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Regulation of Leptin Promoter Function by Sp1, C/EBP, and a Novel Factor*

MARK M. MASON, YUFANG HE, HUI CHEN, MICHAEL J. QUON, AND MARC REITMAN†

Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, and Hypertension-Endocrine Branch, National Heart, Lung, and Blood Institute (H.C., M.J.Q.), National Institutes of Health, Bethesda, Maryland 20892-1770

ABSTRACT

Leptin is a hormone produced in adipose cells that regulates energy expenditure, food intake, and adiposity. To understand leptin's transcriptional regulation, we are studying its promoter. Four conserved and functional regions were identified. Mutations in the C/EBP and TATA motifs each caused an approximately 10-fold decrease in promoter activity. The C/EBP motif bound recombinant C/EBPα and mediated *trans*-activation by C/EBPα, -β, and -δ. Mutation of a consensus Sp1 site reduced promoter activity 2.5-fold and abolished binding of Sp1. Mutation of a fourth factor-binding site, denoted LP1, abolished protein binding and reduced promoter activity 2-fold. Factor binding to the LP1 motif was observed with adipocyte, but not with nonadipocyte extracts. Adipocytes from *fa/fa* Zucker rats transcribed the reporter plasmids more efficiently than did control adipocytes. No effect on the transient expression of leptin was noted upon treatment with a thiazolidinedione, BRL49653, or upon cotransfection with peroxisome proliferator-activated receptor-γ/retinoid X receptor-α or sterol response element-binding protein-1. Mutations of the Sp1, LP1, and C/EBP sites in pairwise combinations diminished promoter activity to the extent predicted assuming these motifs contribute independently to leptin promoter function. Our identification of motifs regulating leptin transcription is an important step in the elucidation of the mechanisms underlying hormonal and metabolic regulation of this gene. (*Endocrinology* **139:** 1013–1022, 1998)

LEPTIN is a hormone produced in adipose cells that is important in the regulation of energy expenditure, food intake, and adiposity (1, 2). Leptin is a signal from adipose tissue to the rest of the body reporting the degree of adiposity; circulating leptin levels correlate best with the amount of body fat (3, 4). Mice lacking a functional *leptin* (formerly *ob* or *obese*) gene become massively obese and develop diabetes mellitus due to overeating and decreased metabolic expenditure (5). These mice are also hypogonadal and hypercorticosteronemic, presumably on a hypothalamic basis. Leptin treatment of *lep*^{ob}/*lep*^{ob} mice reverses all of these abnormalities, and in normal mice causes decreased food intake, increased energy expenditure, and weight loss (6–8).

Mice homozygous for a nonsense mutation in the *leptin* gene (*lep*^{ob}/*lep*^{ob}) show a 20-fold increase in *leptin* RNA levels (1), suggesting that the *leptin* gene is subject to transcriptional regulation. Similarly, mutations in the leptin receptor (*lepr*^{db}/*lepr*^{db} mice and *fa/fa* rats) cause increased leptin RNA. Leptin levels are regulated by factors in addition to adiposity. Protein and RNA levels decrease in response to β-adrenergic agonists or starvation and are increased by glucocorticoids or insulin (9–11). To understand leptin's transcriptional regulation, we isolated the leptin promoter (12) and report here its detailed characterization.

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Address all correspondence and requests for reprints to: Dr. Marc Reitman, Diabetes Branch, Building 10, Room 8N-250, 10 Center Drive, MSC 1770, Bethesda, Maryland 20892-1770. E-mail: mlr@helix.nih.gov.

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Materials and Methods

Plasmids

Standard cloning methods were used (13). Luciferase reporters are derivatives of pGL2-Basic (Promega, Madison, WI); plasmids p(−762)lep-luc, p(−456)lep-luc, p(−161)lep-luc have been described and were previously named p(xx)ob-luc (12). Plasmids p(−6900)lep-luc (p1613) and p(−3800)lep-luc (p1618) were constructed from p(−762)lep-luc digested with either *Xba*I/*Kpn*I or *Kpn*I by insertion of the contiguous 6550-bp *Xba*I/*Kpn*I or 3400-bp (*Kpn*I)*Hin*dIII/*Kpn*I genomic fragment of the *leptin* promoter. Clustered point mutations (creating *Hin*dIII or *Nhe*I sites) were introduced into p(−762)lep-luc by a PCR-based method (14). The mutated plasmids (see Figs. 3 and 4 for base changes) are named using the base number of the 3′-most base in the mutation, with the sequence and cap site determined previously (12); there are slight numbering differences between these and those previously reported (15, 16). Our laboratory designations for these plasmids are: m7, p1761; m16, p1760; m21, p1757; m27, p1581; m47, p1649; m52, p1594; m53, p1645; m59, p1647; m67, p1579; m85, p1797; m95, p1578; m109, p1799; m135, p1803; m47,59, p1651; m52,85, p1834; m52,95, p1801; and m85,95, p1848. Plasmids p(−135)lep-luc (p1809), p(−109)lep-luc (p1807), p(−95)lep-luc (p1583), p(−85)lep-luc (p1805), p(−67)lep-luc (p1585), p(−52)lep-luc (p1589), and p(−27)lep-luc (p1587) were constructed by digestion with *Hin*dIII and religation of plasmids m135, m109, m95, m85, m67, m52, and m27, respectively. PCR-generated regions were confirmed by sequencing.

The C/EBPα expression plasmid was provided by C. Vinson (17). Expression plasmids for C/EBPβ (p1607) and C/EBPδ (p1608) were constructed by insertion of an 850-bp *Eco*RI fragment of pMEX-CRP2 (C/EBPβ) or a 850-bp *Bam*HI fragment of pMEX-CRP3 (C/EBPδ) with *Hin*dIII linkers (18) into *Hin*dIII-digested pRc/cytomegalovirus (CMV; Invitrogen, San Diego, CA).

Transient expression

Transient expression in primary rat adipocytes (CD strain, Charles River Laboratories, Wilmington, MA) (12, 19) and luciferase (Promega Luciferase Assay System) and chloramphenicol acetyltransferase (CAT) assays (20) were performed as previously described. Two independent clones were assayed for each construct. Rous sarcoma virus (RSV)-CAT

was used as an internal control. Results are expressed as a percentage of the activity of p(−762)lep-luc in the same experiment (*e.g.* 100 × (luciferase$_{exp}$/CAT$_{exp}$)/(luciferase$_{p(-762)lep-luc}$/CAT$_{p(-762)lep-luc}$) and and are the mean ± SEM of the indicated number of experiments performed in duplicate or triplicate. Results have been normalized to the number of moles of plasmid transfected. To avoid cell breakage, manipulations were performed more gently with adipocytes from *fa/fa* Zucker rats. For example, only gentle shaking every 15 min was used during the collagenase digestion. The electroporation protocol uses a constant volume of cells; thus, the cell number of *fa/fa* Zucker adipocytes transfected was smaller.

