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Analysis of a 762-bp Proximal Leptin Promoter to Drive and Control Regulation of Transgene Expression of Growth Hormone Receptor in Mice

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Transgenic (TG) mice expressing porcine GH receptor (pGHR) directed by a 762-bp proximal leptin promoter were used to analyze the capability of the promoter to drive and regulate pGHR expression *in vivo*. Transgene expression occurred in inguinal, retroperitoneal, and epididymal/parametrial fat depots in both male and female TG mice, but not in wild type (WT) mice. pGHR transgene was also expressed in liver, heart, kidney, muscle, lung, and brain. Levels of pGHR transgene mRNA were higher in tissues other than adipose tissue. Fasting reduced leptin mRNA levels in adipose; however, pGHR transgene expression was not affected in either adipose or muscle. These results suggest (1) the region between +3 and −759 bp of the leptin promoter is able to drive gene expression *in vivo*, (2) this region may not be responsible for adipose tissue specificity of leptin expression, and (3) this region may not be responsible for negative regulation of leptin gene expression during fasting.

Key Words: **transgenic; leptin; promoter; mice.**

Leptin is a hormone that is involved in regulating energy balance. Leptin gene expression is regulated by nutritional status and hormones (1–8). For example, feeding a high fat diet enhances leptin mRNA expression in adipose tissue of rodents (9), whereas fasting decreases the expression (1, 2). The leptin promoter contains binding sites for factors involved in the regulation of gene expression, including CCAAT enhancer binding protein (C/EBP), SP1, GRE and DR+1 (10–13). The C/EBP binding site is located between −55 and −47 and the PPAR binding site is located between −3951 and −3939 (11). Studies have shown that C/EBPα is able to bind to the C/EBP binding site in the proximal leptin promoter and transactivate leptin gene expression (14). Transfection studies in primary rat adipocytes and 3T3-L1 preadipocytes demonstrated that a 762-bp proximal end of the leptin promoter was sufficient to drive the expression of a reporter gene and mutation of the C/EBP binding site resulted in the reduction of reporter gene activity (10). These studies suggest that the proximal leptin promoter may be critical for driving leptin gene expression.

PPARγ plays a negative role in the regulation of leptin expression (11). BRL49653, an activator of PPARγ, is able to reduce leptin expression (15). However, the mechanism of PPARγ's role in negative regulation of leptin gene expression is not well understood. To study the functional character of the leptin promoter segment more completely within a greater variety of tissues and under physiological conditions, we tested the promoter in vivo with transgenic mice. Transgenic mice were generated to have a transgene that consisted of the 762-bp leptin promoter driving sequences that encoded porcine growth hormone receptor (pGHR). The objectives of this study were to determine (1) if a 762-bp leptin proximal promoter would be able to induce pGHR expression in vivo, (2) if a 762-bp leptin proximal promoter would be sufficient for tissue-specific expression of pGHR transgene when incorporated into the genome and (3) whether this proximal promoter had the ability to mimic leptin expression under conditions previously shown (1, 2, 9) to alter leptin expression—fasting or feeding a high fat diet.

MATERIALS AND METHODS

Transgene construct and production of transgenic mice. The construct utilized in this study consisted of four parts, in this order: 1) the 762-base pair leptin promoter region between +3 and −759 bp, 2) the chimeric 5′ intron from pCI-neo plasmid, 3) the full length porcine GH receptor coding sequences, and 4) the SV40 late polyA segment from pCI-neo plasmid. The mouse 762-bp leptin promoter was kindly provided by Dr. Marc Reitman (the Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, Maryland). The full length porcine GH receptor coding sequences was a gift from

