Aquaporin adipose (AQPap) is a putative glycerol channel in adipocytes (Kishida, K., Kuriyama, H., Furahashi, T., Shimomura, I., Kihara, S., Ouchi, N., Nishida, M., Nishizawa, H., Matsuda, M., Takahashi, M., Hotta, K., Nakamura, T., Yamashita, S., Tochino, Y., and Matsuzawa, Y. (2000) J. Biol. Chem. 275, 20896–20902). In the current study, we examined the genomic structure of the mouse AQPap gene and its regulation by insulin. The mouse AQPap gene spanned 12 kilobase pairs in chromosome 4 and consisted of 8 exons and 7 introns. The first two exons, designated exon 1 and exon 1’, are alternatively spliced to common exon 2, and thus the AQPap gene possessed 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, TGGTTTT 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 that 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 the capability of glycerol transport, was undetectable 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.
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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 AQAPap 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-Pase) (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/GA/TTTT/GT, 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 nuclear factor 3, forkhead/winged-helix family, FKHR1 (18–20). 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 AQAPap mRNA was increased in the adipose tissue of insulin-resistant mice, leading to hyperglycerolemia. A high concentration of plasma glycerol has been shown to cause hyperglycemia by enhancing gluconeogenesis (23–26). To identify the regulatory mechanism of AQAPap 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 AQAPap gene, identified two putative IREs in the 5′-flanking region of the AQAPap gene, and introduced the luciferase assay to show that one of the IREs is required for insulin-mediated repression of AQAPap gene transcription in adipocytes. Furthermore, we showed that the PI3K pathway mediates this inhibitory effect of insulin on AQAPap gene transcription. These results suggest a potential mechanism for insulin-mediated inhibition of the AQAPap 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 purchased from Calbiochem.

*Animals and Cells*—Eight-week-old male C57BL/6 and ICR (MCH) mice were purchased from Clea Japan, Inc. (Osaka, Japan). The animals were kept at 22 °C with a 12-h dark-light cycle (light cycle, 8 a.m. to 8 p.m.). They were acclimated to the new environment for a week before the experiment. A mouse ST3-L1 cell line was obtained from Health Science Research Resources Bank (Osaka, Japan).

For the experiment of insulin deficiency, streptozotocin (STZ, Sigma) or phosphate-buffered saline was administered via intraperitoneal injection (100 mg/kg in 0.05 × citrate buffer (pH 4.5)) into 9-week-old male ICR (MCH) mice. On day 3 after STZ treatment, both groups of mice were anesthetized with 5 mg/ml pentobarbital sodium prior to sacrifice and analysis.

For the experiment on fasting and refeeding, 9-week-old male C57BL/6 mice (each group, n = 3) were used. The fasted group was deprived of food for 18 h before sacrifice (fasted group). The refeed group was allowed free access to standard laboratory chow for 12 h (refed group) after 18 h of fasting. All mice were phlebotomized quickly from the vena cava.

Plasma glycerol and insulin were measured by a fluorometric/colorimetric enzyme method (27) and a double-antibody sandwich enzyme immunoassay using a Glazyme Insulin EIA Kit (Sanyo, Chemical Industries, Ltd., Japan).

