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Insulin Induces the Expression of the *SHARP-2/Stra13/DEC1* Gene via a Phosphoinositide 3-Kinase Pathway*

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Transcription of the rat fatty acid synthase (*FAS*) gene in the rat liver can be regulated by feeding a high carbohydrate diet. A carbohydrate response element (ChoRE) located on the rat *FAS* gene promoter has been identified. Using multiple copies of the ChoRE as the bait in a yeast one-hybrid system, a rat liver cDNA library was screened, and the cDNA of ChoRE-binding proteins was cloned. A positive clone that encodes a basic helix-loop-helix protein, enhancer of split- and hairy-related protein-2 (SHARP-2), was obtained. Northern blot analysis revealed that the levels of SHARP-2 mRNA increase when a high carbohydrate diet is fed to normal rats or when insulin is administered to diabetic rats. In primary cultured rat hepatocytes, insulin rapidly induced an accumulation of SHARP-2 mRNA even in the absence of glucose. A time course for the increase in SHARP-2 mRNA levels indicated that it followed by those of FAS and L-type pyruvate kinase mRNAs and that the initial time course of SHARP-2 mRNA was similar to changes in the levels of glucokinase mRNA and phosphoenolpyruvate carboxykinase mRNA. Although wortmannin, LY294002, and actinomycin D blocked the increase in SHARP-2 mRNA levels by insulin, rapamycin, staurosporine, PD98059, okadaic acid, and 8-bromocyclic AMP had no effect. In addition, nuclear run-on assay revealed that transcription of the rat *SHARP-2* gene was induced by insulin. Thus, we conclude that insulin induces the transcription of the rat *SHARP-2* gene via a phosphoinositide 3-kinase pathway.

The feeding of a high carbohydrate diet including dextrin, sucrose, or glucose leads to an elevation in blood glucose levels and the secretion of insulin from pancreatic β-cells. Insulin binds to an insulin receptor on the plasma membrane of target organs including liver, adipose tissue, and muscle, thus activating downstream signaling pathways (1, 2). This results in an increase in the influx of blood glucose into adipose tissue and muscle cells. It has recently been reported that glucose or its metabolites, such as glucose-6-phosphate and glucosamine, also regulate gene transcription in the liver, pancreatic β-cells, and muscle, respectively (3). Therefore, two pathways are activated as the result of feeding a high carbohydrate diet that affect gene expression; one is an insulin-direct pathway and the other a glucose-dependent pathway. Both the L-type pyruvate kinase (LPK)[1] and Spot 14 genes are well known models for glucose-dependent gene regulation in the liver (4, 5). Similar nucleotide sequences in the regulatory region of these two gene promoters have been identified and are designated as the carbohydrate response element (ChoRE) (6, 7). This consists of two copies of an imperfect palindromic E box sequence (5′-CACGGG-3′) separated by 5 bp and is able to confer glucose-dependent transcriptional activation to the heterologous promoters (4). Uyeda and coworkers (8) purified a ChoRE-binding protein (ChREBP) and identified it as the Williams-Beuren syndrome critical region 14 protein. We also purified a protein that binds to the ChoRE of the rat *LPK* gene promoter (9). However, the issue of whether our purified protein is identical to ChREBP is unclear because the amino acid sequence of the protein has not yet been determined. The mapping of ChoRE on the rat fatty acid synthase (*FAS*) gene distal promoter has recently been reported, and its nucleotide sequence resembles the ChoRE of both the *LPK* and *Spot 14* genes (10).

Generally, the E box sequence (5′-CANNTG-3′) is found in the transcriptional regulatory region of a number of genes and controls the transcription of these genes in biological events, including development, cell differentiation, cell growth, and oncogenesis (11). The basic helix-loop-helix (bHLH) transcriptional factors, with or without a leucine zipper motif, bind to the E box and regulate transcription positively or negatively (11). The rat enhancer of split- and hairy-related protein-2 (SHARP-2) belongs to the bHLH transcription factor family (12). Mouse and human orthologs have also been cloned and are referred to as the stimulation of retinoic acid 13 (Stra13) and differentiated embryo chondrocytes 1 (DEC1), respectively (13, 14). These proteins are expressed ubiquitously and bind to the E box sequence. It has been reported that both Stra13 and DEC1 act as transcriptional repressors via interactions with the histone deacetylase HDAC1 or with the basal transcription actor TFIIB (15, 16).

