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Pref-1, a Protein Containing EGF-like Repeats, Inhibits Adipocyte Differentiation

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Summary

With the aim of identifying novel regulators of adipocyte differentiation, we have cloned and characterized preadipocyte factor 1 (pref-1), a novel member of the epidermal growth factor (EGF)-like family of proteins. Pref-1 is synthesized as a transmembrane protein with six tandem EGF-like repeats. In preadipocytes, multiple discrete forms of pref-1 protein of 45–60 kd are present, owing in part to N-linked glycosylation. While pref-1 mRNA is abundant in preadipocytes, its expression is completely abolished during differentiation of 3T3-L1 preadipocytes to adipocytes. Moreover, constitutive expression of pref-1 in preadipocytes, which in effect blocks its down-regulation, drastically inhibits adipose differentiation. This indicates that pref-1 functions as a negative regulator of adipocyte differentiation, possibly in a manner analogous to EGF-like proteins that govern cell fate decisions in invertebrates.

Introduction

The differentiation of preadipocytes to mature adipocytes involves striking morphological and biochemical changes. Fibroblastic preadipocytes cease dividing, undergo differentiation, and attain the biochemical profile and rounded, lipid-filled morphology typical of mature adipocytes. Studies of adipocyte differentiation have been facilitated by the use of preadipocyte cell lines such as 3T3-L1 that differentiate in vitro in a process that closely resembles in vivo adipocyte differentiation (Green and Kehinde, 1974, 1976; Green and Meuth, 1974). Differentiation is accompanied by significant alterations in the levels of over 100 proteins (Sidhu, 1979). These include increases in the levels of those proteins and mRNAs required for the specialized metabolic role of the adipocyte in lipid metabolism (Paulauskis and Sul, 1988; Wise et al., 1984; Ntambi et al., 1988; Spiegelman et al., 1983), lipid transport (de Herreros and Birnbaum, 1989; Bernlohr et al., 1985), and hormone responsiveness (Rubin et al., 1978). In addition, extracellular matrix (ECM) secretion alters during differentiation (Aratani and Kitagawa, 1988; Calvo et al., 1991), and actin and tubulin mRNAs decrease preceding the expression of adipocyte-specific enzymes (Spiegelman and Farmer, 1982). Cell rounding, typical of the differentiation process, occurs even when triglyceride accumulation is blocked (Kuri-Harcuch et al., 1978).

Adipocyte differentiation can be modulated both by diffusible growth factors and by cell adhesion molecules. For example, insulin-like growth factor 1 substitutes for essential serum factors required for differentiation (Smith et al., 1988), and insulin accelerates lipid accumulation (Green and Kehinde, 1975). Conversely, transforming growth factor B and tumor necrosis factor α inhibit conversion to adipocytes (Ignotz and Massague, 1985; Torti et al., 1985). Growth of preadipocytes on fibronectin prevents morphological changes that occur during differentiation and inhibits their differentiation (Spiegelman and Ginty, 1983). Several cDNAs with regulatory roles in adipocyte differentiation have been identified. The transcription factor CCAAT/enhancer-binding protein (C/EBPα), present in various tissues, has a temporal expression pattern indicative of a role in adipocyte differentiation (Birkenmeier et al., 1989; Umek et al., 1991) and transactivates adipocyte-expressed genes in vitro (Christy et al., 1989; Herrera et al., 1989) but appears dispensable in transgenic mice models (Graves et al., 1991). Expression of C/EBPα antisense mRNA inhibits conversion (Lin and Lane, 1992), while overexpression of C/EBPα in the presence of inducing agents accelerates it (Umek et al., 1991) and alleviates the inhibition of adipocyte differentiation imposed by c-myc (Freytag and Geddes, 1992). C/EBP and myc are thought to affect cell division and thereby terminal differentiation of adipocytes. Several less well characterized genes are also postulated to have a regulatory role (Dani et al., 1989; Wenz et al., 1992); however, their protein products offer no insight as to their function.

The epidermal growth factor (EGF)–like family of proteins function in cell growth and differentiation in an astonishing array of biological settings, with protein–protein interaction as the unifying theme (for review, see Laurence and Gusterson, 1990). Members of the EGF-like family of proteins are soluble and/or transmembrane molecules that contain at least one 35- to 40-residue EGF-like repeat. This motif, initially described in the mitogen EGF (Carpenter and Cohen, 1990), is distinguished by the conserved spacing of six cysteine residues that form three disulfide bonds (Cooke et al., 1987). Growth factor members of this family, which are generated by cleavage of transmembrane precursors and act through the EGF receptor, include transforming growth factor α (Massague, 1990), amphiregulin (Plowman et al., 1990), and heparin-binding EGF-like growth factor (Higashiyama et al., 1991). Several multidomain ECM and cell adhesion molecules, with a demonstrated role in cell guidance and development, contain EGF-like motifs. These include laminin (Panayotou et al., 1989), versican (Krusius et al., 1987), tenascin (Nies et al., 1991), and the selectin family of adhesion molecules (Siegelman et al., 1990; Johnston et al., 1989; Bevilacqua et al., 1989). The importance of the motif in differentiation and development is perhaps best illustrated by the invertebrate EGF-like genes *glp-1* (Yochem and Greenwald, 1989) and *lin-12* (Greenwald, 1985) of Caenorhabditis elegans and *Notch* (Wharton et al., 1985) and *Delta* (Kopczynski et al., 1988) of Drosophila. Genetic and biochemical evidence suggests that cells expressing the proteins Notch and Delta interact via their EGF-like domains to

direct cell fate decisions (Fehon et al., 1990; Rebay et al., 1991). The identification of Xenopus (Coffman et al., 1990), human (Ellisen et al., 1991), and rat (Weinmaster et al., 1991) *Notch* homologs illustrates the universality of this motif and suggests that similar mechanisms may control cell fate determination in vertebrates.

We report here the cDNA cloning, sequence, and expression pattern of a novel member of the EGF-like family of proteins, which we refer to as preadipocyte factor 1 (pref-1). Pref-1 is regulated in and regulatory for adipocyte differentiation. Pref-1 mRNA and protein is expressed abundantly in preadipocytes, and its expression is completely abolished during adipocyte differentiation. Moreover, constitutive overexpression of pref-1 in preadipocytes blocks their conversion to adipocytes and suggests that pref-1 may function to maintain the preadipose phenotype.

