Induction of mRNAs for the Growth Hormone Receptor Gene During Mouse 3T3-L1 Preadipocyte Differentiation

Lanling Zou, Ram K. Menon, and Mark A. Sperling

Adipose tissue is a growth hormone (GH)-responsive tissue in which GH regulates energy metabolism. GH exerts its effect by interacting with its specific GH receptor (GHR). In rodents, alternative splicing of the nascent transcript from the GHR gene produces two major transcripts: GHR mRNA and GHR binding protein (GHBP) mRNA. These two transcripts share the common extracellular ligand-binding domain, but differ in the C-terminal sequence. Since GHR plays an important role in mediating the actions of GH in adipose metabolism, we initiated these studies to examine GHR gene expression in the course of mouse 3T3-L1 preadipocyte-adipocyte conversion. GHR and GHBP transcripts were detected by RNase protection assay (RPA) using the antisense riboprobes complementary either to the specific sequence of the GHR or to the sequence shared by both GHR and GHBP mRNAs. After stimulation of differentiation, mRNA abundance increased 28-fold and reached a maximal level by day 7 of adipogenesis. The GHR mRNA:GHBP mRNA ratio was 1.1 ± 0.12 and remained unchanged during differentiation. The decay rate for both mRNAs, estimated by treating the cells with actinomycin D, was approximately 24 hours and showed no significant difference between preadipocytes and adipocytes. Thus, GHR gene expression is dramatically upregulated during preadipocyte-adipocyte differentiation.

GROWTH HORMONE (GH) receptors (GHRs) mediate numerous biological actions of GH that are essential for postnatal growth and several metabolic processes. Two major products are generated from the GHR gene: a membrane-bound GHR[1] and a circulating soluble GHR binding protein (GHBP).[2] In man and rabbit, GHBP is thought to arise by proteolytic cleavage of the GHR protein, resulting in the release of the extracellular hormone-binding domain into the circulation.[3] However, in rodents, GHBP is generated by the alternative splicing of a single nascent transcript of the GHR gene at exon 7 and exon 8a.[4] Thus, in rat and mouse, GHBP consists of a ligand-binding domain that is identical to the extracellular portion of the GHR, and a hydrophilic carboxyl-terminal domain unique to the GHBP with the absence of transmembrane and intracellular domains of the GHR.[5,6]

Both GHR and GHBP are widely expressed in tissues such as the liver, kidney, muscle, and adipose tissue. In rat and mouse, both GHR and GHBP mRNAs are detected in adipose tissue.[7,8] It is believed that both isoforms of the GHR gene product play an important role in mediating the actions of GH in adipose tissue. The short form of the rat GHR (GHBP) remains associated with the cell membrane, implicating a possible paracrine/autocrine physiological role of GHBP in many tissues where GHBP and GHR are coexpressed.[8-10] It has been well established that mouse GH stimulates lipolysis in adipose tissue.[9] However, the regulation of GHR expression in these cells has not been examined. In this study, we determined the levels of GHR and GHBP mRNAs in the mouse fibroblast cell line 3T3-L1, which can differentiate into adipocytes under certain experimental conditions.[11] Cumulative data suggest that the induction of preadipocyte differentiation involves several second-messenger signaling pathways including the insulin-like growth factor-I (IGF-I)–activated tyrosine kinase pathway, the glucocorticoid pathway, the insulin-activated tyrosine kinase pathway, and the cyclic adenosine monophosphate–dependent protein kinase pathway.[12] GH induces preadipocyte differentiation and regulates lipid metabolism in these cells, possibly via the IGF-I pathway.[9,13,14] In this report, we demonstrate that both GHR and GHBP transcripts are significantly upregulated during differentiation, possibly due to increased expression of the GHR gene.

From the Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Submitted May 23, 1996; accepted July 26, 1996.

Supported by the Children's Hospital of Pittsburgh and the Vira I. Heinz Foundation at Children's Hospital of Pittsburgh.