In this study, we report on the isolation of SHARP-2 cDNA as a ChoRE-binding protein of the rat *FAS* gene using a yeast

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[1] The abbreviations used are: LPK, L-type pyruvate kinase; ChoRE, carbohydrate response element; ChREBP, ChoRE-binding protein; FAS, fatty acid synthase; bHLH, basic helix-loop-helix; SHARP-2, enhancer of split- and hairy-related protein-2; Stra13, stimulation with retinoic acid 13; DEC1, differentiated embryo chondrocytes 1; PI3K, phosphoinositide 3-kinase; STZ, streptozotocin; dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; FKHR, forkhead in rhabdomyosarcoma; SREBP, sterol regulatory element-binding protein.

one-hybrid system. Hepatic expression of SHARP-2 mRNA both *in vivo* and *in vitro* under various nutritional and hormonal conditions was analyzed. The findings indicate that SHARP-2 is an insulin-inducible transcription factor and that the transcriptional stimulation of the rat *SHARP-2* gene by insulin is mediated by a phosphoinositide 3-kinase (PI3K) pathway.

EXPERIMENTAL PROCEDURES

Materials—The yeast one-hybrid system, pACT2, and ExpressHyb hybridization solution were purchased from Clontech (Palo Alto, CA). Streptozotocin (STZ) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Humalin N, human insulin, was purchased from Eli Lilly Japan K. K. (Kobe, Japan). Collagenase was purchased from Yakult (Tokyo, Japan). Type I collagen-coated dishes were purchased from Asahi Techno Glass (Chiba, Japan). Williams' medium E, dexamethasone (dex), Dulbecco's modified Eagle's medium (DMEM), insulin, wortmannin, LY294002, rapamycin, staurosporine, PD98059, okadaic acid, 8-bromo-cAMP, and actinomycin D were purchased from Sigma. The TRIZOL reagent and Superscript II were purchased from Invitrogen. The Biodyne membrane was obtained from PALL (ICN Biomedicals, Inc., Glen Cove, NY). [α-^{32}P]dCTP (110 TBq/mmol) and [α-^{32}P]UTP (110 TBq/mmol) were purchased from Amersham Biosciences. The BcaBest DNA labeling kit and pUC119 were purchased from Takara BIOMEDICALS (Kyoto, Japan). The pGEM-T Easy plasmid was purchased from Promega (Madison, WI). The Big Dye terminator cycle sequencing kit FS was purchased from Applied Biosystems Japan (Tokyo, Japan). The pBluescript II SK(+) was purchased from Stratagene (La Jolla, CA).

Library Screening—The pRW95–1 was a generous gift from Dr. M. Schweizer (17). The pLacZiB1 plasmid was described previously (18). Oligonucleotides 5′-CTAGCTTCCTGCATGTGCCACAGGC GTGTCACCCTC-3′ and 5′-CTAGGAGGGTGACACGCCTGTGGCACATGCAGGAAG-3′ were annealed, phosphorylated, and ligated into the *Spe*I site of the pRW95–1 or *Xba*I site of the pLacZiB1 plasmids to obtain 6 × FASChoRE-HIS3 and 6 × FASChoRE-LacZ, respectively.

YM4271 yeast cells were sequentially transformed with both reporter plasmids. The construction of the rat liver cDNA library was described previously (18). The reporter yeast strain, yFAS, was transformed with a rat liver cDNA library using a high efficiency transformation method (19). When 2.1×10^6 clones were screened on histidine-, tryptophan-, leucine-, and uracil-free synthetic dextrose plates supplemented with 1 mM 3-aminotriazole, one reproducible *HIS3*- and *LacZ*-positive clone was obtained. A plasmid, pF3, was isolated from the yeast and its nucleotide sequence determined.

One-hybrid System and β-Galactosidase Assays—Reporter plasmids, pRW95–1-LHR, pLacZiB1-LHR, 3 × E box-HIS3, and 3 × E box-LacZ were previously described (18, 20). Oligonucleotides 5′-CTAGCGCCACGGGGCACTCCCGTGG-3′ and 5′-CTAGCCACGGGAGTGCCCCGTGGCG-3′ were annealed, phosphorylated, and ligated into the *Spe*I site of the pRW95–1 or *Xba*I site of the pLacZiB1 plasmids to obtain 6 × LPKChoRE-HIS3 and 6 × LPKChoRE-LacZ, respectively. YM4271 cells were transformed with these plasmids. yLHR harboring both the pRW95–1-LHR and pLacZiB1-LHR, yEbox harboring both the 3 × E box-HIS3 and 3 × E box-LacZ, yLPK harboring both the 6 × LPKChoRE-HIS3 and 6 × LPKChoRE-LacZ, and yFAS were employed as reporter yeast strains. These yeast strains were transformed with the pF3 or pACT2, which expresses the activation domain of GAL4 (GAL4 AD) transcription factor alone, and their β-galactosidase activities were determined. Quantitative β-galactosidase assays, using *o*-nitrophenyl-β-D-galactoside, were carried out on permeabilized cells as described previously (21).

