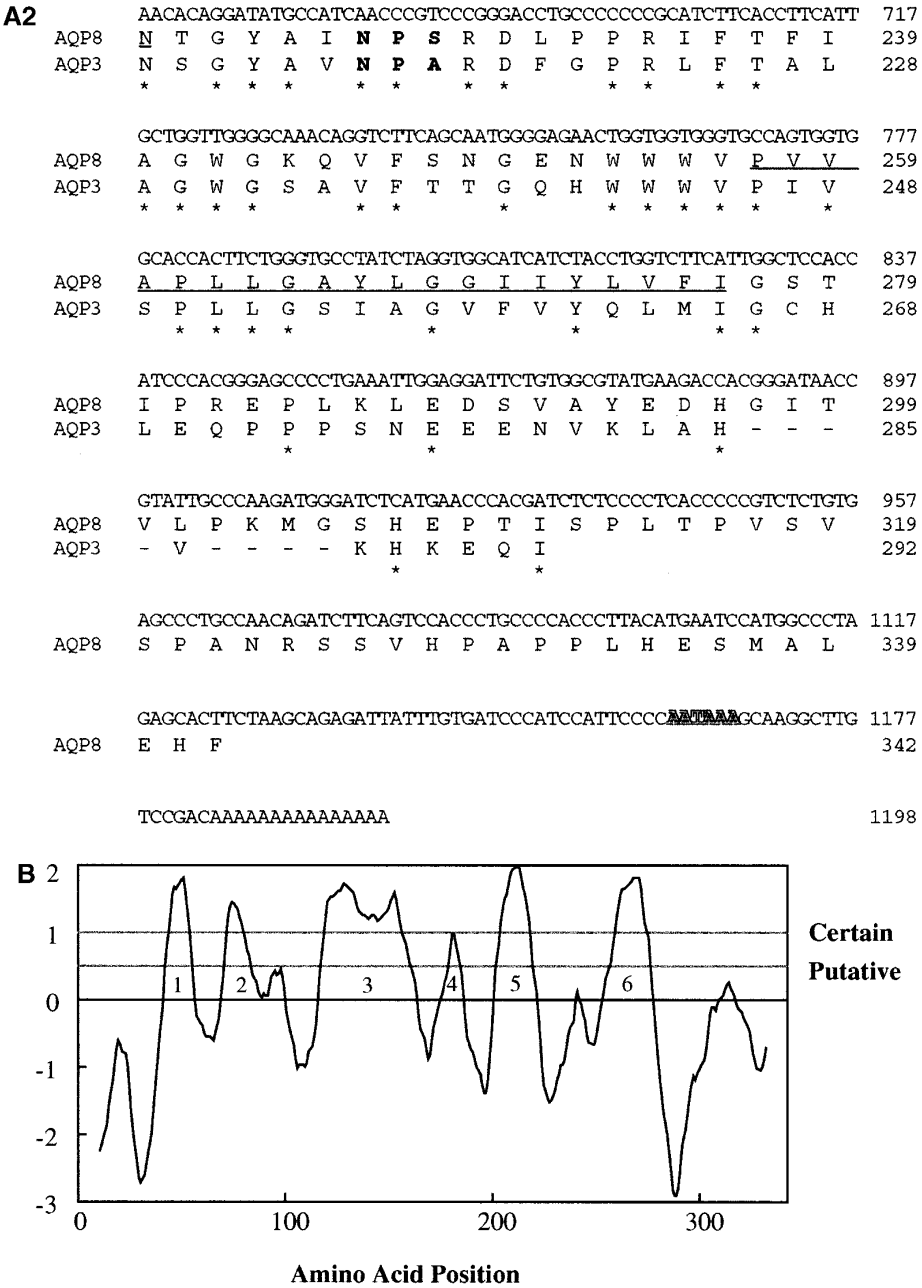

FIG. 1. (A) Nucleotide sequence of human AQP9 cDNA and alignment of its deduced amino acid sequence with that of human AQP3. Asterisks indicate identical amino acids and the putative transmembrane domains are underlined. The NAA or NPS motif corresponding to the NPA motif in other AQPs and the consensus sequences of potential phosphorylation sites for cAMP-dependent protein kinase are shown in bold letters (12). The polyadenylation consensus site is indicated in open-face type. The first ATG was defined as the initiation codon on the basis of Kozak's consensus site (10). (The nucleotide sequence of the AQP9 has been deposited in DDBJ under accession number AB006190.) (B) Hydropathy analysis of the deduced amino acid sequence of AQP9. Membrane-spanning domains were predicted by TopPred II (11) using the GES scale (full window width 21, core window width 11). Putative membrane-spanning domains are numbered from 1 to 6.