**Functional Analysis of Mouse AQAPap cDNA**—Mouse AQAPap cDNA was inserted into the HindIII and SmalI sites of SP64 poly(A) vector (Promega), designated as pSP64-AQAPap. In vitro transcription of the entire encoding of AQAPap and injection of the resulting cRNA into Xenopus oocytes were performed as described previously (3). Oocytes were injected with 10 ng of AQAPap 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 8 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 200 mOsm to 180 mOsm by the addition of 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 (MICD-M2, Imaging Research Inc., Ontario, Canada). Osmotic water permeability (Pf, cm/s) was calculated from the initial rates of swelling, ΔV/Vt, and the oocyte surface-to-volume ratio (Vt/Vs = 50 cm⁻¹), and partial molar volume of water (Vm = 18 cm³/mol) from the relation, Pf = (d(V/Vt)/dt)/(Vt/Vs)(osm₀ – osmₕ). 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 AQAPap Expressed in Xenopus Oocytes**—To determine the stability and size of the AQAPap proteins, eight oocytes were homogenized in 160 μl of homogenization buffer (20 mM Tris (pH 7.4), 0.1 mM HEPES, 0.1 mM NaH₂PO₄, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin and pepstatin) at 4°C on day 3 after injection. Subsequently, the lysates were centrifuged twice for 10 min at 125,000 × g to remove yolk proteins. On day 3 after injection, plasma membranes were isolated from 25 oocytes according to the method of Wall and Patel (25). 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 AQAPap/7-specific affinity-purified polyclonal antibodies (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 AQAPap 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 for 15 h. Washes were performed with 2× SSC and 0.1% SDS at 65 °C and in 0.1× SSC and 0.1% SDS at 65 °C for 10 min, and then exposed to Kodak X-Omat film for 24 h at –80 °C with an intensifying screen. In order to isolate genomic clones, a bacterial artificial chromosome (BAC) mouse II hybridization library was screened using the AQAPap 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 AQAPap cDNA as the probe. These subclones were then isolated and subjected to various restriction enzyme digestions to map the mouse AQAPap 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 (DYE 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 AQAPap 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
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acids and deletion mutant of pG3-L-AQPap luciferase plasmid were constructed using the QuickChange Site-directed Mutagenesis kit (Stratagene). The integrals of all plasmids were verified by DNA sequencing.

Cell Culture, Transfection, and Luciferase Assays—3T3-L1 preadipocytes 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 tissue culture dish in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were grown to confluence and differentiated by incubation in DMEM with 10% FCS containing 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, 5 μg/ml insulin for 48 h. 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 μg of luciferase reporter plasmid (Promega) and [32P]UTP were radiolabeled using the Multiprime DNA Labeling System (Amersham Pharmacia Biotech). After fixation by ultraviolet cross-linking, 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-PCR) was conducted according to the manufacturer's protocol (Marathon® DNA 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'-CACATCTC-CACTGCAAGATGTTGCTCTGATC-3' and nested primer (corresponding to exon 3), 5'-ATTGTGATATGTCTCGACGGACGG-3'. Primer extension was performed according to the method of Ausubel et al. (31) using total RNA and an end-ribolabeled antisense oligo nucleotide probe (5'-TCTCTGAGGTTGGCTCTGTTCTTCCAGGCTTC-3') and an exon 1 oligonucleotide probe (5'-CCCTCTGATCCATCTCTTCTTTACTGCTCCA-3'). Next, 20 μg of total RNA were mixed with 5 × 10^6 copies of primer in a 30-μl volume of hybridization buffer (40 μM MIF, 1 mM EDTA, 0.4 mM NaCl, 50% formamide) and heated to 85 °C for 10 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C. After extension with M-MLV reverse transcriptase (Promega) containing RNase inhibitor and actinomycin D at 37 °C for 30 min and hybridized overnight at 45 °C.

For the RNase 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 an 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% urea. The gel was fixed, dried, and exposed to film.

RESULTS

Cloning of the Mouse AQPap1/7 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 pZERO-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 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 (Research Genetics) according to the manufacturer's instructions, using specific primers (5'-GTGAAAA TTACCTGCTCCGACTG-3' and 5'-GATTGTATGG- TCTCCGACGACAGCT-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 W/MIT 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.
coding region as that of the mouse AQP7 gene obtained from mouse testis (GenBank™ accession number AB010100) but had a longer 5′-untranslated region. A novel mouse AQP7 (Fig. 2A) was detected by 5′-RACE-PCR amplification of white adipose tissue RNA using a primer against the region in exon 3. The transcription start sites of the AQPap gene was determined by 5′-RACE-PCR amplification of white adipose tissue cDNA (around 2.4 kb) of mouse AQPap, estimated from Northern blotting of mouse adipose tissue RNA, 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. In white adipose tissue and 3T3-L1 adipocytes, only the AQPap transcript (350-bp protected fragment) was detected, whereas shorter AQP7 transcript (216-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 was 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.