Address reprint requests to Lanling Zou, PhD, MD, 3811 O'Hara St, University of Pittsburgh Medical Center, WPIC 1413, Pittsburgh, PA 15213.

0026-0495/97/4601-0020$03.00/0

MATERIALS AND METHODS

Cell Culture

3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS). Differentiation was initiated using the method of Bernlohr et al.[15] Confluent cells were switched to differentiation medium (DMEM, 10% FBS, 1 μmol/L dexamethasone, 10 μg/mL insulin, and 0.5 mmol/L isobutyl methyl xanthine) for 2 days. Then, the cells were cultured in postdifferentiation medium (DMEM, 10% FBS, and 10 μg/mL insulin), and the medium was changed every other day. Using morphological criteria for triacylglycerol accumulation in the cytoplasm, differentiation was judged to be maximal (80% to 90% of the cells) by day 7. For inhibition of differentiation, 10 μmol/L retinoic acid (RA) was added to 3T3-L1 cells with the differentiation medium. This concentration of RA has been previously shown to inhibit adipose conversion.[16] After 48 hours, cells were switched to postdifferentiation medium alone. For measurement of mRNA stability, 10 μg/mL actinomycin D (Sigma, St Louis, MO) was added to the medium for 24 hours before RNA extraction. Addition of RA and actinomycin D did not affect cell viability determined morphologically. The cell number per plate was counted under a microscope after trypsinizing and trypan blue staining.

RNA Extraction and Synthesis of Complementary RNA Probes

Total RNA was prepared from the cells using Tri-Agent-LS (MRC, Cincinnati, OH) and quantified by UV absorbance at 260 nm. Ten micrograms of total RNA was used in each reaction, and the assay was performed according to the manufacturer's protocol (RPA II kit; Ambion, Austin, TX). The plasmid containing the entire GHR cDNA

(2.2 kb) sequence was provided by Dr Frank Talamantes (University of California at Santa Cruz).[6] The GHR cDNA sequence is in the opposite orientation to the T7 promoter, and thus transcription with T7 RNA polymerase will synthesize an antisense riboprobe to the GHR mRNA. The most 3′ end sequence was deleted by *Kpn*I restriction enzyme digestion at position +1259. The plasmid was linearized by restriction enzyme digestion at either the *Eco*RV site (+815, probe I) or the *Apa*I site (+650, probe II) (Fig 1) blunt-ended by Klenow treatment, and gel-purified using the QIAquick column (QIAGENE, Chatsworth, CA). The antisense probes (from +1259 to +815 or to +650 of the mouse GHR cDNA) were generated using the T7 Maxiscript kit (Ambion). Probe I (478 nucleotides [nt]) contains the sequence complementary to the GHR sequence from +815 to +1259, and thus the protected fragment by GHR mRNA is 444 nt. Antisense probe II contains the sequence complementary to the GHR sequence from +650 to +1269, including the 161 nt (+650 to +810) of the ligand-binding domain–encoding sequence shared by both GHR and GHBP. Therefore, hybridization between the antisense probe II and GHBP mRNA protected a fragment of 161 nt (part of the ligand-binding domain) during RNase digestion, whereas hybridization of the same riboprobe II to GHR mRNA protected a 609-nt fragment covering part of the ligand-binding domain, the transmembrane domain, and part of the intracellular domain. A 250-nt mouse β-actin riboprobe (Ambion) was used as an internal standard in RNase protection assay (RPA).

RPA

RPA was performed using the RPAII kit (Ambion) according to the manufacturer's instructions. Total RNA (10 μg) was hybridized to the gel-purified riboprobe (2 to 5 fmol) at 42°C overnight. Following digestion with RNase A and RNase T1, the protected riboprobe fragments were fractionated on a 6% urea–polyacrylamide gel. The probe was present in molar excess over the target RNA in the hybridization reaction. To normalize the loading variations, a 1-kb DNA fragment was end-labeled with ^{32}P and added to the reaction mixture after RNase treatment. ^{32}P-labeled RNAs of varying length transcribed from a mixture of DNA templates (Ambion) using T7 Maxiscript were used as size markers. The bands corresponding to the fragments protected by GHR, GHBP, or β-actin mRNA were quantified using a phosphor imaging processor (Molecular Dynamics, Sunnyvale, CA), and the signal was normalized to the 1-kb DNA signal and expressed as arbitrary densitometric units.