Animals and Treatment—Male Sprague-Dawley rats (6-weeks-old, 160–180 g body weight) were used. Rats were housed in a photoperiod of 12 h of light and 12 h of darkness (from 20:00 through 8:00). To determine effects of a high carbohydrate diet, the rats were starved for 48 h and then refed a high carbohydrate diet containing 81% dextrin and 10% casein for 3, 6, and 16 h. For an analysis of circadian rhythm, rats given laboratory chow at *ad libitum* or starved rats were sacrificed starting from 6:00 at 6-h intervals. For the insulin study, STZ (60 mg/kg) was intraperitoneally administered to rats, and the onset of diabetes mellitus was then confirmed by determining the level of blood glucose. Four days after the administration of STZ, insulin was intraperitoneally injected with Humalin (40 units/kg), and the rats were sacrificed at 30 min, 1 h, and 2 h. At all times, treatment of the animals followed National Institutes of Health guidelines.

Primary Culture of Hepatocytes—Hepatocytes were isolated from Sprague-Dawley male rats using a collagenase perfusion method (22, 23). Cells (10^5 cells/cm^2) were plated on type I collagen-coated dishes and cultured in Williams' medium E supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.5 μM dex at a 37 °C, 5% CO_2 incubator. After 4 h, the medium was replaced with serum- and glucose-free DMEM supplemented with 2 mM sodium pyruvate, antibiotics, and 0.5 μM dex. After 24 h, the medium was changed to the indicated one in the presence or absence of insulin. For signaling experiments, 0.2 μM wortmannin, 0.1 mM LY294002, 0.1 μM rapamycin, 0.1 μM staurosporine, 25 μM PD98059, 10 nM okadaic acid, 1 mM 8-bromo-cAMP, or 0.8 μM actinomycin D was added to the medium, respectively, then incubated for an additional 3 h. All reagents were added to the medium at 5 min before the addition of insulin.

Preparation and Analysis of RNA—Total RNA was prepared from rat livers or primary cultured hepatocytes using the TRIZOL reagent. Total RNA (10 μg/lane) was subjected to a 0.8% denaturing agarose gel electrophoresis and then transferred to a Biodyne membrane. The filter was prehybridized in ExpressHyb hybridization solution at 68 °C for 30 min and then hybridized with a ^{32}P-labeled probe and 20 μg/ml heat-denatured herring testis DNA for 1 h. After washing at 50 °C in 0.1 × 0.15 M NaCl/0.015 M sodium acetate (SSC), 0.1% SDS, the filter was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected and quantified with the FUJIX BAS-2000 image analyzing system.

The pUC119-FAS was a generous gift from Dr. Nobuko Iritani (Tezukayama Gakuin University, Sakai, Japan). A 1.1-kb *Eco*RI/*Mlu*I fragment of the pF3, a 1.3-kb *Eco*RI fragment of the pGK5, a 1.9-kb *Pst*I/*Dra*I fragment of the pLPK57, a 1.8-kb *Eco*RI fragment of the pUC119-FAS, and a 1.2-kb *Pst*I fragment of the pUC18-PEPCK were used as the probes for SHARP-2, glucokinase (GK), LPK, FAS, and phosphoenolpyruvate carboxykinase (PEPCK), respectively (23–25). For cDNA cloning mouse 36B4, a ribosomal protein, reverse transcription-polymerase chain reaction was carried out using oligonucleotides, 5′-ATGCCCAGGGAAGACAGGGCGACC-3′ and 5′-TTAGTCGAAGAGACCGAATCCCATA-3′ as primers and mouse liver cDNA as a template (26). The product was subcloned into the pGEM-T Easy to give pGEM-T Easy 36B4. A 954-bp *Eco*RI fragment was used as a probe. Probe DNAs were labeled with [α-^{32}P]dCTP using the BcaBest DNA labeling kit.

Nuclear Run-on Assays—Nuclei were isolated from diabetic rat livers with or without the administration of insulin for 2 h. Run-on assays were performed as described previously in the presence of [α-^{32}P]UTP (24). An *Eco*RI/*Mlu*I fragment of the pF3 was subcloned into the pBluescript II SK(+) to give pBSII-SHARP-2-EM. The plasmids used for hybridization were pBluescript II SK(+), pBSII-SHARP-2-EM, pUC119, and pUC-PEPCK. After linearization, ten μg of these plasmids was bound to nylon membranes, prehybridized at 50 °C for 2 h, and hybridized at 50 °C for 24 h without or with the same amount of labeled RNA (5×10^6 cpm) in 50% formamide, 5 × Denhardt's solution, 5 × SSC, 250 μg/ml herring testis DNA, 0.5% SDS, and 10 ng/ml poly(A). The filters were washed under high stringent conditions (27). Hybridization signals were detected and quantified with the FUJIX BAS-2000 image analyzing system.