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 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 3T3-L1 cells. Probe 1′ (for exon 1′, 2, and 3) detected a major transcript with a size of 213 bp, with a trace
amount of 263-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 main 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 glycerol release from adipose tissues (9). Plasma insulin decreased after 12 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 glycerol levels were also elevated in the fasted mice and decreased in the refeed 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 glycerol 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 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/ATT/TT/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-

**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, MapToper version 2.2 (transfac.gbf.de/cgi-bin/matteSearch/matteSearch.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/HinFI 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 (***) 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 activities. 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.
Effect of insulin on AQPap mRNA expression in fasting/refeeding, streptozotocin treatment, and 3T3-L1 adipocyte. A, AQPap mRNA expression during fasting and refeeding in white adipose tissues of C57BL/6J mice. Mice were sacrificed after 18 h of fasting or 12 h of refeeding after 18 h of fasting, and then white adipose tissues were removed for analysis of AQPap mRNA. Northern blotting was performed as described in the legends to Fig. 2D. A representative autoradiograph showing the 2.4-kb AQPap mRNA band and a photograph of the same gel after ethidium bromide staining (showing 2S ribosomal RNA, below) are shown. Plasma glycerol and insulin were measured by a fluorometric/colorimetric enzyme method and a double antibody sandwich enzyme immunoassay as described under “Experimental Procedures.” Data are represented by mean ± S.E. B, AQPap mRNA expression in white adipose tissue of insulin deficiency. Insulin-deicient mice were generated by STZ treatment as described under “Experimental Procedures.” Data are represented by mean ± S.E. C, AQPap mRNA expression in white adipose tissue of insulin deficiency. Insulin-deicient mice were generated by STZ treatment as described under “Experimental Procedures.” Data are represented by mean ± S.E. D, schematic presentation of the plasmid constructs used to identify the insulin response sequence in the promoters of the AQPap gene. Deletion sequences of the constructs are shown. 3T3-L1 adipocytes were transfected with the indicated constructs and assayed for luciferase activities as described under “Experimental Procedures.” Luciferase activities of these constructs in the presence of 50 nM insulin are shown as percentages of the control (mean ± S.E. for 4–6 assays). An asterisk denotes a significant difference (p < 0.01, Student’s t test) between the control group and the insulin-treated group. The value for non-insulin-treated pGL3-AQPap luciferase activity was arbitrarily set as 1.0. C, dose curve of insulin-mediated inhibition of AQPap promoter activity in incubated transfected 3T3-L1 adipocytes. 3T3-L1 adipocytes were co-transfected with pRL-SV40 plasmids and either pAQPap (wild)-luciferase (open circle) or pAQPap (ΔIRE1)-luciferase (open circle) for 18 h and incubated for 12 h with the indicated concentration of insulin before harvesting. The cells were harvested for measurement of luciferase activities. The value for pAQPap (wild)-luciferase activity in the absence of insulin was arbitrarily set as 1.0. Each value represents the average of triplicate incubations. This experiment was performed three times with similar results. D, time course of insulin-mediated inhibition of AQPap promoter activity in transfected 3T3-L1 adipocytes. 3T3-L1 adipocytes were co-transfected with pRL-SV40 plasmids and either pAQPap (wild)-luciferase (closed circle) or pAQPap (ΔIRE1)-luciferase (closed circle) for 18 h (zero time for the experiment) and incubated with 10 nM insulin for the indicated time before harvesting. The value for pAQPap (wild)-luciferase activity in the absence of insulin was arbitrarily set as 1.0. Each value represents the average of triplicate incubations. This experiment was performed three times with similar results.
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Fig. 6. Single point mutation analysis of the IRE1 sequence in mouse AQPAp promoter. 3T3-L1 adipocytes were transfected with either wild-type or mutant plasmids and then incubated with or without insulin for 12 h. The calculated value for percent inhibition by insulin for each plasmid is also shown (Fig. 6, lower panel). As expected, the activity of the wild-type AQPAp promoter was reduced by 46% in the presence of insulin. All the mutations in the core element had lower promoter activities in the absence of insulin. Mutations in base pairs 2–5 of the heptanucleotide sequence blocked the insulin-sensitive suppression of transcription. As a result, the promoter activity was nearly equal in the absence and presence of insulin. Mutations in base pairs 6–7 markedly reduced the basal promoter activity without insulin, but insulin sensitivity was fairly well retained. Therefore, the AQPAp promoter required base pairs 2–5 of the heptanucleotide to achieve a response to insulin.

