Transcriptional Regulation of p90 with Sequence Homology to
Escherichia coli Glycerol-3-phosphate Acyltransferase*

Dong-Hoon Shin, Joseph D. Paulauskis, Naima Moustaid, and Hei Sook Sul†

From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts 02115

We have previously isolated cDNA clones for several mRNAs that are dramatically increased in livers of fasted mice refed a high carbohydrate diet. We report here the sequence and regulation of one such mRNA; the 6.8-kilobase mRNA has an open reading frame of 2481 nucleotides, and the coded protein contains 827 amino acid residues (Mr of 90,000) with a 30% identity and an additional 42% similarity in an approximately 300-amino acid stretch to Escherichia coli glycerol-3-phosphate acyltransferase. The p90 mRNA is highly expressed in liver and in adipose tissue. When previously fasted mice were refed a high carbohydrate, fat-free diet, the liver mRNA level for p90 was increased about 20-fold at 8 h. Administration of dibutyryl cAMP at the time of refeeding prevented the increase in the p90 mRNA by 70%. In addition, there was no increase in the p90 mRNA level when previously starved streptozotocin-diabetic mice were refed. In diabetic animals, the p90 mRNA level increased by 2-fold 1 h after insulin injection and reached a maximum of 19-fold after 6 h. The increase in transcription rate of the p90 gene preceded that of steady state mRNA level caused by fasting/refeeding, and cAMP abolished the increase in transcription. Expression of the p90 gene was not detectable in either fasted or refed streptozotocin-diabetic mice, but increased 4-fold 30 min after insulin administration and further increased up to 8-fold at 2 h. On-going protein synthesis was necessary for this increase.

In the course of cloning specific genes which are under hormonal and nutritional control, we have isolated cDNA sequences to a novel protein p90 (1). The p90 mRNA level was elevated dramatically in the liver of previously fasted mice which were refed a high carbohydrate diet. The dietary manipulation of fasting/refeeding is known to induce lipogenesis, associated with increased circulating insulin and decreased glucagon. We have also reported that insulin and cAMP independently affect the mRNA level for p90; treatment of mature 3T3-L1 adipocytes with insulin elicited a 3-fold increase in p90 mRNA and dibutyryl cAMP decreased the level to 10% of control. Overall, the pattern of expression of the p90 mRNA was similar to that of fatty acid synthase. These observations led us to believe that p90 mRNA may code for a protein which is involved in lipogenesis. Glycerol-3-phosphate acyltransferase (EC 2.3.1.15) catalyzes the committed step of triacylglycerol and phospholipid biosynthesis by generating lysophosphatic acid in mammals (2, 3). Glycerol-3-phosphate acyltransferase activity is present in the microsomal membrane fraction which is the principal site for glycerolipid synthesis and also in the outer mitochondrial membrane (4–6). In liver, 50% of total activity is found in the mitochondrial fraction, while in most other tissues microsomal glycerol-3-phosphate acyltransferase activity is about 10 times that of the mitochondrial fraction (4, 7). The partitioning of the fatty acids for esterification from those for oxidation is partly carried out by glycerol-3-phosphate acyltransferase and is known to be under nutritional and hormonal control. The glycerol-3-phosphate acyltransferase activity is thought to be increased by insulin and decreased by starvation, and the effect is presumably greater on the N-ethylmaleimide-insensitive mitochondrial enzyme (8, 9). In spite of the important roles it may play in the regulation of triacylglycerol and phospholipid biosynthesis, mammalian glycerol-3-phosphate acyltransferase has not been purified or characterized. The structural gene for the 83-kDa E. coli anglycerol-3-phosphate acyltransferase (plgB), however, has been identified and sequenced (10, 11).

In the present paper, we report cloning, structure, and hormonal regulation of a murine protein p90 with sequence homology to E. coli glycerol-3-phosphate acyltransferase. The deduced amino acid sequence of the cDNA for the 6.8-kb mRNA revealed a 30% identity and an additional 42% similarity in the 322-amino acid residue stretch with Escherichia coli glycerol-3-phosphate acyltransferase. The p90 mRNA was expressed in high levels in lipogenic tissues, such as liver and adipose tissue. Insulin causes a marked and rapid induction of the p90 mRNA in diabetic mice. In addition, the hepatic p90 mRNA induction by fasting/refeeding is prevented by cAMP and by streptozotocin diabetes. Furthermore, we report here that these hormonal effects are on the transcription rate of the gene coding for p90.

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† To whom correspondence and reprint requests should be addressed: Dept. of Nutrition, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. Tel.: 617-432-0938. Fax: 617-432-2435.