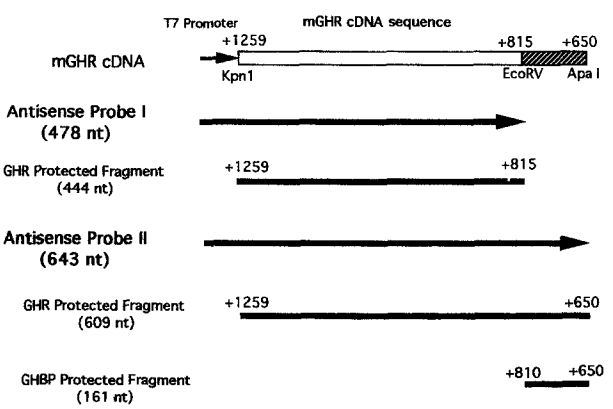

Fig 1. Schematic representation of mouse GHR antisense probes and the protected fragments of GHR and GHBP mRNAs in RPA. Positions of GHR sequences are numbered relative to the translation start site ATG (+1) according to the published sequence.[4,6] Restriction sites used to prepare cDNA templates are indicated.

RESULTS

GHR mRNA Was Induced During Mouse 3T3-L1 Preadipocyte Differentiation

To examine GHR gene expression during preadipocyte differentiation, 3T3-L1 preadipocytes were grown to confluence and then exposed to the differentiation medium for 2 days. After 7 days, 80% to 90% of the cells were rounder and loaded with fat, consistent with differentiation to adipocytes. The cell numbers determined microscopically after trypsinizing and trypan blue staining were the same between confluent predipocytes and mature adipocytes. Total RNA determined by UV absorbance was threefold to fivefold more abundant in adipocytes than in preadipocytes. We measured the abundance of GHR mRNA using the antisense riboprobe I, which specifically detects the GHR mRNA. Figure 2A shows that the level of GHR mRNA started to increase after day 3 and reached the maximal level by day 7. The abundance of GHR mRNA was 28-fold greater in adipocytes than in preadipocytes. In contrast, the β-actin mRNA, an internal control, exhibited a slight decrease during the process. Thus, the expression of GHR gene is specifically upregulated during the process of preadipocyte differentiation.

Induction of GHR mRNAs Was Blocked by RA

RA has been shown to inhibit differentiation of 3T3-L1 cells.[16] Figure 2B shows that simultaneous treatment of preadipocytes with RA and differentiation medium dramatically inhibited the induction of GHR mRNA that was observed after exposure to the differentiation medium alone. The signal for control β-actin RNA was not affected by the addition of RA (data not shown). This result suggests that the increased GHR gene expression is truly part of the differentiation program, and is not an artifact due to the addition of differentiation agents. The slight increase of GHR mRNA in the presence of RA may be due to the incomplete blockade by RA, since 10% to 20% of the cells still exhibited fat accumulation in the presence of 10 μmol/L RA.

Both GHR and GHBP mRNAs Were Induced During 3T3-L1 Preadipocyte Differentiation

Since GHR gene expression in the mouse generates both GHR and GHBP mRNAs, it was of interest to examine whether the GHBP mRNA exhibited a similar change during the process of preadipocyte-adipocyte conversion. To test this possibility, we designed a second RPA probe (antisense probe II) and measured the abundance of both mRNAs simultaneously. Figure 3 is a representation of the RPA gels showing a 609-nt fragment protected by the GHR mRNA and a doublet of about 160 nt that represents the fragments protected by GHBP mRNA. As suggested by Ilkbahar et al,[17] the likely cause of the doublet is hybrid breathing at an A-U–rich region at the junction of the ligand-binding domain and the hydrophilic tail of GHBP mRNA. Both bands were considered to represent GHBP mRNA and were quantified together to assess the abundance of this transcript. The ratio of GHBP mRNA to GHR mRNA in the preadipocytes was 1.1 ± 0.12 (mean ± SE) and remained unchanged during differentiation, suggesting that the synthesis