Single Point Mutation Analysis in the IRE1 Sequence of Mouse AQPAp Promoter—To determine further the roles of individual nucleotides in the heptanucleotide consensus sequence, we prepared a series of AQPAp-luciferase plasmids with single transversion mutations in or around the core element. The plasmids were introduced into 3T3-L1 adipocytes by transfection, and luciferase activity was measured after incubation for 12 h, in the absence or presence of 50 nM insulin (Fig. 6, upper panel). The calculated value for percent inhibition by insulin for each plasmid is also shown (Fig. 6, lower panel). As expected, the activity of the wild-type AQPAp promoter was reduced by 46% in the presence of insulin. All the mutations in the core element had lower promoter activities in the absence of insulin. Mutations in base pairs 2–5 of the heptanucleotide sequence blocked the insulin-sensitive suppression of transcription. As a result, the promoter activity was nearly equal in the absence and presence of insulin. Mutations in base pairs 6–7 markedly reduced the basal promoter activity without insulin, but insulin sensitivity was fairly well retained. Therefore, the AQPAp promoter required base pairs 2–5 of the heptanucleotide to achieve a response to insulin.

Fig. 7. Northern blot analysis and promoter activity showing the effect of PI3K or mitogen-activated protein kinase inhibitor on insulin-mediated suppression of mouse AQPAp gene. A. effect of LY294002 and PD98059 on insulin-mediated suppression of AQPAp mRNA. 3T3-L1 adipocytes on day 9 after differentiation-induction were preincubated with DMEM containing 0.5% BSA for 12 h, after which the indicated inhibitors were added: control, no inhibitor (C), 50 mM LY294002 (LY), and 50 mM PD98059 (PD). After 60 min of pretreatment, the cells were incubated with DMEM containing 0.5% BSA without or with 50 nM insulin for 6 h. Northern blotting was performed as described in the legend to Fig. 2D, using a mouse AQPAp cDNA probe. The autoradiographic signals were normalized for ribosomal RNA content (based on the combined area under the 28 S curves). Abundance of mRNAs was determined by densitometric analysis and represented by arbitrary units. The effect of insulin treatment on relative AQPAp mRNA abundance (% inhibition, mean ± S.E. (n = 3)) is shown for each treatment group in the lower panel. Activity in the absence of insulin in control is taken as 100%. * p < 0.01, with insulin versus without insulin. Student’s t test. B, effect of LY294002 and PD98059 on insulin-mediated suppression of AQPAp promoter activities. 3T3-L1 adipocytes were cotransfected with pGL3-AQPap (WILD) (1.0 µg) and pRL-SV40 (10 ng). After 24 h, the medium was replaced with fresh serum-free medium containing 0.5% bovine serum albumin alone (C), supplemented with LY294002 (50 µM; LY), or PD98059 (50 µM; PD). Sixty min later, the dishes were incubated with or without insulin (50 nM) for 12 h before harvesting for the luciferase assay. Relative luciferase activity and % inhibition are plotted for the control, LY294002, and PD98059-treated samples. Activity in the absence of insulin in the control is taken as 100%. Each value represents the average of triplicate incubations. This experiment was performed three times with similar results.
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 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 (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 IGFBP-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 (data 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 (data not shown). It remains to be elucidated whether or not AQPap IRE involves FKHr-mediated regulation. In H4IIE rat hepatoma cells, it was shown that insulin binding to its receptor activates phosphorylation of Akt/protein kinase B through the PI3K pathway, FKHR-mediated regulation. In H4IIE 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 blockade 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 (TATTTTG) at −152/−146 and atypical IRE (TGTTTTC) at −629/−625 in human AQPap promoter (data 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 AQP family that show glycerol permeability (2). Both glycerol and free 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 (TGTTTTTTC) 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 down-regulated in response to a high concentration of insulin in the plasma, resulting in deterioration of insulin signaling and induction of gluconeogenic genes such as PEPCk and Glc-6-Pase, which lead to hyperglycemia (43). The combination of reduced IRS-2 and increased gluconeogenic 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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