HeLa cells were transiently transfected using Lipofectamine (Life Technologies, Grand Island, NY) as described by the manufacturer. The internal control was pRL-CMV (Promega), and the dual luciferase assay system (Promega) was used.

Electrophoretic mobility shift analysis

Electrophoretic mobility shift assays were performed as previously described (21, 22) except for the following. Adipose cells were lysed (23) (without the Polytron), and nuclei and nuclear extracts were prepared (24). Protein concentrations were determined (Bio-Rad Protein Assay, Hercules, CA), and binding reactions were performed in 25 mM HEPES (pH 7.5), 16 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 2 μM $ZnCl_2$, 1 mM dithiothreitol, 40 μg/ml BSA, 0.01% Nonidet P-40, and 8% glycerol. Electrophoresis in 4% or 6% polyacrylamide gels used 0.5 × TBE. Polyclonal antisera to Sp1, Sp3, and Sp4 (1 μg; Santa Cruz Biotechnology, Santa Cruz, CA) was added after the DNA and then incubated for 60 min (4 C) before electrophoresis. Figure 4 describes the wild-type oligonucleotide sequence and mutations used in the C/EBPα binding assays. Other oligonucleotides are (only one strand is shown): aP2 (25) (x312/x313), 5′-AACCAAAGTTGAGAAATTTCTATTAAAAAC; wt95 (x314/x315), 5′-GCCCGCTGGGTGGGGCGGGAGTTGGCGCTC; m95 (x267/x271), 5′-GCCCGCTGGGTGaaGCttGAGTTGGCGCTC; wt85 (x414/x415), 5′-AGTTGGCGCTCGCAGGGACTGGGGCTGGCC; wt85a (x490/x491) 5′-GGGGCGGGAGTTGGCGCTCGCAGGGACTGG; and m85 (x408/x409), 5′-GGGGCGGGAGTTaagctTCGCAGGGACTGG. Methylation interference analysis was performed essentially as previously described (13).

Results

Identification of leptin regulatory regions

To identify the DNA elements contributing to leptin expression, a series of reporters with varying amounts of 5′-sequence from the murine gene was constructed. These plasmids were tested for activity by transient expression in primary rat adipocytes (Fig. 1). Seven plasmids containing between about 6700 and 109 bp of 5′-sequence showed a 2.2-fold range in reporter activity. More dramatic decreases in promoter activity were observed on deletion from −109 to −95, from −85 to −67, and from −67 to −52. Deletion from −52 to −27 may also decrease expression, but this could not be assessed due to the already low activity of the −52 deletion. These data are consistent with small, but significant, effects on expression from regions upstream of −109 and identify three promoter regions between −109 and the TATA motif that contribute strongly to promoter activity.

Another potential source of leptin regulation is the RNA structure itself: A small fraction of leptin RNAs have an extra 93-bp exon in the 5′-untranslated region (12). When this exon was placed in its native configuration in the 5′-untranslated region of a leptin-luciferase construct, no effect on expression was seen. (Expression was 99 ± 19% (n = 4) that of p(−762)lep-luc, which does not contain the 93-bp exon but is otherwise identical.)

Leptin promoter point mutants

To define more accurately the specific bases contributing to proximal promoter function, a series of clustered point mutants was tested for promoter activity. In the regions implicated by the deletion mutants, sequences conserved