Results

Isolation of cDNA for Pref-1

Differential hybridization screening was employed to isolate candidate cDNAs with a regulatory role in the differentiation of 3T3-L1 preadipocytes. A 3T3-L1 preadipocyte library was prepared from cells 40 hr after the start of dexamethasone and methylisobutylxanthine (dex/mix)–induced adipocyte differentiation (Rubin et al., 1977) and was differentially screened using two reverse-transcribed probes. The first was derived from the same RNA used in library construction (3T3-L1 cells, 40 hr post-dex/mix) and the second from 3T3-C2 cells, a related but nondifferentiating cell line (Green and Kehinde, 1975), that had been subjected in parallel to the dex/mix differentiation protocol. Five differentially hybridizing phage plaques were determined to represent cDNAs restricted in expression, as they failed to hybridize to reverse-transcribed probes of mouse liver or c2c12 myoblast RNA. Further analysis revealed one cDNA clone that had a higher mRNA level in 3T3-C2 than 3T3-L1 cells, contained an EGF-like motif, and was down-regulated during adipocyte differentiation. This cDNA and its encoded protein, pref-1, was therefore characterized in detail.

cDNA and Predicted Amino Acid Sequence of Pref-1

The cDNA sequence of pref-1 is shown in Figure 1A and reveals a 1589 bp cDNA with several potential polyadenylation sequences (AATAAT) (Jansen et al., 1983) just upstream from the poly(A) tail. The first ATG conforms well to the Kozak consensus sequence for an initiator methionine (Kozak, 1984), and the open reading frame (ORF) encodes a 372 amino acid protein with a calculated molecular weight of 39.4 kd. Two hydrophobic stretches are predicted by a Kyte–Doolittle plot (Kyte and Doolittle, 1982). The first is located within the first 20 amino-terminal residues and has characteristics of a signal sequence (von Heijne, 1985). The second spans amino acids 300–322 and presumably functions as a transmembrane anchor. The most striking structural feature of pref-1 is the presence of six tandem EGF-like repeats in the putative extracellular domain of the protein. These begin shortly after the signal sequence and encompass 60% of the protein. The sixth repeat is followed by a dibasic sequence that, in other proteins, can serve as a proteolysis site (Barr, 1991). However, we have no evidence that it does so in pref-1, despite our efforts to detect a soluble form of pref-1 in conditioned media from 3T3-L1 cells or from pref-1-transfected COS-7 cells (data not shown). A juxtamembrane region, with no detected homology to other motifs, precedes the putative transmembrane domain. Three consensus sites for N-linked glycosylation are present, all in the putative extracellular domain. The short cytoplasmic domain is rich in serine, threonine, and proline and contains several potential phosphorylation sites (Kemp and Pearson, 1990). In addition, these 3 amino acids, along with glutamine, are present in PEST sequences and may modulate protein turnover (Rogers et al., 1986). A GenBank data base search revealed that the cDNA sequence of pref-1 has a high degree of homology to a human adrenal-specific cDNA (Helman et al., 1990) at the nucleotide level but no homology at the predicted amino acid level. It is possible that sequencing errors caused the incorrect assignment of the start codon and reading frame shifts in the adrenal-specific cDNA.

The alignment of the six pref-1 EGF-like repeats and their comparison with those in other proteins are shown in Figures 1B and 1C. The pref-1 repeats maintain the overall cysteine spacing and other amino acids characteristic of this motif. Because none of the pref-1 EGF-like repeats has the exact cysteine spacing or those residues critical for binding to the EGF receptor (Burgess et al., 1988; Ray et al., 1988; Campbell et al., 1990), it is unlikely that pref-1 functions through the EGF receptor. Rather, the tandem nature of the repeats, the cysteine spacing, and the presence of proline preceding the second cysteine show striking similarity to the EGF-like repeats of the Drosophila cell fate–determining proteins Notch and Delta.

Pref-1 mRNA Expression in Adult Tissues and Mouse Embryo

To gain further insight into pref-1 function, the expression of pref-1 mRNA was examined by Northern analysis and in situ hybridization. Figure 2A illustrates that the 1.7 kb pref-1 mRNA is readily detected in 3T3-L1 preadipocytes; however, of a panel of adult mouse tissues examined, it is present only in adrenal gland. This highly restricted distribution of pref-1 mRNA in adult tissue and its presence in embryo-derived 3T3-L1 cells led us to address pref-1 mRNA expression in mouse embryo. As shown by the Northern blot in Figure 2B, pref-1 mRNA is first detected in day E8.5 mouse embryo and continues until the last time point, E18.5. Figure 2C shows an autoradiograph of in situ hybridization analysis of pref-1 mRNA in sagittal sections of 13-day mouse embryo. Bright- and dark-field microscopic examination of the embryo sections revealed that pref-1 signal was most prominent in the pituitary and the mesenchymal regions of the developing vertebra and was also present in the tongue, lung, and liver. This pattern