† The abbreviation used is: kb, kilobase(s).
screened by plaque hybridization with the two EcoRI-PstI fragments of previously isolated 5.3-kb cDNA sequence, first labeled with $^{32}$P by random priming (13).

cDNA Sequencing—Phage DNA inserts of the isolated clones were subcloned into pBluescript SK+ (Stratagene). Nucleotide sequence analysis was carried out on alkali-denatured double-stranded templates by the chain termination method (14) using Sequenase (U. S. Biochemical Corp.) and universal primers or oligonucleotides synthesized by standard phosphoamidite techniques on a BioSearch synthesizer. Also employed were the overlapping deletion subclones of pBluescript insert constructed by successive exonuclease III and multilinkage nuclelease digestions (15). Nucleotide sequence analysis was carried out from both strands or from multiple independent overlapping clones on one strand.

Animal Treatment, RNA Preparation, and Northern Blot Analysis—Diabetes was induced in 6-8-week-old CD-1 male mice by three weekly intraperitoneal injections of streptozotocin (10 mg/100 g body weight) (17). Mice were used 7 days after the last injection. Diabetes was confirmed by high fasting blood glucose level (>250 mg/ml) and urinary glucose by Clinistix (Ames). Insulin was administered to diabetic mice at a combined dose of regular insulin (3 units/100 g; Sigma) intraperitoneally and Lente Insulin (30 units/100 g; Lilly) subcutaneously. Dibutyryl cAMP (6 mg/100 g) and theophylline (3 mg/100 g) were given intraperitoneally to the previously fasted mice at the start of refeeding of a high carbohydrate, fat-free diet. Streptozotocin-diabetic animals and normal animals injected with dibutyryl cAMP were employed as controls. Total RNA was isolated from five pooled livers using the modified phenol chromatography. Poly(A)+ RNAs were electrophoresed in 0.7% (unless otherwise indicated) formaldehyde-agarose gels (5 μg/μl per lane) and blotted onto nitrocellulose filters as described previously (1). The filters were hybridized with p13 with a 5.3-kb insert labeled with $^{32}$P in 50% formamide, 5X SSC at 42 °C and were washed at 65 °C in 0.1 × SSC, 0.1% sodium dodecyl sulfate.

Nuclear Run-on Transcription Assays—Livers from three mice were homogenized in 5 volumes of buffer containing 0.32 M sucrose, 3 mM MgCl$_2$, 5 mM Hepes (pH 6.9), and 0.5 mM β-mercaptoethanol. Nuclei collected by centrifugation were washed once by centrifugation through a 2.1 M sucrose cushion at 20,000 rpm for 60 min in a Beckman SW 28 rotor. The nuclei were stored in liquid nitrogen in 50 mM Tris (pH 7.9), 5 mM MgCl$_2$, 0.5 mM β-mercaptoethanol, and 40% glycerol. Run-on transcription was carried out at 25 °C for 45 min in a reaction mixture containing 10$^5$ nuclei and 100 μCi of [α-32P]UTP (3000 Ci/mmol) in a final volume of 0.5 ml. Labeled RNA was isolated and hybridized to 5 μg of plasmids fixed on nitrocellulose as described previously (17).

RESULTS AND DISCUSSION

Isolation of Overlapping cDNA Clones Coding for p90—We have previously described the isolation of one cDNA clone (p13) coding for a novel mRNA which is induced by refeeding (1). We utilized differential hybridization screening of a partial cDNA library constructed with mRNA fractions larger than 28 S RNA in sucrose density gradient centrifugation (1). When we used a synthetic poly(A)-tailed RNA ladder ranging from 0.24 to 9.5 kb in size instead of the ribosomal RNAs as standards in Northern blot analysis, this 5.3-kb cDNA sequence hybridized to an mRNA with a size of 6.8 kb (Fig. 1).

The DNA sequence determination showed that the 5.3-kb insert of the cDNA clone p13 contains a poly(A) tail. To obtain sequences corresponding to the 5’ region of this mRNA and also to verify the sequences in the 3’ region, two additional libraries were constructed from fasted/refed liver and 3T3-L1 adipocyte (day 5) mRNAs by a modified Gubler and Hoffman method. These libraries were screened with the 0.7-kb most 5’ sequence of p13 and the 5.3-kb total p13 insert. Overlapping cDNA clones were isolated and characterized. The sequence at the 5’-end of the p90 mRNA, however, was present only in a single cDNA clone (Fig. 2).

cDNA Sequence of p90—The 6650-nucleotide cDNA sequence for the 6.8-kb mRNA was determined by dideoxy nucleotide chain termination sequencing in Bluescript SK+ vector and is presented in Fig. 3. The longest open reading frame of 2481 nucleotides precedes the 3240 nucleotides of the 3’-untranslated sequence. This open reading frame starts at the Met codon of nucleotide position 1 designated in Fig. 3. The sequence immediately preceding the assigned initiator ATG exhibits characteristics of translation start sites in eukaryotic mRNAs; ATG is preceded by CTGCC with one nucleotide diverged from the consensus sequence of CC(G/A)CC (18). The sequence of 926 nucleotides upstream from the ATG codon was also established from the available cDNA clones. The unusually long leader sequence may participate in the regulated expression of this gene. Although it is likely that the initiator ATG is correctly assigned, final proof will require purification and amino acid sequence analysis of p90.