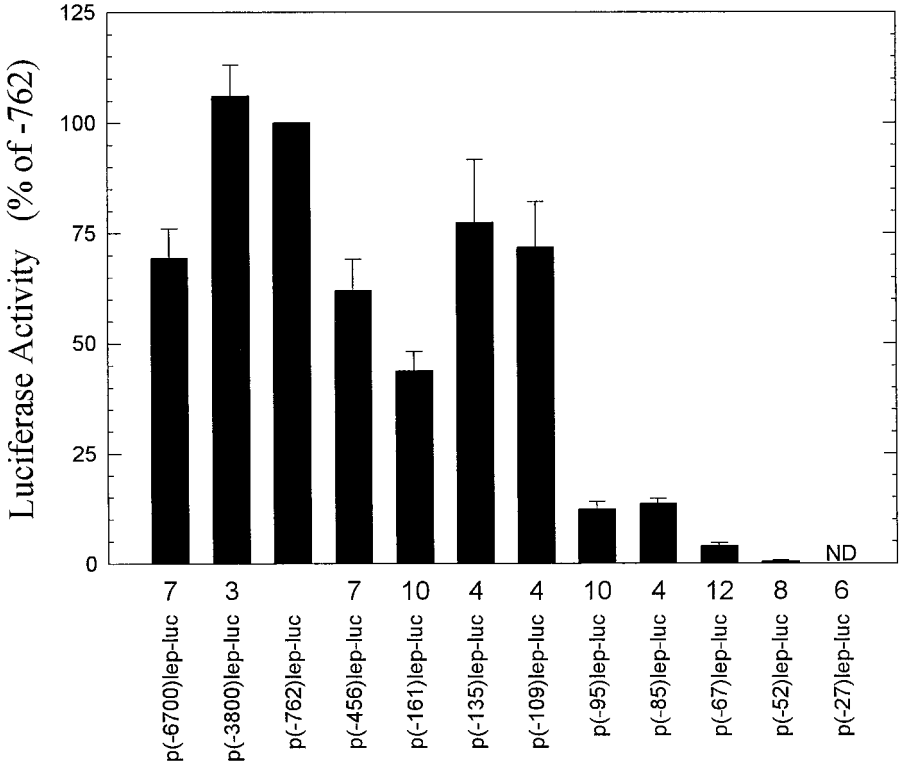


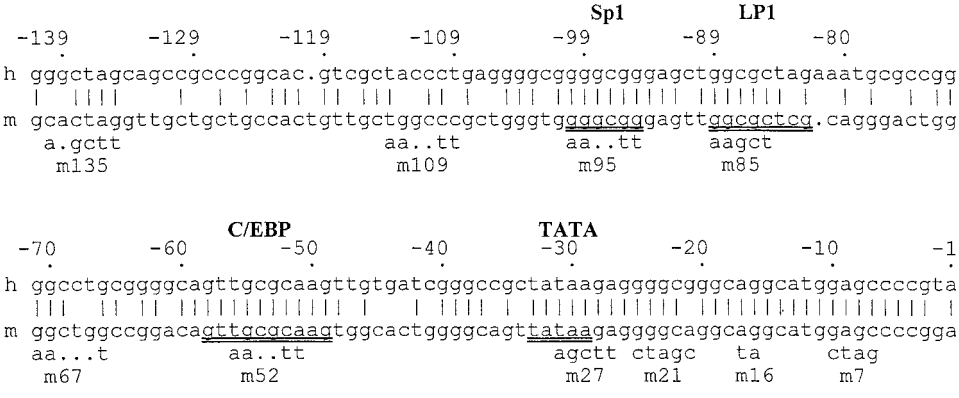
FIG. 1. Activity of leptin promoter deletions. Transient expression in primary adipose cells from CD rats was performed as described in *Materials and Methods*. Plasmids are named for the number of bases included upstream of the start of exon 1. Data are presented as a percentage of the activity of p(−762)lep-luc. *Bars* are the mean ± SE, with the number of independent determinations given *underneath*.

between mouse and human were chosen for mutation (Fig. 2). The region between the TATA and cap sites was strikingly conserved (more so than exon 1 or other promoter regions), but mutations in this region (m7, m16, and m21) did not have a large effect on promoter activity. Presumably our transient expression assay is insensitive to the conserved function(s) of this region. Mutations in two nonconserved regions (m67 and m135) served as controls and had little effect on promoter activity.

In contrast, mutants m27 and m52 had particularly severe effects on promoter performance, each decreasing activity approximately 10-fold. The TATA box is mutated in m27, whereas a C/EBP motif is mutated by m52. A less drastic effect, a 2.5-fold drop in activity, was produced when a putative Sp1 motif (TGGGGCGGGA) was disrupted in m95. In m85, a 2-fold decrease in activity was caused by changing the conserved region centered at −87, hereafter denoted LP1. The LP1 sequence is not an obvious match to known transcription factor-binding motifs.

Transient expression in fa/fa Zucker adipocytes

To look for adiposity-mediated regulation of leptin expression, we transfected the leptin promoter constructs into adipose cells from *fa/fa* Zucker rats. These rats have a mutated leptin receptor (26), greatly increased adipose stores, and increased leptin RNA levels. Due to their larger size, fewer *fa/fa* cells are contained in the volume used for transfection. Consistent with the fewer number of cells, luciferase and CAT activities were proportionately lower in the *fa/fa* cells. To allow comparison between these two cell types, each assay included samples transfected with RSV-luc and CMV-luc. However, the RSV-luc and CMV-luc reporters were expressed at different levels in the *fa/fa* and CD cells. In Table 1, we present the leptin promoter activity normalized to



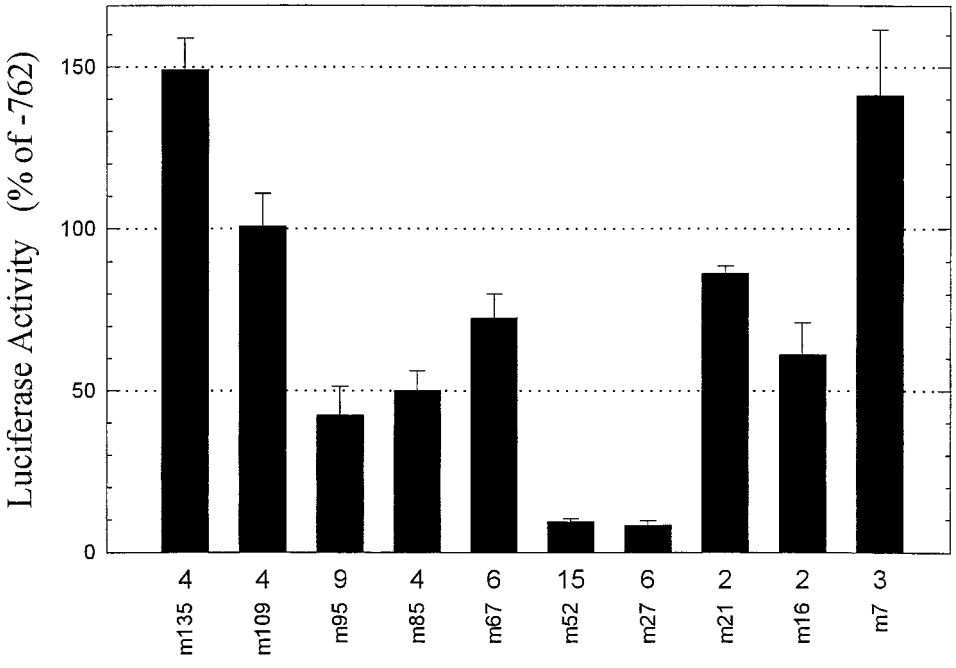


FIG. 2. Effect of leptin promoter point mutations. Comparison of the murine (m) and human (h) leptin promoters is shown at the *top*, with the Sp1, LP1, C/EBP, and TATA motifs labeled and *double underlined*. Shown *underneath* the murine sequence are the base changes made to create the indicated point mutants. Transient expression in primary rat adipose cells was performed as described in *Materials and Methods*. Data are presented as a percentage of the activity of the unmutated p(−762)lep-luc. *Bars* are the mean ± SE, with the number of independent determinations given *underneath*. The murine and human sequences are from GenBank files U36238 and U43589.

TABLE 1. Promoter activity in Zucker *fa/fa* adipocytes

Plasmid	Raw luciferase CD adipocytes (arbitrary units)	Raw luciferase *fa/fa* adipocytes (arbitrary units)	*fa/fa*::CD (to RSV-luc)	*fa/fa*:CD (to CMV-luc)
p(−762)lep-luc	22 ± 12	6.5 ± 1.9	1.98	7.20
RSV-luc	464 ± 112	87 ± 50	(1.00)	3.34
CMV-luc	6702 ± 2396	153 ± 81	0.35	(1.00)

Adipocytes from the indicated rats were transfected, and luciferase activity was measured (arbitrary units) in the same manner for all samples. Raw luciferase data (mean ± SEM; n = 3) are per μg transfected DNA, but have not been normalized to the internal RSV-CAT control. Due to the larger size of the *fa/fa* cells, 5- to 10-fold fewer cells were used for the *fa/fa* transfections. The ratio of *fa/fa*:CD luciferase activity was calculated after normalization to the RSV-CAT. For example, the p(−762)lep-luc *fa/fa*:CD to RSV-luc ratio was calculated as follows: $(\text{luciferase}_{fa/fa,\text{p(}-762\text{)lep-luc}}/\text{CAT}_{fa/fa,\text{p(}-762\text{)lep-luc}})/(\text{luciferase}_{\text{CD,p(}-762\text{)lep-luc}}/\text{CAT}_{\text{CD,p(}-762\text{)lep-luc}}) \times (\text{luciferase}_{\text{CD,RSV-luc}}/\text{CAT}_{\text{CD,RSV-luc}})/(\text{luciferase}_{fa/fa,\text{RSV-luc}}/\text{CAT}_{fa/fa,\text{RSV-luc}})$. RSV-luc is pRSV-L (57) and CMV-luc is pCIS2-luc (19).