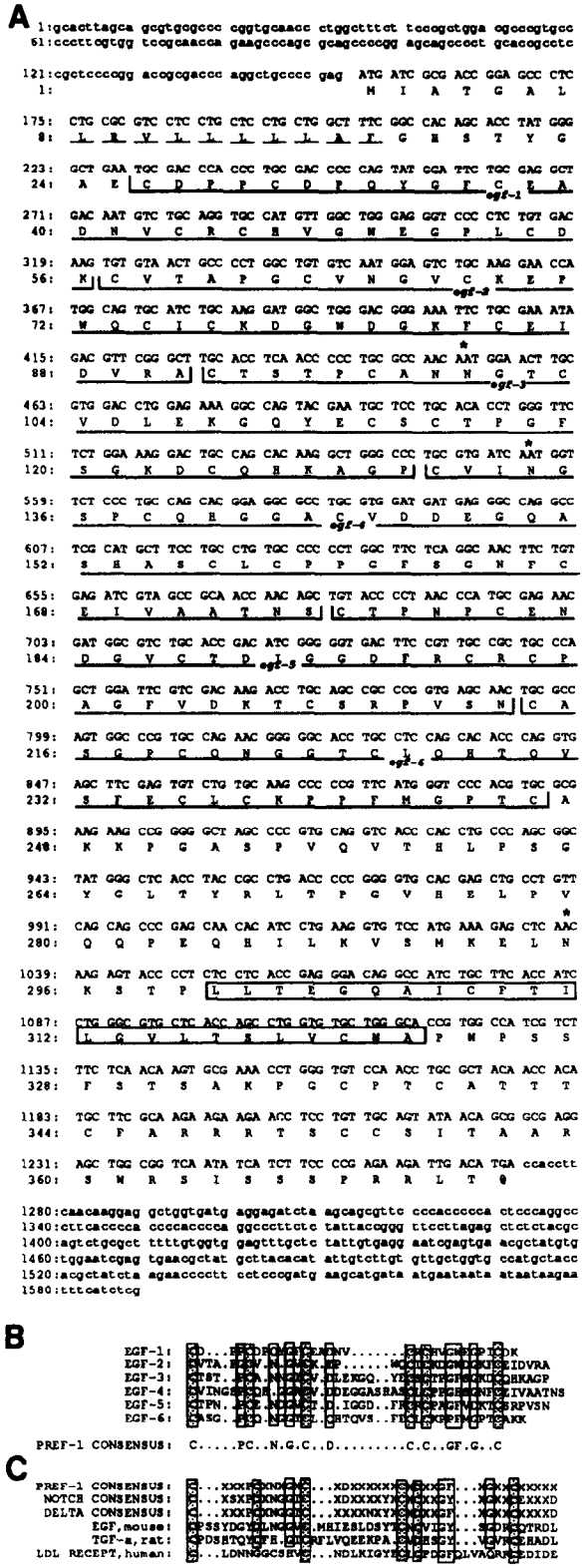

Figure 1. Nucleotide and Predicted Amino Acid Sequence of Pref-1 cDNA

(A) Predicted amino acid sequence of pref-1 is shown below the nucleotide sequence. The putative signal peptide is indicated by dashed underlining, and the transmembrane domain is boxed. The six tandem EGF-like repeats are underlined and numbered. The three potential N-linked glycosylation sites (N-X-S/T, X ≠ P) are indicated by asterisks. (B) Alignment of pref-1 EGF-like repeats. The six conserved cysteines are in stippled boxes; other residues conserved in this motif are in open boxes. Gaps are indicated by periods. The consensus sequence includes residues appearing in 4 or more of the 6 EGF-like repeats. X, nonconserved amino acid.

(C) Alignment of the pref-1 consensus EGF-like repeat with the repeats of Notch (Wharton et al., 1985), Delta (Kopczynski et al., 1988), murine EGF (Gray et al., 1983), rat transforming growth factor α (Lee et al., 1985), and LDL receptor (Sudhof et al., 1985). Gaps (periods) were introduced to maximize alignment. X, nonconserved amino acid.

of pref-1 expression was confirmed by Northern blot of mRNA prepared from embryonic tissues (data not shown). It is of interest to note that two tissues, lung and liver, that express pref-1 in the embryo (Figure 2C) do not express it in the adult (Figure 2A). This suggests that pref-1 is down-regulated during the maturation of these tissues and recalls Drosophila *Notch* mRNA, which is widely expressed in the embryo but highly restricted in expression in the mature organism.

Pref-1 Expression during Adipocyte Differentiation

To determine whether pref-1 expression is regulated during adipocyte differentiation, pref-1 mRNA and protein levels were examined in preadipocytes before and after their differentiation to adipocytes. Under the standard in vitro differentiation protocol, a mixed population of cells is apparent at 7 days postinduction; approximately 75% of the initial cell population has differentiated to rounded, lipid-filled mature adipocytes, while the remaining 25% fails to differentiate and continues to grow as preadipocytes. RNA was prepared from preadipocytes and from the mixed population of adipocytes and residual preadipocytes present 7 days after the start of the differentiation protocol. To specifically address pref-1 mRNA expression in adipocytes, adipocytes were purified from differentiated cultures by centrifugation in bromobenzene. The Northern blot in Figure 3A confirms that the purified cells were adipocyte enriched, as indicated by higher levels of two adipocyte-specific mRNAs: fatty acid binding protein (FABP) and stearoyl coenzyme A (CoA) desaturase. In marked contrast with the signal for these adipoctye-specific mRNAs, pref-1 mRNA was readily detected in preadipocytes and decreased in proportion to their differentiation to adipocytes. Moreover, pref-1 mRNA was undetectable in purified mature adipocytes, which correlates with the absence of pref-1 mRNA in adult fat (see Figure 2A).

Pref-1 protein levels during adipocyte differentiation were determined by Western analysis of crude membrane fraction protein from 3T3-L1 preadipocytes and differentiated adipocytes. Purified adipocytes were not used because degradation of membrane proteins, and therefore an artifactual decrease in pref-1 protein expression, could occur during the purification procedure. Use of a pref-1-specific antibody prepared against a TrpE–pref-1 fusion protein detected discrete multiple forms of pref-1 in preadipocytes, ranging from 45 to 60 kd. Tunicamycin treatment resulted in a reduction in size of the pref-1 protein in 3T3-L1 cells and pref-1-expressing COS-7 cells and demonstrated that the multiple forms of pref-1 protein were due in part