A search of the GenBank and EMBL protein data bases with the deduced amino acid sequence revealed homology with glycerol-3-phosphate acyltransferase from E. coli as shown in Fig. 4. The alignment of deduced amino acid sequence of this novel murine protein p90 to the 806-residue-long E. coli glycerol-3-phosphate acyltransferase (10, 11) starts with amino acid residue 153 and ends with residue 444 with a 1 residue gap at position 417, two 2-residue gaps at positions 210 and 396, and a 6-residue gap at position 281 of
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Fig. 3. Nucleotide sequence of the p90 cDNA and the deduced amino acid sequence. The numbers on the left correspond to the nucleotide sequence. The A residues in the initiation codon are in uppercase. The translation termination codon TAG that follows the C-terminal leucine is indicated with stars. Sequences in small letters designate 5' and 3' noncoding sequences.
amino acids between the two sequences; were transferred to nitrocellulose filters and hybridized with \( ^{32}P \)-labeled poly(A+) RNA (S.SEQ1) with \( ^{32}P \)-labeled p13. Colons, matched amino acids; transferase activity may be regulated by phosphorylation and

In Vivo Hormonal Regulation of p90 mRNA in Liver—We have examined the hormonal and nutritional regulation of the p90 mRNA level in mouse liver (Fig. 6). The p90 mRNA level increased 20-fold after 8 h and further increased to 30-fold after 16 h of refeeding previously starved mice with a high carbohydrate, fat-free diet, a state in which lipogenesis would be high. Since fasting/refeeding causes an increase in insulin secretion and a decrease in circulating glucagon level, the effects of diabetes and cAMP in the nutritional induction of p90 mRNA were examined. When previously starved streptozotocin-diabetic mice were given a high carbohydrate diet for 8 h, there was no significant increase in the p90 mRNA level. In addition dibutyryl cAMP, administered at the time of refeeding to normal starved animals, inhibited by 70% the level of p90 mRNA observed in fasting probably was caused by increased circulating glucagon. This indicates that the very low level of p90 mRNA observed in fasting probably was caused by increased circulating glucagon.

Effect of Fasting/Refeeding and cAMP on Transcription Rate of the Gene Coding for p90—We have measured the transcription rate for the p90 gene in liver of normal mice previously fasted and then refed a high carbohydrate, fat-free diet. The transcription rate increased 2.5-fold at 6 h, 7-fold after 8 h, and reached 22-fold after 16 h of refeeding (Fig. 7).
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FIG. 7. Effect of fasting/refeeding, cAMP, and diabetes on the transcription for p90. Normal or streptozotocin-diabetic mice were fasted for 48 h and refed a high carbohydrate, fat-free diet. At the designated times, nuclei were isolated from pooled livers of three mice for each group and used for run-on transcription and hybridization with p13 (p90, ♂) and pAM 91 (actin, ♀) as described under "Experimental Procedures." Open symbols represent values obtained from the transcription carried out in the presence of α-amanitin. Autoradiograms were subjected to quantitative densitometric scanning. Essentially identical results were obtained from two separate experiments.

FIG. 8. Effect of insulin on steady state mRNA level for p90 in streptozotocin-diabetic mice. Poly(A+) RNA was prepared from livers of streptozotocin-diabetic mice killed at the indicated times after insulin administration. Saline-injected mice were also used at 8 h as a control (8C). Northern blot analyses were carried out as described for Fig. 5 and under "Experimental Procedures." The autoradiogram of the Northern blot was scanned densitometrically. The data were normalized to the values obtained prior to insulin administration and are presented as -fold increases. The level of actin mRNA remained the same through the experiment. Similar results were obtained from two separate experiments.

Increase in mRNA level and transcriptional rate for p90 caused by fasting/refeeding was similar but somewhat slower than that for fatty acid synthase gene we have reported previously (17). When dibutyryl cAMP was given at the start of refeeding of normal animals, there was no increase in transcription, indicating a negative transcriptional regulation of p90 mRNA by cAMP. Furthermore, we did not detect any appreciable transcription for p90 in fasted or refed streptozotocin-diabetic animal liver, indicating the requirement of insulin for transcriptional induction by fasting/refeeding.