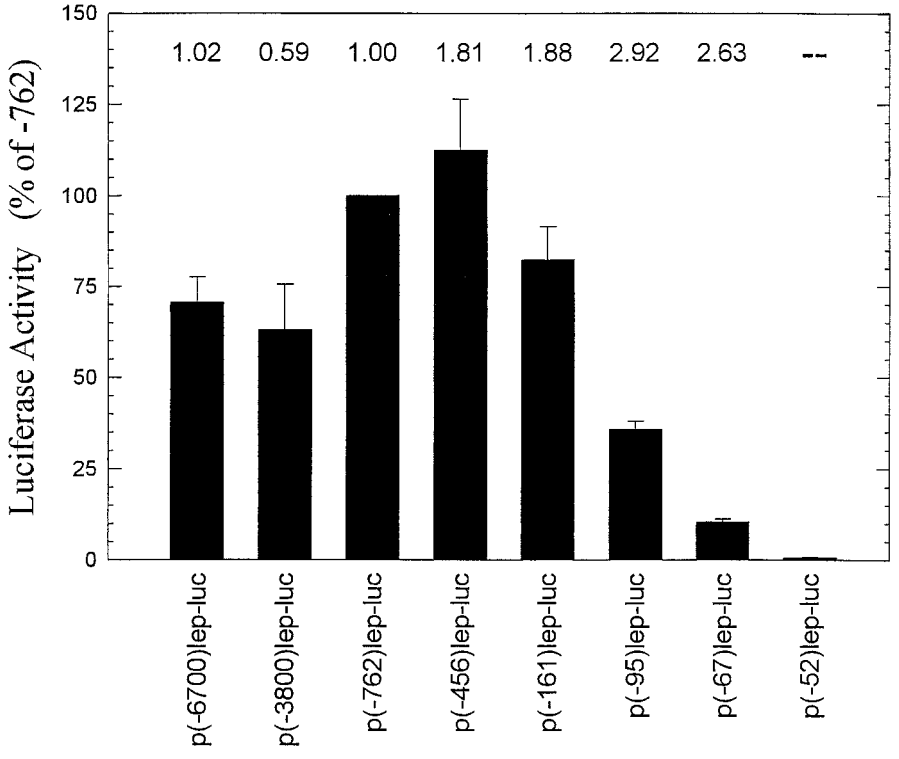


FIG. 3. Expression in *fa/fa* Zucker adipocytes. Transient expression in primary adipose cells from *fa/fa* Zucker rats was performed as described in *Materials and Methods* and Fig. 1. Data are presented normalized to the expression of p(−762)lep-luc in *fa/fa* adipocytes. The *number above each bar* is the ratio of activity in *fa/fa* Zucker to CD cells (setting the RSV-CAT-normalized $\text{luciferase}_{\text{fa/fa, p(}-762\text{)lep-luc}}$:$\text{luciferase}_{\text{CD, p(}-762\text{)lep-luc}}$ ratio equal to 1). The activity of p(−52)lep-luc was too low to calculate ratios accurately. Data are the mean ± SE of five experiments.

RSV-luc and CMV-luc, as it is not clear which is the appropriate choice. Leptin promoter activity was 2- or 7-fold higher in the *fa/fa* cells (depending on whether normalization was to RSV-luc or CMV-luc).

To look for specific elements causing the increased expression, we measured the activity of the promoter deletions in *fa/fa* cells (Fig. 3). The numbers at the *top* of Fig. 3 are the ratio of mutant promoter activity in *fa/fa* cells relative to that in CD cells, normalized so that the ratio of p(−762)lep-luc is 1, thus removing any assumptions about transfection efficiency or relative strength of the control reporter. The shorter promoters were expressed relatively better in *fa/fa* cells. This suggests that shorter promoters are sufficient for maximal activity in the *fa/fa* cells, whereas the CD cells need a longer promoter. To examine the role of the C/EBP and Sp1 motifs, the promoter activity of point mutations in these motifs was tested. Expression from plasmids m52 and m95 was reduced to 11.5 ± 1.8% (n = 5) and 31.9 ± 1.1% (n = 4) that of p(−762)lep-luc, respectively. Thus, individual C/EBP or Sp1 mutations in the context of the 762-bp promoter had similar effects in *fa/fa* Zucker and CD adipocytes. Taken together, these data suggest that the leptin promoter is transcribed more efficiently in *fa/fa* cells, with a disproportionate contribution from the proximal promoter (although neither the C/EBP nor the Sp1 motif contributes disproportionately).

Analysis of the C/EBP-binding region

We previously identified the leptin promoter C/EBP motif and reported that C/EBPα coexpression increased leptin promoter activity (12). We have now undertaken a detailed analysis of this motif. Binding of recombinant C/EBPα (17) to this region was studied using electrophoretic mobility shift experiments. The C/EBPα protein bound the leptin C/EBP motif with high avidity, comparable to that for the C/EBP site in the aP2 promoter (25) (Fig. 4a and data not shown). Mutations within the leptin promoter C/EBP motif reduced (m53) or abolished (m52) C/EBPα binding (Fig. 4a). Competition experiments confirmed these results and were consistent with a 10- to 100-fold reduction in C/EBPα bind-

