Identification of a Placental Enhancer for the Human Leptin Gene

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Leptin is a hormone that regulates metabolic efficiency, energy expenditure, and food intake. Leptin is produced chiefly in adipose cells, but in humans, mRNA encoding leptin is also present in the placenta. Here we elucidate the basis for placental leptin production. The same promoter is used for adipose and placental transcription. An upstream enhancer functions in the JEG-3 and JAR choriocarcinoma cell lines but not in adipocytes or HeLa cells. The minimal positive acting region is 60 base pairs in length. This region is within a MER11 repetitive element, suggesting that human placental expression of leptin is the result of insertion of this element. Binding analyses demonstrated three protein binding sites, designated placental leptin enhancer elements (PLE1), PLE2, and PLE3. PLE2 binds Sp1. Enhancer activity was reduced by mutation of the PLE1 or PLE3 sites but was unaffected by alteration of PLE2. Proteins binding to PLE3 were present in JEG-3 and human placental nuclear extracts but not in extracts from non-placental sources. Upon triplication, the PLE3 element was a strong enhancer in choriocarcinoma cells but not in HeLa cells. The protein binding to the PLE3 motif appears to be a novel, placenta-specific transcription factor.

A number of laboratories have studied the regulation of the leptin gene in adipose cells. Adipose leptin mRNA levels are increased by glucocorticoids (15–17) and by insulin (18–20) and decreased by β-adrenergic agonists (17, 21). Three motifs in the leptin promoter, in addition to the TATA box, contribute to leptin transcription: a C/EBP site at –55 (22–26), a site at –87, and an Sp1 motif at –97. The observation that leptin RNA is also made by the human placenta (27) motivated us to examine regulation of leptin transcription in this tissue.

Experimental Procedures

Northern Blot and 5′-Rapid Amplification of cDNA Ends—Northern blots were hybridized (Rapid-hyb, Amersham Corp.) using probes (leptin, bp 463–3426 in GenBank™/EBI accession number U43653, or β-actin; labeled by random priming) and washed twice (0.5 × SSC, 20 min, 65 °C). 5′-RACE was performed using a commercial kit and human placental cDNA (CLONTECH). After amplification, the products were cloned and sequenced.

Cell Lines—JEG-3 (ATCC HTB-36) and HeLa cells (ATCC CCL-2) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Life Technologies, Inc.). JAR cells (ATCC HTB-144) were grown in Waymouth’s MB 752/1 medium (ICN) supplemented as above.

Transient Expression—The luciferase reporter constructs are based on pGL3-basic or pGL3-promoter (Promega) and are shown schematically in Figs. 2–4, 7, and 8. p1774 and p1779 were previously named pGL3/3kb(+) and pGL3/0.3kb(+), respectively (28). Cloning details are available from the authors. Transient expression using electroporation in primary rat adipocytes was performed as described (29). For JEG-3, JAR, and HeLa cells, typically 100 ng of luciferase reporter, 5 ng of pRL-CMV internal control construct (Promega), and carrier plasmid DNA to 1 μg were transfected using 5 μl of LipofectAMINE (Life Technologies, Inc.). The medium was replaced after 5 h, and the cells were harvested 24 h later using 250 μl of passive lysis buffer (Promega). Luciferase activity was measured (Dual-Luciferase Reporter Assay System, Promega) and normalized to the internal control. Transfections were performed in duplicate. Data are the mean ± S.E., usually from three to five experiments.

Electrophoretic Mobility Shift and Methylation Interference Analyses—Binding reactions containing 3–5 μg of nuclear extract protein (30), 20–40 fmol of kinase-labeled probe, 10 mm Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, and 1 μg of poly(dI:dC) were incubated (15 min, 23 °C) and electrophoresed. Competitor oligonucleotides were added prior to the addition of nuclear extract. Polyclonal antiserum to Sp1 (2 μg, Santa Cruz Biotechnology) was added after the probe and incubated for 60 min at 4 °C before electrophoresis. Methylation interference analysis was performed essentially as described (31).