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Allan Robins of BresaGen Limited, Addaide, SA. The leptin-pGHR transgene was constructed in several steps as follows: 1) The chimeric 5′ intron from pCI-neo plasmid was amplified by PCR with primers designed such that the product contained a BamH1 site at its 5′ end and an Xbal site at its 3′ end. The PCR product was digested with BamH1 and Xbal and inserted into the BamH1 and Xbal site of pBluescrip II. 2) The SV40 polyA segment containing an Xbal site at 5′ end and a Sac1 site at 3′ end was generated by PCR from pCI-neo plasmid. The SV40 polyA segment was cut by Xbal and SacI and ligated into the Xbal and SacI site of pBluescrip II with the chimeric intron. 3) The 762-base pair leptin promoter fragment was excised by BamH1 from p1494, subcloned into BamH1-digested pBluescrip II which contained the chimeric intron and the SV40 polyA, and orientation was checked to confirm that 5′ end of the leptin promoter fragment was linked to chimeric intron. 4) The 1.99 kb pGHR coding sequence was excised from pCIS2-pGHR by Xbal and inserted into the Xbal site between leptin promoter and SV40 polyA of pBluescrip II. 5) pBluescrip II with transgene was digested by Sal1 and SacI to release the 3.12 kb transgene segment. The 3.12 kb transgene segment was further purified and used for microinjection into fertilized mouse eggs at the Dr. Carl A. Pinkert's Laboratory in the Department of Comparative Medicine, The University of Alabama at Birmingham, AL. Trangenic founder mice were determined by PCR analysis of tail tissue. The founder mice were mated with line mates and F1 hemizygous offspring were identified.

PCR for detection of pGHR transgene. Mouse tail tissues were incubated with digestion buffer containing 0.1 M NaCl, 0.02 M EDTA, 1% SDS, 0.01 Tris, pH 7.5-8.0 and 0.1 mg/ml protease K at 55°C overnight. The digested tissues were centrifuged at 12,000 rpm for 10 min. DNA from supernatant was purified by phenol-chloroform extraction and ethanol precipitation. PCR reactions were carried out using 50 ng of DNA. DNA was amplified with primers specific for pGHR transgene. The conditions of PCR were as follows: denaturation for 1 min at 92°C, annealing for 1 min at 50°C, and elongation for 1 min at 72°C with 30 cycles.

Animal studies. Selection of both wild type and transgenic breeding stock mice was based on the greater tail length to body weight ratios. In experiment one, 16 WT and 16 TG mice from the same founder were started on the high fat diet (50% protein, 20% fat, modified from PMI 5020 diet) at 4 weeks of age. Animals were individually housed and fed the high fat diet throughout the experiment. Body weight and food intake were monitored at one week intervals for 7 weeks. At 11 weeks of age, the mice were sacrificed, the tail length was recorded, and tissues were collected. In experiment two, twelve mice from each of the two strains (TG and WT mice) were selected for the experiment at 8 weeks of age. After selection, animals were fed a normal mouse diet (PMI 5020 diet) ad libitum until the time of the experiment. Six mice from each strain were fasted for 12 hours prior to collecting tissues.

RT-PCR for pGHR mRNA and leptin. Mice were killed by CO_2, and inguinal, retroperitoneal and epididymal/parametrial fat pads, liver, heart, lung, kidney, brain and gastrocnemius muscle were removed, frozen immediately in liquid nitrogen, and stored at −80°C for determination of mRNA expression for pGHR and leptin. Total RNA samples were treated with RQ1 DNAse at 37°C for 1 hour, followed by extraction with phenol-chloroform and ethanol precipitation. The cDNA primers for the pGHR were designed to specifically detect the PGHR transgene. The sense primer of pGHR was 5′cctgtcccagactatacctcc3′ (located in porcine GHR coding region) and antisense primer was 5′ctgcattctagttgtggtttgtcc3′ (located in SV 40 polyA site) which is unique to pGHR transgene. The cDNA primers for leptin and β-actin were 5′agtgcctatccagaaagtcc3′ (sense) and 5′ggagattctccaggtcattg3′ (antisense) and 5′gaagagctatgagctgcctgac3′ (sense) and 5′gtactcctgcttgctgatccac3′ (antisense), respectively. The reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific pGHR RNA from 1g of total RNA were performed separately using RT and PCR system (Promega, Madison WI). The reverse transcription reaction was performed at 42°C for 60 min, followed by 95°C for 5 min to inactivate the AMV RT enzyme. For PCR amplification, two sets of primers for pGHR and β-actin were added in the same PCR reaction. Samples were denatured at 94°C for 30 sec, annealed for 1 min at 56°C (pGHR gene) or 50°C (leptin), and extended for 2 min at 68°C. Amplification was carried out using 25 to 30 cycles. Final extension was carried out at 68°C for 10 min. The PCR products were run in a 2% agrose gel stained with ethidium bromide.