RESULTS

Molecular Cloning of ChoRE-binding Protein cDNA—We attempted to clone the cDNA for a protein(s) binding to the ChoRE of the rat *FAS* gene using the yeast one-hybrid system. Six copies of the ChoRE sequence were inserted upstream of the *gal1*-minimal promoter of the *HIS3*- or *LacZ*-based reporter plasmids. Using a reporter yeast, yFAS, that harbors both reporter plasmids, 2.1×10^6 clones of a rat liver cDNA library were screened. We obtained a *HIS3* and *LacZ* double positive clone and isolated a plasmid named pF3. We first determined the binding specificity between the ChoRE and a protein expressed from the pF3 using yeast one-hybrid assays. Two yeast strains, yFAS and yLHR (which harbors six copies of the early growth response gene-1-binding site of the rat luteinizing hormone receptor gene promoter), were used as the reporter. The pACT2, which expresses the activation domain of GAL4 alone, and pF3 were employed as the prey plasmids. When these reporter yeasts were transformed with the pACT2, they showed low β-galactosidase activities (Fig. 1). When yFAS was transformed with the pF3, high β-galactosidase activity was observed. In contrast, when the yLHR reporter yeast

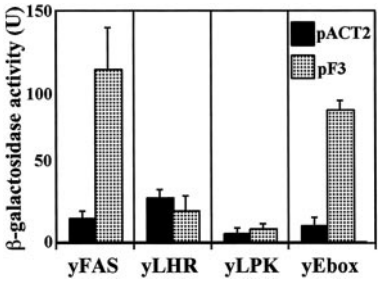

FIG. 1. **Specific binding of a protein expressed from the pF3 to the cognate reporter sequence.** Four reporter yeast strains shown at the *bottom* were transformed with the pACT2 or pF3, respectively. β-galactosidase activities of these yeasts were then determined. The yFAS, yLHR, yLPK, and yEbox containing both *HIS3* and *LacZ* reporter plasmids inserted six copies of the ChoRE of the rat *FAS* gene, the early growth response gene-1-binding site of the rat luteinizing hormone receptor gene, the ChoRE of the rat LPK gene, and three copies of the E box sequence, respectively.

was transformed with the pF3, the yeasts continued to show a low β-galactosidase activity (Fig. 1). These results indicate that a protein that is expressed from the pF3 specifically binds to the ChoRE of the rat *FAS* gene.

We then determined the nucleotide sequence of the pF3 and searched for similar sequences in the GenBank™ data base. The results showed that it was similar to the nucleotide sequence of rat SHARP-2, mouse Stra13, and human DEC1 cDNAs (12–14). The nucleotide sequence of the pF3 contained from 240 base upstream of the initiation codon to 964 base downstream that of rat SHARP-2. Two bases, "ga," in the coding region were replaced with "ag." The differences cause the replacement of the lysine residue at 133 with an arginine residue. The rat SHARP-2 protein consists of 411 amino acid residues and belongs to the bHLH protein family (12). The region where we cloned the amino acid sequence between residues 1 and 322 contains the bHLH domain. It has been reported that DEC1 binds to the E box sequence (16). We then examined the issue of whether SHARP-2 encoded by the pF3 binds to another ChoRE or the E box sequence. When two reporter yeast strains, yLPK and yEbox, which harbor six copies of the ChoRE of the rat *LPK* gene promoter or three copies of the E box sequence, respectively, were transformed with the pACT2, they showed low β-galactosidase activities (Fig. 1). When the yLPK reporter yeast was transformed with the pF3, the yeasts continued to show a low β-galactosidase activity (Fig. 1). In contrast, when the yEbox was transformed with the pF3, high β-galactosidase activity was observed. These results indicate that SHARP-2 also binds to the E box sequence but not the ChoRE of the *LPK* gene promoter.

Regulation of Gene Expression of SHARP-2 in the Rat Liver—We then examined the expression and regulation of rat SHARP-2 mRNA in the rat liver under various nutritional and hormonal conditions. During starvation for 48 h, both glycolysis and lipogenesis are repressed and gluconeogenesis is induced in the rat liver. In contrast, the administration of a high carbohydrate diet to starved rats induces both glycolysis and lipogenesis and represses gluconeogenesis. As shown in Fig. 2*A*, Northern blot analysis revealed that the level of SHARP-2 mRNA increased in the livers of refed rats. An increase in the level of SHARP-2 mRNA is rapid and was observed at least 3 h after the administration of a high carbohydrate diet. At 3, 6, and 16 h after refeeding a high carbohydrate diet, the levels of SHARP-2 mRNA increased to 3.5-, 4-, and 8-fold, respectively. The time course for the increase was followed by an increase in FAS and LPK mRNA levels, and the initial time course of SHARP-2 mRNA was similar to changes in the levels of GK mRNA and PEPCK mRNA. In contrast, the levels of 36B4 mRNA were unchanged in these rat livers.