at 18°C in Barth's buffer with daily buffer changes. Three days after the injection, the oocytes were transferred from 200 mOsm to 40 mOsm modified Barth's buffer, and the osmotic volume change was observed at 18°C by transmitted light using an Olympus phase-contrast microscope. Oocyte images were recorded at 10 sec intervals, and the oocyte volume was calculated from the images with a C-IMAGING 1280 image processing system. Osmotic water permeability (Pf) was determined from the initial slope of the time course of V/V_0 ($d(V/V_0)$ dt), the initial oocyte volume ($V_0 = 9 \times 10^{-4}$ cm^3), the initial oocyte surface area ($S = 0.045$ cm^2) and the molar volume of water ($Vw = 18$ cm^3/mol): $Pf = [\, V_0 \times d(V/V_0)dt \,] / [\, S \times V_w \times (mOsm_{in} - mOsm_{out})]$.

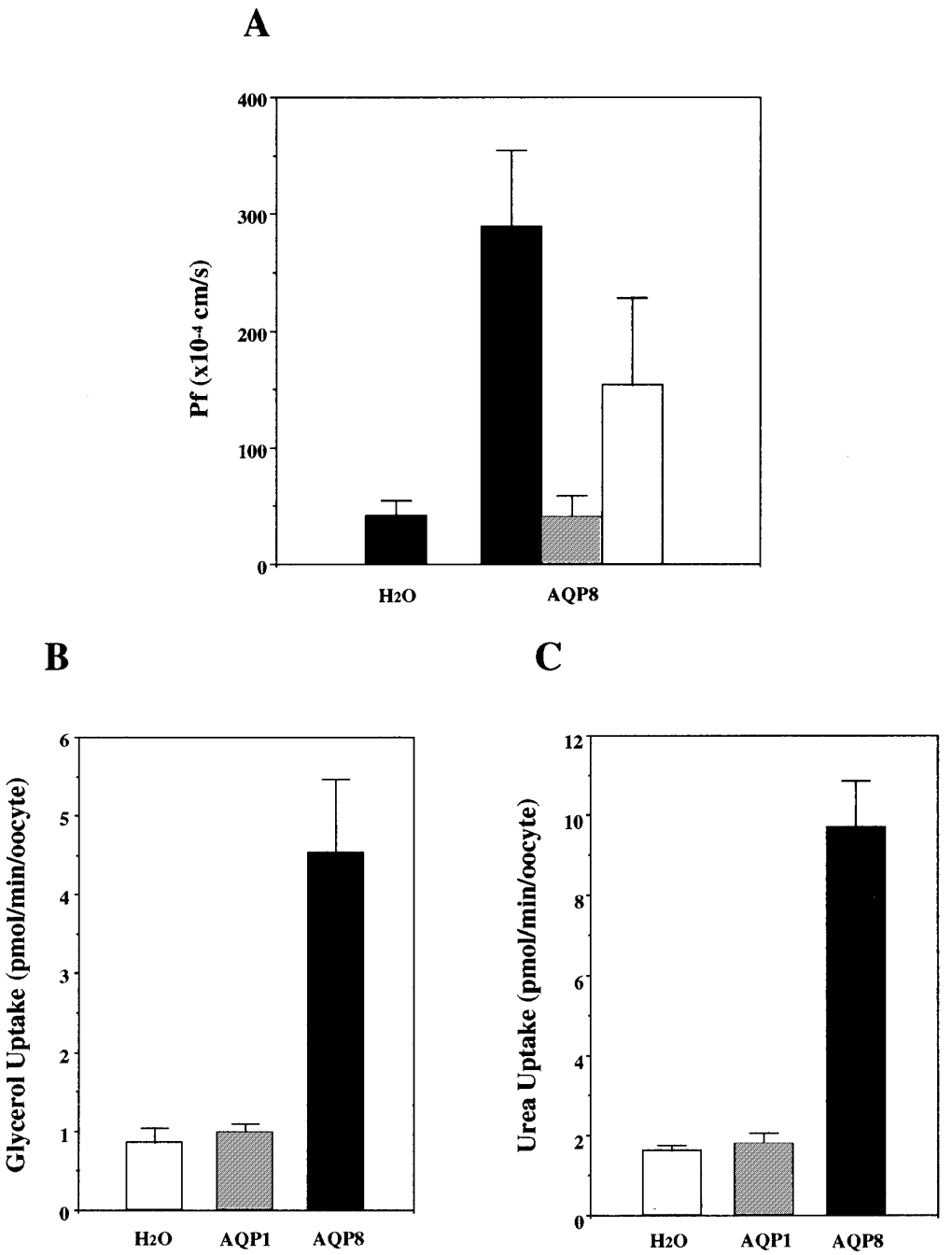

FIG. 2. (A) Osmotic water permeability (Pf) of oocytes injected with 50 nl of water or 10 ng of the indicated cRNAs. Shown are the mean ± SD of 5 oocytes receiving no further treatment (*black bars;* H_2O 40.9 ± 14.0, AQP9 cRNA 288.8 ± 65.6), oocytes incubated for 5 min in 0.3 mM $HgCl_2$ (*stippled bar;* 39.5 ± 18.9), or oocytes incubated for 5 min in $HgCl_2$ followed by 30 min in 5 mM 2-mercaptoethanol (*open bar;* 153.2 ± 75.0). (B) Comparison of [^{14}C] glycerol uptake into oocytes injected with water or AQP1 cRNA or AQP9 cRNA. The oocytes were incubated in Barth's solution with [^{14}C] glycerol for 30 min. Shown are the mean ± SD of 5 oocytes (H_2O 0.86 ± 0.18, AQP1 cRNA 0.99 ± 0.11, AQP9 cRNA 4.52 ± 0.95). (C) Comparison of [^{14}C] urea uptake into oocytes injected with water, AQP1 cRNA or AQP9 cRNA. Shown are the mean ± SD of 5 oocytes (H_2O 1.60 ± 0.14, AQP1 cRNA 1.78 ± 0.28, AQP9 cRNA 9.69 ± 1.16).