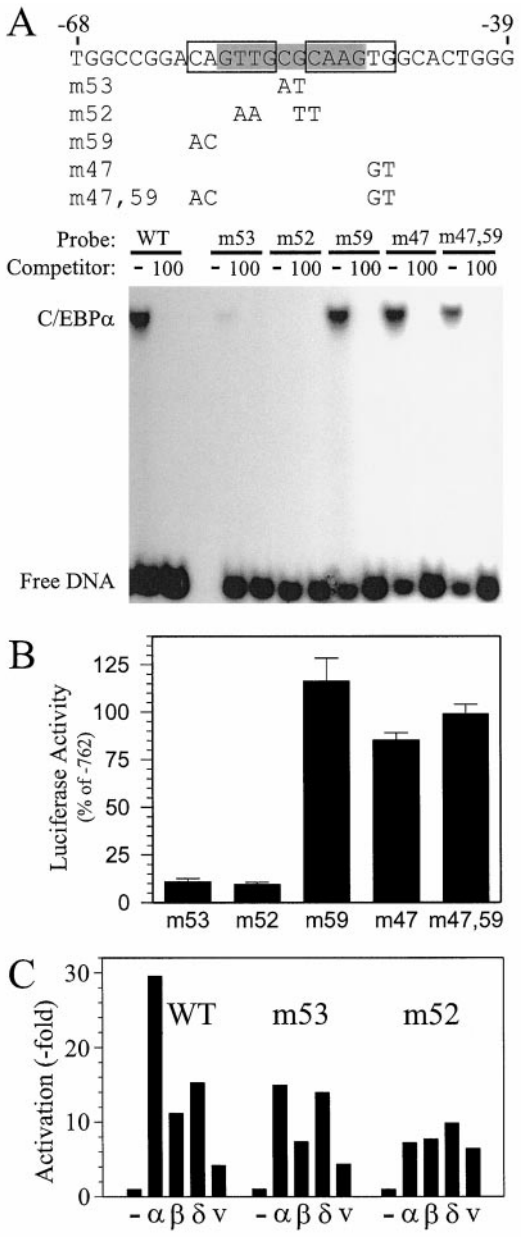


FIG. 4. Analysis of the C/EBP motif. A, Leptin promoter sequence (−39 to −68) showing the C/EBP motif (shaded) and two putative E box motifs (*boxed*). The base changes used to mutate only the C/EBP motif (m53), only the left E box (m59), only the right E box (m47), both E boxes (m47,59), and the C/EBP motif and both E boxes (m52) are indicated *beneath* the sequence. Electrophoretic mobility shift assays (see *Materials and Methods*) were performed with the indicated oligonucleotide probes (m53, m52, m59, m47, and m47,59), labeled with kinase to similar specific activities, using 210 pmol recombinant C/EBPα (17) and 25 fmol probe. Where indicated, a 100-fold molar excess of the unmutated competitor (WT) was included. B, Promoter activity of mutants in the C/EBP region. Mutations were introduced into the p(−762)lep-luc reporter and assayed for function in adipocytes from CD rats. Data are presented as a percentage of the activity of the unmutated p(−762)lep-luc. *Bars* are the mean ± SE, using results from 8, 15, 4, 4, and 4 (*left* to *right*) independent assays. C, *Trans*-activation by C/EBPα, -β, and -δ. The indicated reported vectors [m53, m52, or WT, which is p(−762)lep-luc] were cotransfected with expression vectors for C/EBPα (α), C/EBPβ (β), and C/EBPδ (δ) or with empty pRc/CMV expression vector (v) or pUC18 (−). Data are the average of two independent experiments.

ing by the m53 site (data not shown). Mutations abutting the C/EBP motif (m47, m59, and m47,59) had no effect on C/EBPα binding (Fig. 4a).

Transient expression experiments showed a correlation between C/EBPα binding and promoter activity (Fig. 4b), suggesting that C/EBP factors function at this site in cells. Two E box motifs (CAnnTG), similar to sites used to regulate genes important in metabolism (27), overlapped the C/EBP site. However, mutations of these E boxes (m47, m59, and m47,59) did not affect promoter activity.

We next examined the ability of two other C/EBP family members to *trans*-activate the leptin promoter. Cotransfection with C/EBPβ or C/EBPδ also stimulated transcription (Fig. 4c). Obliteration of the C/EBP site (m52) abolished *trans*-activation, whereas the mutant with a less severely mutated site (m53) could still be *trans*-activated, albeit at a reduced level and with a shifted dose-response curve (Fig. 4c and data not shown). Taken together, these data demonstrate that the C/EBP site is of fundamental importance for leptin promoter activity.

We also tested C/EBPα *trans*-activation of *leptin* promoter deletion constructs. Remarkably, p(−67)lep-luc (the minimal C/EBP and TATA promoter) was *trans*-activated about 800-fold, compared with approximately 25-fold for p(−762)lep-luc (12). Thus, with cotransfected C/EBPα, these two plasmids showed a similar absolute level of luciferase expression. The p(−67)lep-luc plasmid is one of the most C/EBP-responsive constructs known. The strong *trans*-activation by C/EBPα of p(−67)lep-luc suggests that upstream elements may modify C/EBPα action in the intact promoter.

Analysis of the Sp1-binding region

The site centered at −97 is an exact match to the Sp1 core motif sequence. To test for protein binding to this region, the binding of recombinant Sp1 and that of rat adipocyte nuclear extracts was examined. The recombinant Sp1 bound well to the −97 region, but less avidly than to the highest affinity Sp1 sites from the simian virus 40 promoter (Fig. 5, lanes 1–6), probably due to bases −93A (G binds better) and −92G (C or T bind better) (28). Electrophoretic mobility shift assays using adipocyte nuclear extracts showed a complex of the expected mobility for Sp1-DNA and faster migrating complexes (Fig. 5, lanes 7–14). Three independent nuclear extracts gave similar results. Mutation m95 abolished all binding to this region (Fig. 5, lane 15), as did competition with unlabeled wt95 DNA (not shown). To confirm that Sp1 was responsible for the slowest complex, antibody to Sp1 was used to specifically retard the mobility of Sp1-DNA complexes. Most of the putative Sp1-DNA complexes were indeed reactive with anti-Sp1 (Fig. 5, lanes 7–14) and not with other antibodies (to Sp3, Sp4, or chicken globin; data not shown). Taken together, these data suggest that Sp1 is the predominant protein binding to the −97 region of the leptin promoter and that the 2.5-fold reduction in expression in m95 is due to the loss of this factor's contribution.