The following double-stranded oligonucleotides were used in gel mobility shift assays (a lowercase letter indicates an introduced mutation): PLE1, CAGTACCCCTAGGCTTTACAGGTGGTGAAAGCTC; PLE2, AGGGT-GGTGAAAAACTCCGGCCTGTTAATTTTGTGG; mPLE2, AGGGTG-GGTGAAAAACTCCGGCCTGTTAATTTTGTGG and mPLE1, CAGTACCCCTAGGCTTTACAGGTGGTGAAAGCTC; PLE2, AGGGT-GGTGAAAAACTCCGGCCTGTTAATTTTGTGG; mPLE2, AGGGTG-GGTGAAAAACTCCGGCCTGTTAATTTTGTGG and mPLE1, CAGTACCCCTAGGCTTTACAGGTGGTGAAAGCTC.

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3 The abbreviations used are: RACE, Rapid amplification of cDNA ends; MER, medium reiteration frequency repeat; PLE, placental leptin enhancer; bp, base pairs; kb, kilobase(s).
RESULTS

Leptin Expression in Human Placenta—Leptin expression in adipose tissue (both white and brown) has been reported in a number of species. The only other tissue identified as having significant amounts of leptin mRNA is human placenta (27). However, we did not detect leptin mRNA in mouse placenta, so a Northern blot was performed to confirm that human placenta contains leptin mRNA (Fig. 1). Leptin RNA was detected in human placenta at a level roughly 100-fold lower than that in white adipose tissue, 4 kb. Additional confirmation that leptin is expressed in placenta came from the expressed sequence tag database, which contains leptin cDNAs in libraries from both 8–9 week and term placentas.

Having established that human placenta contains leptin mRNA, we performed 5' RACE to determine if placenta and adipose tissue use the same promoter. All six clones extending upstream of exon 2 agreed with the reported sequence for exon 1 from adipose tissue (data not shown). Thus, the same promoter is used for placental and adipose expression.

Leptin Promoter and Enhancer Activity in Human Placental Cell Lines—To search for DNA elements important for placental expression, reporter constructs containing from 218 to 2922 bp of 5' flanking genomic DNA were used in transient expression assays. Expression of these leptin promoter-luciferase reporters was studied in four cell types: primary rat adipocytes, HeLa cells, and the choriocarcinoma cell lines JEG-3 (32) and JAR (33). In each case, all constructs directed transcription at higher levels than the promoterless pGL3-basic vector (Fig. 2). Intriguingly, when the 5' flanking region was lengthened from 21546 to 21951, expression increased significantly in the choriocarcinoma cell lines but not in adipose and HeLa cells (Fig. 2). These data suggest that the 100-bp region from 1946 to 1847 upstream of the promoter is sufficient for partial leptin enhancer activity in JEG-3 cells.

Electrophoretic mobility shift assays were used to identify the sequence elements and proteins responsible for the enhancer activity of the 100-bp DNA fragment. At least two distinct DNA-protein complexes were identified, one of which was competed by an oligonucleotide corresponding to a region of human leptin DNA containing the 100-bp enhancer fragment.

Identification of the Enhancer Elements—To identify the boundaries of the enhancer, deletion constructs were tested for enhancer activity (Fig. 3). Deletions from −1546 to −1951, −1951 to −1546, or −1847 reduced, but did not abolish, luciferase expression. In contrast, any deletion of the 5' end resulted in complete loss of enhancer activity. Thus, the 100-bp region from 1946 to 1847 bp upstream of the promoter contains a placenta-selective enhancer.

Next, the 400-bp region was tested directly for enhancer activity using the heterologous SV40 promoter to drive luciferase. In JEG-3 cells, this DNA caused 8–9-fold enhancement, independent of its orientation, at a distance of 2.1 kb from the promoter (Fig. 3). The 400-bp region could not increase expression in the absence of a promoter and did not have promoter activity itself. These data demonstrate that the DNA from 1951 to 1546 bp upstream of the leptin promoter contains an enhancer.
hancer activity. Four overlapping oligonucleotides (designated PLE1, PLE2, PLE3, and oligo D) were used to screen the 100-bp region. With nuclear extracts from JEG-3 cell and human placenta, protein binding was detected to PLE1, PLE2, and PLE3 but not to oligo D (Fig. 5A). Nuclear extracts from HeLa cells showed abundant binding to PLE2, slight binding to PLE1, and none to PLE3. These data are consistent with the hypothesis that the proteins binding to PLE3, and possibly PLE1, are placenta-specific. Additional support comes from the observation that no PLE3 binding activity was detected by mobility shift assays using nuclear extracts from rat liver, undifferentiated 3T3-L1 preadipocytes, differentiated 3T3-L1 adipocytes, rat adipocytes, or erythroid K562 cells (data not shown).