The uptake of urea or glycerol into the oocytes was measured as previously reported (5). Briefly, the oocytes were incubated at room temperature in Barth's solution with [^{14}C] urea or [^{14}C] glycerol for 30 min, then rapidly rinsed five times in ice-cold Barth's solution after incubation. The oocytes were lysed in 200 ml of 10% SDS overnight, and the radioactivity was measured by liquid scintillation counting.

Northern blot analysis. Total RNA (10 μg) from human adipose tissue and other tissues (Clonetech) was denatured in formamide and formaldehyde at 65°C for 5 min and separated by electrophoresis in agarose gels containing formaldehyde. RNA was blotted onto Hybond N nylon membranes (Amersham) and hybridized with [α^{32}P]-dCTP-labeled cDNA probe using a random priming kit (Amersham).

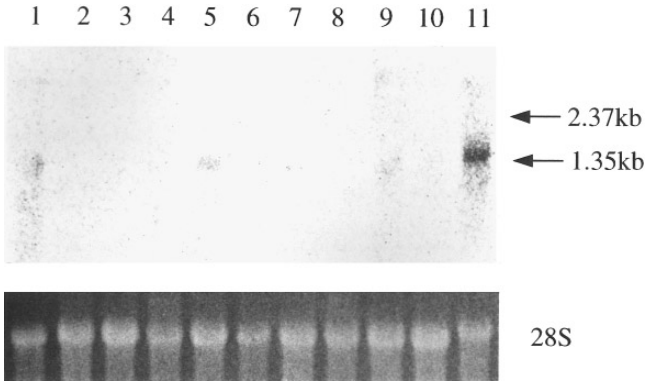

FIG. 3. Northern blot analysis of AQP9 expression in different human tissues (1.heart, 2.brain, 3.lung, 4.liver, 5.kidney, 6.ovary, 7.uterus, 8.placenta, 9.small intestine, 10.skeletal muscle, 11.white adipose), showing that AQP9 transcripts are detected most abundantly in white adipose tissue, while much fainter bands are detected in heart, kidney and small intestine. The 28S ribosomal RNA bands visualized with ethidium bromide are shown in the bottom panel.

