Aquaporin adipose (AQPap) is a putative glycerol channel in adipocytes (Kishida, K., Kuriyama, H., Funahashi, T., Shimomura, I., Katoh, K., Nishizawa, H., Maeda, M., Matsuda, M., Okada, M., Kondo, H., Kuriyama, H., and Funahashi, T.).

The first two exons, designated exon 1 and exon 1', are alternatively spliced to common exon 1, and thus the AQPap gene possesses two potential promoters. The exon 1-derived transcript is dominant in both adipose tissues and adipocytes on the basis of RNase protection assay and promoter analysis. The mRNA increased after fasting and decreased with refeeding. Insulin deficiency generated by streptozotocin enhanced the mRNA in adipose tissue. Insulin down-regulated AQPap mRNA in 3T3-L1 adipocytes. The AQPap promoter contained heptanucleotide sequences, TGTTTTT at -443/-437, similar to the insulin-response element identified previously in the promoters of insulin-repressed genes. Deletion and single base pair substitution analysis of the promoter revealed that these sequences were required for insulin-mediated repression of AQPap gene transcription. The phosphatidylinositol 3-kinase pathway was involved in this inhibition. We conclude that insulin represses the transcription of AQPap gene via insulin response element in its promoter. Sustained up-regulation of AQPap mRNA in adipose tissue in the insulin-resistant condition may disturb glucose homeostasis by increasing plasma glycerol.

Aquaporins (AQPs) are channel-forming integral proteins and function as water channels. To date, at least 10 AQPs have been identified in mammalian tissue (1). Among these, AQP3, AQP9, and aquaporin adipose (AQPap) (2, 3) possess the capability for transporting glycerol as well as water. AQP3 was identified from the kidney (4, 5) and AQP9 (6) from the liver. Recently, we cloned AQPap as a novel cDNA belonging to the AQP family from the human adipose tissue cDNA library, and we showed that its mRNA was highly and almost exclusively expressed in human adipose tissue (3). Therefore, we named it aquaporin adipose (AQPap). AQP7 was independently cloned from rat testis and was a rat homologue for AQPap (7, 8).

Adipose tissue plays an important role in glucose and lipid metabolism in the mammalian body. Adipocytes continuously synthesize and hydrolyze triglycerides in response to energy balance. When energy is required in other organs, triglyceride stored in adipose tissue is hydrolyzed into the glycerol and free fatty acid, releasing both products into the bloodstream (9). Molecules facilitating the transport of free fatty acid were identified and characterized. These include fatty acid translocase (10), fatty acid transport protein (FATP) (11), and plasma membrane fatty acid-binding protein (12). However, the molecule responsible for glycerol release from adipocyte has not been identified. We consider AQPap to be the adipose-specific glycerol channel for the following reasons. 1) AQPap had glycerol permeability and was abundantly expressed in adipose tissue and fully differentiated 3T3-L1 adipocytes. 2) During the differentiation, 3T3-L1 adipocytes increased the epinephrine-stimulated release of glycerol in parallel with the induction of AQPap mRNA. 3) Incubation with HgCl2 totally blocked and addition of mercaptoethanol recovered the epinephrine-stimulated glycerol release in 3T3-L1 adipocytes, which are the phenomena generally seen for proteins belonging to the AQP family. 4) The mRNA of AQP3 or AQP9, which are other AQPs with similar characteristics, was not detectable in adipose tissue or cultured adipocytes (2). All these results support the notion that AQPap is the major transporter of glycerol in adipose tissue.

The levels of AQPap mRNA were regulated nutritionally. Fasting enhanced and refeeding suppressed the levels of AQPap mRNA in adipose tissue, leading to increased levels of plasma glycerol with fasting and a decrease with refeeding.
**Insulin-mediated Inhibition of Aquaporin Adipose Gene**

The mechanism controlling the expression of this putative glycerol channel has not been clarified. Insulin is one of the factors implicated in the mediation of these regulations of AQPAp mRNA. There have been several genes reported to be suppressed by insulin (13), including the genes encoding phosphoenolpyruvate carboxykinase (PEPCK) (14), insulin-like growth factor-binding protein-1 (IGFBP-1) (14), glucose-6-phosphatase (Glc-6-Phase) (15), apolipoprotein CIII, insulin receptor substrate-2 (IRS-2) (16), and FATP (17). The promoter regions of these genes contain the consensus heptanucleotide sequence, T/G/A/T/TTT/G/T, designated as insulin-response element (IRE). Several candidate factors have been proposed as insulin-responsive transcriptional factors, including hepatic nuclear factor 3, forkhead/winged-helix family, FKHR, and FKHLR1 (18–20), although the direct interaction of these factors with IRE has not been fully elucidated.