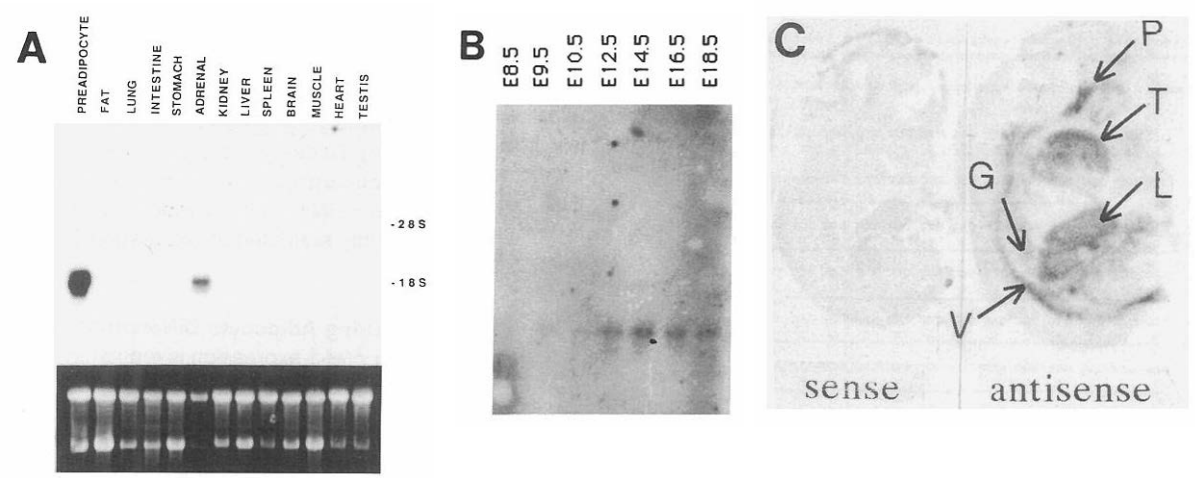

Figure 2. Pref-1 mRNA Distribution in Adult Tissues and Embryo

(A) Northern blot of adult tissues. Ten micrograms of total RNA (except adrenal, 2.5 μg) was run per lane of 1% Northern gels and hybridized to ^{32}P-labeled pref-1 probe. Positions of 28S and 18S RNA are shown at the right of the upper panel. Lower panel shows ethidium bromide staining of the gel.

(B) Northern blot of mouse embryo. Northern blot containing 10 μg of total RNA per lane of indicated embryonic day was hybridized to ^{32}P-labeled pref-1 probe and processed as described.

(C) In situ hybridization of 13-day mouse embryo. Sagittal sections of 13-day mouse embryo hybridized to either sense (left) or antisense (right) ^{33}P-labeled pref-1 riboprobes. The resultant autoradiographs are shown. Examination of the sections by microscopy revealed that the strongest pref-1 signal is in the pituitary (P) and the developing vertebra (V), with signal also present in the tongue (T), liver (L), and lung (G).

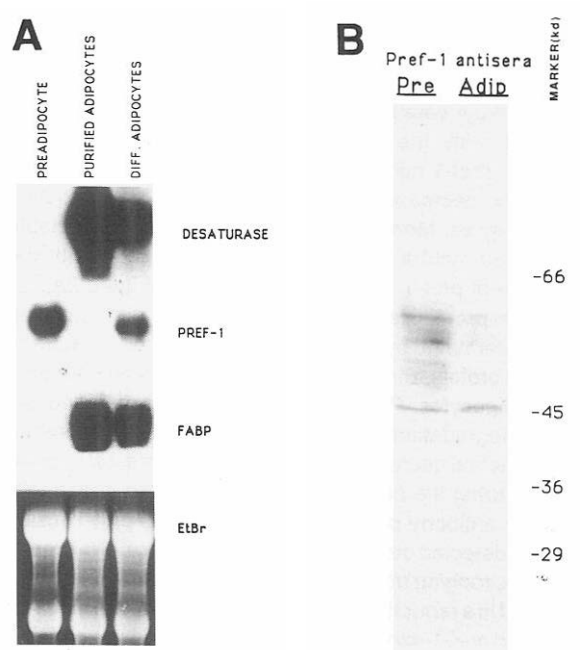

Figure 3. Down-Regulation of Pref-1 in Adipocyte Differentiation

(A) Pref-1 RNA. Twenty micrograms of total RNA from preadipocytes, purified adipocytes, or a mixed population of differentiated adipocytes consisting of adipocytes and residual preadipocytes was analyzed by Northern blotting. The same filter was hybridized to each of the indicated ^{32}P-labeled probes (from top of top panel: stearoyl CoA desaturase, pref-1, FABP). Bottom panel shows ethidium bromide staining of the gel.

(B) Pref-1 protein. Thirty micrograms of crude membrane fraction protein of 3T3-L1 preadipocytes (Pre) or adipocytes at 7 days postdifferentiation (Adip) was run on SDS–polyacrylamide (10%) gels and analyzed by Western blotting utilizing pref-1-specific antisera. The band at 45 kd is nonspecific.

to N-linked glycosylation (unpublished data). As shown in Figure 3B, all forms of the pref-1 protein decreased upon differentiation to adipocytes. The Northern and Western analyses indicated that pref-1 is unusual, as we know of no previously identified preadipocyte gene product whose expression is abolished during adipocyte differentiation.

Differential Expression of Pref-1 mRNA in Cell Lines and by Serum

If pref-1 down-regulation is required for adipocyte differentiation, this should be reflected by increased expression of pref-1 mRNA in cells and/or culture conditions in which adipocyte differentiation is inhibited and by decreased pref-1 expression under conditions that promote differentiation. Figure 4A compares pref-1 mRNA levels in differentiation-competent 3T3-L1 preadipocytes with levels in the closely related but differentiation-defective 3T3-C2 cells. 3T3-C2 cells express pref-1 mRNA at approximately 3-fold higher levels than 3T3-L1 cells. This is consistent with the hypothesis that lowered pref-1 levels are a prerequisite for differentiation. Fetal calf serum (FCS) and/or serum factors are required for adipocyte conversion. Regulation of pref-1 by serum was addressed by Northern analysis of RNA from 3T3-L1 preadipocytes cultured for 2 days in 0.5% FCS, followed by growth for 24 and 48 hr in the presence of 20% FCS. Figure 4B shows that while actin mRNA was unaffected by FCS concentration, the level of pref-1 mRNA is approximately two-thirds lower in the presence of 20% FCS than in 0.5% serum. These data indicate that serum decreases pref-1 mRNA levels and support the hypothesis that pref-1 down-regulation acts either as a primary signal or as a signaling point in preadipocyte differentiation.