Analysis of the LP1 site

As mutation of the conserved LP1 region at −87 of the leptin promoter resulted in a 2-fold drop in promoter activ-

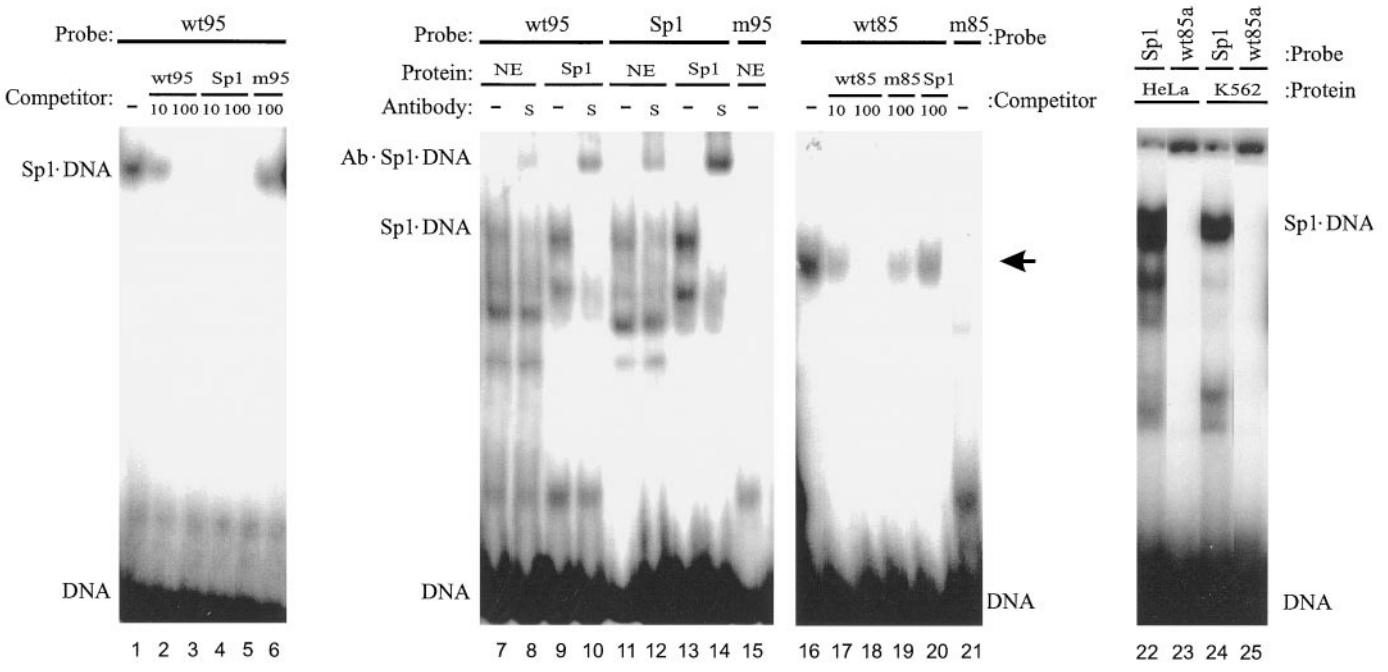


FIG. 5. Protein binding to the −87 and −97 regions. Electrophoretic mobility shift assays were performed with 25 fmol of the indicated oligonucleotide probes (labeled with kinase to similar specific activities; see *Materials and Methods*). Rat adipocyte nuclear extract (210 ng) was used in lanes 7, 8, 11, 12, and 15–21. Recombinant Sp1 (2.5 ng; Promega) was used in lanes 1–6, 9, 10, 13, and 14. HeLa nuclear extract (2.5 μg; Promega) was used in lanes 22 and 23. K562 nuclear extract (a gift from Adam Bell) was used in lanes 24 and 25. Where indicated, a 10- or 100-fold molar excess of unlabeled competitor was included. Preincubation with anti-Sp1 antibody (1 μl) was performed in lanes 8, 10, 12, and 14. The mobilities of free oligonucleotide (DNA) and Sp1-DNA and antibody-Sp1·DNA complexes are indicated.

ity, this region was examined for factor binding. Incubation of adipocyte nuclear extract with the wt85 oligonucleotide produced a protein-DNA complex (Fig. 5, lanes 16–21). Similar patterns were obtained with three independent nuclear extracts and with the corresponding region of the human leptin promoter. Complex formation was inhibited by unlabeled oligonucleotide, but not by an oligonucleotide containing an Sp1 motif. Mutation m85 abolished protein binding (Fig. 5, lane 21), and anti-Sp1 antibodies did not affect complex formation (data not shown). Weak binding was observed with nuclear extracts from undifferentiated 3T3-L1 preadipocytes, which was unchanged in differentiated 3T3-L1 adipocytes (data not shown). A protein-DNA complex was not seen when nuclear extracts from HeLa and K562 cells was used (Fig. 5, lanes 23 and 25). These data are consistent with an adipose specificity for binding to this site.

Binding at the LP1 site was examined further using methylation interference (Fig. 6). Methylation of residues at positions −81, −82, −83, −85, −86, −88, −89, and −90 relative to the cap site inhibited factor binding. This binding site (GGCGCTCGC) is not an obvious match to known consensus sequences.

Independent contributions of the Sp1, LP1, and C/EBP motifs to promoter activity

To examine interactions among the Sp1, LP1, and C/EBP sites of the leptin promoter, constructs containing pairwise mutations were assayed for activity. The promoter activity of each of the double mutants was lower than that of the constituent single mutants and approximated the level expected from the constituent single mutations (Table 2). These data suggest that the Sp1, LP1, and C/EBP sites contribute independently to promoter activity; one does not require another for function.

Lack of regulation by peroxisome proliferator-activated receptor-γ (PPARγ) and sterol response element-binding protein-1 (SREBP)

PPARγ is a steroid superfamily transcription factor that promotes differentiation into adipocytes (29). The thiazolidinedione class of antidiabetic drugs is made up of activating ligands for PPARγ (30). There are a number of reports that thiazolidinediones decrease leptin expression (31–35). We investigated the roles of PPARγ and thiazolidinediones in leptin transcription using transient expression in rat adipocytes. Luciferase activity of p(−762)lep-luc and m52 was not significantly changed in the presence of the thiazolidinedione ligand BRL49653 at 10 μM (127% and 115%, respectively, of the untreated control value; mean of two experiments). Cotransfection with PPARγ and retinoid X receptor-α (RXRα), its dimerization partner, either with or without BRL49653 was also performed (Table 3). Two PPARγ-responsive reporters showed high basal levels of expression. These data suggest that the adipocytes have high levels of endogenous factors acting via the PPAR (DR+1) motif. Inclusion of PPARγ/RXRα (with or without BRL49653) did not alter expression of the leptin reporters, but, as expected, did increase expression of thymidine kinase-PPREx3-Luc control. Similar results were obtained in HeLa cells (Table 3). In these transient expression systems,