Glycerol, produced and released from adipose tissue, is an important substrate for gluconeogenesis in the liver and kidney, both of which have glycerokinase to convert the glycerol into glycerol-6-phosphate for de novo synthesis of glucose (21, 22). Previously, we showed that AQPAp mRNA was increased in the adipose tissue of insulin-resistant mice, leading to hyperglycemia. A high concentration of plasma glycerol has been shown to cause hyperglycemia by enhancing gluconeogenesis (23–26). To identify the regulatory mechanism of AQPAp mRNA in adipose tissue is important for understanding the physiological and pathological significance of glycerol release from adipose tissue. In the current studies, we determined the genomic structure of the mouse AQPAp gene, identified two putative IREs in the 5′-flanking region of the AQPAp gene, and introduced the luciferase assay to show that one of the IREs is required for insulin-mediated repression of AQPAp gene transcription in adipocytes. Furthermore, we showed that the P13K pathway mediates this inhibitory effect of insulin on AQPAp gene transcription. These results suggest a potential mechanism for insulin-mediated inhibition of the AQPAp gene and thereby help to understand regulation of the amount of glycerol in plasma in the normal and insulin-resistant status.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Bovine pancreatic insulin was obtained from Sigma. LY294002, an inhibitor of phosphatidylinositol 3-kinase, and PD98059, an inhibitor of mitogen-activated protein kinase kinase, were purified using the Qiagen plasmid kit. A mutation of single nucleic acid was inserted into the HinDIII and Smal sites of pSP64 poly(A) vector (Promega), designated as pSP64-AQPAp. *In vitro* transcription of the entire encoding of AQPAp and injection of the resulting cDNA into Xenopus oocytes were performed as described previously (3). Oocytes were injected with 10 ng of AQPAp cRNA (0.5 μg/μl) and incubated in modified Barth’s buffer (NaCl 96 mM, KCl 2 mM, CaCl2 1.8 mM, MgCl2 1 mM, HEPES 5 mM) at 18 °C. After 48 h of incubation, osmotic water permeability and uptake of glycerol was measured as described previously (3). Briefly, for measurement of osmotic water permeability, the oocytes were transferred from Barth’s buffer containing 200 mM mannitol to Barth’s buffer containing 200 mM sucrose and the swelling was monitored with a Nicon phase-contrast microscope equipped for video recording. The oocyte volume was calculated from the recorded images with a microcomputer-imaging device (MCID-M2, Imaging Research Inc., Ontario, Canada). Osmotic water permeability (Pf, cm/s) was calculated from the initial rates of swelling, diffusion, and the oocyte surface-volume ratio (V/Vs = 50 cm⁻¹), and partial molar volume of water (Vw = 18 cm³/mol) from the relation, Pf = (d(V/Vs)/dt)/(V/Vs)(osm∞ - osm∞). The relation Pf = (d(V/Vs)/dt)/(V/Vs)(osm∞ - osm∞) was 180 mOSm. For measurement of the uptake of glycerol, groups of 5–8 oocytes were incubated in Barth’s buffer containing 2 μCi/ml [U-¹⁴C]glycerol (Amersham Pharmacia Biotech, nonradioactive glycerol was added to give a 1 mM final concentration at room temperature). After 20 min of incubation, oocytes were rapidly rinsed five times in ice-cold Barth’s buffer. The oocytes were lysed in 400 μl of 5% SDS overnight, and the radioactivity was measured by a liquid scintillation counter.

**Immunodetection of AQPAp Expressed in Xenopus Oocytes—**To determine the stability and size of the AQPAp proteins, eight oocytes were homogenized in 160 μl of homogenization buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 0.5 μM nonylphenol ethoxylate, 1 mM PMSF, 1 mM threitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and pepstatin) at 4 °C on day 3 after injection. Subsequently, the lysates were centrifuged twice for 10 min at 125 × g to remove yolk protein. On day 3 after injection, plasma membranes were isolated from 25 oocytes according to the method of Wall and Patel (26). Lysates or plasma membranes equivalent to 8 oocytes were denatured for 30 min at 37 °C in sample buffer (2% (w/v) SDS, 50 mM Tris (pH 6.8), 12% (v/v) glycerol, 100 mM dithiothreitol), electrophoresed through a 12.5% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (Schleicher & Schuell) as described previously (2). For immunodetection, the membrane was incubated with a 1:500 dilution of rabbit anti-rat AQPAp antibody prepared against a specific amino acid peptide (Chemicon international, Inc.). As a secondary antibody, a 1:750 dilution of affinity-purified anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) was used. Proteins were visualized using the ECL™ system (Amersham Pharmacia Biotech).

**Genomic Southern Blots and Isolation of Genomic Clones—**A 10-μg quantity of mouse genomic DNA (ICR, Swiss mouse (Promega)) was digested overnight with BamHI, EcoRI, or XhoI, respectively, and size-fractionated by electrophoresis on 0.8% agarose gel. Gels were blotted onto nucleic acid transfer membrane (Hybond™-N+, Amersham Pharmacia Biotech) and hybridized with mouse AQPAp cDNA probe (exon 7–8 regions). The probes were labeled using the Multiprime DNA labeling system (Amersham Pharmacia Biotech) with [α-³²P]CTP. Hybridization was carried out with the QuickHyb® hybridization solution (Stratagene) at 65 °C with an intensifying screen. In order to isolate genomic clones, a bacterial artificial chromosome (BAC) mouse II hybridization library was screened using the AQPAp cDNA probe (Genome Systems, Inc.).