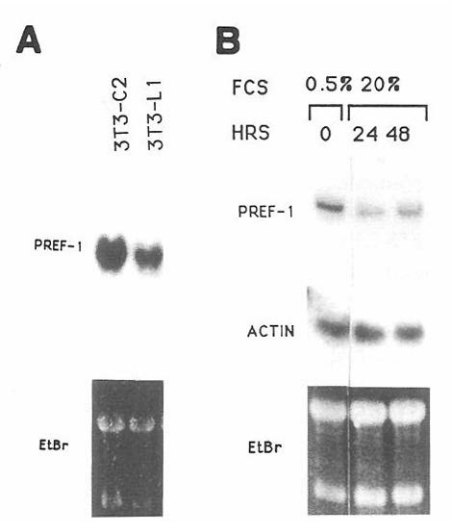

Figure 4. Differential Expression of Pref-1 mRNA in Cell Lines and by Serum

(A) Top panel shows Northern analysis of pref-1 mRNA in 3T3-C2 and 3T3-L1 cells. Ten micrograms of total RNA from 3T3-C2 and 3T3-L1 cells was fractionated per lane of 1% agarose gels and hybridized to ^{32}P-labeled pref-1 probe. Lower panel indicates ethidium bromide staining of the gel.

(B) Effect of FCS on pref-1 mRNA. Northern blot containing 10 μg of total RNA from 3T3-L1 preadipocytes prepared after 2 days' growth in 0.5% FCS and after 24 or 48 hr in media containing 20% FCS. The filter was sequentially hybridized to ^{32}P-labeled pref-1 and actin probes. Lower panel shows ethidium bromide staining.

Inhibition of Adipocyte Differentiation by Constitutive Pref-1 Expression

The decrease of pref-1 mRNA and protein levels during adipocyte differentiation, the down-regulation of pref-1 mRNA by serum, and the higher levels of pref-1 mRNA in nondifferentiating 3T3-C2 cells than in 3T3-L1 cells are consistent with a regulatory role for pref-1 in adipocyte differentiation. We investigated whether persistent pref-1 expression in 3T3-L1 preadipocytes, which would thus in effect block down-regulation, would affect their conversion to adipocytes. Pools of stably transfected cells were used to minimize the effects of plasmid integration and ensure that pref-1 expression was, in essence, the only variable that could affect differentiation. Two independently generated stable pools of approximately 400 clones each were used for both the correct and reverse orientations of the pref-1 ORF.

The Northern blot in Figure 5A shows the relative levels of pref-1 mRNA expression in untransfected cells and the stable pools. Constitutively expressed pref-1 mRNA was predicted to be approximately 1.9 kb and was therefore not distinct from the 1.7 kb endogenous form. Instead, an upward expansion of the signal resulted; this expansion was present in both pools expressing the correct orientation of the pref-1 ORF but not in the reverse orientation pools or in untransfected cells. Since untransfected preadipocytes express pref-1 mRNA at relatively high basal levels, the constitutively expressed form contributed at most 50% to the total level of pref-1 mRNA in these cells. More important than the absolute level of pref-1 mRNA expressed is the fact that these cells could no longer completely abolish pref-1 mRNA expression, which normally occurs during adipocyte differentiation. Western analysis of pref-1 protein in the transformants was difficult to interpret because of the high level and multiple forms of endog-

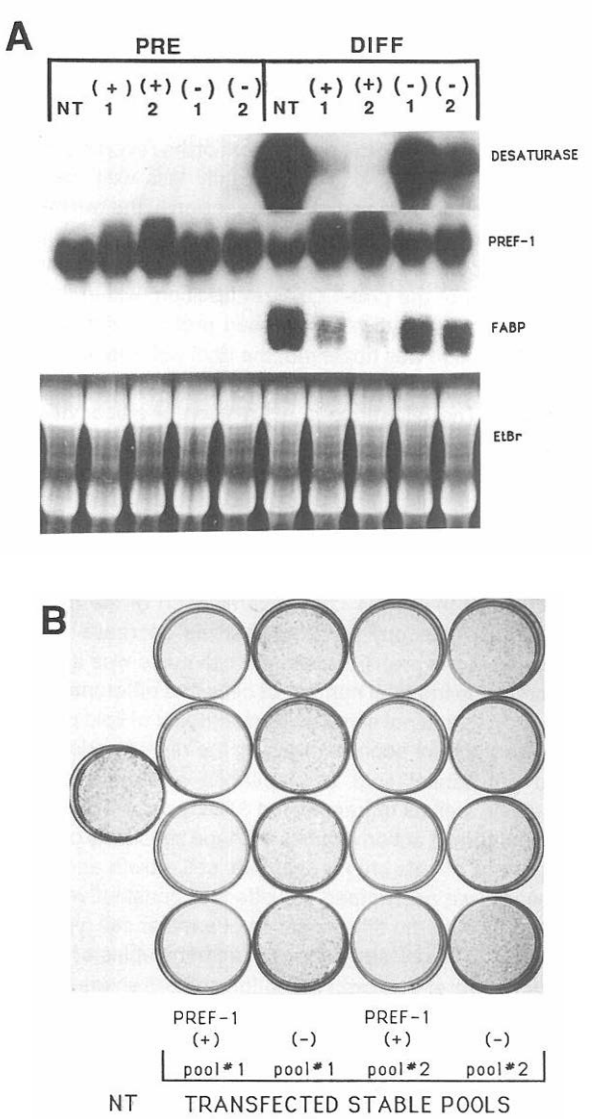

Figure 5. Constitutive Pref-1 Expression Inhibits Preadipocyte Differentiation

(A) Northern analysis. RNA was prepared from four independent stable pools of 3T3-L1 cells transfected with the correct ([+]1 and [+]2) or reverse ([−]1 and [−]2) orientation of the pref-1 ORF at confluence (PRE) or 7 days following differentiation (DIFF). In addition, RNA was prepared from nontransfected 3T3-L1 cells (NT) differentiated and processed simultaneously. Top panel shows hybridization of the same Northern blot containing 20 μg of RNA per lane to, from top: stearoyl CoA desaturase, pref-1, and FABP ^{32}P-labeled cDNA probe. Lower panel shows ethidium bromide–stained gel.