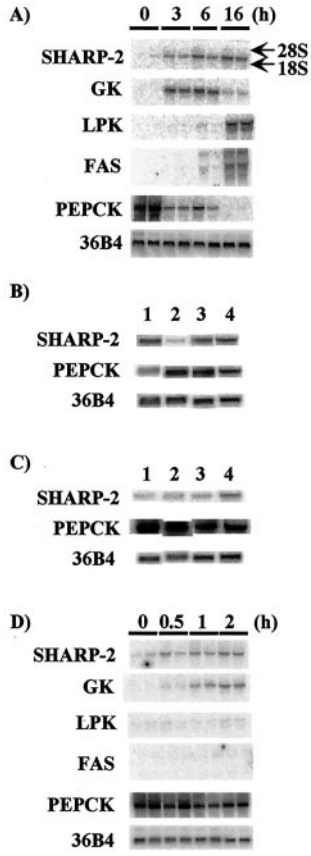

FIG. 2. **Northern blot analysis of expression of rat SHARP-2 mRNA in the rat liver.** Ten μg of total RNA from rat livers were subjected to a 0.8% denaturing agarose gel electrophoresis and then transferred to a Biodyne membrane. The membranes were hybridized with ^{32}P-labeled probes (*left*). Each experiment was carried out in duplicate. *A*, rats were starved for 48 h, then refed a high carbohydrate diet. Rats were sacrificed at the indicated times (*top*). Positions of 28S and 18S rRNA are shown on the *right*. *B*, rats given laboratory chow at *ad libitum* and (*C*) rats starved for 24 h were sacrificed at 6:00 (*lane 1*), 12:00 (*lane 2*), 18:00 (*lane 3*), and 24:00 (*lane 4*), respectively. Total RNAs were isolated from the pooled livers of two rats in each condition (*B* and *C*). *D*, diabetic rats were sacrificed at the indicated times (*top*) after insulin treatment. *SHARP-2*, enhancer of split and hairy-related protein-2; *GK*, glucokinase; *LPK*, L-type pyruvate kinase; *FAS*, fatty acid synthase; *PEPCK*, phosphoenolpyruvate carboxykinase; *36B4*, ribosomal protein 36B4.

We next examined the issue of whether expression of SHARP-2 mRNA is regulated in a circadian fashion. Rats fed freely with laboratory chow or starved rats were sacrificed at 6:00, 12:00, 18:00, and 24:00, and the levels of hepatic SHARP-2 mRNA were determined. As shown in Fig. 2*B*, the level of SHARP-2 mRNA was low at 12:00 and gradually increased, reaching a maximum level at 6:00. In contrast, the level of PEPCK mRNA was low at 6:00, reached a maximum level at 12:00, and then gradually decreased. In the case of the starved rat livers, the levels of SHARP-2 mRNA were lower than those of the freely fed rat livers (Fig. 2*C*). Only the level was increased at 24:00. In contrast, the levels of PEPCK mRNA were high. The levels were gradually and slightly decreased at 24:00. In these rat livers, the levels of 36B4 mRNA were unchanged. These results suggest that the expression of the *SHARP-2* gene is mainly regulated by diet, although diet-independent regulation was observed.

We then determined whether insulin regulates the level of SHARP-2 mRNA. Diabetes mellitus was induced in rats by administrating STZ. In the diabetic rat liver, gene expressions of glycolytic and lipogenic enzymes are suppressed and that of