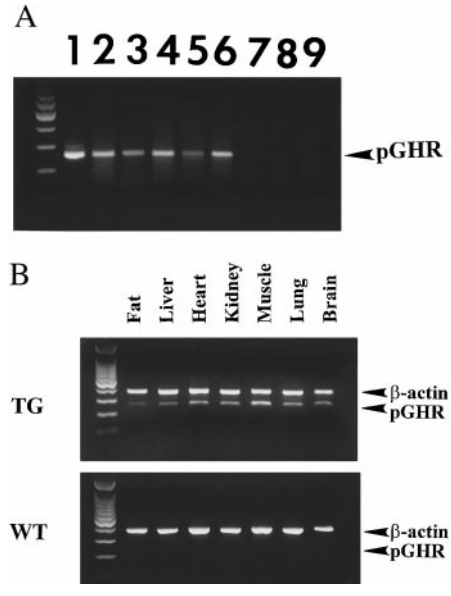

FIG. 1. Expression of pGHR transgene mRNA in fat depots and multiple tissues of TG and WT mice (Panel A). RT-PCR was performed using a set of specific primers for pGHR transgene. Lane 1: a product of PCR using pGHR construct as a template (positive control). Lane 2: inguinal fat pad from male TG mice. Lane 3: epididymal fat pad from male TG mice. Lane 4: retoperitoneal fat pad from male TG mice. Lane 5: parametrial fat pad from TG female mice. Lane 6: inguinal fat pad from female TG mice. Lane 7: retroperitoneal fat pad from female WT mice. Lane 8: parametrial fat pad from female WT mice. Lane 9: inguinal fat pad from female WT mice. (Panel B) Tissue distribution of pGHR mRNA expression in TG mice fed a standard diet.

Statistical analysis. Density of bands was quantified by image system (ChemiImage 4000, Alpha Innotech Corporation). The quantities of pGHR and leptin mRNA were normalized to the levels of β-actin mRNA and expressed as a percentage of pGHR or leptin versus β-actin mRNA levels. Data for body weight and fat pad weights were subjected to an analysis of variance (ANOVA) procedure of the Statistical Analysis Systems.

RESULTS

Transgene expression was examined by RT-PCR using specific primers for the pGHR transgene. Transgene expression in the different fat depots of transgenic and wild type mice is shown in Fig. 1A. The pGHR transgene was expressed in retroperitoneal, epididymal/parametrial and inguinal fat pads of male and female TG mice, while pGHR transgene expression was not detected in any fat pad of WT mice. As shown in Fig. 1B, transgene mRNA expression was also de-