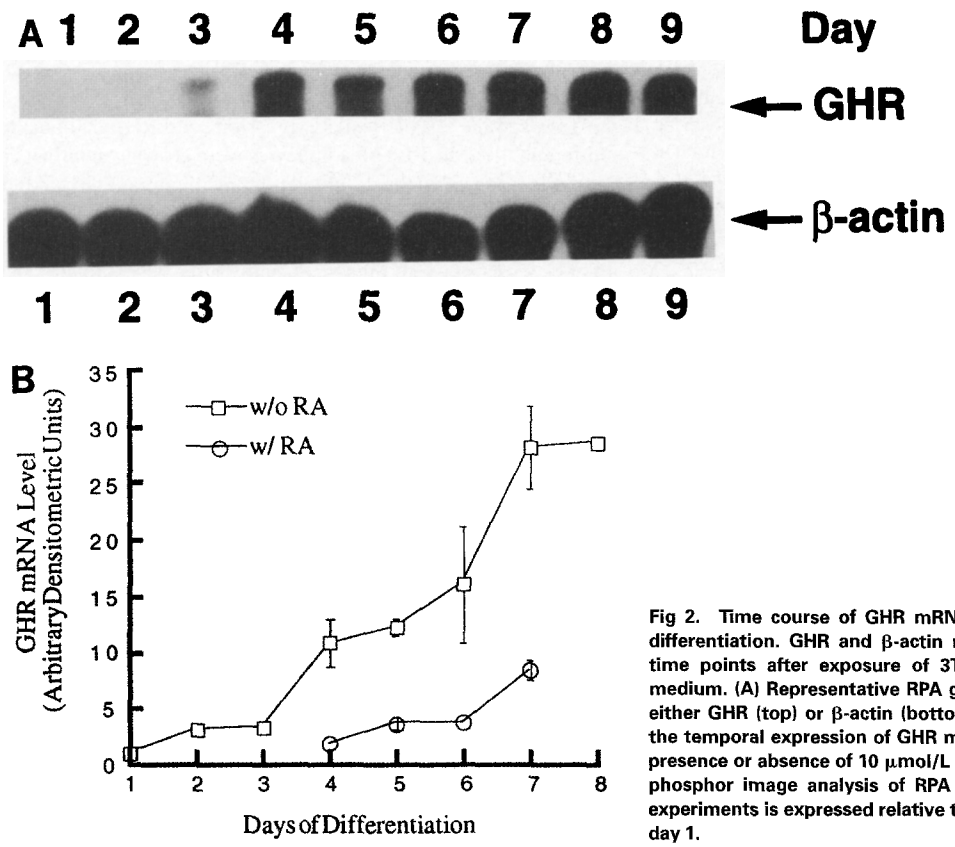

Fig 2. Time course of GHR mRNA induction during preadipocyte differentiation. GHR and β-actin mRNAs were assayed at varying time points after exposure of 3T3-L1 cells to the differentiation medium. (A) Representative RPA gel using the antisense probes for either GHR (top) or β-actin (bottom). (B) Graphic representation of the temporal expression of GHR mRNA during differentiation in the presence or absence of 10 μmol/L RA. Data points were obtained by phosphor image analysis of RPA gels. The mean ± SE of 3 to 6 experiments is expressed relative to the basal level of GHR mRNA at day 1.

of GHR and GHBP mRNAs increased at a similar rate. In addition, the abundance of GHBP mRNA also decreased to the same degree as GHR mRNA in the presence of RA (data not shown).