**Restriction Mapping, Determination of Exon/Intron Boundaries, and DNA Sequencing—**The restriction fragments that were digested with EcoRI or XhoI were purified and ligated into the corresponding sites of the pZERO-1™ vector (Invitrogen). The ligated product was used for transformation into the Escherichia coli DH10B, and plasmid DNA was isolated. Positive clones were identified by Southern blot analysis of the plasmid DNA using mouse full-length AQPAp cDNA as the probe. These subclones were then isolated and subjected to various restriction enzyme digestions to map the mouse AQPAp gene. Double-stranded sequencing of denatured plasmid DNA was performed to determine the intron/exon boundaries and also to obtain sequence information of the promoters by sequencing (DYEnamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech), PerkinElmer Life Sciences, ABI PRISM® 377 Automated DNA Sequencer and Genetic Analyzer 310 Sequencer).

**Plasmids—**Plasmids containing various lengths of the mouse AQPAp promoter were amplified by PCR with restriction sites engineered for subcloning into the MluI and XhoI site of the promoterless pGL3-basic luciferase expression vector (Promega). Plasmids for transfection were purified using the Qiagen plasmid kit. A mutation of single nucleic
acids and deletion mutant of pG3L-AQPap luciferase plasmid were constructed using the QuickChange Site-directed Mutagenesis kit (Stratagene). The integrities of all plasmids were verified by DNA sequencing.

**Cell Culture, Transfection, and Luciferase Assays—**3T3-L1 preadipocyte cells were grown to confluence and induced to differentiate into adipocytes according to the modified method of Rubin et al. (29). Briefly, 3T3-L1 cells were grown on a 9-cm culture dish in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were then maintained in DMEM containing 10% FCS. Differentiated cells were maintained in DMEM containing 10% FCS until the transfection experiments. Typically, for each 12-well culture plate, 1 μl of firefly (Photinus pyralis) luciferase plasmid constructed from pG3L-basic luciferase expression vector and 10 ng of a sea panay (Renilla reniformis) luciferase control vector, pRL-SV40 (Promega), were complexed with LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s protocol and used for transfection. An equal volume of 20% fetal calf serum in DMEM was added 3 h later. The transfection mixture was removed 24 h later after transfection, and the cells were maintained in DMEM containing 10% fetal calf serum for 24 h before cell lysis for reporter assays. Luciferase activities were assayed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

**Synthesis of Mouse White Adipose Tissue cDNA Library and RNA Analysis—**Total cellular RNA was isolated using the TRIZOL reagent kit (Life Technologies, Inc.) (30). The white adipose tissue cDNA library was generated using Superscript choice system (Life Technologies, Inc.) and the Lambda ZAP II Vector kit (Stratagene) according to the manufacturer’s protocol. Northern blot analysis was performed as described previously (2). Briefly, 10-μg quantities of total RNA from various tissues were electrophoresed on 1% agarose/formaldehyde gel and transferred to a nylon acid transfer membrane (Hybond-N+, Amersham Pharmacia Biotech). After fixation by ultraviolet cross-linking, the filter was prehybridized with the QuikHyb hybridization solution (Stratagene) at 65°C for 0.5 h. Mouse AQPap cDNA, obtained by reverse transcription-polymerase chain reaction to mouse adipose tissue RNA, was used as a probe for Northern blot analysis. The probes were radiolabeled using the multiprime DNA labeling system (Amersham Pharmacia Biotech) with α-[32P]CTP and used for hybridization (10 × 10^6 cpm/ml). Hybridization was carried out with the same solution at 65°C for 1 h after adding 0.2 mg/ml of denatured salmon sperm DNA. Washes were performed in 2× SSC and in 0.1% SDS at 65°C for 30 min, followed by 0.1× SSC plus 0.1% SDS at 65°C for 15 min, and the filter was exposed to Kodak X-Omat x-ray film for 24 h at −80°C with an intensifying screen.

**Determination of Transcription Initiation Site by 5′-RACE-PCR, Primer Extension, and Ribonuclease Protection Assay—**In order to identify the 5′-end of the mouse AQPap gene, rapid amplification of the cDNA (RACE) was conducted according to the manufacturer’s protocol (Marathon™ cDNA amplification kit (CLONTECH) and 5′-RACE system (Life Technologies, Inc.)) using the following primers: first primer (corresponding to the region in exon 6–7), 5′-CAGCATCT-CCAGTCGAAATGCTCTC-CATC-3′ and nested primer (corresponding to exon 3), 5′-GATTTGATATGTCCTCAGCAGACTT-3′. Primer extension was performed essentially according to the method of Ausubel et al. (31) using total RNA and an end-radioabeled antisense exon 1 oligonucleotide probe (5′-TCTTCTAGAAATGTCCTCTTGTTCAAGCGCTTCC-3′) and an exon 1′ oligonucleotide probe (5′-CCTCTATGCTCCTCCTTCTGAGCTGCCA-3′). Next, 20 μg of total RNA were mixed with 5 × 10^6 cpm of primer in a 30-μl volume of hybridization buffer (40 mM PIPES, 1 mM EDTA, 0.4 mM NaCl, 50% formamide) and heated to 85°C for 10 min and hybridized overnight at 45°C. After hybridization with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37°C for 30 min and at 42°C for 30 min, the products were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel containing 8 μg urea. An M13mp18 sequencing reaction (Amersham Pharmacia Biotech) and radiolabeled dX174 DNA/HpaII dephosphorylated probe (Promega) were used as a size standard.