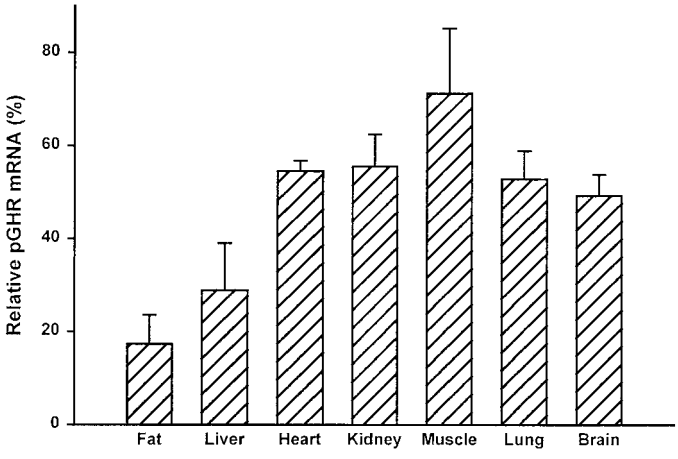

FIG. 2. Tissue distribution of pGHR mRNA expression in TG mice fed a high fat diet. Total RNA isolated from various tissues of TG or WT mice fed a high fat diet was used to perform RT-PCR using two sets of primers for pGHR and β-actin in the same PCR reaction. RT-PCR results were quantified using image system and expressed as a percentage of abundance relative to β-actin.

tected in liver, heart, kidney, muscle, lung and brain of transgenic mice, but not WT mice. The levels of pGHR mRNA expression relative to β-actin were relatively lower in adipose tissue when compared to other tissues that do not expressed endogenous leptin.

Studies have shown that a high fat diet elevates leptin mRNA expression in adipose tissue of rodents (9). To test if the leptin proximal 762-bp promoter harbors elements that may respond to a high fat diet, transgenic mice were fed a high fat diet for 7 weeks and tissues were tested for transgene expression. Transgene expression in the different tissues of the TG mice fed a high fat diet is shown in Fig. 2. The pattern of transgene expression in fat and other tissues was not altered by feeding a high fat diet. In addition, the levels of pGHR transgene mRNA expression were higher in tissues other than adipose tissue. In particular, muscle expressed the transgene at the highest level.

Body weight and fat pad weights in WT and TG mice fed a high fat diet for 7 weeks are shown in Table 1. Animals were started on a high fat diet at 4 weeks of age. At 11 weeks of age, TG mice were reconfirmed based on the pGHR transgene expression in the adipose tissue. There was no significant difference in body weight at any week between WT and TG mice. Furthermore, no difference in fat pad weights between WT and TG mice was observed.

To determine if the 762-bp leptin promoter region contains important inhibitory regulatory elements, we examined the effect of fasting on expression of both leptin and the pGHR transgene in adipose tissue. Figure 3A shows the effect of fasting on leptin mRNA expression in adipose tissue of both TG and WT mice with quantification of leptin mRNA levels using image analysis presented in panel B. Fasting, as expected, reduced leptin mRNA expression in adipose tissue of TG and WT mice. If the 762-bp leptin promoter contains the same negative response elements as the native leptin promoter, then fasting should decrease the expression of pGHR; however, fasting did not decrease pGHR mRNA levels in adipose tissue of TG mice (Figs. 4A and B). Moreover, in muscle, which does not express endogenous leptin, transgene expression was also not affected by fasting (Fig. 5, quantified mRNA levels of the transgene).

DISCUSSION

The performance of the leptin promoter in gene expression at the transcriptional level has been investigated in cell culture in several independent experiments. Studies show that the transcription factors C/EBP, SP-1 and a novel factor (binding to an LP-1 motif) are able to bind to response elements in the 762-bp leptin promoter and play a role in the functional regulation of leptin expression (10–13). The C/EBP and SP-1 transcription factors and their corresponding response elements are quite common between genes and cell types and inactivation of the LP-1 element only reduces expression by half in fat cells (12). Thus it is difficult to understand how the proximal leptin promoter could account for tissue specificity and physio-

TABLE 1

Changes in Body Weight and Fat Pad Weights of Wild Type and Transgenic Mice Fed a High Fat Diet