(B) Lipid staining. At 7 days following differentiation, cultures of four pools (as in [A]: [+]1, [+]2, [−]1, and [−]2) were fixed with 10% paraformaldehyde in PBS, stained for lipid content by Oil Red O, and photographed. Each pool was assayed in quadruplicate. Nontransfected 3T3-L1 cells (NT), differentiated and processed simultaneously, are shown at the left.

enous pref-1 protein in 3T3-L1 cells. However, transfection of this same plasmid construct in L cells and CHO cells, which lack endogenous pref-1, resulted in expression of pref-1 protein, as determined by Western analysis of crude membrane fractions.

To determine the effect of constitutive pref-1 expression on adipocyte differentiation, cultures were assayed 7 days after the start of the dex/mix differentiation protocol. Extent of differentiation was judged by the expression of two adipocyte-specific mRNAs, FABP and stearoyl CoA desaturase, and by Oil Red O staining of cellular lipids. As indicated by the expression of adipocyte-specific mRNAs shown in Figure 5A, the cells in both of the reverse orientation pools differentiated only slightly less well than untransfected cells. In contrast, expression of the two mRNA markers of adipocyte differentiation was drastically reduced in both of the cell pools transfected with the correct orientation of the pref-1 ORF. In addition, within the two pools that constitutively expressed pref-1, a dose-dependent inhibition was observed; the pool with the higher expression of pref-1 had lower levels of adipocyte-specific mRNAs. The difference in expression of adipocyte-specific mRNAs correlates well with lipid content, as determined by Oil Red O staining of parallel cultures. As shown in Figure 5B, with lipid visualized as dark areas on the dishes, adipose conversion was severely inhibited in both stable pools that constitutively expressed the correct orientation of the pref-1 ORF. Examination of the cultures by light microscopy revealed that the decrease in lipid staining in the pref-1-transfected cell pools was due to a decrease in the total number of cells that differentiated to adipocytes and not in the average amount of lipid per cell. The two control pools expressing the reverse orientation had substantial lipid accumulation and differentiated nearly as well as untransfected 3T3-L1 cells. To address whether pref-1 action exhibits cell-type specificity or is due to general effects on, for example, cell growth and/or adherence, we determined the effect of constitutive pref-1 expression on the differentiation of another cell type, murine c2c12 myoblasts. Although c2c12 myoblasts lack endogenous pref-1, growth cessation and cell shape change are integral to myoblast differentiation. Constitutive expression of pref-1 had no effect on the conversion of myoblasts to myotubes (data not shown). On the basis of these results, we conclude that inhibition of differentiation by pref-1 demonstrated cell type specificity and that pref-1 down-regulation is required for adipocyte differentiation.

Discussion

We have isolated a cDNA for pref-1, a novel member of the EGF-like family of proteins that experiments indicate is regulated and regulatory for adipocyte differentiation. While a large number of mRNAs whose levels increase during adipocyte differentiation have been characterized and their regulation has been studied in detail, very few have been identified that are down-regulated during adipocyte differentiation. Actin and tubulin synthesis decreases during adipose conversion (Spiegelman and Farmer, 1982), and transcription from the simian virus 40 promoter is specifically suppressed (Dijan et al., 1988). The down-regulation of pref-1 is interesting in that its mRNA expression is totally abolished during adipocyte differentiation. Pref-1 may simply no longer be needed in the mature adipocyte, or down-regulation of pref-1 may be required for the differentiation process, in either a permissive or instructive manner. The inhibition of adipocyte differentiation in 3T3-L1 preadipocytes that constitutively express pref-1 argue for the latter.

The function of pref-1 in adipocyte differentiation remains under speculation at this time. Multiple forms of the pref-1 protein are present in 3T3-L1 preadipocytes, owing in part to posttranslational modification by N-linked glycosylation. Each form may have a distinct function dependent either on alternate forms of the primary transcript and thus primary translation product and/or on various post-translational modifications. Although it is of interest to note that EGF has been demonstrated to inhibit adipose tissue development in vivo (Serrero and Mills, 1991), none of the six EGF-like repeats of pref-1 retains the exact spacing of cysteines, nor the presence of additional residues, crucial for binding to the EGF receptor. However, if pref-1 functions as a growth factor, its down-regulation may be involved in the cessation of cell growth required for preadipocyte differentiation (Green and Meuth, 1974).

The presence of EGF-like repeats in the extracellular domain of pref-1 and the demonstrated role of this motif in protein–protein interaction serve as the basis of a number of hypotheses regarding pref-1 function in adipocyte differentiation. Studies with Notch and Delta demonstrate that EGF-like repeats of these two transmembrane proteins govern cell–cell interaction (Fehon et al., 1990; Rebay et al., 1991) and consequently cell fate decisions. Similarly, pref-1 may mediate homotypic or heterotypic interactions with EGF-like repeats or other motifs in a still unidentified preadipocyte molecule. The amount and composition of the ECM is altered during 3T3-L1 differentiation (Aratani and Kitagawa, 1988; Calvo et al., 1991), and several ECM proteins secreted by fibroblasts contain EGF-like repeats. Cell shape change is as integral a part of the conversion process as the expression of adipocyte-specific enzymes (Kuri-Harcuch et al., 1978). Preadipocytes, which express pref-1 as a transmembrane protein, could modulate their interaction with ECM components via the association of pref-1 EGF-like repeats with EGF-like or other domains of ECM components. Such interaction could maintain the preadipocyte fibroblast morphology and decrease their differentiation capacity, similar to that noted when preadipocytes are cultured on fibronectin (Spiegelman and Ginty, 1983).

While we have shown that expression of the full-length transmembrane form of pref-1 inhibits adipocyte differentiation, we have no evidence that a diffusible form of pref-1 exists or functions in adipocyte differentiation. The presence of dibasic residues following the last EGF-like repeat, which could function as a proteolysis site, suggests a mechanism for generation of soluble pref-1. However, we have not detected soluble pref-1 in conditioned media from 3T3-L1 preadipocytes or from pref-1-transfected COS-7 cells. In addition, the observation that adipocyte differenti-

ation occurs in the presence of residual preadipocytes that continue to synthesize pref-1 suggests that the action of pref-1 is cell autonomous, or at most affects neighboring cells, and argues against an inhibitory function for a freely diffusible form of pref-1. Moreover, in an experiment similar to that used to demonstrate the inhibitory effect of transmembrane pref-1, transfection of 3T3-L1 cells with an expression construct encoding the extracellular portion of pref-1 from the start codon through the dibasic site and thus lacking the transmembrane domain had no effect on their differentiation to adipocytes (unpublished data). However, this does not exclude the possibility that soluble pref-1 could be inhibitory under different experimental conditions.