For the RNA protection assay, cDNA fragment (350 bp) in exon 1, 1′, or 8 was subcloned into the plasmid pGEM T-easy (Promega) and linearized with an appropriate restriction enzyme. A radiolabeled antisense cRNA probe was generated using SP6 RNA polymerase from in vitro transcription kit (MAXIscript SP6/T7 kit, Ambion) and [32P]UTP (Amersham Pharmacia Biotech). Next, 20 μg of total RNA were hybridized at 68°C for 10 min and ribonuclease protection assays were performed according to the manufacturer’s protocol (HybSpeed™ RPA, Ambion) (32). The protected fragments were analyzed together with a sequencing reaction on 5% polyacrylamide gel containing 8 μl urea. The gel was fixed, dried, and exposed to film.

**Radiation Hybrid Mapping—**Chromosomal localization was determined by the Gene Bridge 4 Radiation Hybrid Panel (Research Genetics) according to the manufacturer’s instructions, using specific primers (5′-GTGAAAA TTCAATCCTCAGCAGC-3′ and 5′-GATTTGATG-GTTCAGCACAGCAGC-3′) in AQPap exons 3 and 4. The amplification profile consisted of denaturation for 180 s at 96°C, followed by 30 cycles of denaturation for 30 s at 96°C, annealing for 60 s at 60°C, and extension for 60 s at 72°C. The results were analyzed with a WIST mouse radiation hybrid mapper.

**Statistical Analysis—**The results were expressed as mean ± S.E. The significance of the difference between the mean values of the groups was evaluated by Student’s t test.

**RESULTS**

**Cloning of the Mouse AQPap cDNA and Functional Expression of AQPap in Xenopus Oocytes—**Previously, we cloned human AQPap, a new member of the aquaporin family, which was exclusively expressed in human adipose tissue (3). This protein exhibited transport activity of glycerol as well as water. In this study, we have isolated mouse AQPap cDNA from the mouse white adipose tissue cDNA library. This gene encodes a 304-amino acid (GenBank™ accession number AB056099, Fig. 1A), which has 78% homology to human AQPap. To test the function of the mouse AQPap, we injected the entire mouse AQPap cRNA and expressed the protein in the Xenopus oocytes. Immunoblotting of the oocytes membrane fraction detected a 28-kDa protein, corresponding to the predicted molecular mass of AQPap (Fig. 1B). The osmotic water permeability coefficient (Pf) of AQPap cRNA-injected oocytes was 9 times higher than that of water-injected oocytes (Fig. 1C). The oocytes injected with AQPap cRNA also showed 10-fold stimulation of glycerol uptake, which was comparable with human AQPap (Fig. 1C) (3).

**Genomic Structure and Transcriptional Termination of Mouse AQPap 7 Gene—**Genomic Southern blot analysis, using different restriction enzymes and a 1-kb cDNA fragment corresponding to the exon 7 and 8 regions as the probe, detected a single band, which suggested that the mouse AQPap gene is a single copy gene (data not shown). To obtain genomic DNA of mouse AQPap, the BAC mouse II hybridization library (Genome Systems, Inc.) was screened, using mouse AQPap cDNA (coding region) as a probe. Southern blot analysis using three clones (GS20454, GS20455, and GS20456) from the BAC library a probe showed an identical pattern to the result to mouse genomic DNA, indicating that these clones contained the AQPap gene (data not shown). The XbaI/EcoRI-digested fragment of these clones was subcloned into pZEO-1® vector (Invitrogen). Intron/exon boundaries and the intron sizes are determined and summarized in Fig. 2A. All intron/exon boundaries confirmed to the established consensus GT/AG rule (GenBank™ accession numbers AB056092–AB056098, data not shown). The AQPap gene spans around 12 kb and has 8 exons, three of which, exons 1, 1′, and 2, are upstream of the translation initiation site (Fig. 2A). Exon 1 and exon 1′ are alternatively spliced to a common second exon. The translation initiation site ATG was located in exon 3. Chromosomal localization was determined by the Gene Bridge 4 Radiation Hybrid Panel using specific primers to amplify exon 3 and 4 regions. Mouse AQPap gene was revealed to be localized at D4 Mit 236 (23.7 centimorgans) of the mouse chromosome 4 (data not shown). Several genes related to adipocyte biology, including adipocyte differentiation-related protein, leptin receptor, tumor necrosis factor receptor 1, and brain natriuretic peptide, were previously reported to be localized closely to the site for AQPap (33). The mouse AQPap gene had exactly the same
To determine the relative amount of exon 1- and exon 1′-derived transcript, RNase protection assay using a 268-bp cRNA against the region for exons 1–3 or a 263-bp cRNA against the region for exon 1′, 2, and 3 as the probe was performed (Fig. 3C). The cRNA probe 1 (for exons 1–3) gave one major protected fragment (268 bp) from the total RNA of mouse white adipose tissue and mature adipocytes. Probe 2, specific for AQPap, also detected a major transcript with a size of 213 bp, with a trace