Stability of GHR and GHBP mRNAs Did Not Change Significantly During Preadipocyte Differentiation

One possible mechanism for the increased mRNA abundance is increased stability of mRNA in adipocytes. To examine whether the decay rate for GHR and GHBP mRNAs differs between preadipocytes and adipocytes, we treated both preadipocytes (day 0) and adipocytes (day 7) with actinomycin D (10 μg/mL) to inhibit new RNA synthesis, and measured the abundance of GHR and GHBP mRNAs. After 24 hours of drug treatment, GHR mRNA decreased to 52% ± 3.5% in preadipocytes and to 43% ± 4.1% in adipocytes, and GHBP mRNA decreased to 43% ± 3.8% and to 58% ± 4.6% in preadipocytes and adipocytes, respectively (Table 1). Two-sample *t* test showed that the difference among these values is not statistically significant. We did not attempt to treat the cells with actinomycin D for a longer period, due to the toxic effects of the drug. These results showed that the decay rate for both GHR and GHBP mRNAs was about 24 hours and did not change significantly during differentiation. Thus, the dramatic increase in the abundance of GHR and GHBP mRNAs during preadipocyte-adipocyte differentiation did not result from increased stability of the mRNAs.

DISCUSSION

The primary role of adipose tissue is to store energy as triacylglycerol during periods of nutritional caloric excess, and to mobilize this reserve when caloric expenditure exceeds intake. These functions are performed with the full complement of enzymes and regulatory proteins required for both de novo lipogenesis and lipolysis. These processes are regulated in response to hormones that regulate energy metabolism. For example, insulin promotes lipogenesis and inhibits lipolysis, whereas counterregulatory hormones (eg, glucagon, GH, and epinephrine) promote lipolysis. The expression of many enzymes and other proteins involved in the pathways of de novo fatty acid and triacylglycerol biosynthesis and lipolysis and the response to the lipogenic and lipolytic hormones have been demonstrated to be stimulated in adipocytes,[12] and the synthesis of many new proteins results from increased particular mRNAs.[18,19] This is consistent with our finding that the total RNA amount was threefold to fivefold greater in adipocytes than in undifferentiated fibroblasts.

It has been known for some time that GH produces a variety of effects in adipose tissue via GHRs on the cell membrane. Upon binding to the hormone, GHR is known to initiate a phosphorylation cascade that presumably results in expression of many, if not all, of the responses to GH.[20] Studies suggest that the short isoform, GHBP, can be either released into the medium or retained on the cell surface, where it binds GH and modulates cellular functions.[21] GH apparently plays a role in adipocyte differentiation by initiating an autocrine/paracrine mechanism that leads to the secretion of IGF-I.[22-24] The present study has demonstrated that at the mRNA level, GHR is highly expressed in these cells and significantly upregulated as the preadipocytes differentiated into mature adipocytes. Since the GHBP mRNA to GHR mRNA ratio and the decay rate for both mRNAs did not