While the predicted protein structure of pref-1 is dominated by the presence of six tandem EGF-like repeats, pref-1 is unique in its overall structure. It lacks additional recognizable sequence motifs such as the cdc/swi sequences found in several invertebrate EGF-like proteins (Breeden and Nasmyth, 1987) or the kringle or proteolysis domains of some EGF-like clotting factors (Furie and Furie, 1988). The tandem arrangement of the EGF-like repeats in pref-1 and the amino acid sequence within each repeat most resemble those of the EGF-like proteins that function in invertebrate cell differentiation. The fact that we demonstrate a regulatory role for pref-1 in adipocyte differentiation, taken with the demonstrated role of cell shape modulation in this process, suggests that pref-1 could function in an analogous manner: that is, by governing cell–cell interaction and thereby cell differentiation. In addition, evidence to date suggests that pref-1 mRNA expression has parallels to that of Drosophila *Notch* (Hartley et al., 1987). Pref-1 mRNA is detected early in mouse embryogenesis, is present in the embryo-derived 3T3-L1 cells, and is down-regulated as these cells cease dividing and undergo in vitro maturation to adipocytes. Moreover, we detect pref-1 in embryonic lung and liver but not in the corresponding adult tissues. This suggests that, in addition to the specific role we demonstrate for pref-1 in adipocyte differentiation, pref-1 may function in the development of these tissues. Further studies on the role of pref-1 in adipocyte differentiation will offer additional insights into how EGF-like proteins function in mammalian development.

Experimental Procedures

Cell Lines and Culture

Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum and fed with fresh media the day before harvesting for RNA or protein. To induce adipose conversion of 3T3-L1 preadipocytes, cells were treated at confluence (day 0) with dex/mix (0.25 μM dexamethasone and 0.5 mM 1-methyl-3-isobutylxanthine) for 48 hr, at which time the drugs were removed, and cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum until harvesting (Rubin et al., 1977). Typically, by day 6 approximately 75% of the cells had differentiated to adipocytes and the remainder consisted of residual preadipocytes, as judged by microscopic morphology.

cDNA Library Construction and Screening

A cDNA library representing 3T3-L1 cells 40 hr after induction of differentiation was prepared based on the method of Gubler and Hoffman (1983) in the UniZap XR directional cloning vector (Stratagene). Five micrograms of poly(A)$^+$ RNA was used for first-strand synthesis employing an oligo(dT) primer that incorporated an XhoI restriction site and Moloney murine leukemia virus reverse transcriptase. First-strand cDNA was rendered resistant to XhoI via the incorporation of methyl-dCTP. Following second-strand synthesis utilizing RNAase H and DNA polymerase, cDNA was blunt ended with T4 DNA polymerase. After ethanol precipitation, EcoRI linkers (New England Biolabs) were ligated to the double-stranded cDNA via T4 DNA ligase and treated with polynucleotide kinase. The cDNA was digested with XhoI and size selected for that greater than 300 bp with a Biogel A15m column (Bio-Rad Laboratories). Pooled, size-selected fractions were ligated into predigested EcoRI–XhoI UniZap XR vector and packaged with Gigapack Gold (Stratagene) to produce 1.65 × 10^6 primary clones. The library was amplified once prior to screening.

For screening, 300,000 plaques of the 3T3-L1 40 hr cDNA library were plated at low density and screened by differential hybridization of replicate filters (Maniatis et al., 1989). Filters were prehybridized for at least 4 hr at 65°C in 6× SSC, 1× Denhardt's solution, 0.05% SDS, and 100 μg/ml herring sperm DNA. Hybridization was under the same conditions with the addition of an equal number of trichloroacetic acid–precipitable counts of probes produced by reverse transcription of poly(A)$^+$ RNA from either 3T3-L1 or 3T3-C2 cells 40 hr after the start of the differentiation protocol. Final washes of filters were for 1 hr in 0.1× SSC, 0.1% SDS at 65°C. Sixty of the differentially hybridizing clones were taken to plaque purity. To identify clones restricted in expression, a second round of differential screening was conducted on purified populations of plaques, as above, utilizing probes derived from the reverse transcription of murine c2c12 myoblasts and mouse liver poly(A)$^+$ RNA. Phage clones that did not hybridize to either of these probes were subjected to in vivo excision of the pBluescript SK(−) phagemid as described by the manufacturer (Stratagene), and plasmid DNA was prepared by standard methods.

DNA Sequencing

Double-stranded DNA sequencing of plasmid DNA was performed utilizing Sequenase (US Biochemicals) and synthetic oligonucleotide primers via the chain termination method (Sanger et al., 1977).

RNA Preparation and Northern Blot Analysis

Cell monolayers were rinsed twice with phosphate-buffered saline (PBS) and harvested by scraping in guanidium isothiocyanate followed by centrifugation over cesium chloride (Chirgwin et al., 1979). For RNA preparation from purified mature adipocytes, we employed differential centrifugation in bromobenzene (Spiegelman and Farmer, 1982). Differentiated 3T3-L1 cultures were trypsinized, resuspended in PBS, and centrifuged for 5 min at 2500 × g to pellet preadipocytes and immature adipocytes. An equal volume of bromobenzene-saturated PBS was added to the supernatant and centrifuged for 5 min at 2500 × g, and pelleted adipocytes were lysed by addition of guanidium isothiocyanate. For tissues, tissue was excised, frozen in liquid nitrogen, and homogenized in guanidium isothiocyanate with a polytron. For Northern analysis, RNA was size fractionated on 1% agarose gels in 2.2 M formaldehyde, 20 mM MOPS buffer, 1 mM EDTA, stained with ethidium bromide, and transferred to Hybond (Amersham Corporation). Following prehybridization for at least 4 hr in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 50 μg/ml herring sperm DNA at 42°C, filters were hybridized for 16–20 hr under identical conditions with the addition of at least 1 × 10^6 cpm/ml of ^{32}P-random-primed probe. Final washes were for 1 hr in 0.1× SSC, 0.1% SDS at 65°C. Filters were exposed with intensifying screen to Fuji RX film. The mouse embryo blot was kindly provided by Dr. S.-J. Lee (Carnegie Institution of Washington).