coding region as that of the mouse AQP7 gene obtained from mouse testis (GenBank accession number AB010100) but had a longer 3′-end of cDNA in comparison with that of mouse AQP7 (Fig. 2B). A typical polyadenylation site AATAAA, which is used for AQP7, was found 33 bp downstream in the transcript termination site, and a variant polyadenylation site ATTTAAA, which is for AQPap, was 1172 bp downstream (Fig. 2B). A GT-rich downstream element, which is important in defining the polyadenylation site, is found 38 bp downstream of the AQPap cDNA end (Fig. 2B). To confirm the 3′-end of cDNA, we generated two kinds of cDNA probes (Fig. 2C). One (probe 1) spans the cDNA regions in the open reading frame, which is common to both AQPap and AQP7. The other probe (probe 2) hybridizes to 3′-specific region of AQPap. The mRNA size (around 2.4 kb) of mouse AQPap, estimated from Northern blotting of mouse adipose tissue, was consistent with the size of the cDNA obtained from mouse adipose tissue cDNA library (2375 bp) (Fig. 2D). Northern blotting using probe 1 detected a significant amount of the 2.4-kb transcript, which is for AQPap, in white adipose tissue and mature adipocytes. Probe 2, specific for AQPap, detected an identical signal pattern at around 2.4 kb. A 1.8-kb transcript, which is for AQP7, was observed only from the mouse testis RNA. Probe 2 specific for AQPap showed little hybridization with the mRNA derived from this testis. These data suggested that, in white adipose tissue, AQPap was the major transcript form. An RNase protection assay further confirmed the tissue-specific usage of the transcription termination site. The assay was performed using a 350-bp cRNA probe spanning common region (216 bp) in AQPap and AQP7, and specific region for AQPap (Fig. 2C), to determine the relative amount of AQPap and AQP7 in white adipose tissue, 3T3-L1 adipocyte, and testis (Fig. 2E). In white adipose tissue and 3T3-L1 cells, only the AQPap transcript (350-bp protected fragment) was detected, whereas shorter AQP7 transcript (268-bp protected fragment) was in the testis. These data also indicate that mouse white adipose tissue and testis express AQPap and AQP7 mRNAs from the same gene, respectively.

5′-Flanking Region of AQPap Gene and Promoter Analysis—The existence of two differentially spliced isoforms of mouse AQPap was verified by 5′-RACE PCR amplification of white adipose tissue RNA using a primer against the region in exon 3. It suggested that the two different cDNA species arise from alternative splicing of 5′-untranslated region, exon 1 and exon 1′. The transcription start sites of the AQPap gene were determined by 5′-RACE PCR, RNase protection, and primer extension assay. We identified four independent 5′-RACE clones differing in the length of exon 1 (GenBank accession number AB056092, Fig. 3A). An RNase protection assay, using total RNA from white adipose tissues and a 350-bp RNA probe containing the exon 1 region and 5′-flanking region, gave four protected fragments, whose size was consistent with the result obtained from 5′-RACE (date not shown). The smallest fragment (98 bp) was most intense and corresponded to the shortest clone obtained from 5′-RACE. This start site predicted by 5′-RACE and RNase protection assay is designated +1 (Fig. 3A). Analysis of a 1097-bp length of the 5′-flanking sequence for potential transcription factor-binding sites revealed several clustered consensus sequences. Conserved consensus sequences to note were CAAT, hepatic nuclear factor 3β, and CCAAT enhancer-binding protein (C/EBP) α and β (Fig. 3A). Primer extension of total RNA from white adipose tissues was also performed with each primer complementary to exon 1 or exon 1′ of the cDNA (Fig. 3B). Exon 1 primer produced four extended DNAs, the size of which was attributed to the four transcription initiation sites identified by 5′-RACE and RNase protection assay. Exon 1′ primer produced a single extended DNA, and the size is 156 bp for the size of exon 1′. This results indicated that there is no transcript reading through exon 1 and exon 1′ together.

Fig. 1. cDNA cloning of mouse AQPap and functional expression of mouse AQPap in Xenopus oocytes. A, nucleotide sequence and deduced amino acid sequence of AQPap isolated from the cDNA library of mouse white adipose tissue (GenBank accession number AB056092). B, AQPap protein expression after injection of cRNA into oocytes. Oocytes were injected with water or 50 ng of mouse AQPap cRNA as described under “Experimental Procedures.” Membranes prepared from eight oocytes were loaded in each lane. The immunoblots were performed with anti-AQPap antiserum. C, osmotic water permeability (P) and [14C]glycerol uptake of oocytes injected with water (open bar) or 50 ng of mouse AQPap cRNA (closed bar). Each assay was conducted, as described under “Experimental Procedures.” Bars show mean ± S.E. of 4–5 determinations of oocytes.
Insulin-mediated Inhibition of Aquaporin Adipose Gene