The membranes were washed in 2× SSC-0.1% SDS, then in 0.1× SSC-0.1% SDS at 65°C for 15 min each and visualized by autoradiography with Kodak XR5 film at −80°C using an intensifying screen for 48 h.

RESULTS

Molecular cloning of human AQP9 cDNA. In the cDNA library containing the 3′ regions of cDNA constructed from human adipose tissue, a novel gene, represented by the fragmentary sequence GS3340 was cloned. A 1.3-kb insert was obtained from a cDNA library constructed from human adipose tissue and sequenced. The result is shown in Fig.1A. The insert consists of a 172-bp 5′-untranslated sequence preceding an initiation site consensus (10) and a 1026-bp open reading frame, followed by a 3′-untranslated sequence with a polyadenylation signal. The open reading frame encoding 342 amino acids was surveyed in the GenBank and found to be a novel member of the human aquaporin family of water-selective membrane channels found in plants and animals. It contains six putative bilayer-spanning domains (11) (Fig. 1B), and an NPA (Asn-Pro-Ala) motif, which is highly conserved in the AQP family (4). In this case, however, the first NPA has been replaced by NAA (Asn-Ala-Ala) and the second NPA by NPS (Asn-Pro-Ser). The known mercury inhibitory sites (Cys) of AQPs are located just in front of the 2nd NPA motif (4, 6), but AQP9 does not have Cys in such a position. The NH_3-terminal domain contains the sequence RRST (Arg-Arg-Ser-Thr), matching the cAMP-protein kinase phosphorylation consensus site (12) found in AQP2, AQP4 and AQP5 (4). A dbEST search showed that the sequence is almost identical (96-99%) to several independent ESTs (EST156430, 153310, 186500 and 186588) that have been derived from human breast. Since the breast is a mixture of mammary glands and adipose tissue, these ESTs are likely to have originated from the adipose tissues in the source material. These combined sequences also showed that the second NPA was replaced by NPS. We therefore named this novel aquaporin gene, AQP9. (The nucleotide sequence of the AQP9 has been deposited in DDBJ under accession number AB006190.)

Functional analysis of AQP9. We examined the properties of AQP9 through its expression in *Xenopus* oocytes. When the oocytes were injected with AQP9 cRNA, the coefficients of osmotic water permeability (Pf) increased approximately 7-fold (Fig. 2A), a behavior typical of aquaporin. As with most AQPs (4), this increase in Pf was reduced to 14% by the addition of 0.3 mM $HgCl_2$, and this inhibition was eliminated with the addition of 2-mercaptoethanol (Fig. 2A). Since the AQP9 sequence shows high homology with AQP3 (45% identical at the amino acid level) and AQP7 (76% identical in the region of amino acid residues 20 to 281 of AQP9) which permeate small molecules, including glycerol and urea (4-7) (Fig. 1A), we examined the uptake of glycerol and urea. The [^{14}C] glycerol uptake in AQP9 cRNA-injected oocytes was 5.3 or 4.6 times higher, respectively, than in water- or AQP1 cRNA-injected oocytes (Fig. 2B). The AQP9 cRNA-injected oocytes also showed a 6.1- or 5.4-fold greater [^{14}C] urea uptake, respectively, compared with the water- or AQP1 cRNA-injected oocytes (Fig. 2C).

Tissue Distribution of AQP9 mRNA. To observe the distribution of this gene product among various human tissues, we performed Northern blot analysis. The results in Fig. 3 show that AQP9 mRNA (1.4 kb) is expressed strongly in adipose tissue. Much fainter bands were detected in the heart, kidney and small intestine.

DISCUSSION

We have cloned a novel water channel (AQP9) that also makes cells permeable by glycerol from human adipose tissue. In mammals, AQP3 has long been the only aquaporin with glycerol and water permeability. Recently, another aquaporin, AQP7, has been cloned from rat sperm that also facilitates glycerol permeation. Although AQP7 has significant similarity to AQP9, it is insensitive to mercury chloride, unlike AQP9.