Regulation of p90 mRNA by Insulin—As shown above, when we carried out fasting/refeeding experiments with streptozotocin-diabetic animals to examine the effect of insulin on p90 mRNA, there was no detectable mRNA or transcription for p90 in livers of refeeding animals indicating an insulin requirement for the induction of p90 by fasting/refeeding (Figs. 6 and 7). To determine whether insulin plays a direct physiological role in regulating the p90 mRNA level, the time course of the steady state mRNA level was followed after insulin administration in streptozotocin-diabetic mice (Fig. 8). The p90 mRNA level increased 2-fold 1 h after injection of insulin and further increased to attain a maximum level of 19-fold by 6 h. The magnitude and time course of p90 mRNA induction by insulin in diabetic animals was similar to our previous reports on that for fatty acid synthase, an enzyme crucial for lipogenesis (17). It is known that glucagon secretion is de-
poly(A+) RNA was prepared from livers and Northern blot analyses were carried out as described in Figs. 5 and 8. The level of actin mRNA was increased when insulin is administered to diabetic animals (21). It cannot be ruled out that the decreased circulating glucagon may have contributed to the increased mRNA level for p90 by insulin administration. However, it is likely that insulin plays a positive role in increasing the p90 mRNA level, considering the rapid increase in transcription shown in this experiment and the independent effect on the p90 mRNA level observed in 3T3-L1 adipocytes (1).

The dramatic increase in mRNA level for p90 by insulin may be due to the insulin effect on the transcriptional rate or on post-transcriptional events. The insulin effect on the mRNA level for p90 in diabetic animals, however, was abolished by actinomycin indicating transcriptional regulation by insulin (data not shown). Therefore, we carried out the transcription run-on analysis in isolated liver nuclei from diabetic animals at various times after insulin administration (Fig. 9). There was a rapid and marked increase in the transcription rate for p90 when insulin was given to the streptozotocin-diabetic mice; the transcription rate increased 4-fold after 30 min and attained a maximal increase of 8-fold at 2 h (Fig. 9, Exp. 2). The increase in transcription preceded and probably contributed to the increase in p90 mRNA level observed in Fig. 8. The time course of the increase in the transcription rate for p90 elicited by insulin was similar to that for the fatty acid synthase gene we previously reported (17). We tested whether the insulin effect on this specific transcription requires ongoing protein synthesis and/or a regulatory protein with a short half-life. As shown in Fig. 10, the increase in p90 mRNA level was not observed in the presence of cycloheximide after 6 h of insulin administration to streptozotocin-diabetic mice liver. Also abolished by cycloheximide was the increase in transcription for p90 by insulin (Fig. 9, Exp. 1). We observed that cycloheximide also abolished the effect of insulin on the fatty acid synthase gene transcription (data not shown). It appears that a similar mechanism is involved in insulin regulation of these genes. Stumpo and Blackshear (22) reported a transient increase in c-fos mRNA in 3T3-L1 cells treated with insulin and proposed a possible role for c-fos in the induction of lipogenic enzymes. In addition, these investigators showed that the insulin effect on c-fos is through the insulin receptor, and the serum-response element in the c-fos promoter is necessary for the increase in c-fos transcription by insulin (23). We did not detect any change in c-fos transcription from 30 min to 12 h after insulin administration in diabetic mice (Fig. 9, Exp. 1). The transcription rate measured in these experiments was by RNA polymerase II as indicated by the inhibition shown by α-amanitin. The p90 gives us a good model system for studying positive transcriptional control by insulin and negative control by cAMP at a molecular level.

p90, which is up-regulated by insulin and down-regulated by cAMP, is expressed highest in the tissues where and when lipogenesis is high. The marked effects of insulin and cAMP on the mRNA level and the transcription rate of this gene indicate the possibility that p90 may be the mitochondrial enzyme. There are conflicting reports on the hormonal regulation of glycerol-3-phosphate acyltransferase activities. Both mitochondrial and microsomal enzyme activities have been shown to decrease in adipose tissue by streptozotocin diabetes, and insulin administration restored them significantly (8, 9). On the other hand, the glycerol-3-phosphate acyltransferase activity of the mitochondrial form, but not the microsomal form, has been shown to decrease in starvation and by anti-insulin serum treatment in liver (8). Nevertheless, the mitochondrial glycerol-3-phosphate acyltransferase is generally thought to be more sensitive to hormones (2, 3). Antibodies against this glycerol-3-phosphate acyltransferase-like protein will make it possible to characterize and to demonstrate positive identification and subcellular localization of p90.

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