Fig. 2. Organization of the mouse AQPap/7 gene. A, exon/intron organization and restriction enzyme map of BamH1 (B), EcoR1 (E), and XhoI (X) in mouse AQPap gene. Exon numbers are indicated, and the relative positions of introns and exons are drawn to scale. Exons are represented by boxes, and solid areas indicate coding regions, and open areas indicate non-coding regions. Exon 1 and Exon 1' are alternatively spliced to exon 2. B, the sequence of exon 8 and its 3'-flanking sequence are shown. The coding region is shown in italicized letters (translation stop nucleotide, *). The 3'-untranslated region is shown in non-italic bold letters (AQP7 cDNA end nucleotide, open circle). The polyadenylation signal is boxed, and GT-rich elements are underlined. C, schematic illustration shows the termination of cDNAs isolated from white adipose tissue cDNA library (AQPap) and from testis cDNA library (AQP7) (GenBank accession number AB010100: Ishibashi et al. (8)). Northern blotting probe, probe 1 (common in AQPap and AQP7) and probe 2 (specific to AQPap). A 350-bp cDNA fragment containing the common region for AQPap and AQP7 and specific region for AQPap was subcloned into plasmid pGEMT-easy, and the linearized plasmid was used to generate a 350-bp cRNA probe for RNase protection analysis. E, D, Northern blotting of AQPap and AQP7 in various tissues. Total RNAs (10 μg/lane) from mouse various tissues were subjected to Northern blot analysis. The blots were hybridized with AQPap/7 (probe 1 or probe 2, refer to C) cDNA probes. The lower panel represents the ethidium bromide-staining of 28 S ribosomal RNAs. E, RNase protection assay to evaluate the expression levels of AQPap and AQP7 in adipose cells and testis. The 350-bp cRNA fragment described in A was hybridized with total RNAs (10 μg/lane) from mouse white adipose tissues, 3T3-L1 adipocytes, and testis and subjected to RNase protection assay.

Amount of 283-bp protected fragment. These results show that exon 1-derived transcript is much more abundant than the exon 1'-derived transcript in both white adipose tissue and 3T3-L1 adipocytes.

Transient transfection assay was performed to test whether the 5'-flanking region of the AQPap gene (exon 1- and exon 1'-derived types) exhibits promoter activity in adipocytes (Fig. 3D). A series of luciferase constructs containing progressive 5'-deletions of the AQPap 5'-flanking sequence was transfected into undifferentiated 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocytes. Trace amounts of promoter activities were observed in 3T3-L1 preadipocytes. Luciferase activities in differentiated 3T3-L1 adipocytes transfected with the three kinds of promoter-containing constructs was more than 5-fold higher than that of empty vector. The promoter activity of exon 1'-flanking region spanning between -242 and +179 was almost similar to that of the empty vector. These data confirmed that the major transcription of AQPap is driven by the 5'-flanking region of exon 1 in adipocytes.

Inhibitory Effect of Insulin on AQPap mRNA Expression in White Adipose Tissue and 3T3-L1 Adipocytes—Fasting activates lipolysis and accelerates glyceral release from adipose tissues (9). Plasma insulin decreased after 18 h of fasting and increased after 12 h of refeeding (Fig. 4A). The AQPap mRNA in adipose tissue was regulated to mirror the changes of plasma insulin. AQPap mRNA increased after fasting and decreased with refeeding (Fig. 4A). Plasma glyceral levels were also elevated in the fasted mice and decreased in the refead mice, in parallel to the change in adipose AQPap mRNA. We also examined the amount of adipose AQPap mRNA in the presence or absence of insulin. AQPap mRNA levels in white adipose tissue were compared between the control and STZ-treated insulin-deficient mice (Fig. 4B). AQPap mRNA levels in white adipose tissue of insulin-deficient mice were 2.5-fold higher than those of control phosphate-buffered saline-treated mice. Plasma glyceral concentration was elevated in a similar pattern to AQPap mRNA in insulin deficiency. Inhibitory effect of insulin on AQPap mRNA was confirmed in 3T3-L1 cells (Fig. 4C). AQPap mRNA levels in 3T3-L1 adipocytes were down-regulated by insulin in dose-dependent and time-dependent manners (Fig. 4C).

Negative IRE in the Promoter of Mouse AQPap Gene—In the promoter of the mouse AQPap gene, we identified two regions identical or similar to the core negative IRE (TG/ATTTT/GT) which were found previously in the promoters of the genes such as PEPCK, IGFBP, and Glc-6-Pase (Fig. 5A) (14, 15). These two
core regions were designated IRE1 and IRE2, respectively (Fig. 5A). To define the specific region required for the repression of AQPap transcription by insulin, various mutants of mouse AQPap promoter were subcloned into luciferase vectors (Fig. 5B). The first construct (wild type) contained native regions, having both IRE1 and IRE2, and showed 50% inhibi-

![Figure 3](image)