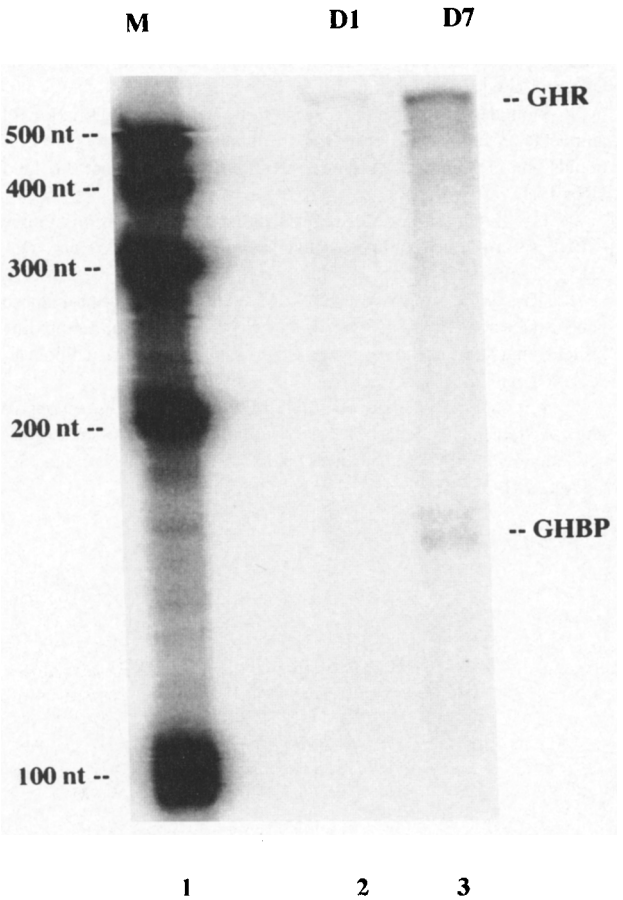

Fig 3. Representative phosphor image of RPA gels using the antisense riboprobe II. RPA was performed with 10 μg RNA. Lane 1, RNA size markers; lanes 2 and 3, fragment protected by GHR and GHBP mRNAs in preadipocytes (D1) and adipocytes (D7).

Table 1. Effect of Exposure to Actinomycin D for 24 Hours on the Levels of GHR and GHBP mRNAs

Cells	GHR mRNA (%)	GHBP mRNA (%)
Preadipocytes (day 0)	52 ± 3.5	43 ± 3.8
Adipocytes (day 7)	43 ± 4.1	58 ± 4.6

NOTE. RNA prepared from 3T3-L1 cells at day 0 (preadipocytes) and day 7 (adipocytes) was analyzed by RPA. Values are expressed relative to the level obtained just before addition of actinomycin D at a concentration of 10 μg/mL. Values are the mean ± SE of 3 to 4 individual experiments. Two-sample *t* test showed that differences between preadipocytes and adipocytes were not statistically significant.

change significantly during the process of differentiation, we propose that the increased GHR and GHBP mRNA levels resulted mainly from the increased transcription of the gene. This finding further supports the concept that GH acts on adipose tissue via its multiple receptors.

Several studies have indicated that the process of preadipocyte differentiation is regulated by a family of C/EBP (CCAAT/enhancer binding protein) transcription factors that have a basic region/leucine zipper (bZIP) DNA binding domain.[12] Expression of a member of the C/EBP family, C/EBPα, increases just before the coordinate expression of adipocyte-specific genes,[25] and C/EBPα stimulates gene transcription by interacting with the promoter elements of the target genes.[26,27] The time course of GHR mRNA accumulation suggests a possible regulatory effect of GHR gene expression by C/EBPα. The direct confirmation of this hypothesis requires the analysis of GHR promoter activity, and the molecular mechanisms involved in the actions of GHR/GHBP in adipose physiology remain to be elucidated.

ACKNOWLEDGMENT

We thank F. Talamantes for supplying mouse GHR cDNA.

REFERENCES

1. Roupas P, Herington AC: Cellular mechanisms in the processing of growth hormone and its receptor. Mol Cell Endocrinol 61:1-12, 1989

2. Baumann G: Growth hormone–binding proteins. Proc Soc Exp Biol Med 202:392-400, 1993

3. Spencer SA, Hammonds RG, Henzel WJ, et al: Rabbit liver growth hormone receptor and serum binding protein. J Biol Chem 263:7862-7867, 1988

4. Edens A, Southard JN, Talamantes F: Mouse growth hormone–binding protein and growth hormone receptor transcripts are produced from a single gene by alternative splicing. Endocrinology 135:2802-2805, 1994

5. Baumbach WR, Horner DL, Logan JS: The growth hormone–binding protein in rat serum is a spliced form of the rat growth hormone receptor. Genes Dev 3:1199-1205, 1989

6. Smith WC, Kuniyoshi J, Talamantes F: Mouse serum growth hormone (GH) binding protein has GH receptor extracellular and substituted transmembrane domains. Mol Endocrinol 3:984-990, 1989