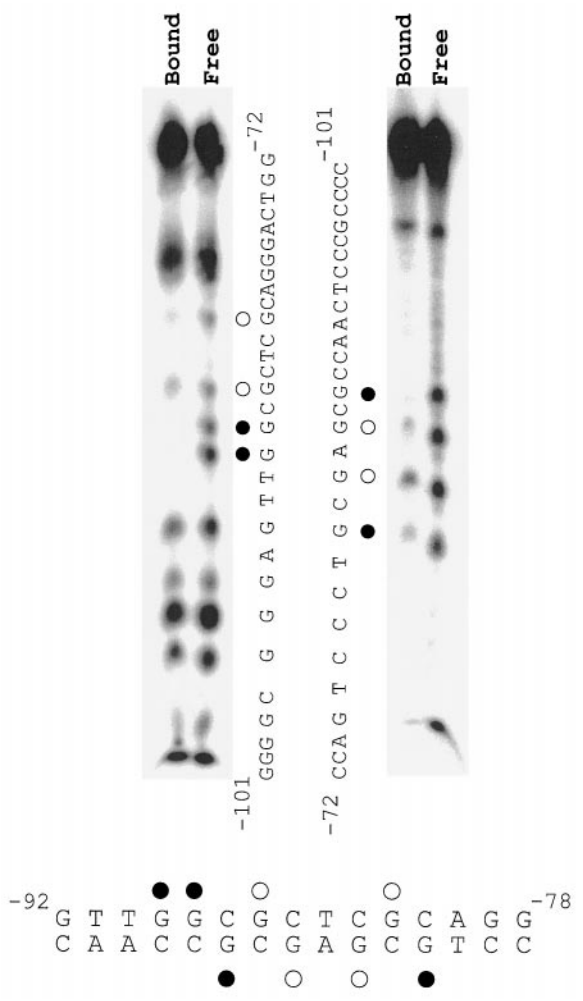


FIG. 6. Methylation interference analysis of the −87 region. Electrophoretic mobility shift using a partially methylated DNA probe (wt85a) was performed, then the methylation pattern of the free and bound DNA was determined. Bases whose intensity was reduced in the bound fraction by more than 50% but less than 85% are indicated (○) as are those reduced more than 85% (●).

TABLE 2. Promoter activity of double mutants

Plasmid	Motifs mutated	Luciferase activity	
		Mean ± SEM (n)	Calculated
m52	C/EBP	7.6 ± 0.9 (5)	
m85	LP1	51.3 ± 3.4 (7)	
m95	Sp1	33.6 ± 2.5 (5)	
m52,85	C/EBP, LP1	2.5 ± 0.5 (6)	3.9
m52,95	C/EBP, Sp1	3.3 ± 0.5 (6)	2.6
m85,95	LP1, Sp1	15.4 ± 1.6 (4)	17.2

Transient expression in primary adipose cells from CD rats was performed as described in *Materials and Methods*. The calculated activities were obtained by multiplying the activities of the constituent single mutants (*e.g.* for m52,85: 3.9% = 7.6% × 51.3%).

we did not find a major role for a thiazolidinedione or for exogenous PPARγ in the regulation of leptin.

SREBP-1, also known as adipocyte determination- and differentiation-dependent factor 1 (27, 36), is an important transcriptional regulator of cholesterol metabolism and adipocyte genes. Under high cholesterol conditions it is membrane anchored and inactive, but at low cholesterol levels it is proteolytically cleaved, releasing an active transcription factor (36, 37). Coexpression of SREBP did not affect leptin transcription, but greatly increased expression of the positive control (Table 4). Expression of the activated transcription factor protein, SREBP-(1–410), increased leptin expression about 2-fold, but also increased RSV-CAT expression slightly while massively increasing expression of the positive control. Thus, in this system, SREBP is not a major regulator of leptin expression.

Discussion

Three types of mechanisms are likely to control leptin RNA levels

First, leptin production is tissue specific, implicating tissue-selective transcription factors. Second, hormonal and metabolic regulators influence leptin RNA levels via paracrine or endocrine (noncell autonomous) routes. Third, it is believed that the leptin RNA content in each cell reflects its adiposity. For example, larger fat cells have more leptin RNA than smaller ones (38). This suggests that a cell autonomous mechanism(s) exists for sensing adiposity and converting the signal to regulation of leptin RNA levels.

To examine these mechanisms, it is necessary to understand the leptin promoter. We show that a 109-bp promoter is as effective as longer promoters in directing leptin transcription in transient expression assays. Four elements in the proximal 109 bp contribute to leptin promoter activity: the TATA box at −30, a C/EBP motif at −53, the LP1 region at −87, and an Sp1 motif at −97. The data are consistent with a small effect on adipose expression of more distant regions. No distant elements with a large effect on adipose expression have been identified, although a placental enhancer is found upstream of the human leptin promoter (39). It seems plausible that a distant element(s) with a large effect on adipose expression also exists.

PPARγ ligands have been shown to have a small negative influence on endogenous leptin expression. In transient expression assays, a slight decrease in leptin expression by PPARγ ligands has been observed (34, 35). Our inability to see this effect could be due to the small magnitude of the effect, subtle differences between the reporter plasmids, or other differences between the model systems.

C/EBPα regulation of the leptin promoter

C/EBPα is a basic region/leucine zipper transcription factor important for the transcription of most adipocyte genes and of other genes involved in energy metabolism (40). Before adipocyte differentiation, C/EBPα, -β, and -δ levels are low. During differentiation, first C/EBPβ and -δ rise transiently, and then C/EBPα levels rise and remain high in the mature adipocyte (41). Forced expression of C/EBPα promotes adipogenesis (42, 43), and mice with a nonfunctional C/EBPα gene do not deposit lipid in their adipose tissue (40).

Since the suggestion that C/EBPα stimulated leptin expression via the −53 motif (12), supporting evidence has come from a number of studies (15, 16, 34, 44). Here we have expanded these observations by showing a correlation between C/EBP binding affinity to the −53 site and the degree

TABLE 3. Effects of PPARγ and BRL49653 on leptin expression

Additions:	Luciferase activity		
	None	PPAR/RXR	PPAR/RXR, BRL
Rat adipocytes			
Reporter plasmid			
p(−762)lep-luc	100	74 ± 13 (5)	79 ± 16 (4)
p(−6700)lep-luc	82 ± 11 (4)	64 ± 11 (4)	93 ± 8 (3)
m52	7.9 ± 1.2 (4)	6.9 ± 2.6 (4)	9.7 ± 2.5 (3)
tk-PPREx3-Luc	362 ± 107 (3)	347 ± 65 (3)	1135 ± 681 (2)
AcoGLuc	2130 ± 478 (3)	1615 ± 631 (3)	2782 ± 641 (2)