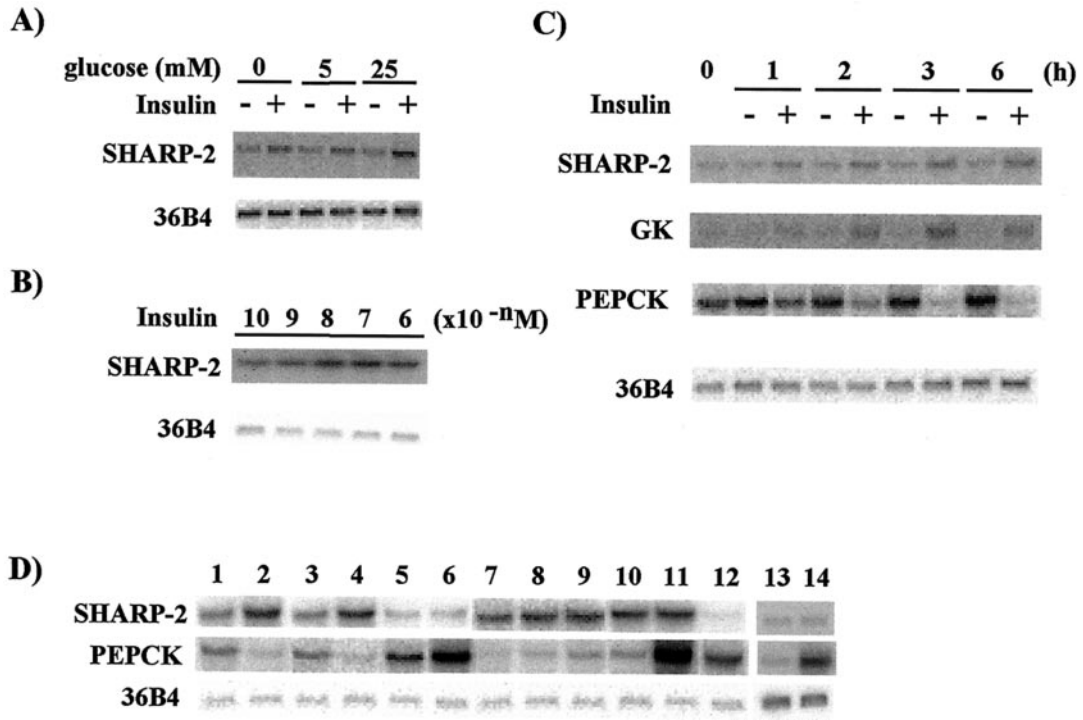

FIG. 3. **Northern blot analysis of expression of rat SHARP-2 mRNA in the primary cultured rat hepatocytes.** The procedures and abbreviations are the same as those in the legend of Fig. 2. Each experiment was carried out at least twice. Hepatocytes were plated at a density of 10^5 cells/cm^2 in a type I collagen-coated dish and after 4 h medium was replaced with serum- and glucose-free DMEM supplemented with 2 mM sodium pyruvate, antibiotics, and 0.5 μM dex for 24 h. *A*, medium was changed to one containing various concentrations of glucose shown at the *top* in the absence (−) or presence (+) of 10 nM insulin and cultured for another 3 h. *B*, dose-dependent effects of insulin on the accumulation of SHARP-2 mRNA. Hepatocytes were cultured for 3 h in serum- and glucose-free DMEM supplemented with 2 mM sodium pyruvate in the presence of the indicated concentration of insulin. *C*, time course of alterations in the level of SHARP-2 mRNA by insulin. Hepatocytes were cultured in serum- and glucose-free DMEM supplemented with 2 mM sodium pyruvate and 0.5 μM dex in the absence (−) or presence (+) of 10 nM insulin for indicated times (*top*). *D*, primary cultured rat hepatocytes were treated for 3 h with various inhibitors of proteins mediating insulin-signaling pathway, cAMP, and actinomycin D. *Lane 1*, control; *lane 2*, 10 nM insulin; *lane 3*, Me_2SO; *lanes 4* to *12*, Me_2SO plus 10 nM insulin; *lane 5*, 0.2 μM wortmannin; *lane 6*, 0.1 mM LY294002; *lane 7*, 0.1 μM rapamycin; *lane 8*, 0.1 μM staurosporin; *lane 9*, 25 μM PD98059; *lane 10*, 10 nM okadaic acid; *lane 11*, 1 mM 8-bromo-cAMP; *lane 12*, 0.8 μM actinomycin D; *lane 13*, control; *lane 14*, 1 mM 8-bromo-cAMP. Probes are shown on *left*.

gluconeogenic enzyme is active. The administration of insulin to the diabetic rats causes alterations of these gene expressions. As shown in Fig. 2*D*, the level of SHARP-2 mRNA was low in the diabetic rat liver and increased in insulin-administered diabetic rat liver. At 0.5, 1, and 2 h after administrating insulin, the level of SHARP-2 mRNA increased to 2-, 2-, and 3-fold, respectively. In these processes, the level of GK mRNA was induced and that of PEPCK mRNA was decreased. The levels of both LPK and FAS mRNAs were marginal at this time. In these rat livers, the levels of 36B4 mRNA were unchanged.