	Genotype		*P* values		
Parameters measured	Wild type	pGHR	Genotype	Sex	Genotype × sex
Mouse numbers	16	10			
Terminal body wt. (g)	25.09	26.18	0.44	<0.01	0.66
Tail length (mm)	80.69	79.60	0.38	0.69	0.48
Fat depots (g)					
Retroperitoneal	0.13	0.18	0.33	0.03	0.74
Epididymal/parametrial	0.48	0.57	0.55	0.01	0.92
Inguinal	0.25	0.33	0.26	0.01	0.42

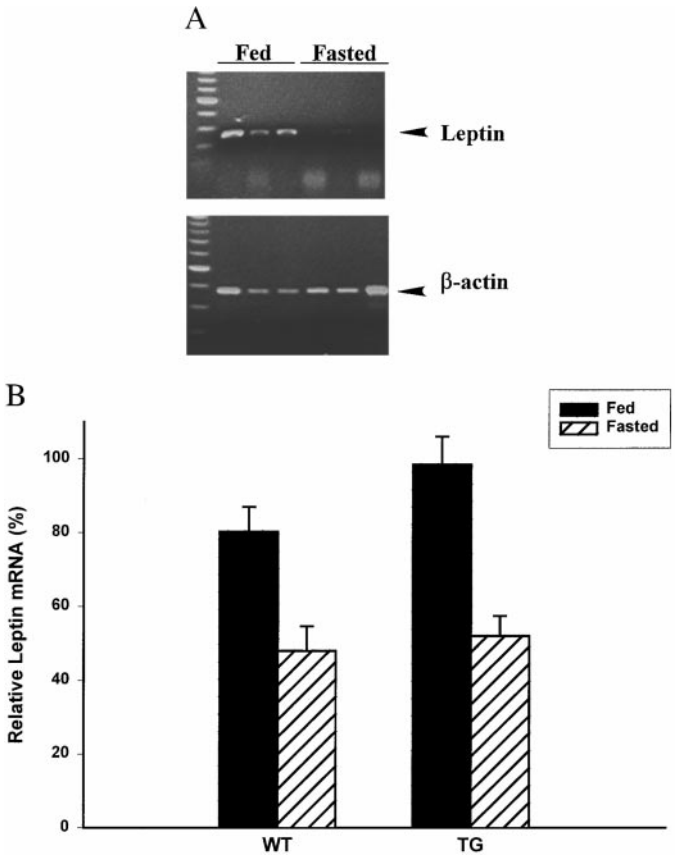

FIG. 3. The effect of fasting on leptin expression in adipose tissue of WT and TG mice. Total RNA isolated from adipose tissue of WT and TG mice fed or fasted for 12 hours was used to perform RT-PCR using a set of primers for leptin. RT-PCR results were quantified using image system and expressed as a percentage of abundance relative to β-actin. (Panel A) Leptin mRNA expression by RT-PCR in adipose tissue of fed or fasted TG mice. (Panel B) Quantified leptin mRNA in adipose tissue of fed or fasted TG and WT mice.

logical responses unless it contains unrecognized elements that provide these functions.

Although cell culture systems are useful for investigating leptin promoter roles in gene expression, gene regulation related to complex physiological changes, such as fasting or high fat diets, can not be easily mimicked in cell culture. Moreover, only a limited number of cell types are usually compared in cell culture to determine cell specificity. To study gene regulation in a physiological context, transgenic mice are a very useful model, particularly for leptin, a hormone that acts as an endocrine factor. In this study, we used transgenic mice to determine if the 762-bp leptin proximal promoter region could demonstrate adipose tissue specificity and respond to positive or negative regulatory factors when integrated into a permissive region of the genome. Activity was assessed by comparison to native leptin gene expression.