Since the PLE2 oligonucleotide contains a canonical Sp1 sequence (CCGCCC; Ref. 34), we tested whether Sp1 is responsible for the observed protein binding to PLE2. An oligonucleotide with an authentic Sp1 site gave the same pattern as PLE2 upon protein binding (Fig. 5A). Mutation of the Sp1 motif in PLE2 (mPLE2) abolished protein binding to PLE2 (Fig. 5A). Finally, antibodies to Sp1 caused a decreased mobility of the protein-PLE2 complex (Fig. 5B). Thus, Sp1 is able to bind the PLE2 sequence and appears to account for most of the binding activity to PLE2 in JEG-3 cells.

To identify specific residues in the PLE1 and PLE3 binding sites, methylation interference analysis was performed. Methylation of guanines at positions -1946, -1943, -1942, -1941, -1939, -1937, -1936, and -1935 in PLE1 and at -1994, -1992, -1988, and -1887 in PLE3 reduced binding to these elements (Fig. 6A). This information was used to design mutations of the PLE1 and PLE3 binding sites (Fig. 6B), which were incorporated into oligonucleotides (mPLE1 and mPLE3, sequences shown under “Experimental Procedures”). As expected, these mutant oligonucleotides were unable to form the PLE1 and PLE3 complexes (not shown) and were unable to compete for protein binding to the unmutated sites (Fig. 6C). Binding to PLE1 was not competed by PLE2, PLE3, or Sp1. Binding to PLE3 was not competed by PLE1, PLE2, or the following binding sites for transcription factors known to regulate placental genes: Sp1 (35), AP1 and CREB (36–39), thyroid response element (40), Pit-1 (41, 42), C/EBP (43, 44), DF3 and DF4 (45), FREAC (46), or GATA (39, 47) (Fig. 6C, and data not shown). Taken together, these results demonstrate that the leptin enhancer contains three independent protein-binding sites: an Sp1 site (PLE2) and two novel placenta-selective binding sites, PLE1 and PLE3.

Contribution of Individual Elements to Leptin Enhancer Activity—Since no protein binding to the 3' 40 bp of the 100-bp enhancer was detected, a 60-bp enhancer comprising the 5' end was tested for enhancer activity by transient expression in JEG-3 cells. Luciferase activity was normalized to pGL3-promoter. Mutations of the Sp1 site at PLE2 had no effect on activity (Fig. 7). However, mutation of PLE1 reduced enhancement by 56%, and mutation of PLE3 abolished
enhancer activity. Similarly, constructs containing only PLE1 (p1870) or PLE3 (p1871, p1877) did not have enhancer activity. Triplication of the PLE3 motif created a construct which showed 32-fold enhancement. Thus, as measured in JEG-3 cells, the PLE3 motif is essential for enhancer activity and when multimerized is a strong enhancer.

To examine the tissue specificity of the leptin enhancer, the reporter plasmids were tested for activity in four different cell types (Fig. 8). Transient transfection expression experiments confirmed that the enhancer works in the choriocarcinoma cell lines (JEG-3, JAR) but not in adipocytes or HeLa cells. The triplicated PLE3 element stimulated transcription in JEG-3 and JAR, but not in HeLa cells. These results suggest that the enhancer and the transcription factor binding to the PLE3 site are placenta-selective.