The driving force for water movement through AQPs is thought to be an osmotic pressure gradient between the inside and outside of the cell (4), and all but one of the previously reported AQPs have direct contact with body fluids that are susceptible to rapid osmotic pressure change (4-7). The only exception is AQP4 in the hypothalamus that functions as an osmoreceptor (4, 13). In this context, AQP9 is exceptional if it primarily

functions as a water channel, although such a possibility cannot ruled out easily.

On the other hand, triglycerides in adipocytes are hydrolyzed to glycerol and fatty acids by hormone-sensitive lipase and other enzymes in response to a demand for energy (8). It is well known that fatty acids are transported by several proteins (14-16). Although a substantial amount of glycerol obviously passes through the adipocyte membrane rapidly (17-19), the mechanism for glycerol transport has remained obscure (20-22). From the facts that AQP9 is almost exclusively and abundantly expressed in adipose tissue and the AQP9 protein actually facilitates glycerol permeability, it is natural to speculate that this protein transports glycerol when a gradient is created between cell membrane by lipolysis (17-20). Further study is required to understand the *in vivo* function of this protein.

ACKNOWLEDGMENTS

We would like to express our appreciation for the helpful assistance of Kazuhisa Maeda, Kenta Nakai and Wakako Adachi.

REFERENCES

1. Okubo, K., Hori, N., Matoba, R., Niiyama, T., Fukushima, A., Kojima, Y., and Matsubara, K. (1992) *Nature Genet.* **2,** 173–179.
2. Maeda, K., Okubo, K., Shimomura, I., Mizuno, K., Matsuzawa, Y., and Matsubara, K. (1997) *Gene* **190,** 227–235.
3. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa Y., and Matsubara, K. (1996) *Biochem. Biophys. Res. Commun.* **221,** 286–289.
4. King, L. S., and Agre, P. (1996) *Annu. Rev. Physiol.* **58,** 619–648.
5. Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T., and Marumo, F. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6269–6273.
6. Ma, T., Yang, B., Kuo, W.-L., and Verkman, A. S. (1996) *Genomics* **35,** 543–550.
7. Ishibashi, K., Kuwahara, M., Gu, Y., Kageyama, Y., Tohsaka, A., Suzuki, F., Marumo, F., and Sasaki, S. (1997) *J. Biol. Chem.* **272,** 20782–20786.
8. Nilsson, N. O., Strålfors, P., Fredrikson, G., and Belfrage, P. (1980) *FEBS Lett.* **111,** 125–130.
9. Taylor, M. A., Johnson, A. D., and Smith, L. D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6586–6589.
10. Kozak, M. (1987) *Nucletic Acids Res.* **15,** 8125–8132.
11. Claros, M. G., and von Heijne, G. (1994) *Cabios* **10,** 685–686.
12. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 15555–15558.
13. Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 13052–13056.
14. Schaffer, J. E., and Lodish, H. F. (1994) *Cell* **79,** 427–436.
15. Baxa, C. A., Sha, R. S., Buelt, M. K., Smith, A. J., Matarese, V., Chinander, L. L., Boundy, K. L., and Bernlohr, D. A. (1989) *Biochemistry* **28,** 8683–8690.
16. Ibrahimi, A., Sfeir, Z., Magharaie, H., Amri, E. Z., Grimaldi, P., and Abumrad, N. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2646–2651.
17. Arner, P., and Bolinder, J. (1991) *J. Int. Med.* **230,** 381–386.
18. Jansson, P. A., Larsson, A., Smith, U., and Lönnroth, P. (1992) *J. Clin. Invest.* **89,** 1610–1617.
19. Arner, P., Kriegholm, E., Engfeldt, P., and Bolinder J. (1990) *J. Clin. Invest.* **85,** 893–898.
20. Maurel, C., Reizer, J., Schroeder, J. I., Chrispeels, M. J., and Saier, M. H., Jr. (1994) *J. Biol. Chem.* **269,** 11869–11872.
21. Lin, E. C. C. (1977) *Ann. Rev. Biochem.* **46,** 765–795.
22. Luyten, K., Albertyn, J., Skibbe, W. F., Prior, B. A., Ramos, J., Thevelein, J. M., and Hohmann, S. (1995) *EMBO J.* **14,** 1360–1371.