In Situ Hybridization Analysis

Single-stranded RNA probes were prepared by in vitro transcription of linearized template DNA to yield either the antisense or sense strands, corresponding to a 338 bp portion of pref-1 (nucleotides 969–1307) that includes the 3′ end of the ORF but not the 3′ untranslated region or the EGF-like repeat region. Probes were synthesized in a 20 μl reaction containing 1 μg of DNA template, 40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 500 μM (each) ATP, GTP, CTP with 80 μCi of ^{35}P UTP (1900 Ci/mmol,

New England Nuclear), and 1 U/μl T7 or T3 RNA polymerase for 1 hr at 37°C. Following synthesis, template DNA was removed by acid–phenol extraction, and probe size was reduced to an average length of 100 bases by alkaline hydrolysis. Slides containing 5–7 μm paraffin sagittal sections of 13-day mouse embryo were pretreated by successive 5 min room temperature incubations with 0.2 M HCl, 1 μg/ml proteinase K, and 0.25% acetic anhydride, 0.1 M triethanolamine–HCl (pH 8.0). Sections were prehybridized at 50°C for 3 hr in 50% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 50 μg/ml heparin, 10 mM dithiothreitol, 10% polyethylene glycol 7500, and 1 × Denhardt's solution. Hybridization was continued for 18–24 hr with the addition of 0.5 mg/ml carrier DNA, 0.5 mg/ml tRNA, and equal amounts of either antisense or sense riboprobes. Following hybridization, washing was for 30 min with 2 × SSC at 50°C, 30 min at 37°C with 20 μg/ml RNAse A, 30 min at 50°C in 2 × SSC/50% formamide, and two 30 min washes in 1 × SSC, 1% sodium pyrophosphate at 50°C. Slides were first exposed to Cronex MRF 34 film (Du Pont) prior to coating with emulsion (NTB-2; Kodak). Following development, tissue was counterstained with Giemsa stain and analyzed by bright- and dark-field microscopy.

Antibody Production and Western Analysis

The details of TrpE–pref-1 fusion protein preparation and antibody production will be described elsewhere. In brief, a NcoI–BglII fragment (nucleotides 368–1307) encoding all but the 72 amino-terminal residues of pref-1 was subcloned into SmaI–BamHI-cut pATH11 vector. Fusion protein expression was initiated by tryptophan starvation and extracted as described (Dieckmann and Tzagoloff, 1985). Approximately 100 μg of fusion protein in water was emulsified with an equal volume of complete Freund's adjuvant and used to immunize 6-week-old female New Zealand white rabbits with booster injections at 3- to 4-week intervals. The antiserum was determined to be specific for pref-1, since it recognized pref-1 protein in COS-7 cells transfected with the correct but not the reverse orientation of the pref-1 ORF, and since detection of pref-1 protein in 3T3-L1 cells was competed by preincubation of the pref-1 antisera with bacterially produced TrpE–pref-1 protein but not with TrpE protein alone. In addition, no signal was detected in 3T3-L1 cells utilizing either normal sera or antiserum directed against an unrelated TrpE fusion protein or when the pref-1 antiserum was used on crude membrane fractions prepared from mouse L and CHO cells.

3T3-L1 preadipocytes and differentiated cultures were prepared for Western analysis by rinsing monolayers twice with PBS and scraping cells into PBS containing 2 mM phenylmethylsulfonyl fluoride. The cell suspension was subjected to three freeze–thaw cycles, and the crude membrane fraction was recovered by centrifugation at 13,000 × g for 25 min at 4°C. The pellet was dissolved in lysis buffer (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride) on ice for 30 min and clarified by brief spinning in a microfuge, and protein content was determined (Bio-Rad Laboratories). SDS–polyacrylamide (10%) gels were electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore Corporation) using 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol transfer buffer. For immunodetection of proteins, membranes were blocked for 1 hr at room temperature in 1 × NET (145 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100, and 50 mM Tris–HCl [pH 7.4]), followed by overnight incubation at 4°C with primary antisera. Detection of the antigen-antibody complexes was accomplished via goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad Laboratories), and the peroxidase conjugate was developed with 0.015% H_2O_2, 16% methanol, 8.3 mM Tris–HCl (pH 7.4), and 0.05% (w/v) 4-chloro-1-naphthol.

Plasmid Constructs and Stable Transfection

For cloning into mammalian expression vectors, the majority of the 5′ and 3′ untranslated regions of the pref-1 cDNA were removed by digestion of the full-length cDNA with RsrII and BglII at nucleotides 132 and 1307, respectively, which flank the pref-1 ORF. The resultant 1175 bp fragment was rendered blunt ended with Klenow enzyme and ligated to blunt-ended plasmid vector. For stable transfection, the plasmid pMSXND (Lee and Nathans, 1981), which confers resistance to the antibiotic geneticin (G418), was blunt ended at the XhoI site. Supercoiled plasmid DNA of both orientations of the pref-1 cDNA was prepared by banding in cesium chloride. For stable transfection, 3T3-L1 preadipocytes were plated at 10^6 cells per 100 mm dish 24 hr prior to transfection and media were changed 3 hr before transfection. Cells were transfected with 20 μg of plasmid DNA per dish via the calcium phosphate method and selected for 2–3 weeks in 400 μg/ml active G418. All colonies (approximately 400) from a single dish were pooled by trypsinization and replating. Four independent pools were prepared, two each for the correct and reverse orientations of the pref-1 ORF. For assay of differentiation ability, stable pools of cells were plated at 1.3 × 10^5 per 60 mm dish and upon confluence were subjected to the dex/mix differentiation protocol. At 7 days postconfluence, four dishes of each pool were stained for lipids with Oil Red O, and six dishes of each pool were harvested together for RNA preparation and Northern analysis.

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GenBank Accession Number

The accession number for the pref-1 sequence reported in this paper is L12721.