**DISCUSSION**

Leptin is expressed predominantly in adipocytes where its production correlates with the degree of adiposity. However, leptin is also made by the placenta (27). We identified an enhancer located 1.9 kb upstream of the human leptin gene. It contains three protein binding elements, PLE1–3, and transient expression experiments demonstrate that the enhancer works in choriocarcinoma lines but not in adipose or HeLa cells. The proteins binding to the PLE3 motif and possibly to the PLE1 motif may be placenta-specific and contribute to the
activity of the enhancer in JEG-3 cells. Sp1 binds at the PLE2 motif but does not contribute to enhancer activity in these cells. We concentrated on PLE3 which, when triplicated, constitutes a strong enhancer. Placental enhancers have been identified for the chorionic somatomammotropin-B (45) and glycoprotein hormone α-subunit (48) genes. However, the PLE3 motif does not match the sequence elements (CRE (36), GATA (39), Pit-1 (41, 42), DF3 and DF4 (45), among others) implicated in transcription of these and other placental genes, suggesting that the protein binding to the PLE3 motif is a novel, placenta-specific transcription factor. Verification will require cloning of the transcription factor and analysis of its expression pattern.

The enhancer is located within a MER11 (medium reiteration frequency repeat) element (28). MER11 elements (which can be at least 1100 bp in length) are found 1500–3000 times in the human genome, but are not present in the murine genome (49). This observation provides an explanation of why the human, but not the murine, placenta expresses leptin mRNA. The MER11 insertion introduced DNA elements in a configuration that allowed interaction with the leptin promoter. (This hypothesis can be tested using phylogenetic analysis, scoring placental leptin expression, and the presence of the upstream MER11. Since the MER11 upstream of the leptin gene is only 94% identical to a consensus MER11, it is likely that the insertion event occurred at least several million years ago.) The high degree of sequence similarity between the leptin enhancer and other MER11s suggests that the other MER11s have the potential to be placental enhancers but require an accessible, compatible promoter for realization of this function. For example, 1.2 kb upstream of the human P450C17 gene (50) is a MER11 that matches the leptin PLE1–3 motifs exactly, yet the P450C17 gene is not expressed in the placenta (51). Insertion of repetitive elements in germline DNA is one of the major forces in evolution. Presumably, most insertions do not affect gene expression although sometimes insertion will disrupt a gene (52). Least commonly, introduction of a repetitive element results in a gain of function (53) as apparently is the case with the human leptin enhancer. Thus, human placental production of leptin appears to be an example of evolution in action; ectopic expression of a gene is acquired coincidentally and then is available for integration into the physiology of the tissue.

The biology of leptin during gestation is not well understood. In humans, the serum leptin concentration is increased 1.7-fold at the end of pregnancy (corrected for fat mass; Ref. 54). Placental production is a likely explanation for this increase although increased synthesis by maternal adipose tissue is also possible. One might have expected that a low leptin would be found during gestation since the metabolic effects of high leptin levels are not observed (decreased food intake) or would be undesirable (decreased metabolic efficiency) when the mother is putting great effort into nutrition of the fetus and into preparation for lactation. There is no information on either the bioactivity/bioavailability of leptin, or on the responsiveness of the body to leptin during pregnancy. If pregnancy is indeed a state of leptin resistance, then the observed increase in leptin could be a compensatory response. Alternatively, the increase in leptin levels might be selectively important for the reproductive system, rather than for general energy metabolism.

Some information about the role of leptin in pregnancy comes from studies using the ob/ob mouse. Leptin is needed for reproductive system maturation and fertility (11, 14) although this requirement can be bypassed and pregnancy induced (inefficiently) by gonadotropin treatment (10). Ongoing leptin treatment is not necessary to maintain pregnancy, but leptin appears to be needed for parturition and nursing (11). However, comparison between humans and mice is not straightforward. In humans, the serum leptin concentration is increased 1.7-fold (54) compared with 20-fold in mice. In mice there is also an increase in expression of a high-affinity binding protein, which has not been observed in humans. While the increase in leptin during pregnancy in humans may be a random evolutionary event without functional significance, another possibility is that, in humans, placental leptin has a paracrine function, for example preparing the uterus for parturition. This hypothesis has the advantage of unifying the need for leptin during parturition in the mouse, with the apparent attenuation of its systemic metabolic effects.

In summary, we have identified an enhancer of the human leptin gene and a placenta-selective element within the enhancer. This enhancer mediates placental expression of leptin, which could explain the increased leptin levels during human pregnancy. The biologic functions of leptin in gestation are not known, but a role in the control of pregnancy, parturition, or establishment of the lactating state seems likely and merits further investigation.

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