**FIG. 3. Structure of 5′-flanking region, determination of transcription initiation sites, and promoter activity of the mouse AQPap gene. A,** the sequence of the mouse AQPap promoter and 5′-flanking region. Putative transcription factor-binding sites are predicted by the sequence motif search program, MatInspector version 2.2 (transfac.gbf.de/cgi-bin/matSearch/matsrch.pl.). The four transcription start sites determined by 5′-RACE-PCR, RNase protection (data not shown), and primer extension assays for exon 1 are represented by solid arrows. The start site predicted by the shortest fragment is designated as +1. The exon region is shown in bold letters. The transcription start site for exon 1 is shown as a broken arrow. **B,** transcription start sites examined by primer extension assay. Each end-labeled oligonucleotide, which is complementary to exon 1 or exon 1′ of the AQPap cDNA, was used to prime reverse transcription of 20 μg of total RNA from mouse white adipose tissues (lanes 6 and 8) and control yeast-tRNA (lanes 7 and 9) by primer extension. Nucleotide sizes are indicated to the left of the M13mp18 sequencing ladder and X174 DNA/HinII dephosphorylated markers, which serve as a size standard. Specific bands indicating each transcription start site are shown as solid arrows for exon 1 and a broken arrow for exon 1′. **C,** RNase protection assay to estimate the usage of exon 1 and exon 1′ in adipose tissue and adipocytes. The plasmid pGEMT-easy and a 268- or 263-bp fragment containing exon 1/2/3 or exon 1/2/3 were ligated and designed to generate a 268 (*), 263 (**), or 213-nucleotide (***, exon 2/3) length of protected fragments for RNase protection assay. Total RNAs (10 μg/lane) from mouse white adipose tissues, 3T3-L1 adipocytes, and yeast tRNA were subjected to RNase protection assay. **D,** promoter activities of AQPap in 3T3-L1 preadipocytes and adipocytes. Firefly luciferase constructs containing serial deletions of the mouse AQPap promoter of exon 1 (closed bar), exon 1′ (hatched bar), or control pGL3basic (open bar) were co-transfected with pRL-SV40 into 3T3-L1 preadipocytes (left) or mature 3T3-L1 adipocytes (right) and assayed for luciferase activity. Transcription and luciferase assay were conducted as described under “Experimental Procedures.” Normalized luciferase activities are shown as mean ± S.E. (n = 4) for the results that are represented by a column and bar graph. Similar results were obtained in three independent experiments. Representative data are indicated. The value for pGL3-basic luciferase activity was arbitrarily set as 1.0.
tion of luciferase activity after the treatment with insulin. The second (−497/+63) and third (−595/+63, ΔIRE1) constructs lacking IRE1 were totally resistant to the inhibitory effect of insulin on the promoter activities. The fourth construct (−595/+63, AIRE2) lacking only the IRE2 region showed insulin-mediated suppression of luciferase activity, similar to the wild-type construct (−595/+63). These results demonstrate that the IRE1 sequence (−443/−437) is required for mediating the suppressing effect of insulin on AQPap transcription. We examined the detailed analysis of the promoter activity between wild-type (−595/+63) and IRE1-deleted mutant (−595/+63, ΔIRE1) (Fig. 5, C and D). Insulin suppressed the wild-type luciferase activity in 3T3-L1 adipocytes, in dose- and time-dependent
Insulin was arbitrarily set at 1.0. In the lower panel, results are plotted as the mean inhibition of AQPap-mediated luciferase activity in mutant constructs each of which was conducted in triplicate.

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open bar closed bar

50 nM insulin for 18 h at 37 °C, the serum-free DMEM containing 0.5% BSA was supplemented in the absence (open bar) or presence (closed bar) of 50 nM insulin for each mutant construct is plotted above the corresponding mutated base pair. The bases in the heptanucleotide core element had lower promoter activities in the absence of insulin, but insulin sensitivity was fairly well retained. Therefore, the AQPap promoter required base pairs 2–5 of the heptanucleotide sequence to achieve a response to insulin.

PI3K Mediates Insulin Inhibition of AQPap mRNA and Promoter Activity—The effects of specific inhibitors of phosphatidylinositol 3-kinase (PI3K) (LY29402) or mitogen-activated protein kinase kinase (PD98059) on insulin-mediated inhibition of AQPap mRNAs and promoter activities were determined in 3T3-L1 adipocytes by Northern blotting (Fig. 7A) and the luciferase assay (Fig. 7B). In the absence of inhibitors, incubation with 50 nM insulin decreased AQPap mRNA levels by 50% and promoter activity by 62%. Insulin inhibition was not disturbed by incubation with PD98059. By contrast, the insulin-mediated suppression of AQPap mRNA and the promoter activity were greatly abolished when the adipocytes were incubated with PI3K inhibitor, LY29402. These results indicate that the PI3K pathway mediates insulin inhibition of AQPap mRNA and promoter activity in 3T3-L1 adipocytes and
that the mitogen-activated protein kinase pathway is not involved in the inhibition.