It is known that insulin directly regulates gene expression via the insulin receptor signaling pathway and that glucose or its metabolites also regulate gene expression (3). *In vivo* analysis does not distinguish whether an accumulation of SHARP-2 mRNA is dependent upon either insulin or glucose or both. Because of this, we employed primary cultured rat hepatocytes to determine whether insulin or glucose is involved in the accumulation of SHARP-2 mRNA. As shown in Fig. 3*A*, in primary cultured rat hepatocytes the levels of SHARP-2 mRNA in the presence of 0, 5, and 25 mM glucose were increased by 2-, 2-, and 3-fold by insulin, respectively. Insulin led to an increase in the level of SHARP-2 mRNA even in the absence of glucose. This indicates that insulin directly induces the accumulation of SHARP-2 mRNA. In addition, this effect was dose-dependent and the optimum concentration of insulin was 10 nM (Fig. 3*B*). In contrast, the level of 36B4 mRNA remains unchanged under all conditions. We then examined the time course for the induction of SHARP-2 mRNA under optimum conditions. An increase in the level of SHARP-2 mRNA by insulin was observed from 1 to 6 h. An increase in GK mRNA and a decrease in PEPCK mRNA were observed in similar time course (Fig. 3*C*). The levels of 36B4 mRNA remained unchanged.

Insulin Stimulates Accumulation of SHARP-2 mRNA via a PI3K Pathway—Insulin binds to the insulin receptor on the plasma membrane, induces receptor tyrosine kinase activity, and phosphorylates insulin receptor substrates; the signal is then conferred downstream of the PI3K and ras/raf-1/mitogen-activated protein kinase pathways. We then examined the issue of which insulin signaling pathway is involved in an accumulation of SHARP-2 mRNA. Inhibitors of protein kinases or phosphatase involved in the insulin signal transduction pathways were added to the medium in primary cultured rat hepatocytes. Although both wortmannin and LY294002 blocked the accumulation of SHARP-2 mRNA, rapamycin, staurosporine, PD98059, and okadaic acid had no effects (Fig. 3*D*). Both wortmannin and LY294002 also blocked the down-regulation of PEPCK mRNA by insulin. In contrast, the level of 36B4 mRNA was unchanged by treatment with all reagents. These results indicate that a PI3K pathway mediates the accumulation of SHARP-2 mRNA by insulin.

We then examined the issue of whether cAMP represses an accumulation of SHARP-2 mRNA by insulin. By the addition of 8-bromo-cAMP in the absence or presence of insulin to primary cultured rat hepatocytes, the levels of PEPCK mRNA were increased but those of both SHARP-2 and 36B4 mRNAs were unchanged. In addition, actinomycin D, an inhibitor of RNA polymerase II, inhibited the accumulation of SHARP-2 mRNA. These results suggest that cAMP neither stimulates the accumulation of SHARP-2 mRNA nor interferes with the effects of

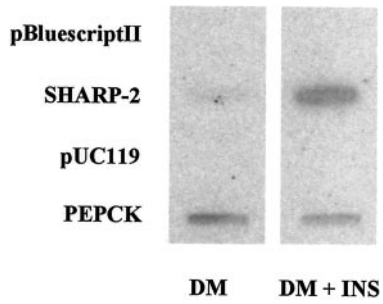

FIG. 4. **Insulin stimulates transcription of the rat *SHARP-2* gene.** Diabetic rats were treated without or with insulin. Control (*DM*) and insulin-treated rats (*DM+INS*) were sacrificed at zero time and 2 h after insulin treatment, respectively. Nuclei were isolated from the pooled livers of two rats in each group. Nuclear run-on assays were carried out as described under "Experimental Procedures." Three independent experiments were carried out. *SHARP-2*, enhancer of split- and hairy-related protein-2; *PEPCK*, phosphoenolpyruvate carboxykinase.

insulin and that insulin may act at the transcription level of the rat *SHARP-2* gene.

Insulin Stimulates Transcription of the Rat SHARP-2 Gene—We further examined the issue of whether the rapid accumulation of SHARP-2 mRNA as the result of treatment with insulin is regulated at the transcription level of the rat *SHARP-2* gene. We compared the transcription rate of the rat *SHARP-2* gene between control diabetic rat liver and insulin-treated diabetic rat livers for 2 h. By treatment with insulin, transcription rate of the rat *SHARP-2* gene was induced by 5.1-fold (±2.9) (Fig. 4). Under the same conditions, the transcription rate of the rat *PEPCK* gene was decreased to ~47% (±3.3%). This result indicates that insulin acts at the transcription level of the rat *SHARP-2* gene.

DISCUSSION

We cloned SHARP-2 cDNA as a ChoRE-binding protein of the rat *FAS* gene. The ChoRE of the *FAS* gene is nearly identical to that of the *LPK* gene, and a common factor is suggested to bind to the ChoRE of the two genes (10). However, the yeast one-hybrid assay showed that SHARP-2 interacts with the ChoRE of the *FAS* gene but not with that of the *LPK* gene. Theses results suggest that SHARP-2 is not involved in the common regulation of both genes. Further studies will be required to address this problem.