Growth hormone (GH) is known to cause reduced fat to muscle ratio both by limiting fat deposition and by increasing the mobilization of fat depots as a result of membrane bound GH receptors on the adipocytes (20). However, the results of this study were not as expected. No physiological effects of overexpressing pGHR on body weight and fat pad weights were observed. Body weight and fat pad weights were not different between WT and TG mice. Two explanations are possible. First, this leptin promoter does not drive a high level of pGHR expression in adipose tissue. Second, this could be attributive to the possible presence of mutation of pGHR sequence, since the pGHR sequence was generated by PCR cloning. Regardless, pGHR can be used as a reporter gene to analyze the leptin promoter activity in the present study.

The tissue specificity of the 762-bp promoter which we used in this study has been examined using transient expression in primary rat adipose cells and the erythroid K562 cell line (10). The levels of reporter gene activity directed by the 762-bp leptin promoter were significantly higher in adipose cells than in K562 cells (10), indicating that this region of promoter can drive gene expression specifically in adipose cells but not in K562 cells. Our results however, showed that

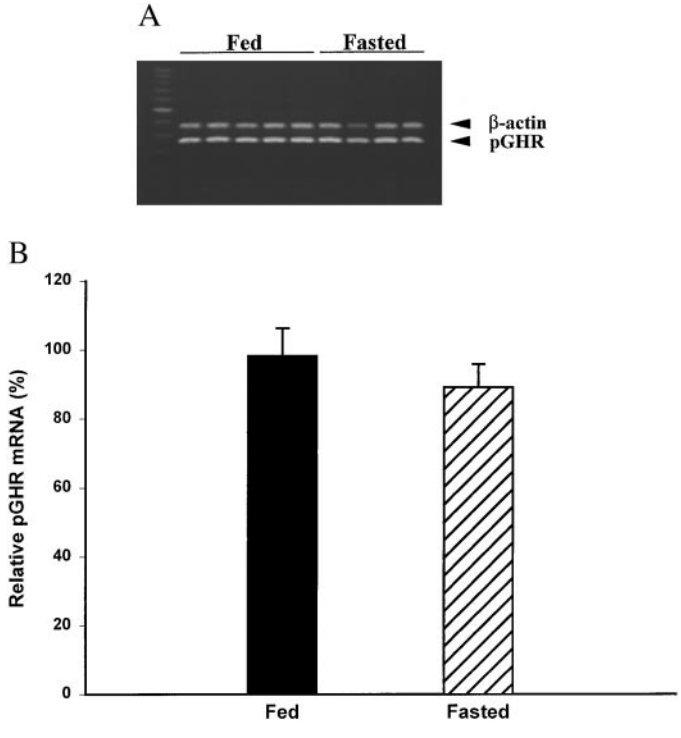

FIG. 4. The effect of fasting on pGHR transgene expression in adipose tissue of TG mice. Total RNA isolated from adipose tissue of TG mice fed or fasted for 12 hours was used to perform RT-PCR using two sets of primers for pGHR and β-actin in the same PCR reaction (Panel A). RT-PCR results were quantified using image system and expressed as a percentage of abundance relative to β-actin (Panel B).

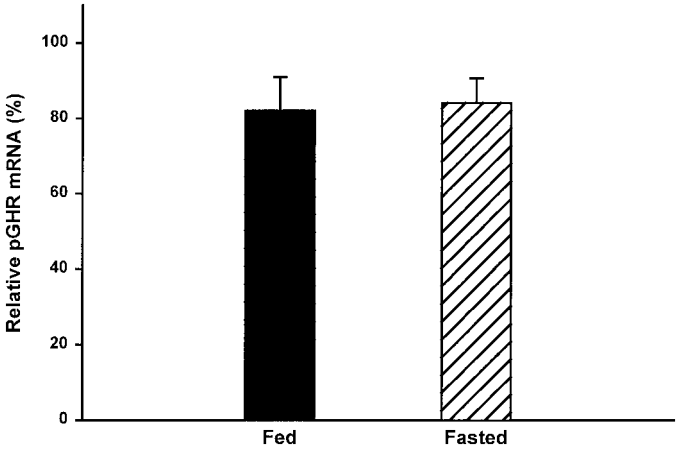

FIG. 5. The effect of fasting on pGHR transgene expression in muscle of TG mice. Total RNA isolated from adipose tissue and muscle of TG mice fed or fasted for 12 hours was used to perform RT-PCR using two sets of primers for pGHR and β-actin in the same PCR reaction. RT-PCR results were quantified using image system and expressed as a percentage of abundance relative to β-actin.