**DISCUSSION**

In the current study, we determined the genomic structure of the mouse AQPap gene. AQPap, isolated from the mouse adipose tissue cDNA library, had exactly the same coding region as AQP7, the sequence of which was submitted as the transcript expressed in mouse testis (GenBank accession number AB010100) (8). Northern blotting using the coding region as a probe detected a 2.4-kb transcript in white adipose tissue and differentiated 3T3-L1 adipocytes and a 1.8-kb transcript in the testis. The different sizes of these transcripts were due to the different lengths of the untranslated region of cDNA from the same gene, confirmed by Northern blotting using a specific probe and RNase protection assay. Mouse AQPap yielded cDNAs with two different 5′-ends. The divergence resulted from the alternative splicing of the first exons, designated exon 1 and exon 1′, to a common exon 2. The exon 1-derived transcript was much more abundant in both white adipose tissue and differentiated 3T3-L1 adipocytes. In *in vivo* and *in vitro* studies showed insulin-mediated repression of AQPap mRNA, and its effect was due to transcriptional regulation through the IRE in the 5′-flanking region of the AQPap gene. IRE in the AQPap gene promoter had a heptanucleotide consensus sequence similar to those in the promoters of the rat PEPCk, Glc-6-Pase, and IRS-2 (14–16) genes. The deletion mutant of this IRE decreased basal transcription activity in the absence of insulin and abolished insulin-mediated repression.

How insulin mediates its signal to IRE has not been fully clarified. Based on studies with other IRE-containing promoters, it is possible that the factor, which binds to and activates the AQPap IRE, belongs to the forkhead/winged-helix family of transcriptional factors. Studies with the promoter of IGBP-1 using HepG2 cells demonstrated that FKHR, one member of this family, directly bound to its IRE and stimulated its promoter activity in IREs in a sequence-specific manner (34). Furthermore, this enhancement was blocked when the cells were incubated with insulin. We analyzed the expression levels of FKHR mRNA in various mouse tissues (date not shown). FKHR mRNA was ubiquitously expressed, but more abundant expressions were observed in adipose tissue and muscle. We also found induced expression of FKHR mRNA during the differentiation of 3T3-L1 adipocytes (date not shown). It remains to be elucidated whether or not AQPap IRE involves FKHR-mediated regulation. In H4IE rat hepatoma cells, it was shown that insulin binding to its receptor activates phosphorylation of Akt/protein kinase B through the PI3K pathway, and the activated Akt phosphorylates the Ser-253 residue of FKHR, resulting in the repression of FKHR’s enhancement of IRE (35–40). Our results, showing selective blockage of insulin-mediated repression of AQPap mRNA and transcription by the PI3K inhibitor, also imply that PI3K pathway mediates insulin’s signal to suppress transcription of the AQPap gene. We also identified typical IRE (TATTTTC) at −152/−146 and atypical IRE (TGTTTTC) at −629/−625 in human AQPap promoter (date not shown). These findings suggest that insulin represses the transcription of the AQPap gene through IRE in the mouse and human.

To date, AQPap is the only aquaglyceroporin known to be expressed in adipocytes, among all members of the AQF family that show glycerol permeability (2). Both glycerol and fatty acid, which are end products of triglyceride hydrolysis through the function of hormone-sensitive lipase, are released into the bloodstream (9). Adipose FATP1, one of the fatty acid transport proteins, was demonstrated previously (17, 41) to show similar transcriptional regulation on AQPap. Adipose FATP1 mRNA increased and decreased during fasting and refeeding, respectively, and incubation with insulin decreased FATP1 mRNA levels in differentiated 3T3-L1 adipocytes (41). The 5′-flanking region of the mouse FATP1 gene also had an insulin-response element (TGTTTTC) at −1353/−1347 (41). Physiologically coordinated regulation of AQPap and FATP1 gene by insulin should be efficient for supplying energy in accordance with nutritional alterations. However, in the adipose tissue of insulin-resistant animals, AQPap and FATP1 mRNA levels were increased, despite high concentrations of plasma insulin, leading to higher plasma glycerol and free fatty acid levels (2, 42). Increased influx of glycerol and free fatty acid into the liver enhances hepatic glucose production and output. Insulin negatively regulates hepatic genes containing IRE in their promoters, including PEPCk, Glc-6-Pase, and IRS-2 (14–16). All these genes are master regulators of hepatic glucose homeostasis. In normal liver, insulin markedly reduced the transcriptions of these genes via its IRE (13). However, at the severe insulin-resistant stage, the regulation of these genes was puzzling. In the livers of insulin-resistant mice, IRS-2 levels were downregulated in response to a high concentration of insulin in the plasma, resulting in deterioration of insulin signaling and induction of gluconeogenetic genes such as PEPCk and Glc-6-Pase, which lead to hyperglycemia (43). The combination of reduced IRS-2 and increased glucogenic mRNAs aggravates insulin resistance and diabetes. Similar situations may also be true for adipose tissue. In normal fat, AQPap and FATP1 mRNAs are negatively regulated by insulin in accordance with the nutritional condition (2, 41). However, reciprocal increase in AQPap and FATP1 mRNAs despite hyperinsulinemia in an insulin-resistant animal may increase the hepatic glucose and lipid output in insulin-resistant syndrome by supplying more glycerol and fatty acid. Elucidation of the detailed mechanism how these IRE-containing genes are regulated should be helpful for understanding the pathophysiology of insulin-resistant metabolic syndrome.

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