7. Smith WC, Linzer DIH, Talamantes F: Detection of two growth hormone receptor mRNAs and primary translation products in the mouse. Proc Natl Acad Sci USA 85:9576-9579, 1988

8. Vikman K, Carlsson B, Billig H, et al: Expression and regulation of growth hormone (GH) receptor messenger ribonucleic acid (mRNA) in rat adipose tissue, adipocytes, and adipocyte precursor cells: GH regulation of GH receptor mRNA. Endocrinology 129:1155-1161, 1991

9. Fielder PJ, Talamantes F: The lipolytic effects of mouse placental lactogen II, mouse prolactin, and growth hormone on adipose tissue from virgin and pregnant mice. Endocrinology 121:493-497, 1987

10. Goodman HM, Frick GP, Tai LR: The significance of the short form of the growth hormone receptor in rat adipocytes. Proc Soc Exp Biol Med 206:304-308, 1994

11. Green H, Kehinde O: Sublines of mouse 3T3 cells that accumulate lipid. Cell 1:113-116, 1974

12. Cornelius P, MacDougald OA, Lane MD: Regulation of adipocyte development. Annu Rev Nutr 14:99-129, 1994

13. Zezulak KM, Green H: The generation of insulin-like growth factor-1–sensitive cells by growth hormone action. Science 233:551-553, 1986

14. Gleen KC, Shieh JJ, Laird DM: Characterization of 3T3-L1 storage lipid metabolism: Effect of somatotropin and insulin on specific pathways. Endocrinology 131:1115-1124, 1992

15. Bernlohr DA, Angus CW, Lane MD, et al: Expression of specific mRNAs during adipose differentiation: Identification of an mRNA encoding a homologue of myelin P2 protein. Proc Natl Acad Sci USA 81:5468-5472, 1984

16. Stone RL, Bernlohr DA: The molecular basis for inhibition of adipose conversion of murine 3T3-L1 cells by retinoic acid. Differentiation 45:119-127, 1990

17. Ilkbahar YN, Wu K, Thordarson G, et al: Expression and distribution of messenger ribonucleic acids for growth hormone (GH) receptor and GH-binding protein in mice during pregnancy. Endocrinology 136:386-392, 1995

18. Cook KS, Hunt CR, Spiegelman BM: Developmentally regulated mRNAs in 3T3-adipocytes: Analysis of transcriptional control. J Cell Biol 100:514-520, 1985

19. Bernlohr DA, Bolanowski MA, Kelly TJJ, et al: Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J Biol Chem 260:5563-5567, 1985

20. Campbell GS, Christian LJ, Carter-Su Z: Evidence for the growth hormone receptor–associated tyrosine kinase in actions of growth hormone. J Biol Chem 268:7862-7867, 1993

21. Frick GP, Goodman HM: Characterization of the short isoform of the growth hormone receptor synthesized by rat adipocytes. Endocrinology 131:3083-3090, 1992

22. Doglio A, Dani C, Fredrikson G, et al: Acute regulation of insulin-like growth factor-I gene expression by growth hormone during adipose cell differentiation. EMBO J 6:4011-4016, 1987

23. Gaskins HR, Kim JW, Wright JT, et al: Regulation of insulin-like growth factor-I ribonucleic acid expression, polypeptide secretion, and binding protein activity by growth hormone in porcine preadipocyte culture. Endocrinology 126:622-630, 1990

24. Nougues Y, Reyne Y, Barenton B, et al: Differentiation of adipocyte precursors in a serum-free medium is influenced by glucocorticoids and endogenously produced insulin-like growth factor-I. Int J Obes 17:159-167, 1993

25. Cao Z, Umek RM, McKnight SL: Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538-1552, 1991

26. Christy RJ, Yang VW, Ntambi JM, et al: Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. Genes Dev 3:1323-1335, 1989

27. Kaestner KH, Christy RJ, Lane MD: Mouse insulin-responsive glucose transporter gene: Characterization of the gene and *trans*-activation by the CCAAT/enhancer binding protein. Proc Natl Acad Sci USA 87:251-255, 1990