pGHR transgene was expressed in all tissues examined. While it is unlikely that our results are completely due to positional effects, we cannot rule out this possibility. If the 762-bp promoter was inserted in a position that was enhanced by flanking promoter/enhancer sequences from another generally expressed gene, then expression of the transgene might be expected to be found in more than one cell type. However, it should be noted that regardless of feeding a high fat diet, the transgene was expressed at the lowest level in fat cells where one would expect the C/EBP, LP-1 and SP-1 response elements to be most active. These observations indicate that the 762-bp leptin promoter does not have an adipose tissue-selective enhancer element which is strong enough to restrict expression to adipose tissue, and this proximal promoter is not sufficient for adipose tissue specific transgene expression *in vivo*. The C/EBP family and PPAR isoforms are transcription factors that play important roles in adipocyte differentiation and adipocyte-specific gene expression (21). The complete leptin promoter contains several binding sites, such as C/EBP, DR+1 and SP1. Several lines of evidence from cell culture studies indicate that the C/EBP binding site, which is located between -47 to -55, is essential for reporter gene expression (11). C/EBPα was able to activate the promoter of the leptin gene, and the promoter activity in adipose cells was not reduced by deletion of regions upstream of -161-bp (10). However, point mutations of C/EBP binding site resulted in reductions in reporter gene expression (14). PPAR, on the other hand, plays a negative role in leptin gene expression. Several studies showed that thiazolidinedione (TZ), a high affinity ligand for PPAR isoforms, reduced leptin mRNA and serum leptin levels (22). A binding site (DR+1) for PPAR has been found between -3951 and -3939 of the leptin promoter (11). This binding site in the leptin promoter can act as a PPAR response element, since PPARγ translated in cells was able to strongly bind to this element (11). However, negative regulation of leptin expression by PPARγ was not dependent on the binding of PPARγ to the DR+1 site. Transfection studies in primary rat adipocytes showed that TZ reduced activities of all leptin promoter constructs including a very short leptin promoter (-65 to $+9$), sequence which possesses only a C/EBP binding site (11). These observations from cell culture studies suggest that negative regulation of the leptin promoter by PPARγ may be mediated by antagonizing C/EBPα function.

Fasting, which has been known to reduce leptin expression, was used in this study to determine if the 762-bp promoter mediates negative regulation of transgene expression. We showed that fasting decreased leptin mRNA expression in adipose tissue of TG and WT mice, but did not reduce transgene expression in adipose tissue and muscle. These results suggest that the 762-bp promoter does not contain the response elements that mediate negative regulation of leptin expression by fasting. Since fasting does not down-regulate the 762-bp leptin promoter, antagonism of the C/EBPα function does not occur through PPARγ. Therefore, antagonism of C/EBPα function could not be the mechanism for down-regulation of leptin expression by fasting. This concept is supported by a recent report in which leptin mRNA levels were decreased while C/EBPα remained unaltered in response to fasting (23). Therefore, it is likely that C/EBPα may only be responsible for up-regulation of leptin expression and inhibitory elements that mediate negative regulation of leptin expression are contained upstream from the 762-bp leptin promoter. Although PPARγ was considered to be a negative factor for leptin expression, PPARγ expression was decreased during fasting (24). It is possible that the mechanism involved in negative regulation by PPARγ and by fasting could be different. Nevertheless, the results of the present study indicate that the 762-bp proximal promoter is not sufficient for adipose tissue specificity of leptin expression in vivo and does not respond to fasting-induced down-regulation found in native leptin.

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