SHARP-2 is involved in the control of the proliferation and/or differentiation of chondrocytes, nerve cells, fibroblasts, and T cells (12–14, 28). It has been reported that the human form, DEC1, and another isoform, DEC2, are expressed in the suprachiasmatic nucleus in a circadian fashion and function as regulators of the mammalian molecular clock (29). Furthermore, the expression of SHARP-2 mRNA was induced by cAMP, hypoxia, serum starvation, and TGF-β (15, 16, 30–33).

This study focused on an analysis of the regulation of hepatic expression of the rat *SHARP-2* gene. The level of SHARP-2 mRNA increased by refeeding a high carbohydrate diet to normal rats or the administration of insulin to diabetic rats. The time course for the increase in SHARP-2 mRNA levels showed that it was followed by an increase in the levels of FAS and LPK mRNAs; the initial time course of SHARP-2 mRNA was similar to changes in the levels of GK mRNA and PEPCK mRNA (Fig. 2). Insulin alone induced the level of SHARP-2 mRNA via a PI3K pathway in primary cultured rat hepatocytes (Fig. 3). The downstream signal may be protein kinase B but not atypical protein kinase C and the mammalian target of rapamycin, because inhibitors of the latter two signaling molecules were not effective. In addition, the administration of insulin to diabetic rats stimulated the transcription rate of the rat *SHARP-2* gene in the liver (Fig. 4). Therefore, SHARP-2 is an insulin-inducible bHLH transcription factor.

It is known that both the forkhead in rhabdomyosarcoma (FKHR) and the sterol regulatory element-binding protein (SREBP)-1c mediate insulin action in the liver (34). In the case of FKHR, it mediates insulin action directly or indirectly (35, 36). Insulin causes the phosphorylation of the serine residue at 256 of FKHR via a PI3K/protein kinase B/Akt pathway. Phosphorylated FKHR dissociates from the cognate sequence, is translocated from the nucleus to the cytoplasm, and represses the transcription of the target gene promoter, such as the insulin-like growth factor-binding protein-1 gene (35). It has been also reported that FKHR functions as a corepressor of hepatocyte nuclear factor 4 (HNF4) and that phosphorylation of FKHR by insulin causes the dissociation of HNF4, which then activates transcription (36). In contrast, SREBP-1c, a bHLH transcription factor, is selectively expressed in liver and fat tissue (37). The overexpression of SREBP-1c both *in vivo* and *in vitro* causes the stimulation of glycolysis and lipogenesis via the activation of the *GK* gene (37, 38). However, the mechanism for this remains unknown (39, 40). It has been reported that insulin acts at the transcription level of the rat *SREBP-1c* gene and induces the level of SREBP-1 c mRNA in primary cultured rat hepatocytes. The time course for the induction was slower than that of SHARP-2 mRNA (41). Thus, this raises the possibility that the *SREBP-1c* gene as well as the *GK* and *PEPCK* genes are downstream genes of SHARP-2. Indeed, an E box sequence was found in accessory element 3 of glucocorticoid response unit of the rat *PEPCK* gene. Two imperfect E boxes separated by 5 bp also exist just downstream of the transcription initiation sites of the gene (42, 43). A functional E box sequence is also present in the regulatory region of both the rat *GK* and *SREBP-1c* genes, respectively (44, 45). Further studies will be required to address the issue of whether SHARP-2 directly represses transcription from the rat *PEPCK* gene promoter or activates transcription from the rat *GK* and *SREBP-1c* gene promoters. It has been reported that SHARP-2 physically interacts with the components, such as TBP and TFIIB, of the basal transcription machinery and recruits the histone deacetylase 1-Sin3A-NcoR corepressor complex through their carboxyl-terminal repression domains (15). SHARP-2 also interacts with the DNA-binding domain of upstream stimulatory factor (USF), a bHLH protein, and inhibits DNA-binding and USF-mediated transactivation (46). Therefore, SHARP-2 may regulate the transcription of these genes via mechanisms in which it not only binds to the E box sequence but also interferes with the activity of other bHLH proteins. Recently, *Stra13*-deficient mice were generated, and it has been shown that Stra13 is a key regulator of lymphocyte activation that is vital for maintenance of self-tolerance and the constraint of autoimmunity (28). Thus far, whether gene expressions of metabolic enzymes and insulin-related transcription factors are normal or not has not been reported.

The transcription rate of the rat *SHARP-2* gene was stimulated by insulin. Thus, it is likely that the promoter region of the rat *SHARP-2* gene contains the insulin response sequence. The molecular cloning of the rat *SHARP-2* gene and detailed analysis of the regulatory elements of the gene will be required to address these questions.

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