Additions:	None	BRL	PPAR	PPAR, BRL
HeLa cells				
Reporter plasmid				
p(−762)lep-luc	100	100 ± 23 (3)	117 ± 31 (3)	109 ± 26 (3)
p(−6700)lep-luc	128 ± 17 (3)	109 ± 11 (3)	267 ± 104 (3)	109 ± 21 (3)
tk-PPREx3-Luc	774 ± 277 (3)	921 ± 233 (3)	1112 ± 224 (3)	4774 ± 1059 (3)

Values are the mean ± SEM (n). Transient expression in primary adipose cells from CD rats was performed using 2 μg (leptin constructs) or 0.1 μg (tk-PPREx3-Luc, ACo-G-Luc) reporter and 2 μg of each of the expression plasmids (PPARγ and RXRα) as described in *Materials and Methods*. Transient expression in HeLa cells used 250 ng reporter and 10 ng PPARγ expression plasmid. Luciferase activity was corrected for transfection efficiency (using the internal RSV-CAT control in rat adipocytes or pRL-CMV in HeLa cells) and then normalized to p(−762)lep-luc. Where indicated, the thiazolidinedione BRL49653 (BioMol) was included at 10 μM. The tk-PPREx3-Luc [R. Evans (58)] and ACo-G-Luc [K. Ozato (NIH), derived from ACo-G-CAT (59)] are plasmids known to be PPARγ responsive. To ease comparison, the tk-PPREx3-Luc and ACo-G-Luc results have been adjusted to 2 μg plasmid. The PPARγ plasmid is PPARγ2-SPORT [B. Spiegelman (60)], and the RXR plasmid is pCMX-hRXRα [*Kpn*; R. Evans (61)].

TABLE 4. Effect of SREBP on leptin expression

Expression plasmid:	Reporter activity		
	None	SREBP	SREBP-(1-410)
Reporter plasmid			
p(−762)lep-luc	100	110 ± 9	212 ± 30
p(−6700)lep-luc	96 ± 13	96 ± 8	167 ± 31
LDL-CAT	<1	23 ± 4	17 ± 1
RSV-CAT	100	139 ± 4	156 ± 18

Transient expression in primary adipose cells from CD rats was performed using 2 μg reporter (except 4 μg LDL-CAT) and 4 μg expression plasmid, as described in *Materials and Methods*. Within each assay, the luciferase results were normalized to p(−762)lep-luc, and the CAT results were normalized to RSV-CAT. Results are the mean ± SEM of three independent experiments (each in quadruplicate). SREBP is hamster SREBP-1 (American Type Culture Collection no. 87012) (37), and SREBP-(1-410) is an activated form of hamster SREBP-1 [M. Brown and J. Goldstein (37)]. LDL-CAT [M. Brown and J. Goldstein, their p1471 (36)] is a reporter plasmid known to be responsive to SREBP.

of *trans*-activation. Furthermore, we demonstrate that mutation of adjacent nucleotides has no effect, and that C/EBPβ and -δ, in addition to α, can *trans*-activate via this motif. These results suggest that the −53 C/EBP motif contributes to the tissue-specific expression of the leptin gene. As C/EBPα is the predominant C/EBP family factor in mature adipocytes, it is likely that *in vivo* this factor acts at this C/EBP site. However, there are at least eight C/EBP-related proteins (45), so it is possible that other family members also function at this site *in vivo*.

Sp1 regulation of the leptin promoter

The site at −97 of the leptin promoter is conserved in evolution, binds Sp1 present in adipocyte nuclear extracts, and contributes to promoter activity. Although these data cannot rule out the possibility that other C2H2 transcription factors might also act at this site, the simplest interpretation is that Sp1 is *trans*-activating the leptin promoter via this motif.

In a hepatocyte cell line cotransfected with C/EBPα, de la Brousse *et al.* (16) did not observe a decrease in promoter activity upon deletion of the Sp1 site. When we cotransfected C/EBPα in adipocytes, we obtained similar results. However, in our experiments without C/EBPα cotransfection, we saw a decrease in activity upon either deletion of this region or point mutation of the Sp1 motif. These data suggest that overexpression of C/EBPα obscures the contribution of the Sp1 element to leptin transcription.

Other promoters [*e.g.* GLUT4 (46), CYP2D5 (47), and C/EBPα (48, 49)], like leptin, are regulated via both C/EBP and Sp1-like motifs. However, none of these appears similar enough to the leptin promoter to allow inferences about its regulation.

Regulation of the leptin promoter by the LP1 region

The sequence of the −87 region of the leptin promoter is conserved between mouse and human, suggesting that this site is functional. Indeed, mutation of the region caused a decrease in expression, and this site bound a factor present in preadipocytes and adipocytes but not in other cell types. The binding motif does not match that of other known transcription factors. Thus, the data suggest that the LP1 region binds a novel *trans*-activating factor that is present in adipose cells but not in the other cells examined.

Regulation of leptin expression by adiposity, metabolites, and hormones

Transiently expressed leptin reporters showed increased activity in *fa/fa* Zucker adipocytes. These data are consistent with cell autonomous regulation of leptin expression by increased adiposity. Higher levels of promoter activity in *fa/fa* adipocytes have been observed for other genes [*GLUT4* (50)

and *GAPDH* (51)]. The increased fatty acid synthetase expression in *fa/fa* adipocytes is due to inhibition of expression in lean cells by a factor binding to a Sp1 site (52). Our data are not consistent with such a mechanism for regulation of the leptin promoter. Indeed, other than an increased contribution to leptin expression by the proximal promoter, we have not been able to identify specific sequence motifs involved in the increased expression in *fa/fa* adipocytes.

The identification of three functional motifs in the leptin promoter raises an obvious question. Does regulation of leptin expression by hormones and metabolites occur via modification, in amount or activity, of the factors that bind to these sites? One hint that it may is the observation that expression and phosphorylation of C/EBP family members is regulated by both glucocorticoids and insulin (53, 54). We do not yet know whether the increase in leptin expression by these hormones is accomplished by modification of C/EBPα expression or activity.

Sp1, another potential target for regulatory cascades, is also a phosphoprotein. Increased Sp1 phosphorylation has been reported to facilitate transcription (55), whereas Sp1 dephosphorylation has been reported to increase its binding affinity for DNA (56). The factor(s) binding to the LP1 motif may also be a target of the regulatory signals converging on the leptin promoter.

The elucidation of the functional DNA elements of the leptin promoter and their cognate transcription factors presented here is a significant step toward a detailed understanding of the transcriptional regulation of the *leptin* gene.

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